INDUCED SYSTEMIC RESISTANCE IN POTATO (*SOLANUM TUBEROSUM* L.) FOLLOWING LOCALISED POTATO VIRUS X INFECTION

by

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DECLARATION

I hereby declare that the work presented in the following thesis is my own, except where otherwise acknowledged, and that the thesis is of my own composition.
No part of this thesis has been previously presented for any other degree.

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SUMMARY

The aim of this project was to investigate the resistance response of potato to potato virus X (PVX) and the possible correlation between resistance and the occurrence of induced systemic resistance (inferring greater resistance to a second challenge from PVX) at a molecular level. The investigation used a number of methods to look at the changes in gene expression which accompanied the invasion of PVX in a restricting potato cultivar. The objective was to identify genes involved in PVX resistance and to characterise them; looking at their expression in comparison to healthy and mock-inoculated controls.

Group 2 strains of PVX, when inoculated onto potato cultivars carrying the *Nb* gene, form local lesions but do not spread systemically. In comparison, Group 3 strains of PVX are able to infect such plants systemically (Cockerham, 1970). Cultivar Pentland Ivory, which carries the *Nb* gene, was inoculated with the Group 2 PVX strain KEBRS and developed local lesions after six days. When non-inoculated upper leaves of these plants were inoculated with PVX-KEBRS, or a Group 3 PVX strain (Gp3RS), 20 days after the first inoculation, the plants expressed a greater level of resistance than control plants which had not been earlier inoculated. Reduced lesion number on plants challenged with a Group 2 virus strain, or a reduced systemic infection when challenged by a Group 3 virus strain, indicated increased resistance. However, the level of resistance induced by pre-inoculation with PVX-KEBRS in comparison to mock-inoculation with water did not show a statistically significant difference.

RNA was extracted from PVX-KEBRS-inoculated, mock-inoculated (water) and healthy uninoculated (control) plants, from both the inoculated and upper leaves of Pentland Ivory plants, at various times after inoculation. The RNA was translated *in vitro*. The polypeptides were separated using one and two-dimensional PAGE systems.

The results obtained from one-dimensional gels showed that significant changes were first observed 7 days after inoculation, both locally and systemically to the point of inoculation with PVX-KEBRS, compared to both the control treatments. Using two-dimensional gels the *in vitro* RNA translation polypeptides (from RNA samples taken 7, 8, 9 and 12 days after inoculation) were further resolved. This identified that most mRNA induced changes in PVX-KEBRS-inoculated leaves were seen 7-8 days after inoculation while the greatest number of systemic changes were seen 9-12 days after inoculation. There were also several mRNAs which were induced both locally and systemically by PVX-KEBRS-inoculation and mock-inoculation. Thus inoculation of Pentland Ivory plants with PVX-KEBRS, or merely mock inoculating them with water, induced the synthesis of several mRNAs both locally and systemically to the inoculated leaf.

Poly A^+ RNA was extracted from non-inoculated leaves of Pentland Ivory plants, 12 days after inoculation with PVX-KEBRS, and used to construct a cDNA library in the lambda vector NM1149. The library was screened with a PR-1a and a thaumatin-like cDNA from tobacco and this identified mRNA showing homology to the two clones at a frequency of 0.1% and 0.05% respectively. Twenty-eight thousand plaques from the library were screened with a combination of subtractive and differential ss cDNA probes made from PVX-KEBRS-inoculated, mock-inoculated and healthy plants from the non-inoculated leaves 12 days after inoculation. This screening resulted in the identification of six clones which were induced systemically by PVX-KEBRS-inoculation. Three of these six clones were further investigated using Northern blot analysis. This identified a clone which was strongly induced by either PVX-KEBRS or mock-inoculation in the inoculated leaf. A second clone was systemically induced by PVX-KEBRS inoculation, while a third clone was found to be poorly differentially expressed between treatments. Therefore, the clones investigated so far have identified a range of mRNAs which are induced locally or systemically by PVX-KEBRS or mock-inoculation. These may be involved in the phenomena of induced systemic resistance.

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CONTENTS

ABBREVIATIONS

ABA	Abscisic acid
ACC	Aminocyclopropane-1-carboxylic acid
AlMV	Alfalfa mosaic virus
AMV	Avian myeloblastosis virus
APE	ATP polymerising enzyme
AR	AnalaR
ATP	Adenosine-5'-triphosphate
AVF	Anti-viral factor
BCMV	Bean common mosaic virus
BMV	Brome mosaic virus
bp	Base pairs
BSA	Bovine serum albumin
BSMV	Bushy stunt mosaic virus
C	Centigrade
CAD	Cinnamyl alcohol dehydrogenase
CA4H	Cinnamic acid hydroxylase
cDNA	Complementary DNA
CHI	Chalcone isomerase
CHS	Chalcone synthase
4CL	4-coumarate:CoA ligase
cm	Centimetre(s)
CMV	Cucumber mosaic virus
cv.	Cultivar
D	Days
dATP	2'-Deoxy-adenosine-5'-triphosphate
dCTP	2'-Deoxy-cytidine-5'-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	2'-Deoxy-guanosine-5'-triphosphate
DMSO	Dimethylesulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
d.p.m.	Decays per minute
ds	Double stranded
DTT	Dithiothreitol
dTTP	2'-Deoxy-thymidine-5'-triphosphate

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EtBr	Ethidium bromide
Ethephon	2-chloroethylphosphoric acid
g	Gravity
gm	Grams
GRP	Glycine rich protein
GTP	Guanosine-5'-triphosphate
h	Hours
HAP	Hydroxyapatite
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethonesulphuric] acid
HMGR	3-hydroxy-3-methylglutaryl co-enzyme reductase
HP	Phosphate hybridisation buffer
HPRI	Human placenta ribonuclease inhibitor
HR	Hypersensitive response
HRGP	Hydroxproline rich glycoproteins
IEF	Isoelectric focusing
ISR	Induced systemic resistance
IVR	Inhibitor(s) of viral replication
K	Thousand revolutions per minute
Kb	Kilobase
Kbp	Kilobase pair
KDa	Kilodalton
Kv	Kilovolts
LSD	Least significant difference
m	Metre
M	Mole
mA	Milliamps
mins	Minutes
ml	Millilitre
mm	Millimetre

mM	Millimole
MOPS	3-[N-morpholino]propane sulfonic acid
mRNA	Messenger RNA
MW	Molecular Weight
Na_4PP_i	Tetra-sodium pyrophosphate
ng	nanogram
nm	nanometre
NP40	Nonidet-P40
nt	Nucleotides
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
p.f.u.	Plaque forming unit
PLRV	Potato leaf roll virus
Poly A^+RNA	Polyadenelated RNA
PPO	2-5-Diphenyloxazle
PR	Pathogenesis related
pv	Pathovar
PVP	Polyvinylpyrrolidone
PVX	Potato virus X
PVY	Potato virus Y
RFLP	Restriction fragment length polymorphisums
RH	Relative humidity
RNA	Ribonucleic acid
r.p.m.	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
SA	Salacylic acid
SBMV	Southern bean mosaic virus
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Second
ss	Single stranded

TCA	Trichloroacetic acid
TEMED	N'N'N'N'-Tetramethylethylenediamine
TMV	Tobacco mosaic virus
TNV	Tobacco necrosis virus
ToMV	Tomato mosaic virus
Tris	Tris (Hydroxymethyl) aminomethane
TRV	Tobacco rattle virus
UV	Ultra violet
µCi	Micro Curie
µg	Microgram
µl	Microlitre
v/v	Volume/volume
w/v	Weight/volume
~	Approximately
°	Degrees
>	greater than
%	Per cent

DEDICATION

This thesis is dedicated to Jacqueline Smith
and
Barbara and Benjamin Barrett

Chapter 1

INTRODUCTION

1.1. A HISTORICAL RECORD OF *SOLANUM TUBEROSUM*.

The genus *Solanum* consists of approximately two thousand species, potato being found in the section *Tuberarium* which consists of approximately 170 species and includes all the tuber-bearing species. These are all indigenous to Latin America in the areas of Venezuela, Colombia, Ecuador, Peru, Bolivia, North West Argentina and Chile, south to 45° (Hawkes, 1978).

Fewer than ten species are relevant to the evolution of the crop plant. The selection of wild potato species as a crop plant, in the regions of Peru and Colombia, is thought to have started approximately 2,000-5,000 B.C. Selection was for species which were less bitter, as they contained fewer toxic alkaloids, and were therefore more palatable. By the time the Spanish had arrived in Latin America the potato crop had developed into a collection of varieties (Hawkes, 1978). The Spanish brought these back to Spain around 1537 (the first recorded shipment was in 1570). Requiring a tropical short-day (12 h) to induce tuber formation these potatoes were the *Solanum tuberosum* sub-species *andigenum*, which was named *Solanum tuberosum* in 1656. The potato was grown as a novelty throughout Europe, mainly in Botanic Gardens, until some two hundred years later, when through selection, it had evolved to a long-day variety with shorter, more bushy haulms and fewer but larger, better shaped tubers. These features facilitated its spread as a crop plant during the 18th century throughout Europe, and from Europe to the rest of the world (introduced in 1719 to North America and also to Australia and South Africa). It is now recognised as *Solanum tuberosum* sub-species *tuberosum* (Simmonds, 1976).

Though many cultivars existed, it was not until the devastation of the potato crop in 1845 by *Phytophthora infestans* that concern was given to breeding for **disease resistance**. This involved the use of closely related species from the Andean region which were highly resistant to certain diseases. Examples include *Solanum*

demissums which carries hypersensitivity to certain races of *P. infestans*, *Solanum acaule* which carries resistance to potato virus X (PVX) and *Solanum stoloniferum* which carries resistance to potato virus Y (PVY). *Solanum spegazzinii* and *Solanum vernei* carry resistance to cyst nematode (*Globodera* spp.).

1.2. POTATO CYTOGENETICS.

Wild potato species have a great range of ploidy levels consisting of diploids, triploids, tetraploids and pentaploids. The diploids tend to be outbreeders, while the polyploids tend to inbreed. The basic chromosome number is thought to be x = 12, however due to secondary associations between chromosomes in meiosis some think it could be x = 6 (Simmonds, 1976).

S. tuberosum is an autotetraploid (2n = 48), being similar to diploid species which are self-compatible, though they are reported to be intolerant to inbreeding in some cultivars as a result of shared common S alleles.

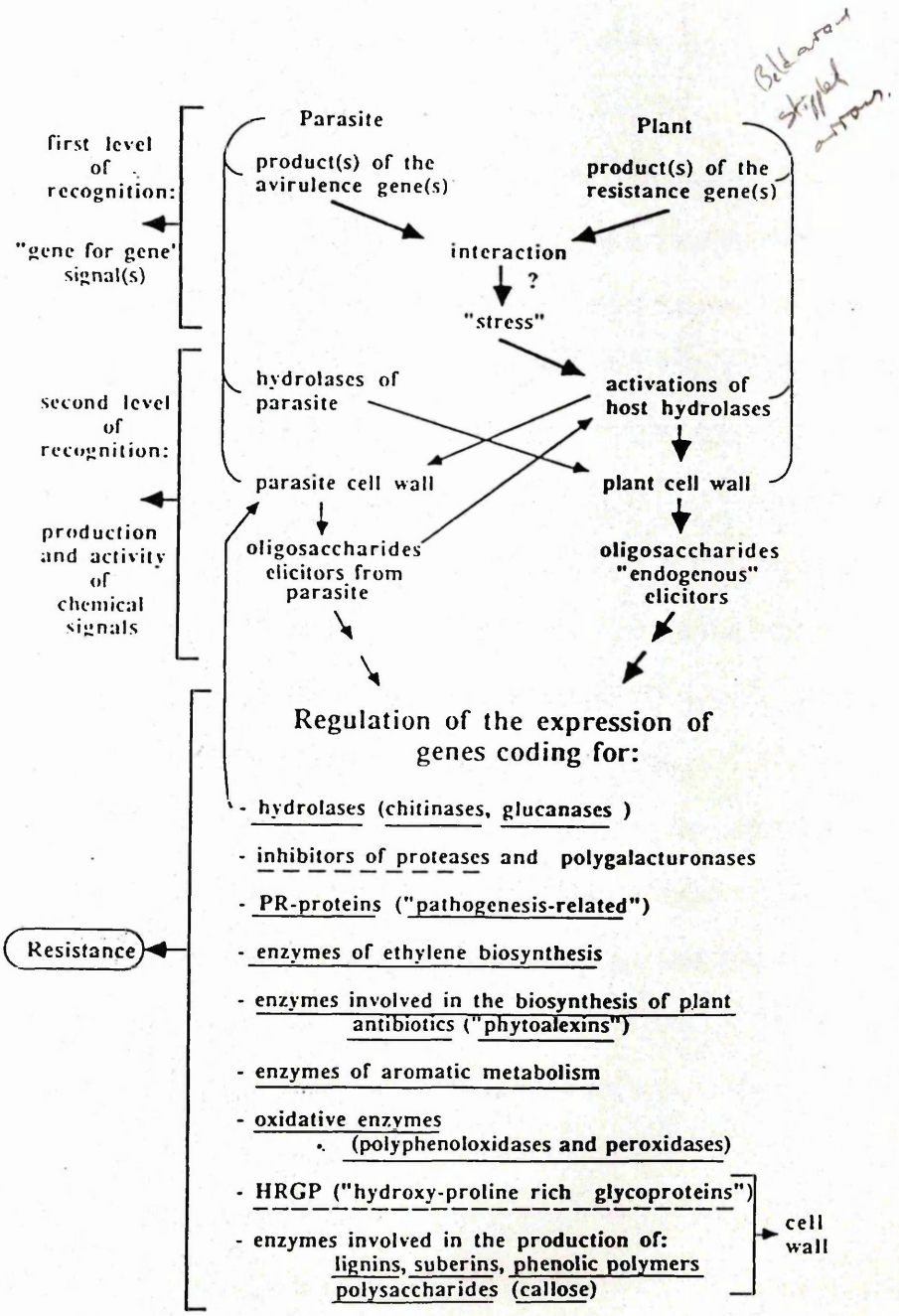
1.3. PLANT RESISTANCE TO PATHOGEN ATTACK.

Plant resistance to the majority of potential pathogens is of the passive type (Fritig *et al.*, 1987). In many cases this involves a physical barrier to the infection. However, when infected, the plant can engage a number of other specific responses which result in a range of resistance levels, from complete resistance to weak or no resistance. The defence reactions may be referred to as active, when they involve the increased expression of some gene products and/or the induction of new gene products solely involved in disease resistance (Fig. 1.1). If protein synthesis is inhibited at this critical time when a plant is infected, then these active defence reactions may fail (Fritig *et al.*, 1987).

Many resistance reactions involve multicellular host responses, for example tobacco mosaic virus (TMV) multiplication in a localising host (Otsuki *et al.*, 1972). Virus multiplication starts rapidly and is then gradually reduced as infected cells stimulate neighbouring cells to induce the resistance responses prior to viral invasion of that cell. This results in the virus being restricted to the local lesion. In contrast,

Fig. 1.1. Possible mechanisms of elicitation of defence reactions in incompatible plant-pathogen interactions. Metabolic alterations that have been demonstrated in the case of hypersensitivity to viruses are underlined with continuous lines; dotted lines indicate proposals that remain to be proven. The hypothetical elicitation scheme operating in virus infections is indicated with **bold** face arrows.

Modified from Fritig *et al.* (1987).



single cells from resistant hosts are often susceptible to virus infection (Otsuki *et al.*, 1972). The localisation of an invading pathogen to the point of infection is referred to as the hypersensitive response. This response, which is one of the most efficient natural defence mechanisms, is induced by infection (viral, bacterial or fungal). It has two main characteristics; necrosis at and around each point at which the leaf tissue was infected (though in some cases necrosis does not occur), and localisation of the parasite to the region of each initiated infection. The cells surrounding the necrotic area undergo marked metabolic changes which are believed to cause, or at least to contribute to, the resistance observed. Similar, though less marked, metabolic changes are also seen in the rest of the plant. These include cell wall thickening, resulting from production of lignin, subarin and related polymers, and the production of "defence" enzymes and proteins (Bol & van Kan, 1988).

"Defence" enzymes fall into two classes, enzymes that catalyse the production of various metabolites participating in resistance (ethylene, phytoalexins, aromatic compounds, oxidised metabolites, etc.) and enzymes involved directly in degrading the pathogen, e.g. hydrolases (chitinases and glucanases) (Collinge & Slusarenko, 1987). Other toxins are also produced, such as anti-viral proteins and inhibitors of proteases and polygalacturonases (Fig. 1.1) (Sela & Applebaum, 1962; Loebenstein & Gera, 1981; Fritig *et al.*, 1987). It must also be remembered that several of these responses are also induced by wounding, though often to a lower level than is seen with pathogen attack, and that this may confuse the issue (Logemann *et al.*, 1988).

The resistance responses are very complex and usually involve many inter-related reactions (Fig. 1.1). These responses will be considered individually to prevent confusion. The hypersensitive reaction, which is the first visual indication of resistance, will be discussed first. The metabolic changes, which are thought to induce the hypersensitive response, coordinate it and also restrict the pathogen, will then be dealt with. Many of the responses, which are thought to be involved with the hypersensitive response, are also seen to increase systemically throughout the plant

and are thought to be involved in the phenomena of induced systemic resistance, a description of which concludes this section (1.13).

A specific gene-for-gene recognition between plant and pathogen triggers the hypersensitive reaction. What the plant does, thereafter, to defend itself appears to be non-specific and consists of a set of metabolic reactions that are specific only to the host. This suggests that intermediate signals of host origin must be operating in all cases and these are responsible for the induction of the metabolic changes.

1.4. THE HYPERSENSITIVE RESPONSE.

Genetic analysis of plant-pathogen interactions has shown that in most cases active defence, related to incompatibility, is under the control of only one host gene, the resistance gene (Fraser, 1982b; Collinge & Slusarenko, 1987). An interaction between the resistance gene product and the product of a specific avirulence gene in the pathogen is believed to trigger the hypersensitive response (HR). This is usually accompanied by a stress reaction of the plant and by major metabolic changes (Heath, 1981; Bushnell & Rowell, 1981).

The HR can best be described as a rapid localised necrosis, forming a local lesion, associated with limitation of pathogen spread (which can be either viral, bacterial, fungal or nematode). Necrosis is the ordered cell death around the point of infection, though cell death is merely an indication that the HR has occurred. Cell death is characterised by granulation and browning of cytoplasm, which is an energy dependent response (Tomiyama, 1982). Electron-dense deposits in the lesions of potato reacting hypersensitively to *P. infestans* are evident in the lysosomes as well as in the host cell cytoplasm. Oxygen radicals generated in these cells are thought to disrupt the cell membranes of the lysosomes, allowing the release of hydrolases (acid phosphatase) into the cytoplasm. These have been implicated in host cell death (Jordan & De Vay, 1990). This coordinated death of cells is associated with a characteristic set of changes in gene expression and protein synthesis. Cell death is associated with increased expression of phenolase, peroxidase, polyphenoloxidase and other enzymes involved in phenolic oxidative metabolism which oxidise phenols, e.g.

O-dihydroxyphenols, to toxic quinones (Collinge & Slusarenko, 1987; Farkas *et al.*, 1960).

The biochemical changes involved in browning are thought to be associated with the rapid activation of phenylpropanoid metabolism, with increases in the enzyme levels of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and 4-coumarate:CoA ligase (4CL). These are, in all cases, preceded by transient coordinated increases in the amount and translational activity of their messenger ribonucleic acids (mRNAs) (Fritzemeier *et al.*, 1987). These enzymes are involved with the production of toxic phenolic compounds in the cytoplasm and the deposition of macromolecules in the cell wall.

The organised cell death may also be used to regulate gene expression in surrounding healthy cells. In potato, for example, rishitin and related compounds are synthesised in healthy tissue around the lesions formed by an incompatible *P. infestans* invasion. Cell death may be looked upon as a suicide response of the infected cell, killing itself to deny the potential pathogen the opportunity to spread further.

However, with interactions involving viruses, the HR does not always prevent the pathogen from infecting the plant systemically, e.g. in the case of tobacco rattle virus (TRV) infecting *Nicotiana tabacum* cv. Samsun *NN*, beet western yellows vein infecting sugar beet, or tomato bushy stunt infecting *Gomphrena globosa* (Goodman *et al.*, 1986). Conversely, localisation of the virus may occur in the absence of any visible symptoms, e.g. PVX localisation in potato (Adams *et al.*, 1986b) and TMV localised in cucumber cotyledons (Linder *et al.*, 1959; Cooper & Jones, 1983). The virus may be restricted to a local lesion, but this may be chlorotic, with no necrosis occurring at the site of infection, e.g. with cucumber mosaic virus (CMV) in *Zinnia elegans* (Nitzany & Wilkinson, 1960). Therefore, it is important to note that necrosis is not always directly responsible for virus localisation (Zaitlin, 1987).

Many resistance responses to viral infection are controlled by single dominant host genes (Cockerham, 1970; Fraser, 1982b, 1990). The resistance

response consists of reduced viral replication compared to susceptible cultivars, and the development of necrotic, chlorotic or invisible lesions, depending on the type of host-virus interaction. Those genes which have been investigated in detail include the *N* gene (originated in *Nicotiana glutinosa*) in *Nicotiana* spp., conferring resistance to all TMV strains (Holmes, 1965). The *N'* gene identified in *Nicotiana sylvestris* (Valleau, 1943) also confers resistance to TMV strain U2, but not U1. It has been suggested that *N, N'* and *n* are allelomorphic (Fraser, 1985, 1990). In *Phaseolus vulgaris* the *I* gene, which causes a necrotic localising reaction to bean common mosaic virus (BCMV) (Ali, 1950), has also been identified. In *Lycopersicum esculentum* three apparently dominant genes *Tm*-1, *Tm*-2 and *Tm*-2^2 individually confer resistance to TMV, with the latter two being allelic (Pelham, 1972). In potato, the dominant genes *Nx*, *Nb* and *Rx* have been shown to confer resistance to strains of PVX (Cockerham, 1970; Moreira *et al.*, 1980).

When tobacco plants containing the *N* gene are inoculated with TMV, the rate of viral multiplication is initially as great as in susceptible (*nn*) plants (Taniguchi, 1963). Later the virus accumulation in *N* gene hosts slows or ceases, and necrotic lesions form. It has also been shown that *nn* hosts can restrict TMV at 11°C but fail to at 25°C. This may indicate that the product of the *n* gene is a protein of similar function to *N* protein but is temperature sensitive (Gianinazzi *et al.*, 1977; Gianinazzi & Schneider, 1979). The *N* gene itself loses its ability to restrict TMV at elevated temperatures (Weststeijn, 1981). Otsuki *et al.* (1972) showed TMV replication takes place in protoplasts isolated from tobacco leaves from plants carrying the *N* gene with no necrosis reaction, indicating resistance is at the tissue level. This has recently been supported by the work of Deom *et al.* (1991) which showed that the presence of the *N* gene prevented the transport protein of TMV opening the plasmodesmata, so restricting the cell to cell spread of TMV. The *N* gene has also been linked to the induction of two putative compounds, the anti-viral factor (AVF) (Sela, 1981a) and the inhibitor of viral replication (IVR) (Loebenstein & Gera, 1981).

The *Tm*-1 gene of tomato prevents the formation of mosaic symptoms by strain O-type isolates of TMV (Pelham, 1972). The *Tm*-1 gene also restricts viral

multiplication by 70% in heterozygous plants, and by 95% in plants homozygous for the *Tm*-1 gene (Fraser & Loughlin, 1980). Thus the effect of TMV multiplication is gene dose dependent in contrast to the straight dominance of the effect on symptoms. The *Tm*-1 gene is seen to operate at the cell level restricting TMV multiplication in protoplasts, while *Tm*-2 and *Tm*-2^2 gene products are thought to suppress viral movement between cells (Motoyoshi & Oshima, 1977; Taliansky *et al.*, 1982). The resistance conferred by *Tm*-1 is not involved at the level of uncoating the virus as TMV RNA infection is also restricted (Motoyoshi & Oshima, 1979). The *Tm*-1 resistance decreases with increased temperature with no detectable resistance occurring at 33°C (Fraser & Loughlin, 1982), though symptoms do not develop as resistance to TMV multiplication breaks down. The failure of the resistance gene to function at high growth temperatures is also seen in other cases, for example the *N* gene and *I* gene systems, suggesting the gene products are temperature sensitive. However, the resistance genes *Nx*, *Nb* and *Rx* in potato do not lose function to PVX with increased temperature (Adams *et al.*, 1984, 1986b). Many resistance genes seem to operate at the level of recognition of the viral coat protein. The presence of such dominant plant viral resistance genes are well documented. However, their exact mechanism of action in causing resistance is still unclear (Deom *et al.*, 1991; Cruz *et al.*, 1990).

Investigations into the HR in *N. tabacum* cv. Samsun *NN* (diploid for the *N* gene) to TMV, has shown that the hypersensitive resistance to virus infection does not pre-exist, but is induced by the infection itself. This is also true for fungi, bacteria and nematode infection when they are restricted by various host plants (Konate *et al.*, 1982; Konate & Fritig, 1983, 1984). Hypersensitive resistance appears at approximately the same time as the necrotic symptoms. Its efficiency increases with time after infection, reducing the level of viral replication compared to the level found in a systemically infected host (Fritig *et al.*, 1987). Surrounding the necrotic lesion is a ring of cells, 1.0-1.5 mm wide, containing virus particles which are not undergoing replication. These cells have been shown to be highly resistant to viral infection (Ross, 1961a). In these cells dehydrogenases accumulate and these are thought to

protect the cells from the toxic phenol oxidation products and therefore limit the spread of the necrotic lesion.

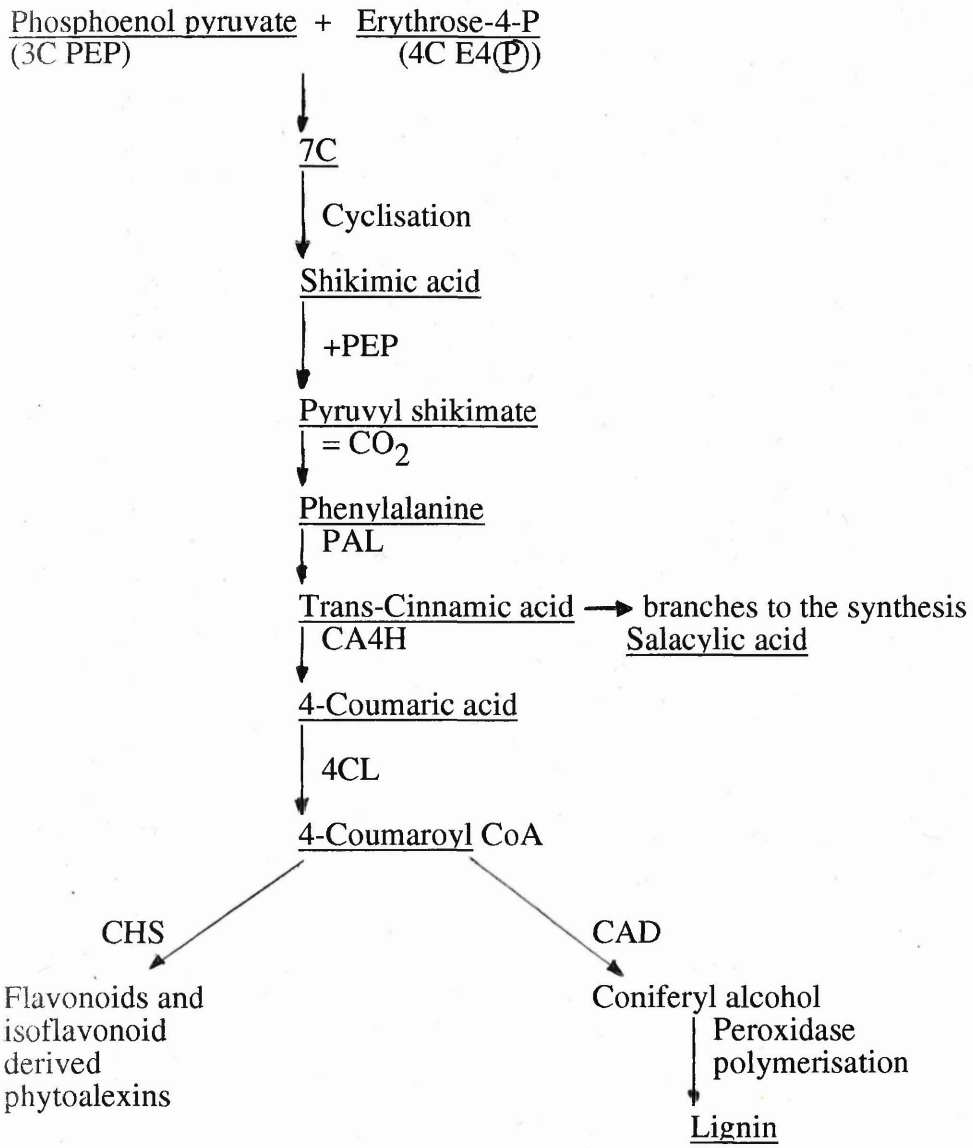
The cell damage, which results from pathogen attack, and later necrosis, is thought to release constitutive elicitors into the surrounding healthy cells, which then respond by synthesising phytoalexins and other defence products (Bailey, 1982). This would explain why different cultivars and physiological races of a pathogen give different plant responses. The biotrophic phase of infection escapes the effects of gross cell injury and so avoids recognition and, therefore, the induction of the HR in the compatible combination (Keen *et al.*, 1981).

It has been suggested that necrosis is the result of the HR, and that the HR itself is a set of earlier reactions which are actually responsible for the expression of resistance. Indeed, bean (*P. vulgaris*) inoculated with *Pseudomonas syringae* pv. *phaseolicola* (Slusarenko & Longland, 1986; Slusarenko *et al.*, 1986) undergoes a HR with necrosis occurring within 21-25h. Early changes in gene expression are seen 2 h after inoculation, PAL mRNA levels are increased 6-9 h after inoculation, peaking at 12 h. Messenger RNA is induced and translated 4 h after *Brassica campestris* has been inoculated with *Xanthomonas campestris* pv. *vitians* and this precedes the visible HR which is not seen until 8 h following inoculation (Collinge & Milligan, 1987). These results clearly show that several changes in gene expression precede both phytoalexin biosynthesis and necrosis in cells inoculated with avirulent pathogens. Contradictory evidence is found, however, with hypersensitive cell death in potatoes infected with *P. infestans*, where the inhibition of protein synthesis after infection does not prevent the HR (Doke & Tomiyama, 1975). But on the other hand, it has been shown that inhibiting PAL results in larger lesions developing, while in plants with high PAL activities the resulting lesions are smaller. Therefore, the HR may indeed be dependent on the early rapid activation of defence genes to restrict pathogen replication and invasion of healthy tissue. The evidence, however, is rather circumstantial and contradictory.

1.5. ETHYLENE.

Ethylene has been shown to decrease the susceptibility of the tissue to infection (Rhodes, 1985). The HR results in an increased localised production of ethylene. De Laat & van Loon (1983) investigated the infection of *N. tabacum* cv. Samsun *NN* with either TMV or tobacco necrosis virus (TNV), and showed the HR was accompanied by a sharp rise in the production and accumulation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), followed by rapid evolution of ethylene. It was shown that both RNA and protein synthesis are required for the virally stimulated production of ACC and ethylene. None of these changes occurred in systemically reacting *N. tabacum* cv. Samsun *nn*. The induction of ethylene production during the HR is restricted to the cells surrounding the necrotic lesion. It is these cells which actively localise the virus. The increase in ethylene production occurs 6 h before the onset of necrosis and so is not determined by necrosis but a much earlier event. Indeed, it has been suggested (van Loon, 1977) that ethylene may induce the HR, as pricking Samsun *NN* with a needle soaked in the ethylene releasing compound 2-chloroethylphosphonic acid (ethephon) resulted in necrotic spots, resembling virus-induced local lesions, developing around the wound. Moreover, treatment with ethephon resulted in the stimulation of plant defence genes, including those for endochitinases, genes involved in lignin biosynthesis, soluble phenolics and pathogenesis related (PR) proteins (van Loon & Antoniw, 1982; Broglie & Gaynor, 1986). These changes act to increase the plant's resistance to the invading pathogen and they are comparable to the changes which occur in Samsun *NN* tobacco reacting hypersensitively to TMV. There may, therefore, be a causal link between ethylene production, symptom expression and viral localisation. Ethylene production may also be involved in the induction of induced systemic resistance (ISR) (Pennazio & Roggero, 1990), as a result of the increased capacity of the tissue throughout the plant to convert ACC to ethylene after initial inoculation with a localising virus. This would result in the more rapid production of ethylene after a second challenge inoculation with TMV or TNV. This rapid local ethylene production may be responsible for limiting lesion enlargement in tobacco leaves exhibiting induced

Fig 1.2. The biochemical pathway involved in lignin and phytoalexin synthesis (Goodwin & Mercer, 1972).



PAL = phenylalanine ammonia-lyase
CA4H = cinnamic acid hydroxylase
4CL = 4-coumarate: CoA ligase
CHS = chalcone synthase
CAD = cinnamyl alcohol dehydrogenase

systemic resistance (De Laat & van Loon, 1983; Pritchard & Ross, 1975). However, when *Glycine max* is inoculated with TNV and treated with inhibitors of ethylene biosynthesis, local lesion formation and TNV localisation is not inhibited. Consequently, one must be careful not to draw general conclusions from specific host-pathogen interactions (Pennazio & Roggero, 1990).

1.6. PHYTOALEXINS.

Phytoalexins were discovered fifty years ago and consist of low molecular weight, antimicrobial compounds, which plants synthesise and which accumulate after exposure to microorganisms (Darvill & Albershein, 1984). Phytoalexins are synthesised by pathways branching off those involved in phenylpropanoid metabolism (Fig. 1.2) and their synthesis in potato is via the acetate-mevalonate pathway (Oban *et al.*, 1985). One enzyme involved in the synthesis of phytoalexins is 3-hydroxy-3-methylglutaryl co-enzyme reductase (HMGR), which catalyses the rate limiting step in isoprenoid biosynthesis, converting 3-hydroxy-3-methylglutaryl co-enzyme A to mevalonate. This is the first committed step in the synthesis of isoprenoid compounds that are used in the biosynthesis of phytoalexins and steroid glycoalkaloids. Inoculation of potato tubers with *Erwinia caratova* sub. sp. *caratovora* (or wounding) induces accumulation of HMGR mRNA, starting within 8 h of inoculation and continuing through 22 h (Yang *et al.*, 1991). Application of mevinolin, a highly specific inhibitor at HMGR, to elicitor-treated potato tuber tissue produces a large decline in lubimin accumulation but does not markedly alter rishitin accumulation. This indicates that HMGR has a role in the complex regulation of sesquiterpenoid phytoalexin accumulation in potato (Stermer & Bostock, 1987).

Phytoalexins have primarily been shown to inhibit fungal and bacterial growth with no reported response to viral infection. However, phytoalexins in many plant pathogen interactions have been studied and are worthy of some note. Phytoalexins have been shown to accumulate in many plant species, e.g. potato (*S. tuberosum*), parsley (*Petroselenium crispum*), French bean (*P. vulgaris*), broad bean

(*Vicia faba*), soya bean (*G. max*), castor bean (*Ricinus communis*) and ground nut (*Arachis hypogaea*) (Coolbear & Threlfull, 1985; Collinge & Slusarenko, 1987; Stermer & Bostock, 1989; Hain *et al.*, 1990). The phytoalexins found in each of these species usually form a small family of biochemically-related compounds. The sesquiterpenoid phytoalexins in potato include rishitin, lubimin, phytuberin, phytuberol, solavetivone and other structurally-related sesquiterpenes (Kuc, 1982).

Phytoalexins are thought to inhibit specific biochemical steps in the pathogen's metabolism, e.g. mitochondrial respiration, and may also interfere non-specifically with membrane function.

Although phytoalexins are synthesised in potato tubers, the role of phytoalexins in potato leaves must be questioned. When leaves were infected with non-compatible fungi, no phytoalexin synthesis was seen even though phenylpropanoid metabolism increased (Rohwer *et al.*, 1987; Fritzemeier *et al.*, 1987).

1.7. MECHANICAL BARRIERS.

Mechanical barriers are employed to physically restrict the spread of a pathogen, to prevent hydrolase activity and to contain the phenolic compounds used to disrupt the membranes and enzymes of the potential pathogen. Synthesis of the compounds forming these barriers is massively induced by pathogen attack (fungi, bacteria and viruses) (Faulkner & Kimmins, 1978). These compounds include polysaccharides such as callose (Faulkner & Kimmins, 1975), cellulose and β-1,3-polyglucan, which are actively synthesised and found in several cellular structures (cell walls, intercellular space and plasmodesmata). In the *Gramineae* incorporation of silicon into mechanical barriers is widespread (Collinge & Slusarenko, 1987).

Callose and β-1,3-glucan have been found surrounding local lesions following some viral infections and may help to prevent spread of the virus (Bell, 1981). Callose synthesis may be mediated by an influx of Ca^{2+} ions into the cell, which directly activates the enzyme β-1,3-glucan synthase rather than by *de novo* gene expression (Köhle *et al.*, 1985).

Aromatic macromolecules such as lignin, suberins (Kimmins & Wuddah, 1977; Faulkner & Kimmins, 1975) and other phenolic polymers, that are produced in large amounts, are incorporated into the host cell wall and restrict the spread of pathogens and their hydrolysing enzymes from the lesions' site. Lignin is a complex polymer, formed by the random condensation of phenylpropanoid units. It is an integral component of secondary cell walls, being resistant to microbial attack. Lignification occurs in many species of plant after pathogen attack, being deposited locally and systemically to the point of attack. An example is the lignification and increased PAL synthesis in potato tubers reacting hypersensitively to a *P. infestans* inoculation (Henderson & Friend, 1979). The increase in lignin and other phenolics is associated with the increased synthesis of mRNA for enzymes involved in the macromolecule synthesis. There are, for example, increases in cinnamyl alcohol dehydrogenase (CAD) activity (the first committed enzyme of lignification) after pathogen attack on *P. vulgaris* (Friend, 1985; Grand *et al.*, 1986). Lignin biosynthesis has also been shown in dwarf bean leaves 24 h to 120 h after TMV, TNV or southern bean mosaic virus (SBMV) inoculation. One complicating feature is that control plants when abraded (wounded) also showed increased cell wall (lignin) synthesis compared to control untreated plants. However, this increase was not as great as that induced by viral inoculation (Kimmins & Wuddah, 1977; Sunderesan & Kimmins, 1981).

Another biochemical change contributing to mechanical barriers is the increased *de novo* synthesis of the hydroxyproline-rich glycoproteins (HRGP) (Kimmins & Brown, 1973; Sunderesan & Kimmins, 1981). HRGPs are linear proteins, rich in basic amino acids, which form a homologous group between different plant species (Esquerré-Tugayé & Lamport, 1979; Chen & Varner, 1985a; Smith *et al.*, 1986; Corbin *et al.*, 1987). HRGPs are induced following pathogen attack (fungi, bacteria and viruses) and wounding (Showalter *et al.*, 1987; Esquerré-Tugayé *et al.*, 1979) and become insoluble soon after their synthesis at the point of infection, being deposited into the cell wall and intercellular space (Benhamou *et al.*, 1990). HRGPs form intermolecular cross-links as a result of peroxidase activity, resulting in a

network that serves as a matrix for the linkage of polysaccharides (pectin and cellulose) or polyphenols (lignin, subarin) (Darvill & Albershein, 1984; Hammerschmidt *et al.*, 1984). Enzymes associated with the biochemical pathway for the biosynthesis of HRGPs (e.g. proline 2-oxoglutarate dioxygenase and arabinosyl transferase) have also been shown to increase following pathogen attack (Bolwell *et al.*, 1985a,b). If HRGPs and associated enzymes are inhibited, it has been shown that the plant becomes more susceptible to pathogen attack (Esquerré-Tugayé *et al.*, 1979). Several HRGP transcripts have been identified in *P. vulgaris* with each transcript being encoded by a separate gene present at single or low copy number in the haploid genome. These transcripts exhibit markedly different patterns of accumulation under different stress conditions (wounding or infection), indicating the operation of several distinct intercellular stress signal systems in higher plants (Corbin *et al.*, 1987). HRGPs possess the ability to agglutinate bacteria and may function by immobilising pathogens in the wall (Melon & Helgeson, 1982). HRGPs have also been shown to accumulate after mechanical wounding in carrot (*Daucus carota*) (Chen & Varner, 1985b) and French bean (*P. vulgaris*) (Kimmins & Brown, 1975). HRGPs have been widely reported in potato (*S. tuberosum*), French bean (*P. vulgaris*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*), tobacco (*N. tabacum*), wheat (*Triticum aestivum*), barley (*Hordeum vulgaris*), rice (*Oryza sativa*) and Soya bean (*G. max*) (Fry, 1986; Lawton & Lamb, 1987). Certain plants contain little if any HRGP, instead their cell walls are rich in glycine. These glycine-rich proteins (GRP) are induced in similar ways to HRGP and are suggested to be a cell wall component (Condit & Meagher, 1987; Bowles, 1990).

1.8. PHENYLPROPANOID METABOLISM.

Following pathogen attack many of the enzymes of the phenylpropanoid pathway rapidly increase in activity. Resistance appears to be correlated to the site of these changes (Bell *et al.*, 1986; Rumeau *et al.*, 1990). The increases are first observed within 20 minutes and peak 3-4 h after pathogen attack, as a result of increased transcription of their genes. The phenylpropanoid pathway produces the precursors

required for the synthesis of such compounds as lignin (Fig. 1.2). The main enzymes that have been studied in this system include (i) phenylalanine ammonia-lyase (PAL) (Rumeau *et al.*, 1990) which is the first enzyme of the phenylpropanoid biosynthesis pathway, and (ii) chalcone synthase (CHS), which is the first enzyme of the branch pathway specifically involved in the synthesis of flavenoids and isoflavenoid derived phytoalexins.

Other enzymes induced include cinnamic acid hydroxylase (CA4H), 4-coumarate:CoA ligase (4CL), ortho-diphenol-o-methyltransferase, chalcone isomerase (CHI), peroxidases and alcohol dehydrogenase (Legrand *et al.*, 1976; Borchert, 1978; Fritzemeier *et al.*, 1987; Bol & van Kan, 1988; Cuypers *et al.*, 1988; Matton *et al.*, 1990).

The site of increased activity of these enzymes is localised in the layer of cells surrounding the viral necrotic lesion, e.g. as seen with tobacco reacting hypersensitively to TMV (Fritig *et al.*, 1972). Similarly, *in situ* hybridisation has shown the rapid accumulation of PAL mRNA in a halo of apparently healthy cells around penetration sites of an incompatible race of *P. infestans* on young potato leaf tissue (Cuypers *et al.*, 1988). The increase in enzyme activity, however, also spreads rapidly into surrounding cells in advance of the necrosis. The zone of increased enzymatic activity starts at the point which will become the centre of the lesion and then spreads out. This forms an area which corresponds exactly to the zone of high resistance to virus multiplication (Massala *et al.*, 1987).

Reduction of lignin synthesis correlates well with the weakening of resistance to viral infection, suggesting that the phenylpropanoid pathway and lignin synthesis participate in the HR (Sela, 1981b).

The transcriptional activation of PAL, CHS and related enzymes and HRGP biosynthesis occur not only in directly infected tissue reacting hypersensitively, but also in distant hitherto uninfected tissue (Lawton & Lamb, 1987). These distant reactions may be associated with induced systemic resistance, establishing the early stages of an incompatible interaction, so increasing the plant's ability to limit lesion size and the number of secondary infections. This reaction at a site distant from the

lesion implies the intercellular transmission of some endogenous signal factor from the site of infection to the uninfected area. The signal may be a host gene product (Bell *et al.*, 1986).

Increases in the level of phenylpropanoid compounds may also be induced by wounding though often to a lower level than that seen following pathogen attack (Borchert, 1978; Bell *et al.*, 1986; Yang *et al.*, 1989; Rumeau *et al.*, 1990), indicating that the response produced by wounding and pathogen attack are very similar. It may be that resistance to pathogen attack and tissue repair after wounding share many common features. The phenylpropanoid enzymes are produced in relatively small amounts, 20-50μg per kg fresh weight of infected leaves, but exhibit very high biological activity. It must also be remembered that it is the rapid stimulation of phenylpropanoid metabolism, as it is with most other defence responses, which confers resistance. Several compatible reactions between host and pathogen occur where the defence responses reach the same levels as that seen in the incompatible reaction, but it is thought that a slow response of defence products to pathogen invasion results in susceptibility.

1.9. HYDROLYTIC ENZYMES.

1.9.1. Chitinases.

Endochitinase activity increases in plants after inoculation with fungal, bacterial and viral plant pathogens (Pegg & Young, 1982; Collinge & Slusarenko, 1987; Meins & Ahl, 1989) and after treatment with ethylene or other elicitors (Mauch *et al.*, 1984; Roby *et al.*, 1986). In melon seedlings (*C. melo*) treated with an elicitor from *Colletotrichum lagenarium*, inhibition of ethylene biosynthesis also inhibits chitinase induction (Roby *et al.*, 1986). However, in pea (*Pisum sativum*) inoculated with *Fusarium solani* pv. *phaseoli* ethylene biosynthesis is suppressed without inhibiting the induction of chitinase (Mauch *et al.*, 1984). Thus the link between ethylene production and chitinase induction is not clear. In French bean (*P. vulgaris*) a multigene family encodes chitinases, though only two of the genes are induced by ethylene (Broglie & Gaynor, 1986). Chitin does not occur commonly in plants but is

a common constituent of many fungal cell walls and insect exoskeletons. Chitinases are known to be potent inhibitors of bacterial and fungal growth. Chitinase has also been shown to be able to degrade bacterial cell walls (Schlumbaum *et al.*, 1986; Bol & van Kan, 1988).

In the interaction between French bean and *P. syringae* pv. *phaseolicola*, chitinase mRNA was detected 6 h after inoculation with an avirulent form. In contrast, activity was only detected 24 h after inoculation with a virulent isolate. Again, it seems to be the length of time required for induction of the enzyme that is critical to the expression of resistance.

1.9.2. β-1,3-glucanases.

Increases in β-1,3-glucanase activity have been shown in French beans exposed to ethylene, and in parsley induced by pathogenic fungal elicitors (Abeles *et al.*, 1970; Kombrink & Hahlbrock, 1986; Nichols *et al.*, 1980), and pea induced by *F. solani* pv. *phaseoli*. The activation of this polysaccharide-degrading enzyme is thought to release elicitor-active carbohydrate fractions from β-1,3-glucans (a major component of fungal cell walls) in the fungal cell walls which trigger an incompatible defence response in the host plant (Keen & Yoshikawa, 1983; Keen *et al.*, 1983). In *in vitro* experiments, the combined effects of chitinases and β-1,3-glucanases have been shown to inhibit fungal growth more effectively than when the enzymes are acting separately (Bol & van Kan, 1988).

1.10. DEFENCE PROTEINS.

Proteases, protease inhibitors, polygalacturonase inhibibors and pathogenesis related (PR) proteins enable the plant to limit the action of enzymes that play a key role in the virulence of various pathogens. PR proteins have been shown to accumulate in many host pathogen interactions and some have been assigned functions (section 1.12).

1.10.1. Protease inhibitors.

Polypeptide inhibitors of proteases are widely distributed in all plant tissues. They are thought to have a role in defence against herbivores since they tend to be active against animal but not endogenous proteases (Ryan, 1978, 1988). When leaves

of potato and tomato are wounded by chewing insects, or when other severe mechanical damage occurs, two inhibitors of serine proteases, Inhibitor I (8.1 KDa) and Inhibitor II (12.3 KDa), are rapidly synthesised (Ryan 1978, 1988). The systemic response is induced by the release and transport of a putative "wound hormone", an oligosaccharide released from the site of damage (Cleveland *et al.*, 1987; Ryan, 1972), which induces mRNA synthesis (Keil *et al.*, 1990). The sequences necessary for wound inducibility are present within the first ~1000 bp upstream of the transcriptional start of the Inhibitor II gene in potato (Thornburg *et al.*, 1987).

In tomato, the protease inhibitors account for up to 10% of the soluble leaf protein 48 h after a severe wounding. These inhibitors from potato and tomato show considerable (up to 84%) amino acid sequence homology (Sanchez-Serrano *et al.*, 1986; Collinge & Slusarenko, 1987; Thornburg *et al.*, 1987). mRNAs from other wound-inducible genes characterised in potato, such as *wun* I and *wun* II (Logemann *et al.*, 1988) and *win* I and *win* II (possible chitin binding protein) (Stanford *et al.*, 1989), also first appear 30 minutes after wounding and their accumulation is maximal after 10 h to 20 h.

1.11. POSSIBLE MECHANISMS OF RESISTANCE TO VIRUS INFECTION.

There are two proteins which have received detailed investigation with regard to their potential anti-viral properties. These are the anti-viral factor (AVF) and the inhibitor(s) of virus replication (IVR). They are induced under different circumstances and were first thought to be quite different. More recent work, however, suggests that these two proteins may be the same or at least closely related. They are the same size (26 KDa) and cross-react serologically. The work on these proteins has been restricted to two research groups (see below) and requires further characterisation and investigation before it can be widely accepted.

AVF has been shown to be structurally homologous to human β-interferon (Sela *et al.*, 1987), both are glycoproteins and inhibit virus replication when added to TMV infected tobacco protoplasts (Mozes, 1980). The mode of action of β-interferon and AVF is similar. They induce an adenosine triphosphate (ATP) polymerising

enzyme (APE). This enzyme, 2',5'-linked oligoadenylate (2-5A) synthase, has been characterised in humans (Reichmann *et al.*, 1983; Devash *et al.*, 1984, 1985). The APE enzyme produces oligoadenylates with anti-viral activity. The plant and animal enzymes are again very similar, they cross react serologically, are of the same size and are each composed of 4 subunits.

Using both human β-interferon and 2-5A synthase gene probes, to probe Southern blots of tobacco genomic DNA, homologous genes were found to be present in tobacco. Moreover their expression is induced rapidly following infection with TMV in plants possessing the *N* gene. The presence of AVF has been reported in many *Nicotiana* species infected with TMV, irrespective of whether the infection results in local or systemic infection (Sela, 1981a; Sela *et al.*, 1987). AVF has also been reported to accumulate in several other plant species responding to viral infection (Sela & Applebaum, 1962; Nagaich & Singh, 1970; Nienhaus & Babovic, 1978). It is proposed that the AVF response is common to several plant species infected by viruses. AVF was found at the greatest concentration around the site of the viral local lesion, but it was also found at lower levels systemically throughout the plant after infection (Sela *et al.*, 1987). In *Nicotiana* spp., where the *N* gene is required to be present for the rapid expression of AVF, an AVF message was reported to appear 6 h after inoculation with the virus, to peak at 24 h and to decline by 48 h. At 24 h after inoculation AVF mRNA accounted for 1.5%-2.5% of the total mRNA, and accounted for 50% of new mRNA stimulated by TMV infection. The mRNA encoding the ATP polymerising enzyme was also reported to be induced after inoculation, accounting for 0.03% of the total mRNA population 24 h after TMV inoculation (Sela *et al.*, 1987).

In tobacco species not possessing the *N* gene TMV or alfalfa mosaic virus (AlMV) infection also induced AVF synthesis, but this occurred much later (4-7 days) after inoculation and only reached a low level. AVF mRNA was also reported in healthy plants but at even lower levels. Therefore, although plants appear to have the capacity to synthesise AVF and APE, the *N* gene is required to induce a rapid turn on of these genes. The regulatory role of the *N* gene on the synthesis of AVF has been

confirmed in studies on cultivar Samsun infected with TMV. The cultivar Samsun *NN*, which contains the *N* gene, gave a rapid synthesis of AVF after TMV infection. The cultivar Samsun *nn*, which is genetically related to the above cultivar except for the fact it lacks the *N* gene, does not show a rapid rise in AVF synthesis following TMV infection, and AVF accumulates only slowly. The AVF gene must, therefore, also be weakly induced by mechanisms other than the *N* gene in tobacco. In the systemically infected plants green islands of cells are seen in leaves showing mosaic symptoms. These green islands are free from virus infection and contain AVF. AVF is thought to consist of a family of closely related proteins some of which are phosphorylated (Mozes *et al.*, 1978; Sela, 1986; Edelbaum *et al.*, 1990). Sela *et al.* (1987) proposed that AVF is highly active, only one molecule per cell being required to inhibit virus replication.

The homology between AVF and human interferon has inspired an investigation of the anti-viral properties of human interferon in plant-virus interactions. Orchansky *et al.* (1982) reported a 50% reduction in TMV coat protein accumulation in leaf disks of *N. tabacum* cv. Samsun *nn* which were treated for 7 h with human interferon, starting 1 h after inoculation with TMV (compared to control plants). These results were, however, questioned by Huisman *et al.* (1985) who concluded, using enzyme-linked immunosorbent assay (ELISA) and Northern blot analysis, that human interferon does not inhibit the infection of cowpea (*Vigna unguiculata*) protoplasts by AlMV nor the replication of the AlMV.

In conclusion, it would seem that if AVF or human interferon can protect plant cells from virus infection, this phenomenon is not a general one, although AVF may play a role in determining resistance of *Nicotiana* spp. to TMV (Orchansky *et al.*, 1982; Huisman *et al.*, 1985).

A second anti-viral protein, the inhibitor of virus replication (IVR), has been reported by Loebenstein and Gera (1981). The monomer molecular weight of IVR has been reported to be 23-26 KDa, depending on the preparation. It may also be found as a dimer of 57 KDa (Gera *et al.*, 1989). IVR has reportedly anti-viral properties, inhibiting virus replication of CMV, TMV and PVX in different hosts

(Gera & Loebenstein, 1983). IVR appears not to be host or virus specific. IVR has been isolated from protoplasts, leaf discs and whole plants of *N. tabacum* cv. Samsun *NN* inoculated with TMV. IVR has not been detected in healthy Samsun *NN* or induced in Samsun *nn* following inoculation with TMV. A very similar protein capable of inhibiting virus replication, similar in size and serologically related to IVR, has been reportedly isolated from green-islands in *N. tabacum* cv. Xanthi systemically infected with CMV (Gera & Loebenstein, 1988). IVR reportedly inhibits virus replication when applied to Samsun *NN* or *nn* protoplasts infected with TMV 18 h after inoculation, reducing viral replication by 60-90% (Loebenstein & Gera, 1981).

IVR, like some PR proteins, is transported into the intercellular space where its role is not clear. It could play a part in induced systemic resistance in plants against later virus infection. An inoculated leaf reacting hypersensitively and producing IVR is highly resistant to a second infection (Spiegel *et al.*, 1989). However, unlike PR proteins, only small amounts of IVR are produced (Gera *et al.*, 1990). IVR appears to be constitutively synthesised in the hybrid *N. glutinosa* X *N. debneyi*. This hybrid is highly resistant to TMV infection and TMV inoculation only causes a small further increase in IVR. IVR was extracted from healthy plants of this hybrid and shown to inhibit TMV replication in Samsun *nn* protoplasts or leaf discs inoculated with TMV (Loebenstein *et al.*, 1990). This supports the view that IVR is involved in virus resistance.

1.12. PATHOGENESIS RELATED PROTEINS.

Pathogenesis related (PR) proteins were discovered independently by Gianinazzi *et al.* (1970) and van Loon & van Kammen (1970) in *N. tabacum* cv. Samsun *NN* reacting hypersensitively to TMV. They are a group of proteins which are rapidly synthesised following pathogen attack and accumulate at high levels (Hooft van Huijsduijnen *et al.*, 1988; Takiri-Alaoui *et al.*, 1990). These proteins are induced by a wide range of pathogens, including viruses, e.g. TMV and PVX; viroids, e.g. potato spindle tuber viroid; bacteria, e.g. *P. syringae*; and fungi, e.g.

Phytophthora parasitica and *Thielaviopsis basicola* (Gianinazzi, 1984), in many species including both monocotyledons and dicotyledons (van Loon, 1985; Hooft van Huijsduijnen *et al.*, 1986a, b).

In addition to pathogen attack, treatment with various chemicals has also been shown to induce PR proteins. These chemical inducers include ethephon, salicylic acid, acetylsalicylic acid, benzoic acid, polyanions such as polyacrylic acid, the anti-viral chemical 2-thiouracil, barium and manganese salts and mercuric chloride (White, 1979; Dumas *et al.*, 1987; Bol, 1988; Nasser *et al.*, 1990). This indicates that PR protein induction is not induced by necrosis alone (Parent & Asselin, 1983). All these chemicals (with the exception of salicylic acid) trigger the production of ethylene. Van Loon (1987) observed that ethylene induced a sub-set of PR proteins in tobacco, while tissue damage, as already mentioned (section 1.8), increased PAL levels. Both these responses are induced by viral necrosis. PR proteins may be induced in some plants by plasmolysis (Wagih & Coutts, 1981; Wagih *et al.*, 1983; Singh *et al.*, 1987), the stress of cutting and mechanical injury, and other various environmental and physiological stresses (Fraser, 1981, 1982a; Otsuki & Matsuoka, 1985; Asselin *et al.*, 1985).

The genes of several PR proteins have been cloned and sequenced (Bol, 1988; Matton *et al.*, 1990; Okshima *et al.*, 1990). The sequence upstream of the transcription start point of the genes of PR proteins in tobacco reveals limited homologies in the first 100 base pairs. These homologies may indicate *cis*-acting elements involved in the induction of PR proteins and perhaps other proteins following necrotising pathogen attack (Bol, 1988; Okshima *et al.*, 1989; van Kan *et al.*, 1989; Vanderheen *et al.*, 1990).

At least twenty plant species have been shown to synthesise PR proteins after necrotic pathogen attack. These include *Nicotiana* spp. (*tabacum, glutinosa, rustica, sylvestris, tomentosiformis* and *debneyi*), *S. tuberosum, L. esculentum, C. sativus, Petunia* sp., *V. unguiculata, P. sativum, P. vulgaris, P. crispum, Zea Mays, H. vulgaris, Capsicum annuum, Citrus medica, Apium graveolans, S. demissum, G. globosa, Gynura aurantiaca* and *Chenopodium amaranticolor* (Pennazio *et al.*, 1983;

van Loon *et al.*, 1983; Hogue & Asselin, 1987; Tobins *et al.*, 1989; Redolfi, 1983; White *et al.*, 1987). Corresponding PR proteins from different species have been shown to be serologically related. They clearly form a group of highly conserved proteins.

PR proteins were first isolated on native polyacrylamide gel systems which resulted in the basic isoforms of the proteins being overlooked. PR proteins within plants have been categorised into groups consisting of both basic and acid isoforms. So far, they have all been found to be encoded by multigene families (Payne *et al.*, 1988). Each gene in the family may be expressed at differing levels and the enzymatic properties of the products of closely related genes may have different specific activities. Thus there may be a mixture of enzymes acting on the same substrate, but with different environmental optima and activities (pH, ionic strength, divalent cations, etc.). This is not surprising as many of the acidic PR proteins are exported into the intercellular space, while the basic isoforms tend to collect in the vacuole (Kauffman *et al.*, 1987; Legrand *et al.*, 1987). This flexibility may be important, allowing the plant to respond to challenges in widely varying environmental conditions (Hogue & Asselin, 1987). Common characteristics of PR proteins include (i) low molecular weight (10-20 KDa), (ii) resistance to protelytic digestion, (iii) induction during the hypersensitive response or by specific chemical inducers, eg. salicylic acid, (iv) accumulation in large amounts, (v) synthesis as a precursor with an N-terminal transport sequence enabling transport across the apoplast or vacuole membrane; the transport sequence is then removed leaving the mature protein (Bol & van Kan, 1988; van Loon & Gerritson, 1989).

Many PR proteins induced in Samsun *NN* tobacco following infection by TMV have been identified and characterised (Table 1.1). Several PR proteins have also been isolated in potato (Table 1.2) and other species. Many of the PR proteins have been identified as enzymes with polysaccharide hydrolysing activity. But it is not clear how this would affect virus multiplication. It is possible that the hypersensitive reaction induced by any localised pathogen is a rather general response to attack, which may activate several, separate resistance mechanisms. In this strategy

TABLE 1.1. The major PR proteins induced in Samsun *NN* tobacco by TMV infection.

	Acid PR proteins			Basic PR proteins		
Group	Name	MW KDa	Est. no. genes	MW KDa	Est. no. genes	Function
1	1a 1b 1c (90% homology)	15.8,15.5,15.6	8	19 (67% homology) to PR1a	8	unknown
2	2, N, O	39.7,40.0,40.6	?	33.0	?	β-1,3,glucanase
3	P, Q (65% homology)	27.8, 29.5	4	32.0,34.0	4	chitinase
4	R	13.0,15.0	2	-	-	unknown
5	S	23.0	4	-	-	resemble maize α-amylase/protease inhibitor homology to Thaumatin

The α-amylase/protease inhibitor is reminiscent of the protease inhibitors induced in many plant species after wounding or fungal infection. No PR proteins are so far glycoproteins. Another four unrelated PR proteins have been isolated from Samsun *NN* and it is thought that with improved techniques more minor PR proteins will be isolated (Kauffmann *et al.*, 1990; Bol & van Kan, 1988; van Loon *et al.*, 1987; Payne *et al.*, 1990).

TABLE 1.2. Pathogenesis related proteins induced in potato by different treatments.

Number	Some 16 or so have been identified
MW KDa	Range 14-40 KDa
Homology	Some show homology to tobacco, pea, parsley PR proteins
Induced rapidly by	PVX, TMV, salacylic acid, ethylene, cyst nematode, *P. infestans*. Some accumulate in wounded or healthy plants
Identified function	Three 1-3-β-glucanases, seven chitinases both basic and acid isoforms. One shows homology with α-amylase/protease inhibitor
Genes	Several seem to be encoded in multi-gene families

White *et al.*, 1987; Parent & Asselin, 1987; Marineau *et al.*, 1987; Logemann *et al.*, 1988; Gaynor, 1988; Kombrink *et al.*, 1988; Hammond-Kosack *et al.*, 1989; Matton & Brisson, 1989; Matton *et al.*, 1990; Pierpoint *et al.*, 1990.

the hope would be that one of these resistance mechanisms would be specific to the attacking pathogen or a later pathogen attack (Tables 1.1 and 1.2).

1.12.1. The role of PR proteins in virus restriction.

Salicylic acid, which is thought to mimic or be the intermediate in the induction process, induces a sub-set of PR proteins (PR-1, 2, N, O, P and Q) when sprayed onto leaves of *N. tabacum* cv. Samsun *NN* leaves (Hooft van Huijsduijnen *et al.*, 1986a). Viral RNA synthesis was inhibited by 90% in Samsun *NN* plants induced with salicylic acid when inoculated with AlMV. No necrotic reaction was observed. When Samsun *NN* protoplasts were treated with salicylic acid and then infected with AlMV viral RNA synthesis was inhibited by 99% compared to control untreated protoplasts. This result indicates that salicylic acid acts at the cellular level and does not require a hypersensitive necrosis response. These are some results which suggest that PR-1 may have an anti-viral effect (Hooft van Huijsduijnen *et al.*, 1986a). However, tobacco plants which constitutively expressed PR-1 (and PR-S), introduced as a transgene, showed no more resistance to TMV or AlMV multiplication than did control plants (Linthorst *et al.*, 1989). There may therefore be other unidentified salicylic acid-inducable proteins that have anti-viral properties (Hooft van Huijsduijnen *et al.*, 1986a). This possibility may be supported by the observation that PR-1 is transported out of the cell (which might not be the expected location of an anti-viral protein, although IVR is similarly located).

Salicylic acid treatment has been shown to induce PR proteins in bean and cowpea. Salicylic acid treatment makes the plant more resistant to AlMV, reducing local lesion number by 75% in AlMV-infected bean plants and viral replication by 99% in cowpea protoplasts (Hooft van Huijsduijnen *et al.*, 1986a). It would therefore appear that salicylic acid induces a protein which has specific anti-viral properties. The failure of transgenic plants, expressing PR-1, to show reduced viral replication may be due to incorrect expression of the PR-1 gene, or that resistance might be conferred by a number of PR proteins acting together. Polyacrylic acid has also been shown to induce PR proteins and to induce resistance in tobacco to AlMV and TMV (Bol, 1988).

Some tobacco cultivars do not respond to the chemicals which induce PR proteins in other cultivars and interestingly these do not become resistant to viral attack. This again supports the role of PR proteins in the resistance mechanism (Dumas *et al.*, 1987). In Xanthi nc the level of TMV replication is reduced to 4% of that seen in systemically infected Xanthi, viral replication being inhibited in the ring of cells surrounding the necrotic lesion (Bol, 1988). In these cells the level of PR proteins is elevated. It is possible, therefore, that it is not just the necrotic reaction *per sé* which restricts the viral spread, but rather the resistance induced in the surrounding healthy tissue by the PR proteins.

1.12.2. PR proteins and their role in induced systemic resistance.

In hypersensitively reacting hosts, locally infected leaves and non-infected leaves show increased resistance to further infection (Ross, 1961, 1966) (see section 1.13). In many species PR proteins are also synthesised at lower levels systemically at sites distant from the initial site of attack. An elevated level of PR proteins in tobacco, distant from the site of infection, has been detected from 8 days after infection (Pierpoint *et al.*, 1981). PR protein levels also seem to increase much more quickly after a second infection. Thus PR proteins may be involved in the induced systemic resistance response (Ye *et al.*, 1989). That PR proteins are synthesised in leaves distant from the inoculation site indicates that a signal must be transported through the plant and that this must induce the transcription of the PR protein genes in those leaves. However, compared to localised infection, in systemically infected plants PR proteins only accumulate at relatively low levels (Hooft van Huijsduijnen *et al.*, 1985).

1.12.3. Conflicting evidence for an anti-viral role of PR proteins in necrotic viral infection.

In tobacco, infected with PVY (Roggero & Pennazio, 1988), infection resulted in necrosis which then became systemic. Large amounts of PR proteins were synthesised, but no restriction of virus spread appears to have occurred. Obviously, the mechanisms of viral resistance appear to involve more than just the production of

PR proteins. In *Nicotiana rustica*, TMV is restricted to local lesions at 15°C. However, at 18-20°C the virus can spread systemically to form necrotic lesions elsewhere in the plant. Dumas and Gianinazzi (1986) showed that at both temperatures induced local and systemic resistance and PR protein production was clearly seen. This again emphasises that more than just the PR proteins are required to localise virus to the hypersensitive lesion. In some cases the expression of resistance to viral attack precedes the synthesis of the PR proteins (Kassanis *et al.*, 1974) and the correlation in level of induced resistance and concentrations of PR proteins is poor (Fraser, 1982a; Pennazio *et al.*, 1983).

PR proteins have been shown to accumulate in tobacco during flowering or senescence (Fraser, 1981). Removal of the inflorescence results in larger TMV lesions than in plants where the inflorescences remain. This effect is not seen if senescing leaves are removed (Fraser & Clay, 1983; Sherwood, 1985). Again the correlation between the presence of PR proteins and the expression of viral resistance is poor (Bol, 1988). However, in the majority of cases, the synthesis and accumulation of PR proteins is closely associated with pathogen localisation, PR proteins being produced in the right circumstances (i.e. in localised but not systemic infection) at the right time and in the right place (Bol, 1988).

1.12.4. The function of PR proteins.

Functions have been assigned to a number of PR proteins of tobacco and potato (Tables 1.1 and 1.2). By analogy corresponding PR proteins from other species have only been assigned putative functions.

Glucanases and/or chitinases have been identified among the PR proteins of cucumber (Métraux & Boller, 1986; Métraux *et al.*, 1989), tomato (Christ & Mösinger, 1989), maize (Nasser *et al.*, 1990), French bean, soybean, pea and barley (Bol *et al.*, 1990) on the basis of derived amino acid sequence homologies. This supports the view that PR proteins are largely involved in resistance to fungi, bacteria, the larval stage of nematodes and possibly insects (Boller, 1987). It is not clear how such enzymes could have a direct function in dealing with viruses (Boller, 1987).

A protein with homology to the maize bifunctional α-amylase/protease is found in potatoes. Although there is no direct evidence for this, the enzyme is thought to be involved in defence against insects, acting as an anti-feedant agent (Richardson *et al.*, 1987; Bryngelsson & Green, 1989; Pierpoint *et al.*, 1990).

The hydrolase activity of several other PR proteins may release oligosaccharides from (damaged) cell walls. These may then act as long distance signals, activating "defence" genes in the non-infected leaves (Bol & van Kan, 1988).

In conclusion, the evidence suggests that upon induction of the necrotic hypersensitive response by a pathogen, and to a lesser level by wounding, a number of genes encoding PR proteins are rapidly transcribed and their products are involved in restricting spread of the pathogen. This response also protects the plant from a subsequent pathogen attack. The genes involved are not specific to the particular pathogen attacking the plant. However, specific subsets of PR proteins may be targeted against a limited range of pathogen types. So far, PR proteins have been shown to be most effective in restricting fungal, bacterial and insect pathogens. As yet, no PR protein has been assigned definitive anti-viral properties.

Though a potential anti-viral protein of 12 KDa (mRNA 650 nt) has been found to be strongly induced in tobacco by TMV or salicylic acid, this protein, cluster C, is not classified as a PR protein since it does not have a transit peptide and is not found outside the cell (Hooft van Huijsduijnen *et al.*, 1986b). Its high glycine content suggests that it may be a component of the cell wall where its position might enable it to inhibit virus spread.

Possibly, improved techniques to isolate minor PR proteins and basic PR proteins might lead to the identification of anti-viral PR proteins, that might have been missed against a background of more abundant PR proteins of a similar molecular weight. Alternatively, PR proteins may not have any direct anti-viral activity at all, indeed they are exported from the cell whereas anti-viral protein would be most likely found within the cell.

1.13. INDUCED SYSTEMIC RESISTANCE.

Induced systemic resistance (ISR) was first demonstrated by Chester (1933). More recently ISR has been demonstrated in a variety of plant species, e.g. *Nicotiana* spp., *Cucurbitaceae* spp., cowpea and French bean. In these examples the plant was first inoculated with a pathogen capable of inducing a necrotic hypersensitive response. Viruses (Ross & Bozarth, 1960; Ross, 1961), bacteria and fungi (Hecht & Bateman, 1964; Bergstrom *et al.*, 1982; Rao *et al.*, 1987) have all been used as the initial stimulus. After a lag period of some 3-7 days (depending on the particular host-pathogen interaction) resistance developed throughout the plant, resulting in increased resistance (compared to control plants) to a second inoculation by the same or different pathogen (Ross & Bozarth, 1960; Ross, 1961, 1966; McIntyre *et al.*, 1981; Bergstrom *et al.*, 1982).

Examples of ISR are wide and varied, covering many different localised and systemic pathogens. However, only pathogens which are capable of inducing the hypersensitive response can induce ISR. Examples include TMV, but only in *Nicotiana spp.* containing the *N* gene, inducing greater levels of resistance to later infection with TMV, *Phytophthora parasitica* pv. *nicotianae*, *Peronospora tabacina*, *Pseudomonas tabaci* and the aphid *Myzus persicae* (McIntyre *et al.*, 1982). TNV inoculation also induced resistance to subsequent infection by TMV, TNV and *P. parasitica* in tobacco (Pennazio *et al.*, 1983). In cucumber, *C. lagenarium*, *Pseudomonas lachrymans* and TNV induced increased levels of resistance to CMV, a systemically infecting virus. This increased resistance was manifested as a reduced number of primary chlorotic lesions and a slower rate of systemic infection when compared to control plants (Bergstrom *et al.*, 1982). However, these results, showing increased resistance to the invasion of a systemic virus, have been questioned by Pennazio & Roggero (1988). This study followed the induction of resistance in *N. tabacum* cv. White Burley with TNV or tomato mosaic virus (ToMV) and the elicitation of a necrotic response causing local lesions. When subsequently challenged with PVY, TMV or TRV systemic infection resulted (determined by viral antigen content in leaves) and there was no reduction in infection compared to control plants,

TABLE 1.3. The biological spectrum of induced systemic resistance in cucumber.

	Pathogen	Disease
Fungi	*Colletotrichum lagenarium*	Anthracnose, local lesions
	Cladosporium cucumerinum	Scab, local lesions
	Mycosphaerella melonis	Gummy stem blight, unrestricted lesions
	Fusarium oxysporum f.sp. *cucumerinum*	*Fusarium* wilt
	Verticillum albo-atrum	*Verticillium* wilt
	Fusarium solani f.sp. *cucurbitae*	*Fusarium* root rot
	Sphaerotheca fuliginea	Powdery mildew
	Pseudoperonospora cubensis	Downy mildew
	Phytophthora infestans	Late blight of potato, local necrosis
Bacteria	*Pseudomonas lachrymans*	Angular leaf spot, local lesions
	Erwinia tracheiphila	Bacterial wilt
Viruses	Tobacco necrosis virus	Local lesions
	Cucumber mosaic virus	Local lesions and systemic mosaic

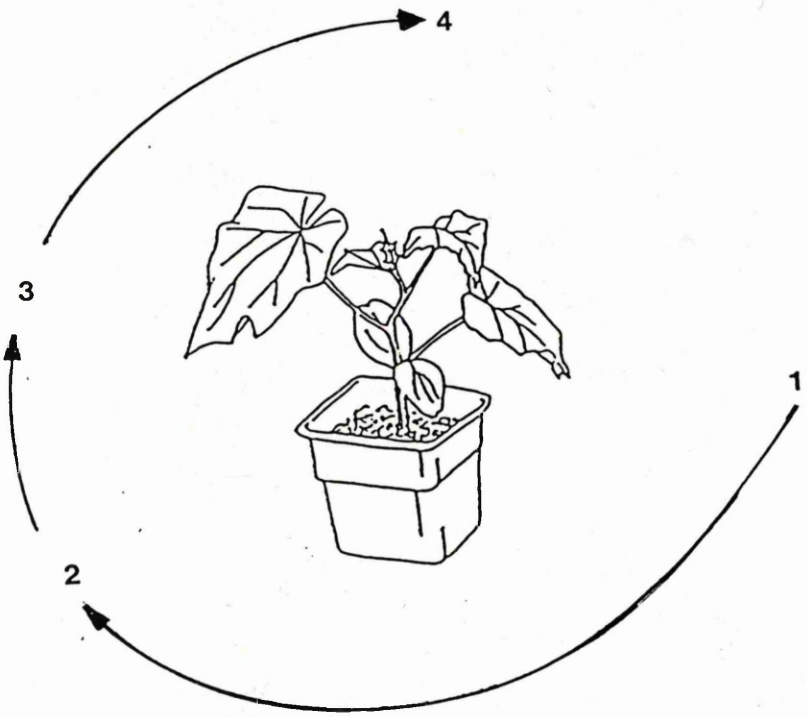
Non-specific resistance in cucumber, water melon and musk melon, has been induced by prior infection with *C. lagenarium* or TNV which is effective against later challenge with at least 13 pathogens listed above (Modified from Dean & Kuc, 1985).

even though similar plants challenged by TNV and ToMV, which are localised, showed reduced lesion size compared to control plants. In cucumber, water melon and musk melon, prior infection with *C. lagenarium* or TNV induced a degree of resistance to a challenge with a broad range of pathogens when compared to control plants (Table 1.3).

The level of induced resistance is not always equal throughout the plant. The leaves which are non-expanded at the time of the first inoculation are often, but not always, protected to a higher level than expanded leaves (Xuei & Kuc, 1984). ISR is manifested as a reduction in number and/or size of the lesions and in the case of fungal pathogens, the extent of sporulation. The diseased area can be reduced by up to 90%, though some host-pathogen interactions induce a much lower level of resistance. For example, TMV lesions in upper leaves of *N. glutinosa* were only reduced by 16% in size in ISR plants compared to control plants (Fraser *et al.*, 1979) but in contrast, in *N. tabacum* cv. Samsun *NN* the TMV lesions were reduced in size by 80% as a result of ISR (Ross, 1966). In both cases ISR was induced by initial inoculation with TMV. Strangely, however, the reduction in lesion size and number correlated weakly with viral content in the leaves (Balazs *et al.*, 1977).

ISR has only been reported as a result of pathogen attack and not in control plants mock-inoculated with carborundum and water (Ross, 1961; Bozarth, 1962; van Loon & Antoniw, 1982) or as a result of necrosis induced by chemicals or dry ice (Ross *et al.*, 1961; Kuc *et al.*, 1975), though carborundum damage has been shown to cause local resistance (Yarwood, 1960). Resistance seems to be associated with lesion development, as with TMV induced resistance against TMV or *P. tabacina* in tobacco. In this case necrotic lesions developed on the second day and resistance was first measurable on the third day. Resistance was maximal at 12 days after infection and persisted for a minimum of 26 days (Ye *et al.*, 1989). In this study, the sizes of lesion caused by *P. tabacina* and TMV were reduced by >80% and >70% respectively. ISR has also been shown to be maintained throughout the plant's growing season in some curcubit species (Dean & Kuc, 1985).

Fig. 1.3. A model for induced systemic resistance in cucumber (modified from Kuc, 1987). 1. Primary treatment (Induction; usually infection of first true leaf) —> metabolic stress and host cell death —> 'Signal' production —> 'Signal' exported from inducer leaf. 2. Systemic 'Signal'movement throughout the plant. 3. 'Signal' processed (mediated by 'Signal' receptors) in systemic tissues —> priming of defence mechanisms (Sensitization). 4. Inoculation of systemic leaves (Challenge; bioassay for protection) —> activation of defence mechanisms.



Obviously, for ISR to occur the initial infection by a pathogen must induce a signal, which results in the protective effect throughout the plant. This signal is not thought to be a PR protein. On the basis of girdling, grafting and tissue culture experiments, the putative signal is believed to be transported in the phloem (Geades *et al.*, 1980; Jenns & Kuc, 1979; Tuzun & Kuc, 1985, 1987; Dean & Kuc, 1986b). The signal is believed to be produced in the inoculated leaf, around the time the lesion forms, and is transported throughout the plant, in some way sensitising the plant to recognise and/or respond more rapidly to the second infection, so increasing resistance (Fig. 1.3) (Garas & Kuc, 1984; Dean & Kuc, 1985, 1986a). There is a direct correlation between the severity of symptoms on the inducer leaf and the level of systemic resistance, up to maximum resistance. A single *C. lagenarium* lesion on cucumber for example induced some resistance, while the response was saturated by five to approximately ten *C. lagenarium* lesions (Kuc & Richmond, 1977). In tobacco, as few as 12 TMV lesions induced significant ISR against *P. parasitica* (McIntyre & Dodds, 1979). So far, no-one has been able to detect the hypothetical signal molecule(s).

The sensitised plants are thought to exhibit reduced lesion number as a result of the tissue being made more resistant to pathogen attack, with a consequent reduction in the number of infection sites. Alternatively, a reduction in lesion size may be due to an earlier and faster hypersensitive reaction than that seen in the control plants (Thomas & Fulton, 1968). As has been mentioned earlier, a number of the gene products eg. the enzymes associated with lignin biosynthesis and PR proteins, which have been related to defence, increase more rapidly in ISR plants following the challenge inoculation than in control plants (Ryan, 1984; Dean & Kuc, 1987; Ye *et al.*, 1989).

1.14. POTATO VIRUS X.

Potato virus X (PVX) is the type member of the potex viruses, a group of distantly serologically interrelated viruses with flexous rod shaped particles (Brandes, 1964). PVX is a highly heat-stable virus, only becoming inactivated when heated to

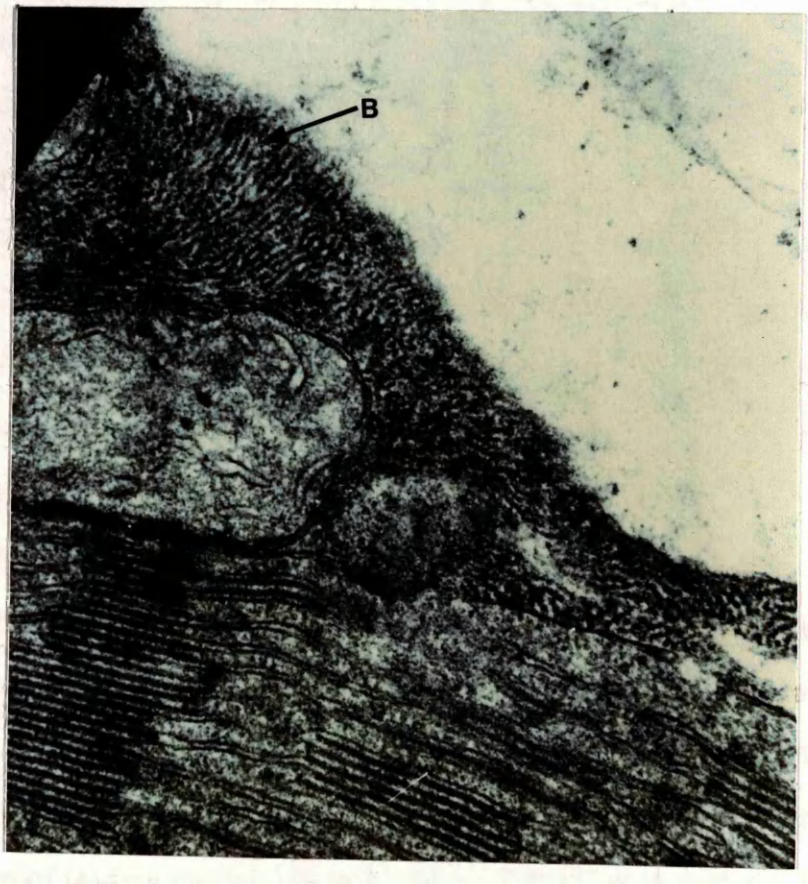
68-76° C (depending on strain) for 10 minutes in tobacco sap. At 20° C it is stable for weeks and this can be extended to a year in the presence of glycerol. It has a genome consisting of a single positive sense RNA strand, which is contained within the core (3.1 nm diameter) of a flexuous filament. The RNA content of the virus is 6% by mass. The PVX filament is formed from repeating units of the coat protein (2.6×10^4 daltons). The coat protein is arranged in a helix of pitch 3.4 nm with 10 sub-units per turn, forming a filament of 515 nm in length and 13 nm in diameter (the apparent size varies slightly depending on electron microscope (EM) preparation). The total PVX particle molecular weight is 35×10^6 daltons with a pI of 4.4 (Reichmann, 1959).

The entire nucleotide sequence of the RNA of two Group 3 isolates of PVX have been published (Huisman *et al.*, 1988; Skryabin *et al.*, 1988) and each comprise 6435 bases. They each have a 3' poly A. tail and are highly homologous. At its 5' terminus the RNA contains a m^7 GpppG cap structure (Sonenberg *et al.*, 1978). PVX virons only contain genomic RNA. In infected tissue two major subgenomic RNAs of 0.9 Kb and 2.1 Kb are present as well as four minor ones of 1.4, 1.8, 3.0 and 3.6 Kb. All these subgenomic RNAs are 3' coterminal (Dolja *et al.*, 1987). It has been suggested that *in vivo* the coat protein is translated from the 0.9 Kb subgenomic mRNA. The PVX genome consists of five open reading frames numbered 1 to 5 which, starting at the 5' terminus of the RNA, encode polypeptides of M_r165588, M_r24622, M_r12324, M_r7595 and M_r25080 (coat protein) (Huisman *et al.*, 1988).

The arrangement of open reading frames within the potexviruses is similar to that of the carlaviruses (Memelink *et al.*, 1990) and these two groups are presumably closely related. There is a degree of sequence homology between the 165 KDa polypeptide and the putative replicases of TMV, bushy stunt mosaic virus (BSMV) and other RNA viruses of the sindbis-like supergroup.

The range of plants infected by PVX is mostly restricted to the *Solanaceae* although some plants in other families, e.g. in the *Amaranthaceae* and *Chenopodiaceae*, are also susceptible (Bercks, 1970, 1978). The virus reaches high concentrations in the systemically infected plant. This aids its ready transmission,

Fig. 1.4. An electron micrograph of a leaf cell from a King Edward plant systemically infected with PVX strain KEBRS. The banded pattern (B) represents an inclusion of PVX virions, each band consisting of several PVX particles arranged parallel to each other. Magnification X 38000


B

which occurs by contact. Some transmission via leaf hoppers and fungi has been reported, but the evidence for this is weak.

PVX infection causes losses in tuber yields of approximately 5 to 15%. However losses can be even higher, as much as 25%, with plants grown from infected tubers (Harrison, 1970). Symptoms range from complete latency to severe necrotic streaks, depending upon the strain of PVX.

When PVX infects potato leaves, virons are first seen in the palisade cells, or less frequently in the epidermis (Kozar & Sheladko, 1969). Particles are distributed diffusely, or in dense, irregularly packed inclusions, or in X-bodies. The inclusions often appear banded (Fig. 1.4), each band consisting of several PVX particles arranged parallel to each other. These inclusions may fill the greater part of the infected palisade cell. Virus particles and spherical inclusion bodies also occur in the plastids. The X-bodies, which occur mainly near the nucleus, are not separated from cytoplasmic material.

PVX, like all RNA viruses, has the capacity to evolve rapidly. RNA viral genomes are typically unstable and this results in a very heterogenous population (this property may enable such viruses to respond rapidly to new host or environmental situations). It has been suggested that this variability is due to the error prone nature of viral RNA polymerase (Steinhauer & Holland, 1987). This enzyme lacks an effective proof reading mechanism and results in many more mistakes being made than with DNA polymerases. The rates of evolution of continuously replicating RNA viruses exceeds that of the DNA based host genome by 10^6-fold. The high incidence of error in RNA replication may limit the length of present day RNA genomes and cellular RNA (Steinhauer & Holland, 1987).

1.15. THE RESISTANCE GENES OF POTATO TO POTATO VIRUS X.

PVX strains have been grouped on the basis of serology (Torrance *et al.*, 1986), thermal inactivation (Fraser, 1986) and interaction with resistance genes in potato. In this thesis, the classification used is that first proposed by Cockerham (1955), who arranged PVX strains into four groups on the basis of their interactions

TABLE 1.4. Examples of potato cultivars containing the hypersensitive genes.

	Genotype	PVX Group 1	PVX Group 2	PVX Group 3	PVX Group 4
Pentland Crown Arran Banner	*nx:nb*	S	S	S	S
King Edward Epicure	*Nx:nb*	N	S	N	S
Pentland Ivory Arran Victor President	*nx:Nb*	N	N	S	S
Pentland Dell Maris Piper Craigs Defiance	*Nx:Nb*	N	N	N	S

N = Hypersensitive reaction: necrotic localisation of the virus at the site of entry or the expression of the lethal top necrotic reaction, according to the circumstances of infection.

S = Susceptible.

Modified from Cockerham, 1955.

with the potato host resistance genes *Nx* and *Nb*. These genes determine the hypersensitive necrotic response which restricts PVX replication (Cockerham, 1955) to local lesions. An additional gene *Rx* is believed to confer absolute immunity to nearly all PVX isolates (Jones, 1985). The genes *Nx*, *Nb* and *Rx* are all dominant genes found at different loci on non-homologous chromosomes and, therefore, segregate independently (Cockerham, 1955, 1970; Solomon, 1985).

In this classification Group 1 strains of PVX induce incompatible, hypersensitive (necrotic) reactions with both genes *Nx* or *Nb*. Group 1 strains only systemically infect cultivars possessing the genotype *nx:nb*, and usually occur in the field in combination with strains of Group 2 or 3. Group 2 strains systemically infect *Nx* genotype potatoes but not *Nb*, while Group 3 strains systemically infect cultivars containing the *Nb* but not the *Nx* gene. Group 4 strains are compatible with both genes. All four strains fail to replicate in potato cultivars carrying the *Rx* immunity gene (Table 1.4). Since the classification was put forward, a fifth type of PVX has been isolated, strain HB, which overcomes *Rx* as well as *Nx* and *Nb* (Moreira *et al.*, 1980). Different susceptibilities to the genes *Nx*, *Nb* and *Rx* may result from differences in the coat protein (Adams *et al.*, 1987), as shown by experiments using *in vitro* recombinants resistance maps to the viral coat protein gene (Cruz *et al.*, 1990; Baulcombe, personal communication).

The Group 3 strain of PVX is the most common strain in countries, such as the UK, which only grow *S. tuberosum* ssp. *tuberosum* (Cockerham, 1970). Apart from an isolate lodged in the Commonwealth Potato Collection there is only one report of a Group 4 strain being found in the UK (Bawden & Sheffield, 1944).

It has been suggested that, at least in those countries operating sophisticated seed potato certification schemes, the ability to overcome the gene *Nx* (i.e. Group 2 strains) may be associated with other characters which diminish general fitness and ability to compete with Group 3 strains (Jones, 1985). Indeed, Group 2 strains do not reach as high a concentration as Group 3 strains in cultivars which react in a non-hypersensitive fashion (Adams *et al.*, 1986b; Aw, 1987). PVX is transmitted in the

field by contact, and most efficient spread occurs when the virus achieves very high concentrations in infected plants.

When *Nx* or *Nb* react to produce the hypersensitive response, viral antigen accumulation is decreased when compared with its accumulation in cultivars reacting non-hypersensitively, regardless of whether or not necrotic symptoms develop. The *Nb* gene is more effective in restricting viral replication than the *Nx* gene (Adams *et al.*, 1986b). In addition, *Nb* completely inhibits systemic movement of Group 2 strains, irrespective of necrotic local lesion formation or symptomless conditions developing from the initial viral infection. In contrast, some systemic spread of Group 3 strains can still occur in *Nx* plants although this is much less than in susceptible cultivars. Although genes *Nx* and *Nb* in isolation have no detectable effect on the accumulation of PVX in the non-hypersensitive situation, when they react together they decrease virus accumulation more effectively than expected. This is an example of synergism. This synergism is so strong in some cultivars that no virus was detected following infection (Adams *et al.*, 1986b).

It would appear that the *Nx* gene operates at the tissue level as Group 3 strains of PVX, which are restricted by the presence of the *Nx* gene in plants of the potato cv. Maris Piper, replicate to high levels in Maris Piper, King Edward, and Pentland Ivory protoplasts (Adams *et al.*, 1985, 1986a). This indicates that the mode of action of the *Nx* gene may be to restrict the transport of the virus between cells. However, conflicting data from Foxe & Prakash (1986) showed that the presence of the *Nx* gene in King Edward protoplasts restricted the replication of the PVX Group 3 strain DX. This point, therefore, requires to be clarified. The *Rx* gene in potato cv. Cara has been shown to act at the cellular level, abolishing the ability of the PVX Group 3 strain DX to replicate in protoplasts of cv. Cara (Adams *et al.*, 1986a). Potato PVX resistance genes do not operate at the level of viral uncoating as resistant potato plants inoculated with PVX RNA react in the same fashion as those inoculated with PVX virus particles.

Examples of viral replication being cell or tissue specific are seen in many other plant-virus interactions (Fraser, 1990). Plants with tissue specific resistance to viral infection include wheat, oats and maize resistant to brome mosaic virus (BMV) (Furusawa & Okuro, 1978). Genes which infer resistance at the cellular level include the potato *Ry* resistance gene to PVY and resistance to CMV in cucumber (Barker & Harrison, 1984; Coutts & Wood, 1977) respectively.

1.16. SYMPTOM EXPRESSION IN POTATOES INFECTED WITH PVX.

Two strains of PVX (*D* and *Xn*) were reported to induce lethal necrosis on potato cultivars possessing neither *Nx* or *Nb* (Cockerham & Davidson, 1963). These are assumed to be aberrant strains. This implies that within cultivated potato varieties additional genes to *Nx, Nb and Rx* also control lethal necrotic reactions to parts of the virus complex.

Group 1 strains only infect systemically those cultivars of potato having the genotype *nx:nb* in which they cause mosaic symptoms. If either of the two hypersensitive genes are present, local lesions and top necrosis appear. Group 2 strains in the presence of *Nb* mainly cause local lesions. The formation of local lesions is temperature dependent, with most lesions forming at 10°C, very few forming at 15-20°C and none at 25°C. Mosaic symptoms occur with Group 2 strains when the *Nb* gene is not present. Group 3 strains cause systemic top necrosis and local lesions in the presence of *Nx*, at temperatures of 15-20°C. Outside this temperature range, at 10°C and 25°C, only local lesions develop, fewer developing as the temperature increases. In the absence of the *Nx* gene mosaic symptoms occur (Adams *et al.*, 1986b).

Group 4 strains, though they seldom occur in nature, have been selectively generated by inoculating potato cultivars which have the genotype *Nx:Nb* with either Group 2 or Group 3 strains (Jones, 1982, 1985) and selecting for mutants which can spread systemically. These Group 4 strains initially cause a necrotic shock reaction on inoculated leaves which later recover; the plant then develops mosaic symptoms.

Natural Group 4 strains of PVX (Cockerham & Davidson, 1963) also cause some necrotic symptoms in *Nx:Nb* cultivars, in the form of mild necrotic mottle. These symptoms of systemic infection of *Nx:Nb* cultivars, in the absence of lethal apical necrosis, are representative of Group 4 infected plants (Rozendial, 1966).

The Group 4 strain of PVX which has been derived from a parental Group 3 strain can revert if not maintained on a selective host (Jones, 1982), although the Group 4 strain derived from a Group 2 PVX isolate appears to be rather stable even when not maintained in a selective host (Jones, 1985). Indeed, if it is not maintained on potato, PVX may even lose the ability to infect potatoes. This genetic drift is an important practical point to note when working with PVX isolates over an extended period of time (Matthews, 1981). Such changes are not unexpected as resistance-breaking properties are often seen to be associated with reduced fitness of the virus to replicate in a non-selectable host (Jones, 1982).

The temperature at which plants are grown after inoculation with PVX can have a marked effect on the rate and extent of viral movement within the plant, symptom expression and viral accumulation. The resistance response by both *Nx* and *Nb* is temperature sensitive, with lesion number reducing with increased temperature although resistance is still maintained even when no lesions are formed. Both Group 2 and Group 3 strains have relatively low optimal temperatures for virus accumulation. This is especially true in the case of Group 2 strains. This suggests that PVX is adapted to multiply under the relatively cool environmental conditions which are optimal for potato growth. The hypersensitive resistance genes are rather stable. They do not break down even when the temperature is raised to 25°C. Indeed they become even more efficient at these elevated temperatures than at 10°C-20°C (Adams *et al.*, 1986b).

Viral movement and symptoms are most prevalent in the temperature range 15-20° C, and these are associated with severe mosaic symptoms. At 10° C or 25° C only mild mosaic symptoms develop in susceptible plants. No symptoms develop above 30° C and no virus replication occurs, irrespective of the potato host. The increase in resistance contrasts with the *N*-gene resistance to TMV in tobacco, where

both localisation and the induction of necrosis are abolished at temperatures above 30°C. When the temperature is reduced, TMV produces systemic necrotic symptoms (Smart *et al.*, 1987).

Though Group 4 strains are easily generated experimentally they have not been isolated in the field in the UK. However, in the area of the Andes, where many land races of potato have both *Nx* and *Nb* genes, Group 4 strains are much more common. As already mentioned, Group 4 strains can be readily selected from Group 2 and 3 strains. However, attempts to isolate mutants which overcome the *Rx* immunity gene by graft inoculating PVX onto *Rx*-containing cultivars have been unsuccessful (Jones, 1985). Thus it appears that it is difficult for PVX strains to overcome the gene *Rx*. Perhaps this is because *Rx*, unlike *Nx* and *Nb*, appears to block replication rather than virus spread. Not surprisingly, the strain HB, which overcomes immunity genes which have previously withstood challenge with all known strains of PVX, has been isolated from a wild Andean potato. HB causes a mild mosaic, systemic chlorotic blotching or symptomless infection in potato, irrespective of the presence of the *Nx*, *Nb* or *Rx* genes (Moreira *et al.*, 1980). The HB strain, in infected sap, shows exceptional stability for a PVX strain, infectivity not being lost over four months when grown in Pentland Crown, which were at the same time infected with a Group 3 PVX strain (Jones, 1985). HB does not lose its virulence towards *Rx*-containing cultivars over long periods of time when grown in either potato or tobacco non-selective hosts. It also reaches high concentrations in infected plants, these features favouring the spread of a contact transmitted virus. Such features are quite unusual for a resistance breaking strain which in general would be expected to compete poorly with other strains of the virus.

1.17. BIOTECHNOLOGICAL STUDIES ON POTATOES.

The potato crop represents the most important non-cereal crop in the world and is proving to be one of the first beneficiaries of biotechnological advances. The amenability of the potato to transformation with Ti and Ri plasmid vector systems and the development of regeneration techniques from potato explant material has enabled

the introduction of several foreign genes into *S. tuberosum*. This has allowed the expression and regulation of these genes to be studied (Matton *et al.*, 1990).

Areas of research involved in the improvement of the potato crop include (a) increased resistance to viral, insect, bacterial and fungal attack, (b) improving the understanding of melanin synthesis, carbon flow and tuber induction and (c) the application of restriction fragment length polymorphisums (RFLP) mapping which it is hoped will identify at a molecular level genes of interest for breeding programmes (Vayda & Park, 1990).

In an attempt to improve resistance against PVX and PVY, the coat protein genes from the two viruses have been transformed into potato using *Agrobacterium tumefaciens* (Tavantzis, 1990). These transgenic plants are at present undergoing field trials. The expression of the coat protein of PVX and PVY has been shown to reduce the accumulation of virus with time and also reduce the final viral titre attained within the plant. However, the use of naturally occurring resistance genes to PVX and PVY seems to offer greater scope in the control of viral infection. At present, information on these putative genes is largely lacking, but the use of RFLP analysis and transposon mutagenesis may offer a way to identify and utilise them (Tavantzis, 1990).

RFLP has been used to physically map important genes to specific areas of the potato genome. This is important as it provides the ability to localise any cloned DNA sequence in the genome, for example through linkage analysis with RFLP markers the cloned genes for potatin have been mapped in potato and tomato. The close relationship between tomato and potato has enabled tomato DNA probes to be used to improve the potato RFLP map.

In potato, RFLP markers have been used to (a) compare variation between closely related species; (b) tag genes of interest in breeding programmes, e.g. resistance genes, so enabling quicker characterisation of a crossed progeny; and (c) to check the stability of potato tissue cultures and forced interbreeding programmes.

Currently, potato RFLPs are being used to identify those genes in *Solanum brevidens* conferring resistance to soft rot and potato leaf roll virus (PLRV) in hybrid

plants with *S. tuberosum* from protoplast fusions. Sexual crosses of the above two species are also being used with RFLP analysis to investigate the genes in *S. brevidens* conferring insect resistance. The identification of gene products and gene localisation by tight linkage with RFLP markers will lead to a better understanding of the genetics, evolution and biochemistry of important trails that are difficult to study by other means, opening the door to new approaches to ultimately clone the controlling genes. Up to now, genes to be cloned must have a protein product which has been characterised. RFLP mapping provides a way to clone genes about which only the effect on phenotype is known, e.g. resistance genes.

Although the potato is an important crop plant, the development of potato genetics has been hampered by the biology of the species, polyploidy, inbreeding depression and a shortage of clones with good male fertility. RFLP techniques should overcome many of these problems associated with conventional breeding (Bonierbale *et al.*, 1990).

Chapter 2

MATERIALS AND METHODS

2.1. POTATO CULTIVARS.

The potato cultivars, Pentland Ivory (*nx Nb*), King Edward (*Nx nb*), Pentland Crown (*nx nb*) and Pentland Dell (*Nx Nb*), were purchased from P.L. Fordyce (Growers) Limited, 6 Hunter Street, Auchterarder, and were super elite grade, 99% free of PVX infection. The tubers were stored at 4°C in the dark until required.

2.2. PROPAGATION OF POTATO PLANTS.

The tubers were placed at 15-20°C, 80-100% relative humidity (RH) under a 16 h light regime of 200 μmoles/m^2/sec of photosynthetically active light, in a seed tray, rose end up, and allowed to sprout. When the sprouts were 0.5 - 1.0 cm in length they were bored out of the tuber using a sterile No.10 cork borer. The sprout was left with 2 cm of tuber below it and placed in dry potting compost (SAI Growers potting and bedding compost) in a 5-inch pot. The sprout was just covered with compost and left for 24 h to allow a periderm to develop. The sprouts were then watered daily from above. The pots were placed on a wire mesh allowing free draining of the potting compost. The sprouts were cultivated under the same conditions as used to sprout the tubers. When the plants averaged 10 cm in height, stunted and sickly plants were discarded. The temperature was reduced to 10°C and 6 days later plants were inoculated on their first fully expanded leaf (leaf one) either with 1:5 weight/volume (w/v) of *Nicotiana X edwardsonii* Christie leaves systemically infected with PVX (ground in water), mock inoculated with water, or the plants were left untreated. Leaf samples were then taken at time points as described in individual experiments.

2.3. POTATO VIRUS X STRAINS.

Six isolates of PVX were obtained from various sources and assessed for their potential in the investigation of potato resistance to PVX infection. The sources of the isolates are shown below. Some independent isolates were supplied with the same name, each one was given a different designated name for this work.

Potato virus X Strains used in this study

Name	Group	Supplier	Name designated in this work	Plant species previously maintained in
KE/BX	2	R. Solomon,Pentlandfield*	KEBRS	Potato, King Edward
KE/BX	2	Rothamsted Experimental Station	KEBX	Tobacco
PVB	2	R. Boulton, Plant Breeding Institute	2PBI	Tobacco
PVX	3	R. Boulton, Plant Breeding Institute	3PBI	Tobacco
PVX3	3	R. Solomon, Pentlandfield*	Gp3RS	Tobacco
PVX^N	3	W.S. Pierpoint, Rothamsted Experimental Station**	XN	Tobacco

* Solomon, 1985
**Ireland & Pierpoint, 1980; Matthews, 1948

2.4. PROPAGATION OF POTATO VIRUS X STRAINS.

N. edwardsonii was used to propagate the virus, as the plants grow quickly from virus-free seed, produce small plants taking up little room, and develop strong, visible PVX symptoms within 10 days of inoculation. This enabled fresh PVX inocula to be generated more quickly than with potato plants which produce poor symptoms. The virus was inoculated onto *N. edwardsonii* grown from seed to a size of 5-7 true leaves. The virus inoculum was ground either in a liquidiser, until the leaves were just macerated, or in a pestle and mortar, depending on the amount of inoculum required. Using a small pad of muslin, the inoculum, diluted ten-fold with water, was rubbed gently onto the tobacco leaves which had been dusted with

carborundum (super-fine 600 grit, BDH). The plants were either grown in the greenhouse or growth room, until symptoms developed. They were then harvested and either used immediately or stored at -70°C.

The various strains of virus were routinely passaged through a susceptible potato host; either cv. King Edward for Group 2 PVX strains or cv. Pentland Ivory for Group 3 PVX strains, in order to ensure that they retained their initial grouping.

2.5. INOCULATION OF POTATO PLANTS WITH POTATO VIRUS X.

The leaves of the potato plant were lightly dusted with carborundum. Systemically infected *N. edwardsonii* leaves were liquidised until macerated, at a ratio of 1 gm leaf to 5 ml of water, and then applied to the potato leaves with a muslin pad.

2.6. GENERAL METHODS USED THROUGHOUT THIS PROJECT.

2.6.1. Sterilisation of equipment, buffers and solutions.

All equipment and solutions to be used with RNA were incubated overnight with 0.1% DEPC (Diethyl Pyrocarbonate) to destroy RNase activity. The DEPC-treated equipment was then sterilised by autoclaving for 30-60 minutes at 1.1 kg/cm^2. Non-heat resistant equipment was rendered nuclease-free by soaking it in 0.5 M NaOH for 30 minutes, then rinsing copiously with sterile distilled water, followed by air drying.

2.6.2. Siliconization.

For procedures using very small amounts of nucleic acid, equipment such as tips, Eppendorf tubes, glass wool etc. were siliconized to prevent losses in yield resulting from the nucleic acid adhering to the surfaces. Clean, dry equipment was placed in a glass beaker in a fume hood, dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane) was poured over it and left for 2-3 minutes. This solution was then poured off and the equipment allowed to dry. It was then rinsed with distilled water to remove traces of hydrochloric acid produced by the treatment.

2.6.3. Preparation of dialysis tubing.

Tubing (1 or 2 cm diameter) was cut into 15 cm strips and boiled for 10 minutes in 2 litres of 2% sodium bicarbonate, 1mM ethylenediaminetetraacetic acid (EDTA). The tubing was then rinsed thoroughly in distilled water and boiled for 10 minutes in 1 mM EDTA. After cooling, it was stored submerged at 4°C. Before use it was washed with a large volume of AnalaR grade, sterile DEPC-treated water. Gloves were always worn when handling dialysis tubing.

2.6.4. Preparation of buffer saturated phenol.

Redistilled phenol (500 ml) was melted at 68°C. 8-hydroxyquinoline was added, to a final concentration of 0.1%, as an antioxidant. An equal volume of 0.1 M Tris-HCl (pH 8.0), which had been warmed to 80°C, was then added and the components mixed vigorously. The phases were allowed to separate and the aqueous phase poured off; more hot 0.1 M Tris-HCl (pH 8.0) was added, and the mixture was again vigorously shaken. The phases were separated once again, excess Tris-HCl was poured off and the phenol stored, at 4°C under ~50 ml of Tris buffer, in a brown bottle. The phenol was used within a month.

2.6.5. Preparation of deionised formamide or acrylamide.

Formamide, or stock solution of acrylamide + bis-acrylamide in H_20 were mixed with Amberlite MB-1 monobed resin (Sigma) at a ratio of 50 ml/2gm. The mixture was stirred for 1 h, filtered through Whatman No.1 filter paper and stored in a brown bottle at 4°C.

2.7. PURIFICATION OF POTATO VIRUS X.

PVX was prepared according to the protocol of Goodman (1975). Infected leaves were harvested from systemically infected *N. edwardsonii* and stored at -20°C in sealed polythene bags until required. The leaves were thawed and 15 gm were liquidised in 50 mM NaH_2PO_4, 10 mM EDTA pH 7.6, using 2 ml per gm of leaf material. The homogenate was strained through muslin and centrifuged at 15000 g for 20 minutes at 4°C in a Beckman J2-21 centrifuge to remove cellular debris. The resulting supernatant was layered over a pad of 30% sucrose, 5mM EDTA pH 7.6

(7.5ml), and centrifuged at 54,000 rpm (195,000 g) for 45 minutes at 4°C in a Sorvall T865 rotor. The resulting glassy pellet of virus was resuspended in 2.5 ml 5mM EDTA pH 7.6 and homogenised briefly in a hand-held glass homogenizer, to disrupt any viral aggregates. The cycle of low speed/high speed centrifugation was repeated and the resulting pellet was resuspended in 0.25 ml 5mM EDTA pH 7.6 and stored at -70°C. Virus concentration was measured spectrophotometrically. Concentrations of virus were determined by measuring absorbance at 260 nm and 280 nm, and comparing calculated values with the published value of A260 = 2.97 for 1 mg per ml for a 1 cm light path (Bercks, 1970). The ratio of absorbance at 260 and 280 nm was also determined and compared with the expected value of 1.2:1.0.

2.7.1. Isolation of Potato Virus X RNA.

RNA was purified from virus by the protocol of Ricciardi *et al.* (1978), with slight modification. Two ml of virus suspension were diluted four-fold with 5mM Tris buffer (5 mM Tris-HCl pH 7.6, 50 mM NaCl, 0.5 mM EDTA, 0.25% SDS). An equal volume of phenol/chloroform was added to the virus solution, vortexed briefly, then shaken gently at 55°C for 10 minutes. The mixture was centrifuged in a MSE microfuge for 10 minutes at 13,000 rpm. The supernatant was recovered, care being taken not to remove any of the protein interface. One hundred µl of sterile DEPC-treated AnalaR H_20 was added to the tube which was re-centrifuged briefly, the supernatant was recovered as before and the aqueous phases were combined. To the combined supernatant, one-tenth volume of 3M K acetate pH 5.5 and 1.1 volume of isopropanol were added and the flask frozen in liquid nitrogen for one minute. The RNA precipitate was centrifuged for 30 minutes in a MSE microfuge at 13,000 rpm. The pellet was washed twice in 70% ethanol and allowed to dry at room temperature under vacuum. When dry it was resuspended in 100 µl of DEPC-treated AnalaR H_20. The optical density of the RNA solution was measured at 260 nm and 280 nm. The extinction coefficient of a 40 µg/ml solution of RNA was taken to be 1.0 cm^{-1} at 260 nm (Spencer, 1971). The ratio of absorbance at 260 nm and 280 nm was determined and compared to the expected value of 2.0:1.0 for pure RNA.

2.8. ENZYME-LINKED IMMUNOSORBENT ASSAY.

Enzyme-linked immunosorbant assay (ELISA) was carried out by the double antibody sandwich method using a kit purchased from Boehringer (Cat. No. 661368), according to the manufacturers instructions. The protocol given was followed with slight modification.

Leaf samples were taken and homogenated in sample/conjugate buffer in a hand-held glass homogeniser. Debris was removed by centrifugation for 30 seconds at 13,000 rpm in a bench top Eppendorf microfuge. A series of dilutions were then made in the same buffer. Standards were prepared, containing known concentrations of purified PVX, in the same buffer.

Two hundred µl of coating solution, containing anti-PVX antibody, were pipetted onto the wells of a microtitre plate (U-well plate, Sterilin Limited, Feltham, England) this was covered with a plate lid and incubated for 2 h at 37°C. The solution from the wells was tipped out and the plate tapped onto filter paper. The wells were washed three times with washing buffer and 200 µl of the appropriate sample was then pipetted into each well. The plate was covered and incubated overnight at 4°C. The sample was then removed and the plate washed three times with wash buffer. Two hundred µl of antibody-AP-conjugate solution was pipetted into each well and the plate was covered and incubated for 4 h at 37°C. The solution was then removed and the plate washed three times as before. The substrate solution, containing 4-nitrophenyl phosphate, was then added to each well (190 µl per well) and allowed to develop for a maximum of one hour. The absorbance of each sample was read, at 405nm, using a Titertek Multiscan MC plate reader, using substrate solution as a blank.

2.9. ELECTRON MICROSCOPY.

2.9.1. Preparation of thin sections.

Samples of either infected or healthy leaf, approximately 1-2 mm square, were infiltrated under vacuum with fixative (3% (v/v) gluteraldehyde in 0.2 M sodium cacodylate buffer pH 7.2) at room temperature for 12-16 h. They were then washed

thoroughly in 0.2 M sodium cacodylate buffer pH 7.2 (four changes over 24h) and post-fixed in 1% (v/v) OsO_4 in 0.2 M sodium cacodylate buffer pH 7.2 for 3 h. The samples were washed in distilled water (three changes over 30 minutes) and then block-stained in 2% (v/v) aqueous uranyl acetate for 2 h. The samples were then dehydrated in ethanol (25%, 50%, 75% for 2 h each and overnight in 100%), embedded in Spurr resin (Spurr, 1969), a low-viscosity epoxy resin embedding medium for electron microscopy, and polymerised at 60°C for 24 h. Sections of approximately 60 nm were cut on a LKB III ultramicrotome using a diamond knife and mounted on 300 mesh copper grids. Sections were stained with saturated uranyl acetate in 50% methanol for 20 minutes followed by lead citrate (Reynolds, 1963) for 5 minutes. They were then examined in a Philips EM301 electron microscope at 60 and 80 kv. Negatives were prepared on Kodak positive release 35 mm film at various magnifications.

2.9.2. Preparation of samples for negative staining.

For rapid diagnosis of PVX infection, sap was generated from leaf tissue by crushing or breaking a leaf petiole. A formvar-carbon coated 200 mesh grid was touched to the sap for 5-10 seconds, grids were jet rinsed with distilled water and drained on a small piece of Whatman No.1 paper. The grids were then touched either to a droplet of 1% aqueous uranyl acetate or a droplet of 1% sodium phosphotungstate or phosphotungstate acid for 5-10 seconds, rinsed with distilled water, drained, dried and examined in the electron microscope at 60 kv (Gibbons & Grimstone, 1960).

2.10. EXTRACTION OF TOTAL RNA FROM PLANT TISSUE.

RNA extraction was carried out by a modification of the procedure of Wallace (1987). Twelve to fifteen gm of leaf tissue were frozen in liquid nitrogen, ground to a powder in a pestle and mortar, and transferred to a 250 ml conical flask containing 3 ml of 5X extraction buffer (0.25 m NaCl, 25 mM sodium acetate, 2.5 mM EDTA, 5% SDS - pH 5.0), 12 ml of AnalaR DEPC-treated water and 15 ml of 1:1 (v/v) phenol/chloroform. The mixture was incubated at 65°C and gently shaken for 3 to 5 minutes, the solution was then centrifuged at 7000 g for 20 minutes at 4°C

in a Beckman J2-21 centrifuge. The aqueous upper phase was placed on ice. The pelleted leaf material and phenol/chloroform was re-extracted with 15 ml of extraction buffer, as above. The aqueous phases were combined and an equal volume of phenol/chloroform added, this was mixed into an emulsion using a Pasteur pipette for 1-2 minutes and centrifuged at 7000 g for 20 minutes at 4°C in a Beckman J2 13.1 swing out bucket rotor. The aqueous solution was re-extracted first with phenol/chloroform and then chloroform to remove any traces of phenol, care was taken not to disturb any of the protein at the aqueous/phenol chloroform interface when removing the aqueous fraction. To the aqueous solution was added 0.2 volume of 1 M acetic acid and 0.7 volume of absolute ethanol and the RNA was allowed to precipitate overnight at -20°C.

The RNA was harvested by centrifugation at 10000 g for 15 minutes at 4°C. The pellet was washed twice with 3 M sodium acetate pH 5.2 at room temperature and then twice for 5 minutes each in cold 70% ethanol. The RNA was vacuum dried at room temperature, when it was just dry it was resuspended in AnalaR DEPC-treated water.

The protocol was scaled down appropriately for smaller amounts of leaf tissue. The yield and purity of RNA was determined spectrophotometrically by measuring absorbance at 260 and 280 nm using a Philips Pye Unicam SP8-500 UV/VIS spectrophotometer (Spencer, 1971).

2.11. PREPARATION OF POLY A^+ RNA.

Poly A^+ RNA was isolated from total RNA by chromatography on oligo (dT)-cellulose according to a modification of the method of Sambrook *et al.* (1989). A 10 ml column (Poly-Prep, Biorad) was siliconised, washed in DEPC-treated water and autoclaved. Oligo (dT)-cellulose (0.2g) (Boehringer Mannheim GmbH) was mixed with 10 ml of loading buffer (10 mM Tris-HCL pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.1% N-Lauroyl-Sarcosine) and poured into the column. This was washed with 10 ml DEPC-treated AnalaR water and then 3 ml 0.1 M NaOH, 5 mM EDTA. The column was washed further with sufficient water until the pH of the eluate fell

below 8.0. The column was then washed with 5 ml of sterile loading buffer. The RNA, in water, was heated to 65°C for 5 minutes, chilled to room temperature, and then mixed in an equal volume of 2X loading buffer. The RNA was loaded to the column at a concentration of more than 2 mg/ml. The eluate, which still contained some unbound poly A^+ RNA, was collected, reheated to 65°C for five minutes, chilled and reapplied to the column. The column was then washed with up to 20 ml of loading buffer, until the A260 of the eluate fell below 0.020 cm^{-1}, showing that most of the unbound RNA had been washed off the column. The poly A^+ RNA was then eluted by adding 3 ml of DEPC-treated AnalaR water in 0.5 ml steps. The eluted RNA was collected and precipitated, by adding one-tenth volume 3M sodium acetate pH 5.2 and 2.5 volume of absolute ethanol and placed at -20°C overnight. The RNA precipitate was collected by centrifugation for 20 minutes at 10000 g at 4°C. The pellet was washed twice with 70% ethanol, allowed to air dry and then resuspended in an appropriate amount of DEPC-treated AnalaR water. Occasionally, the poly A^+ RNA was reapplied to the column and the procedure repeated. However, this step gave a reduced yield of poly A^+ RNA and was usually omitted.

2.12. *IN VITRO* PROTEIN SYNTHESIS IN A CELL-FREE SYSTEM.

A wheat germ *in vitro* translation system was either purchased from Amersham (code RPN.1), in which case the manufacturer's protocol was followed, or was prepared from wheat germ using a modification of the method of Davies *et al.* (1977). Preparations made by the latter method often had a greater activity than Amersham wheat germ. However, some batches of germ were inactive.

2.12.1. Floating wheat germ.

Working in a fume cupboard, 75 ml of cyclohexane were mixed with 250 ml of carbon tetrachloride. Five gm of wheat germ (obtained from General Mills, California) were stirred in and the material which floated was collected using a tea strainer (all the germ sinks if left too long). The collected germ was then spread out on clean filter paper and allowed to air dry. Active floated germ could be stored in a vacuum desiccator at 4°C for several years.

2.12.2. Preparation of translation extract from floated wheat germ.

All operations were carried out on ice in thoroughly clean, sterile equipment. Floated germ (0.5 gm) was ground with the tip of a Pasteur pipette in a chilled mortar. After 15 seconds of grinding, 1 ml of grinding buffer (5 mM HEPES pH 6.4-6.9, 120 mM K-acetate, 5mM Mg-acetate, 1 mM DTT) was added and the wheat germ was ground for a further 30 seconds. A further 1 ml of grinding buffer was added to the thick paste and grinding continued for 1 minute more. To the paste another 1 ml of grinding buffer was added and the slurry was transferred to an ice-cold 15 ml Corex tube, using a further 2 ml of grinding buffer to wash out the mortar. The slurry was centrifuged for 10 minutes at 30,000 g at 4°C. After this the clear yellow-green supernatant was removed using a Pasteur pipette, care being taken to avoid the upper lipid pellicle and the pellet. The supernatant was transferred to sterile dialysis tubing and dialysed overnight against 1 litre of ice-cold dialysis buffer (20 mM Tris-acetate pH 7.6, 120 mM K-acetate, 5 mM Mg-acetate, 1 mM DTT). The dialysis buffer was changed and the sample dialysed for a further 3 h. The contents of the dialysis tubing were transferred to a Corex tube and centrifuged for 5 minutes at 5,000 g in a Beckman JA21 rotor at 4°C. The supernatant was removed, divided into aliquots of 0.5 ml and stored under liquid nitrogen.

Translation reaction

The recipe given below is sufficient for eight translation reactions.

Wheat germ extract	100 µl
HKMS	20 µl
Mix 6	20 µl
^{35}S-methionine	100-200 µCi (approx. 1000 Ci/mM supplied by either NEN-Dupont or ICN).
Sterile AnalaR H_20, add to give a final volume of	160 µl

HKMS

		Final concentration
4 ml	0.5 M HEPES pH7.6	200 mM
2 ml	3M K-acetate	400 mM
0.2 ml	200 mM Mg-acetate	4 mM
10.2 mg	Spermidine	4 mM
3.8 ml	Sterile AnalaR H_2O	

Mix 6

50 µl	30 mM GTP
100 µl	0.1 M ATP
150 µl	1 mM Amino acids minus Methionine
45 µl	0.8 M Phosphocreatine
5 µl	8 mg/ml Creatine phosphokinase
50 µl	Sterile AnalaR H_2O

2.12.3. Translation of mRNA.

Either 2 µg of poly A^+ RNA or 5 µg of total RNA was made up to a final volume of 5 µl and placed in an Eppendorf vial. To this 20 µl of the translation cocktail was added. The contents of each Eppendorf vial were mixed and the vial centrifuged briefly to bring the contents to the bottom. The samples were incubated at 30°C for 90 minutes, then placed on ice. From each vial two samples, of 2 µl each, were removed using a positive displacement pipettor and added to a 15 ml test tube containing 1 ml of freshly prepared 'stop-bleach' (28.5 ml H_2O, 1 ml 10 M NaOH, 0.5 ml 100 vol. H_2O_2 and 10 mg 'cold' methionine). The samples were incubated for 10 minutes at 37°C, then 1 ml of 25% (v/v) TCA and 10 µl of 2% BSA were added to precipitate the polypeptides. The tubes were then placed on ice for 15 minutes. The contents of each test tube were filtered through a Whatman GF/C filter under suction. Each filter was pre-wet with 8% TCA. The filters were washed with two 10 ml

aliquots of 8% TCA and one 10 ml aliquot of 95% ethanol, under suction. The filters were removed, dried for 10 minutes at 80°C under vacuum, each filter was placed in a scintillation vial, 4 ml of "Ecoscint" scintillant was added and the vials were counted using the ^{35}S channel of a LKB 1211 Minibeta liquid scintillation counter.

2.13. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS.

One-dimensional polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemelli (1970) and Hames *et al.* (1981). Gels were formed using 16 cm^2 gel plates which had been washed with Decon, then water and finally alcohol, and allowed to air dry. The plates were then placed in a BRL gel forming apparatus, using a 1 mm spacer, and the appropriate running gel poured between them. The running gel was overlaid with water to create a perfectly flat gel interface. When the gel had polymerised the water was poured off, the stacking gel was poured and the appropriate comb placed in it. The distance between the well bottom and the separating gel was 2 cm.

Polyacrylamide gel electrophoresis stock solutions

Stacking gel buffer

0.5 M Tris-HCl pH 6.8

0.4% (w/v) SDS

Running gel buffer

1.5 M Tris-HCl pH 8.8

0.4% (w/v) SDS

Acrylamide

30% Acrylamide

0.8% Methylene-bis-acrylamide

The acrylamide stock solution was de-ionised and stored at 4°C in a brown bottle.

Electrolyte

25 mM Tris-HCl pH 8.3

0.192 M Glycine

0.1% (w/v) SDS

Denaturation buffer

50 mM Tris-HCl pH 8.0

2% (w/v) SDS

10% (v/v) glycerol

2% (v/v) Mercaptoethanol (added immediately before boiling)

0.1% (w/v) Bromophenol blue

	Stacking gel	Running gel				
		7.5%	10.0%	12.5%	15.0%	22.5%
Stacking gel buffer	5.0ml	-	-	-	-	-
Running gel buffer	-	5.0ml	5.0ml	5.0ml	5.0ml	5.0ml
30% Acrylamide	3.0ml	5.0ml	6.6ml	8.3ml	10.0ml	15.0ml
H_2O	12.0ml	10.0ml	8.4ml	6.7ml	5.0ml	-
10% Ammonium persulphate	200.0µl	200.0µl	200.0µl	200.0µl	200.0µl	200.0µl
TEMED	20.0µl	12.5µl	12.5µl	12.5µl	12.5µl	12.5µl

Gradient gels were formed using a gradient former.

2.13.1. Loading and running SDS PAGE gels.

In vitro translation products were mixed with two volumes of denaturing buffer and placed in a boiling water bath for 2 to 4 minutes, alternatively, samples were heated at 65°C for 1 h. The appropriate amount of each *in vitro* translation mixture was loaded onto the gel to ensure that each lane contained the same amount of TCA-precipitable radioactivity. The gel was then run at a maximum of 25 mA, until the dye front reached 1 cm from the bottom of the gel (about 4 h).

The gel was removed from the plates and the stacking gel cut off. The running gel was then soaked in two changes of 45 minutes each, in 200 ml of 10% acetic acid to fix the proteins and elute out unincorporated methionine. The gel was then either dried down on 3 MM paper or prepared for fluorography.

2.14. FLUOROGRAPHY OF SDS POLYACRYLAMIDE GELS.

Fluorography was carried out according to Bonner and Laskey (1974). Following fixation with acetic acid, the gel was washed in three successive changes of DMSO (500 ml), using 30 minutes per wash, with gentle shaking. The gel was then soaked for 2 h at 37°C in 22% (w/v) PPO in DMSO and finally gently washed in running tap water for 2 h or overnight. The gel was dried on a Biorad gel drier at 60°C for 1.5 h or until dry. The heat was turned off and the gel allowed to cool under vacuum to prevent any cracking.

The PPO-impregnated gel was placed in an X-ray film cassette with a piece of pre-flashed X-ray film and exposed for an appropriate time at -70°C. Films were developed in a Kodak X-omat machine.

2.15. ISOELECTRIC FOCUSING ELECTROPHORESIS.

Isoelectric focusing (IEF) was carried out according to O'Farell (1975), with slight modifications. Gels were poured in 14 cm long, 3 mm diameter siliconised tubes, which were held in place around a bottle containing hot tap water, using rubber bands. The bottom of each tube was sealed with nescofilm.

The IEF gel solution was prepared fresh each time:

5.5 gm	Urea
2 ml	10% NP40
1.33 ml	28.3% Acrylamide, 1.62% bisacrylamide
1.95 ml	H_2O
0.4 ml	Ampholines (pH 5.0-7.0) BDH
0.1 ml	Ampholines (pH 3.5-10.0) BDH

The 10 ml IEF gel mixture was heated gently to dissolve the urea, de-gassed under vacuum for 5 minutes and 10 µl of 10% ammonium persulphate and 5 µl of TEMED were added to polymerise the gels. The solution was immediately poured into the tubes using a long form Pasteur pipette, being careful not to trap air bubbles in the tube. The tubes were filled to 2 cm from the top, overlaid with water and allowed to polymerise for at least 1 h. The water overlay was removed and the gel overlaid with 25 µl of IEF sample buffer (9.5 M urea, 5% (v/v) 2-Mercaptoethanol, 2% (v/v) Nonidet-P40, 1.6% Ampholines pH 5.7-7.0 and 0.4% Ampholines pH 3.5-10.0) and left for 20 minutes to equilibrate. The sample buffer was then removed and the gels placed in a BRL IEF electrophoresis tank with 10 mM H_3PO_4 in the lower reservoir and 20 mM NaOH in the upper reservoir. Any bubbles present in the bottom of the tube were removed using a hyperdermic syringe. Twenty-five µl of fresh sample buffer were layered onto each gel and the gels electrophoresed as follows: 200 volts for 15 minutes, 300 volts for 30 minutes and then 400 volts for 30 minutes.

After pre-electrophoreses, the NaOH was removed and the liquid over the gel was carefully aspirated. Samples (10-25 µl in sample buffer) were loaded onto the top of each gel and overlaid with 25 µl of sample buffer diluted 1:1 with water.

The samples were electrophoresed at 400 volts for 13.5 h, or 300 volts for 18 h and then for one hour at 800 volts to sharpen the bands. The gels were removed from the tubes and equilibrated for 2 h in NEPHGE buffer (2.3% SDS, 5% 2-Mercaptoethanol, 10% Glycerol, 62.5 mM Tris-HCl pH 6.8, 0.1% Bromophenol blue). For the second dimension SDS PAGE gel, tube gels were gently placed on top of the stacking gel of the SDS PAGE gel and electrophoresed. Bevelled gel plates helped to hold the tube gel in place.

2.16. RNase AND DNase TREATMENT OF *IN VITRO* TRANSLATED PROTEIN SAMPLES.

A 5 x stock solution of 1 µg/ml RNase A and 250 µg/ml DNase I was dissolved in 10 mM Tris-HCl pH 7.6 and stored frozen at -20°C.

Following each *in vitro* translation the reaction products were treated with

Fig. 2.1. A simplified, orientation-specific cDNA cloning strategy.

(Dorsser and Postmes, 1987)

Linker Molecules

pGCTTGAATTCAAGC

This linker forms a *Hin*dIII recognition sequence (AAGCTT) when ligated to the oligo (dA) track at the 3' terminal end of the cDNA molecule and an *Eco*RI site at 5' terminus

mRNA 5'_____3'AAA

a) cDNA synthesis

b) methylation with M-*Eco*RI and M-*Alu*I

c) ligation of pGCTTGAATTCAAGC

GCTTGAATTCAAGC _____AAAGCTTGAATTCAAGC

CGAACTTAAGTTCG _____TTTCGAACTTAAGTTCG

d) digestion with *Hin*dIII and *Eco*RI

*Eco*RI *Hin*dIII

AATTCAAGC _____AA

GTTCG _____TTTCGA

e) removal of linkers and insertion in Lambda phage NM 1149

RNase and DNase by adding one volume of the above enzyme stock to each four volumes of sample and incubating at room temperature for 5 minutes. This removed the RNA and DNA contamination which can hinder the separation of *in vitro* translation products in the gels.

2.17. CONSTRUCTION OF A POTATO cDNA LIBRARY IN LAMBDA NM1149.

The strategy used to construct the cDNA library was that of Dorsser & Postmes (1987) and is shown in Fig. 2.1.

2.17.1. cDNA synthesis.

Total RNA was prepared from leaf material (section 2.10). Poly A^+ RNA was prepared from total RNA by Oligo (dT)-cellulose chromatography and used as a template for cDNA synthesis.

The first and second strand cDNA synthesis was carried out using an Amersham cDNA synthesis kit (Cat. No. RPN 1256Y/Z), by a modification of the procedure of Gubler & Hoffman (1983). All steps were carried out in siliconized Eppendorf vials. Reagents sufficient for 1 µg of poly A^+ RNA were assembled in a 1.5 ml Eppendorf vial using the oligo (dT) 12-18 primer to initiate first strand cDNA synthesis. Ten µCi of >3000 Ci/mM^{-1} (α^{32}P) dCTP were included in the reaction mix to allow first and second strand cDNA synthesis to be monitored. Incorporation of nucleotides into DNA was measured using a DEAE-cellulose binding assay according to the protocol described by Amersham. The size of the cDNA was determined by electrophoresis on a 1.5% alkaline agarose gel (Sambrook *et al.*, 1989). A comparison of the sizes of the first and second strand reaction products was used to check that hairpin structures had not formed in the first strand reaction. The cDNA was extracted twice with phenol/chloroform (1:1), the organic phase re-extracted and the combined aqueous phases extracted three times with ether. The cDNA was precipitated by adding 1 vol. 4 M ammonium acetate and 2 vol. ethanol and incubating on ice for 30 minutes. Following centrifugation at 13,000 r.p.m. in a MSE microfuge, the pellet was resuspended in 50 µl of AnalaR H_2O. The precipitation step

was repeated, the pellet rinsed with 70% ethanol, dried and resuspended in 25 µl of AnalaR H_2O.

2.17.2. Filling in 'ragged ends'.

The 'ragged ends' of the cDNA were in-filled to ensure efficient ligation to oligonucleotide linkers (Watson & Jackson, 1986). To the resuspended cDNA was added 1 µl of dNTP stock (each deoxynucleotide triphosphate at 5 mM in 10 mM Tris-HCl pH 7.5), 1 µl of 2 mg/ml BSA (nuclease free), 9 units T4 polymerase and 3 µl of 10X T4 polymerase buffer to give a final reaction volume of 30 µl. The mixture was incubated at 37°C for 30 minutes. The cDNA was then recovered by two phenol/chloroform and two ether extractions, followed by precipitation with 0.1 vol. 3M sodium acetate pH 5.0 plus 2 vol. absolute ethanol. The precipitate was collected by centrifugation as described above.

2.17.3. Methylation of *Eco*RI and *Hin*dIII sites.

The cDNA was resuspended in 20 µl of *Alu*I methylase buffer (50mM Tris-HCl pH 7.5, 10 mM EDTA, 5 mM 2-β mecaptoethanol, 80 µl S-adenosylmethionine). Ten units of *Alu*I methylase were added and the cDNA incubated for 2 h at 37°C. The cDNA was then recovered by phenol/chloroform extraction, ether extraction and ethanol precipitation as described above. It was resuspended in 20 µl of *Eco*RI methylase buffer (100 mM NaCl, 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 80 µM S-adenosylmethionine, 100 µg/ml BSA). Twenty units of *Eco*RI methylase were added and the mixture incubated for 2 h at 37°C. The cDNA was recovered as described above.

This treatment results in the methylation of *Eco*RI and *Alu*I restriction sites in the cDNA. Since the *Alu*I site forms part of the *Hin*dIII recognition sequence in the methylated cDNA, the cDNA is protected from digestion by both *Hin*dIII and *Eco*RI at any internal site.

2.17.4. Addition of linkers and ligation to the vector.

The addition of the *Eco*RI and *Hin*dIII linkers to the cDNA creates an *Eco*RI site at the 5' end and a *Hin*dIII site at the 3' end. This allows the forced directional insertion of the insert into a suitable lambda vector (e.g. Lambda NM1149)

Fig. 2.2. Lambda NM 1149 restriction enzyme map.

LEFT HAND END

	bp from end
*Bgl*II	471
*Bam*I	5559
*Kpn*I	17430
*Kpn*I	18950
*Sal*I	24887
*Sal*I	25395
*Xho*I	25653
*Bam*I	26651
*Hin*dIII	28661
*Eco*RI	28897
*Bgl*II	29799
*Bgl*II	29859
*Bam*I	32822

RIGHT HAND END 39713 bp

which contains adjacent *Hin*dIII and *Eco*RI cloning sites (Fig. 2.2). This directional cloning strategy, proposed by Dorsser & Postmes (1987) (Fig. 2.1) prevents the arms of the lambda vector re-ligating to each other and therefore reduces the background of phage lacking a cDNA insert.

The *Eco*RI and *Hin*dIII linkers were radiolabelled using T4 polynucleotide kinase and gamma [^{32}P]ATP. The reaction was carried out in a total volume of 50 µl and contained 5 µl of dephosphorylated linkers (5 µg), 5 µl of freshly prepared 10X kinase buffer (100 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP), 36 µl water, 1 µl (10 µCi) gamma[^{32}P]ATP (>3000 Ci mM^{-1}) and 3 µl (15 units) of T4 polynucleotide kinase. The mixture was incubated for 1 h at 37°C, extracted with phenol:chloroform and the linkers precipitated as described above.

To ligate the linkers the cDNA was resuspended in 5 µl of 1 x kinase buffer. Five µl of end-labelled linkers (500 ng), 0.2 µl of 22 mM ATP and 10 units (0.5 µl) of T4 DNA ligase were added and the reaction incubated at 14° C overnight (Watson & Jackson, 1986). The cDNA was then digested, first with *Hin*dIII and then *Eco*RI, to remove the excess linkers from the cDNA, leaving an appropriate cohesive terminal site at each end. The cDNA was purified by extraction, once with phenol/chloroform and once with ether, precipitated by adding 0.1 vol. 3 M sodium acetate pH 5.0, 0.6 vol. isopropanol, left at room temperature for 30 minutes and recovered by centrifugation (30 minutes at maximum speed in a microfuge). It is essential that the free linker molecules are all removed prior to ligation with the vector. The use of isopropanol selectively precipitates the cDNA leaving the linkers in the supernatant.

The pellet was resuspended in 20 µl of STE (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and applied to a column of Ultragel AcA34 (Pharmacia), which had been prepared in a siliconized, disposable, 1 ml pipette and thoroughly equilibrated with STE. The sample was eluted with STE, 50 µl fractions were collected and counted. The peak fractions, containing the cDNA, were pooled and precipitated with 2 vol. absolute ethanol. The pellet was resuspended in 20 µl H_2O and the cDNA concentration was determined by spotting 1 µl onto an ethidium

bromide plate (1% agarose, 0.5 µg/ml EtBr) and comparing the fluorescence under UV light with standards containing known quantities of phage lambda DNA.

The cDNA was ligated into the lambda insertion vector NM1149 (Murray, 1983) (Fig. 2.2), using a vector:cDNA molar ratio of 2:1. In practice, 10 µg of *Eco*RI-*Hin*dIII lambda arms (with the small *Eco*RI-*Hin*dIII central fragment removed by cesium chloride gradient centrifugation as described by Sambrook *et al.*, 1989), were co-precipitated with 100 ng of cDNA, resuspended in 7 µl of 10 mM $MgCl_2$ and incubated at 42°C for 15 minutes to allow the cohesive ends of the lambda to anneal. To this were added 1 µl 10 x ligase buffer (600 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM β-mecaptoethanol, 10 mM EDTA), 1 µl 10 mM ATP and 1 unit T4 DNA ligase, and the reaction mixture was incubated at 14°C overnight.

2.17.5. Packaging lambda DNA into the phage coat protein.

The ligated mix was packaged using a "Giga-pack plus" *in vitro* packaging extract (Stratagene). The phage suspension was titred on *E. coli* strain NM621.

2.18. *E. COLI* STRAIN NM621 USED AS A LAMBDA HOST.

NM 621 (*hsd*R-K, *hsd*M^+, *mcr*A-, *mcr*B-, *rec*D-) is derived from wild type *E. coli* K12 which produces a type 1 restriction enzyme, *Eco*K, capable of cleaving DNA containing unmodified recognition sequences (Adams & Burton, 1985). The *hsd* region in *E. coli* K12 encodes the products necessary for this restriction modification activity and consists of three genes *hsd* R, *hsd* M and *hsd* S (Sain & Murray, 1980). To allow a recombinant lambda phage containing an insert to replicate the *E. coli* host must be restriction deficient (r_k^-). The *hsd* R^- strains, such as NM621, are defective in restriction but not modification (r_k^-, m_k^+). Two additional restriction systems *mcr*A and *mcr*B have been described (Raleigh & Wilson, 1986; Raleigh, 1987). The two *mcr* systems (for modified cytosine restriction) each restrict DNA containing 5-methylcytosine. Since it has been reported that the DNA of many organisms contains 5-methylcytosine (Ehrlich & Wang, 1981) attempts to clone this type of DNA in an mcr^+ strain would result in low recombinant yield.

Strain NM621 is also *rec*D$^-$. *rec*D is one of three genes (*rec*B, *rec*C and *rec*D) which codes for Exonuclease V (*Exo*V), an enzyme with multiple activities which promotes genetic recombination. In strain NM621 the exonuclease activity has been removed (*rec* D$^-$), therefore the phage DNA can replicate by the σor rolling circle mode. This system is much more efficient for generating viable phage as (a) linear concatenates are more efficient substrates for the packaging machinery than di or multimeric circles, and (b) it does not rely on the host recombination systems to form multimeric concatenates. These factors undoubtedly enhance recombinant phage recovery compared to a *rec*D$^+$ strain.

2.19. PREPARATION OF *E. COLI* CELLS FOR PLATING WITH PHAGE.

NM621 was streaked out onto an L Broth plate and grown up overnight at 37°C. A single colony was then picked, inoculated into L Broth and used to make a glycerol stock which was stored at -70°C (Sambrook *et al.*, 1989). From the frozen glycerol stock, a loop was scratched over the surface and inoculated into a flask containing 40 ml of L Broth. The cells were grown up in a shaking incubator at 37°C to an absorbance at 650 nm of 0.5cm^{-1}. They were then harvested by centrifugation in a Beckman J22I for 30 seconds at 10000 g. The pellet was collected and resuspended in 0.4 times the original volume of 10 mM $MgSO_4$. The cells were used immediately or stored at 4°C for up to 4 days before use (Sambrook *et al.*, 1989).

2.20. PLATING LAMBDA PHAGE.

Two hundred and fifty µl of plating cells were mixed with 50 µl of phage suspension which had been diluted in SM medium (100 mM NaCl, 8mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatine) to give the appropriate concentration of plaque forming units (p.f.u.). The cells and phage were incubated for 15 minutes at room temperature in a sterile test tube. A 0.7% agarose L Broth overlay was prepared, at a temperature of approximately 45°C (hand hot) and 6.5 ml was poured into the test tube. This was immediately poured over the surface of a 10 x 10 cm petri dish, containing 20 ml of 1.5% agarose-L Broth which had been surface dried and pre-

warmed to 37°C. The 0.7% agarose overlay was spread evenly over the plate by tilting; care was taken to prevent any air bubbles being introduced into the overlay. The plates were then incubated for 8-12 h at 37°C, until plaques had formed. Phage was plated at a density which enabled individual plaques to be seen, unless otherwise stated.

2.21. ANALYSIS OF THE LIBRARY BY PLAQUE HYBRIDISATION.

The plates were placed in a refrigerator for 30 minutes after incubation. Using a template, squares of nylon filter (Amersham Hy-Bond N) were cut out which just fitted inside the square petri dishes. Three sheets of 3 MM paper were placed on the bench with clingfilm placed underneath each individual sheet of paper. The first sheet was soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH). The second sheet was soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) and the third sheet soaked in 4X SET (20X SET is 3 M NaCl, 20 mM EDTA, 0.4 M Tris-HCl pH 7.8). Each sheet of 3 MM paper was rolled with a separate 10 ml glass pipette to remove air bubbles from beneath them. A sheet of Hy-bond N was placed onto the plate using gloved hands and forceps. The filter was placed down in one movement, care was taken not to pick up or reposition and smear the plaque array. It was left for 30 seconds and, using a hypodermic needle, asymmetric holes were punched through the filter into the agarose to enable subsequent orientation of the filter to be determined. After 30 seconds the filter was removed carefully. The subsequent filters were left on the plaque array for a minute each. The filters were placed, DNA side up, for 1 minute onto a piece of 3 MM paper which had been soaked in denaturing solution, transferred for 5 minutes onto 3 MM paper which had been soaked in neutralising solution and finally transferred for 5 minutes to 3 MM paper which had been soaked in 4X SET. The filters were then placed on a piece of dry 3 MM paper and allowed to air dry. They were then oven-baked at 80°C for 2 h on 3 MM paper to fix the DNA irreversibly to the nylon. The nylon filters were stored, if required, at 4°C.

2.22. PRE-HYBRIDISATION OF FILTERS.

The filters were washed in 0.1% Triton X100 for 2 minutes at room temperature to remove any agarose, then washed in 2X SET at room temperature for 2 minutes and blot dried between 3 MM paper. They were placed in a plastic bag and pre-hybridisation solution (10 ml/filter) added. Air bubbles were removed, the bags sealed and incubated at 68°C for a minimum of 2 h.

A maximum of 6 filters for any one particular probe were placed in the same bag.

Pre-hybridisation solution

4X SET

1% SDS

0.005% Na_4PPi

0.1% Ficoll

0.1% BSA

0.1% PVP

30 µg/ml Polyadenylic acid

100 µg/ml sheared Herring sperm DNA (boiled for 10 minutes and chilled on ice before adding).

Following pre-hybridisation, the bags were cut open and most of the pre-hybridisation solution poured off, leaving 0.05 ml per cm^2 of filter in each bag. The probe (prepared as described below in Section 2.23) was boiled for 3 minutes, chilled on ice and then added to the filters. The bag was resealed and incubated at 68°C with gentle shaking for 16 to 72 h, depending on activity of the probe. Each filter was rinsed in 500 ml of 2X SET at room temperature, then washed twice at 68°C in 500 ml of 2X SET, 0.1% SDS and 0.1% Na_4PPi, for 20 minutes. This was followed by two further washes at 68°C for 15 minutes each in 0.1% SET, 0.1% SDS and 0.1% Na_4PPi. At all times during washing the filters were gently shaken to keep them separated. The filters were then blotted on 3 MM paper and wrapped in clingfilm to prevent them from drying out. They were placed in an X-ray cassette with intensifying screens and exposed to pre-flashed X-ray film at -70°C.

2.23. PREPARATION OF SINGLE STRANDED cDNA PROBES.

Single stranded cDNA probes were made using the Amersham cDNA kit, with slight modification (described in Section 2.17.1). One hundred and fifty μCi (approximately 15 μl) of α^{32}P dCTP (3000 Ci/mM) were dried down in an RNase-free siliconized Eppendorf vial under vacuum. The remaining reagents were then added as follows:

4 μl	First strand buffer (250 mM Tris-HCl pH 8.3 (42°C), 700 mM KCl, 500 mM $MgCl_2$, 50 mM DTT, 1 μl 80 mM Na_4PPi)
1 μl	HPRI (Human placenta ribonuclease inhibitor)
2 μl	dATP, dGTP, dTTP each at 10 mM
1 μl	Oligo (dT) 12 - 18 primer
1 μg	mRNA in a volume of 5 μl
5 μl	DEPC treated AnalaR water
1 μl	AMV reverse transcriptase (20 units per 1 μl)

The mRNA was denatured at 65° C for 5 minutes and cooled in ice water before addition to the first strand cDNA reaction mix.

The reagents were incubated at 42°C for 90 minutes, then 1 μl of unlabelled 10 mM dCTP was added and the incubation continued for a further 60 minutes. The RNA was then hydrolysed by adding 21 μl of 0.6 M NaOH, 20 mM EDTA and incubating for 30 minutes at 65°C. Unreacted nucleotides were removed by chromatography on Sephadex G50 (Sambrook *et al.*, 1989). The appropriate fractions, containing the cDNA probe, were counted in the scintillation counter, using Cerenkov counting, and pooled. If two probes were to be used with differing specific activities the amount of probe added to each bag of filters was kept the same. This allowed the quantitative comparison of replica filters. Before placing each probe into the bag it was boiled for 3 minutes and then cooled on ice, to ensure complete denaturation.

2.24. SUBTRACTIVE HYBRIDISATION.

This procedure used hybridisation to remove common sequences between ss cDNA probes, thus allowing only up-regulated sequences to remain. ss cDNA probes were prepared as described in section 2.23. The probe and driver mRNA were heated to 65°C in a siliconized Eppendorf vial for two minutes and then centrifuged at maximum speed in a microfuge for 5 minutes, to remove any oligo (dT)-cellulose present in the mRNA. The probe and driver were then transferred to a fresh 1.5 ml Eppendorf vial and precipitated by adding one-tenth volume 3 M sodium acetate pH 4.0 and 2.5 volumes of ethanol and incubating for 10 minutes on ice. The precipitate was collected by centrifugation at maximum speed for 5 minutes in a microfuge. The supernatant was removed, the pellet dried at room temperature under vacuum, resuspended in 3 µl of water and transferred to a fresh Eppendorf vial. The original tube was washed out with a further 3 µl of water, to remove any remaining nucleic acid, and the wash added to the second Eppendorf vial. Four µl of 2.5X phosphate hybridisation buffer (HP) [1XHP = 120 mM sodium phosphate pH 6.8, 820 mM NaCl, 10 mM EDTA, 30 µg/ml polyuridylic acid] was added to the pooled washes and overlaid with ~200 µl of mineral oil. This was then heated to 95°C for two minutes and incubated at 68°C, for 24-36 h. The sample was recovered and unhybridised cDNA separated from hybrid and driver mRNA by hydroxyapatite chromatography.

2.25. HYDROXYAPATITE COLUMNS.

2.25.1. Preparation of DNA grade Hydroxyapatite.

Approximately 0.5 gm of Hydroxyapatite (HAP) was added to 10 ml of PB40 (40 mM sodium phosphate pH 6.8, 0.05% SDS), shaken and allowed to settle. The milky supernatant was removed. This process was repeated and the HAP resuspended in PB40. The binding capacity is ~100 µg DNA per 0.5 g of HAP (Britten *et al.*, 1974).

2.25.2. Preparation of the HAP column.

The column was constructed from a 5 ml and 1 ml disposable sterile syringe. The 5 ml syringe had two holes made in its side with the tip of a hot Pasteur pipette.

The nozzle of the syringe was also cut off. Into the 5 ml syringe a 1 ml syringe was placed, the nozzle of the 1 ml syringe going through the hole made by removing the 5 ml syringe nozzle and this was sealed with the hot blade of a scalpel. At the other end the rubber tip from the plunger of the 5 ml syringe was pushed over the 1 ml syringe. The two holes in the side of the 5 ml syringe had yellow Gilson tips placed over each, being melted into place to create two nozzles. This resulted in a 1 ml column with a 5 ml water jacket around it. The jacket was connected to a 60°C circulating water bath and run for 30 minutes to check for leaks.

The HAP was added to the column, allowed to settle to form a packed bed volume of 500 µl, then equilibrated with 4 column volumes of PB40. The column was maintained at 60°C throughout its use.

2.25.3. Subtraction of double stranded DNA from the probe.

Following hybridisation, the mixture of probe and driver mRNA was added to the HAP column after removal of the oil layer. Sometimes, 500 µl of PB40 was added and the mixture heated to 68°C to aid separation of the oil layer. The aqueous fraction was loaded to the column. The sample was then allowed to pass through the column. The eluate was passed through the column a second time if more than 5% of the radioactivity failed to bind to the HAP. The column was then washed with six changes of 500 µl PB40 (0.04 M sodium phosphate pH 6.8, 0.05% SDS). The single stranded DNA fraction was then eluted with eight changes of 400 µl PB120 (0.12 M sodium phosphate pH 6.8, 0.05% SDS). Finally, the mRNA:DNA hybrid fraction was then eluted with six changes of 400 µl PB400 (0.4 M sodium phosphate pH 6.8, 0.05% SDS). Any remaining radioactivity was flushed from the column by heating the water jacket to 90°C.

The fractions were then counted using Cerenkov radiation and the appropriate fractions pooled (Britten *et al.*, 1974).

2.25.4. Treatment of material from the subtraction to produce a probe

To the ssDNA 0.1 vol. 3 M sodium acetate pH 5.0 and 2.5 vol. ethanol were added, the mixture was left on ice for 15 minutes and the precipitate collected by centrifugation in a microfuge for 30 minutes at maximum speed. The supernatant was

removed, and checked to ensure that it contained no radioactivity. The pellet in the Eppendorf vial was dried briefly on the bench and resuspended in 200 µl of water. The resuspended probe was then centrifuged at 'low' speed (6500 r.p.m.) through a 0.22 µm nylon membrane in a microfuge. The filter was checked for radioactivity; if any was present the filter was washed with a further 200 µl of water. The probe was either used directly to hybridise to plaque arrays or subjected to a second round of subtraction to further reduce the percentage of common messages. To do this the phosphate was first removed from the ssDNA fraction eluted from the HAP column. The volume of the probe suspension was reduced by adding 1 ml of butanol, shaking and then removing the butanol upper phase. This was repeated until the volume of the aqueous phase was reduced to 250 µl. Any remaining butanol was then extracted by adding an equal volume of chloroform, shaking and then removing the lower chloroform phase. The sample was heated to 37°C for a few minutes to drive off the chloroform and equilibrated with TE (10 mM Tris-HCl, 1 mM EDTA) pH 8.0, 0.05% SDS by gel filtration on Sephadex G50, as described by Sambrook *et al.* (1989).

2.26. RAPID SMALL SCALE ISOLATION OF BACTERIOPHAGE LAMBDA DNA.

This was carried out as described by Sambrook *et al.* (1989). A single plaque was picked, using the tip of a Pasteur pipette, and placed in an Eppendorf vial containing 1 ml of SM (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% (w/v) gelatine) and a drop of chloroform, and left at 4°C for 4-6 h for the phage to diffuse out of the agarose plug into the buffer. One hundred µl of the phage suspension were mixed with 100 µl of plating cells and left at 37°C for 20 minutes in a sterile test tube. Four ml of hand hot molten 0.7% L Broth agarose were then added and immediately poured onto a pre-warmed (37°C) 1.5% L Broth agarose plate. The overlay was spread evenly over the petri dish and incubated overnight at 37° C. At this stage, lysis was almost confluent. Five ml of SM were added to the plate and left for 1-2 h gentle shaking at room temperature. The supernatant, containing the phage, was then collected by centrifugation at 8000 g for 10 minutes at 4°C in a Beckman

J21 rotor. The supernatant was removed and treated with RNase A and DNase I, each at a final concentration of 1 μg/ml, for 30 minutes at 37°C. An equal volume of 20% PEG 6000, 2 M NaCl in SM was added and the phage incubated at 0°C on ice for 1 h. The precipitated phage were harvested by centrifugation at 10000 g for 20 minutes at 4°C. The supernatant was poured off, and the tube inverted over a paper towel to drain. The pellet was resuspended in 0.5 ml of SM, transferred to an Eppendorf vial and centrifuged at maximum speed (13000 r.p.m.) in a microfuge for 2 minutes at 4°C, to remove debris. The supernatant was then transferred to a fresh Eppendorf vial, 5 μl of 10% (w/v) SDS and 5 μl 0.5 M EDTA pH 8.6 were added and the phage incubated at 68°C for 15 minutes. The mixture was then extracted, once with phenol, once with phenol/chloroform (1:1) and then with chloroform, in order to separate the phage proteins from the DNA. The DNA was precipitated by adding 1 volume of isopropanol and incubating at -70°C for 20 minutes. The precipitate was collected by centrifugation as above for 20 minutes and the DNA pellet washed twice with 70% ethanol, allowed to dry under vacuum at room temperature, and resuspended in 50 μl of TE buffer (pH 8.0).

To isolate the cDNA insert from the recombinant phage DNA, 10 μl of DNA was digested with 2 units each of *Eco*RI and *Hin*dIII for 2 h at 37°C to release the insert. The digests were electrophoresed on a 1.5% low melting point TAE (40 mM Tris-acetate, lmM EDTA) agarose gel and stained with ethidium bromide. The bands were visualised on a UV transilluminator and the desired DNA band was cut out with a razor blade and transferred to a pre-weighed 1.5 ml Eppendorf vial. The vial was reweighed and the weight of the gel slice determined. The DNA was purified from the gel slice using a Gene Clean II kit (supplier, Bio 101). The gel slice was broken up using a needle, and 3 vol. of NaI stock solution added. The vial was incubated at 45-55°C until the gel slices had dissolved (about 3-4 minutes). Five μl of Glassmilk was added to the solutions containing 5 μg or less of DNA. An additional 1 μl for each 0.5 μg of DNA above 5 μg was added. The Glassmilk and DNA were gently mixed and placed on ice for 5-10 minutes, with gentle agitation every 1-2 minutes to prevent the Glassmilk from settling out. The silica matrix with the bound DNA was

pelleted by centrifugation at high speed in a microfuge for 5 seconds. The NaI supernatant was removed and the pellet washed three times in NEW buffer. This involved resuspending the pellet with 700 µl of NEW buffer, spinning for 5 seconds to form a pellet, discarding the supernatant and repeating the process. On the final wash the pellet was resuspended in 20 µl TE buffer and incubated for 2-3 minutes at 45°C to 55°C. This eluted 80% of DNA from the silica matrix. The suspension was then centrifuged for 30 seconds and the eluted DNA collected in the supernatant. This wash procedure was repeated a second time yielding a further 10%-20% of the DNA. A third wash was also used occasionally, though this only yielded a further 1% of the DNA.

2.27. *IN VITRO* LABELLING OF DNA WITH ^{32}P.

DNA was labelled by the random-priming method (Feinberg & Vogelstein, 1983). The DNA (prepared as in section 2.26) was first denatured by boiling for 5 minutes and then cooling on ice for 15 minutes. The reagents were added to a 1.5 ml Eppendorf vial in the order below and incubated overnight at room temperature.

30 µl	water
10 µl	Reaction Mix (see below)
2 µl	nuclease-free BSA (10 µg/ml)
2-8 µl	DNA (containing 5-25 ng)
3µl	(30 µCi) α^{32}P - dCTP (3000 Ci/mmole)
1 µl	(2-3 units) Klenow fragment

Reaction Mix

Stock solutions were prepared as below, and stored at -20°C.

Solution A 1 ml 1.25m Tris-HCl pH 8.0, 125 mM $MgCl_2$

18 µl β-mercaptoethanol

5 µl 100 mM each dATP, dTTP, dGTP

Solution B 2 M HEPES adjusted to pH 6.6 with 4 M NaOH

Solution C Hexadeoxynucleotides, 5000 A260 units in 556 µl TE (Pharmacia)

Shortly before use, the final Reaction mix was prepared as below.

Solution A 200 µl

Solution B 500 µl

Solution C 300 µl

After incubation unincorporated nucleotides were separated from DNA by chromatography with Sephadex G50. Fractions containing the DNA were pooled. Usually 80% of the added radioactive label was incorporated into the DNA.

DNA labelled as above was denatured by adding one-tenth volume of 3 M NaOH and leaving at room temperature for 5 minutes. The probe was then neutralised by adding one-fifth (original) volume of 1 M Tris-HCl pH 7.0, followed by one-tenth (original) volume of 3 M HCl.

2.28. ELECTROPHORESIS OF DNA IN NON-DENATURING AGAROSE GELS.

The appropriate amount of agarose was suspended in 1X TBE or TAE buffer and heated in a microwave oven to dissolve the agarose. The mixture was then allowed to cool to 50°C, poured into the gel former with comb in place and allowed to set. The set gel was placed into the gel tank which had already been filled with 1X TBE or 1X TAE buffer (the same as the gel) and run at 100 volts. The samples were loaded in 10X loading buffer. The gel was stained by soaking in 0.5 µg/ml EtBr and examined at 302 nm in a transilluminator.

1X TBE	9 mM Tris-borate pH 7.0
	89 mM Boric acid
	2 mM EDTA
1X TAE	40 mM Tris-acetate
	1 mM EDTA
1X DNA loading buffer	0.041% bromophenol blue
	0.041% xylene cyanol
	2.5% ficoll 400
	made up in H_2O

2.29. SOUTHERN BLOTTING.

Southern blotting was carried out as described by Sambrook *et al.* (1989). The gel was placed in a shallow tray, covered completely with denaturing solution (1.5 M NaCl, 0.5 M NaOH) and left for two periods of 30 minutes, replacing the denaturing solution between treatments. The denaturing solution was replaced with neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) and left for two periods of 30 minutes, changing the solution between treatments. The gel was gently shaken during these washes. After neutralising, excess liquid was blotted off using tissues. A piece of nylon membrane (Hy-bond N, Amersham) was cut to exactly the same size as the gel to be blotted and wetted with distilled water followed by 2X SSC (20X SSC is 3 M NaCl, 0.3 M Na_3 citrate) pH 7.0. A piece of 3 MM paper was then cut to be exactly the same width as the gel but longer, enabling it to form a wick between the buffer reservoir and the transfer apparatus. This wick was placed under the gel and used to transfer the gel onto the bridge apparatus. The bridge was then placed in the buffer reservoir with the ends of the wick dipping into the reservoir. The nylon membrane was placed carefully onto the gel. On top of the nylon membrane were placed three layers of 3 MM paper cut to exactly the same size as the nylon membrane. On top of this were placed 4-5 cm of absorbent towels cut to the same size as the nylon membrane or a little smaller. On top of all of this was placed a 1 kg weight. 20X SSC was added to the buffer reservoir.

The transfer of DNA was allowed to continue overnight. After blotting, the position of the gel wells were marked on the nylon membrane with a blue ballpoint pen. The membrane was removed from the gel,washed in 2X SSC and then allowed to air dry on 3 MM paper. The nylon membrane was then sandwiched between 3 MM paper and baked for 2 h at 80°C to bind the DNA irreversibly to the membrane.

2.30. NORTHERN BLOTTING.

RNA was separated in an agarose-formaldehyde denaturing gel (Sambrook *et al.*, 1989, modified). After electrophoresis the gel was transferred directly to the blotting apparatus and blotted to nylon filters exactly as described above for Southern blotting.

2.30.1. Electrophoresis of RNA in denaturing agarose gels.

A 1.3% agarose gel was cast by mixing 2.08 gm of agarose in 116 ml of water. This was heated in a microwave oven to melt the agarose and then cooled to 60°C in a water bath. Sixteen ml of freshly made 10X MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA) pH 7.0 and 27.6 ml of 37% formaldehyde solution were then added in the fume cupboard, and the gel poured, with the comb in place. The gel, when set, was placed in the gel tank containing 1X MOPS buffer.

The samples of RNA to be loaded onto the gel were prepared as follows. An equal volume of formamide sample buffer (100 µl 10X MOPS, 200 µl deionised formamide, 120 µl formaldehyde) was mixed with the RNA sample to give a final volume of 20 µl containing 2-3 µg poly A^+ RNA or 20 µg of total RNA. This was heated to 60°C for 5 minutes and then quick cooled on ice. One-quarter volume of FDE buffer (0.5 ml of 0.2 M EDTA pH 7.0, 0.2 g Ficoll type 400, 0.01% Bromophenol blue, all made up to 1 ml with water) was added and the sample loaded onto the gel, which was then eletrophoresed at 40 mA until the dye front had migrated three-quarters of the length of the gel.

2.30.2. Staining RNA.

RNA gels or nylon filters were stained with Toluidine Blue (0.2% Toluidine Blue, 0.4 M sodium acetate and 0.4 M acetic acid). The gel or filter was equilibrated

with the stain, in the case of filters for about 5 minutes, and for gels, depending on thickness, 2-3 hours. The gel or filter was then destained with water. Once destained, the RNA bands remained visible for a number of weeks, though they gradually faded with time. The filters were kept in water, gels were dried down if required.

2.31. HYBRIDISATION OF SOUTHERN AND NORTHERN BLOTS WITH RANDOM PRIMED PROBES.

The nylon filters, with bound nucleic acid, were wetted evenly in 2X SSC and then placed in a plastic bag and pre-hybridisation solution (a minimum of 1 ml for 10 cm^2 of membrane was used) poured in.

Pre-hybridisation solution

Stock solutions	Final concentration	Volume for 100 ml solution
20X SSC	5X	25 ml
Formamide	50%	50 ml
Ficoll	0.1%	0.1 g
PVP	0.1%	0.1 g
BSA	0.1%	0.1 g
10% SDS	0.1%	1 ml
5% $NaPP_i$	0.005%	100 µl
10 mg/ml Herring Sperm DNA	200 µg/ml	2 ml
600 µg/ml polyadenylic acid	15 µg/ml	250 µl
H_20		to a final volume of 100 ml.

The Herring Sperm DNA was boiled for 5 minutes and snap cooled to denature it before adding it to the pre-hybridisation solution. The filters were pre-hybridised for 4 - 15 h to overnight at 42°C with gentle shaking. The bags were

then cut open and the denatured random-primed probe added. The filters were probed in a minimum of 0.05 ml pre-hybridisation solution per cm^2 of filter.

After hybridisation for 18 h at 42°C the filters were washed twice for 15 minutes in 500 ml 2X SSC, 0.1% SDS, at 60°C for Northern blots and at 67°C for Southern blots, and then again twice in 0.1% SSC, 0.1% SDS at the same temperatures respectively.

The damp filters were then wrapped in clingfilm and exposed to a preflashed X-ray film in the presence of intensifying screens, at -70°C.

The probes were sometimes re-used, by heating to 80°C for 10 minutes and then snap cooling.

2.32. RE-USE OF FILTERS.

2.32.1. Removal of probe from Southern blots.

Filters were re-used by removing the hybridised probe and hybridising with a different one. The filters were not allowed to dry completely at any time as this binds the probe irreversibly.

The filter was immersed in 30 mM NaOH and incubated at room temperature with gentle agitation for 10 minutes. It was then immersed in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) and incubated at room temperature for 10 minutes, with gentle shaking. The wash was repeated twice and the damp filter wrapped in cling film and exposed to X-ray film to verify that the probe had been removed (Mason & Williams, 1987).

2.32.2. Removal of probe from Northern blots.

The filter was not allowed to dry out at any time. It was placed in a tray containing boiling 0.1% SDS and left until this cooled. This step was then repeated. The filter was removed and exposed to X-ray film to confirm that the probe had indeed been removed (Mason & Williams, 1987).

RESULTS

3.1. SYMPTOM EXPRESSION AND THE SPREAD OF POTATO VIRUS X STRAINS IN THE POTATO CULTIVARS KING EDWARD AND PENTLAND IVORY.

Virus replication and symptom development induced by Group 2 and 3 strains of PVX were examined in the potato cvs. Pentland Ivory (*nx Nb*) and King Edward (*Nx nb*). The aim of the experiments was to establish those PVX/Potato pairs which would be most suitable for an investigation into the molecular basis of resistance and localisation. An ideal strain/cultivar pair should have the following properties:

1. Localisation of PVX to the inoculated leaf only in the resistant potato cultivar.
2. The production of many local lesions on the inoculated leaf in the resistant potato cultivar.
3. Systemic infection of susceptible potato cultivars with the production of obvious mosaic symptoms.

Details of the three Group 2 strains and three Group 3 strains are given in section 2.3. Each strain was initially passaged once on *N. edwardsonii*. All strains induced strong symptoms (Fig. 3.1).

Infected *Nicotiana* leaves were homogenised and then inoculated onto both Pentland Ivory and King Edward plants. These inoculations were carried out simultaneously to allow the observation of both the resistant hypersensitive response and the susceptible systemic mosaic infection. The potato plants were grown as described in Materials and Methods (Section 2.2) until they attained a height of 15cm.

Fig. 3.1. The development of symptoms occurring 14 days after inoculation of *Nicotiana edwardsonii* plants with different strains of PVX.

Plants were inoculated with either KEBRS, KEBX, 2PBI, Gp3RS, XN or 3PBI and grown at 15-20°C. Note the quite different symptoms that have developed as a result of systemic infection by the different PVX strains.

KEBRS

Gp3RS

KEBX

3PBI

2PBI

XN

The temperature was then reduced to 10°C to optimise PVX lesion development (Adams *et al.*, 1986b). After 5 days at 10°C, the plants were inoculated with the appropriate strain of PVX on the first fully expanded leaf above the soil. An inoculum of 1:10 (w/v), 1 gm systemically infected tobacco leaves ground in 10 ml water, was used. For a period of up to 42 days from inoculation the plants were observed visually and the virus coat protein concentration was measured using ELISA. Samples from each time point used for ELISA purposes were generated from a single plant. These observations allowed the progress of PVX infection to be monitored. For each treatment twenty plants were studied. Control batches included healthy (uninoculated) as well as mock-inoculated plants.

3.1.1. The Infectivity of Group 2 Strains.

3.1.1.1. Strain KEBRS inoculated onto Pentland Ivory (*nx Nb*).

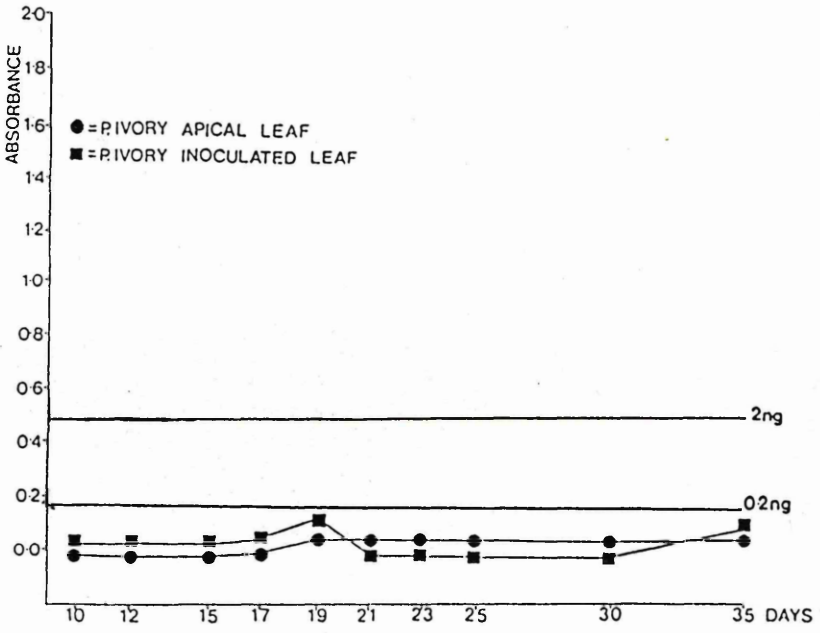
Upper leaves of Pentland Ivory plants inoculated with PVX strain KEBRS on a lower leaf remained healthy in appearance and did not show any visual symptoms of necrosis or mosaic infection. ELISA tests on extracts from these upper leaves did not detect any virus at any time throughout the duration of the experiment (Fig. 3.2). Two to three days after PVX inoculation, the inoculated leaf developed slight light-brown necrosis; this was probably a result of inoculation damage since similar changes were seen in the mock- inoculated plants. The first local lesions were obvious 17 days after inoculation, averaging five lesions on each inoculated leaf. These lesions had typically a diameter of approximately 1 mm, with a chlorotic halo around them (Fig. 3.10). Over the subsequent 18 days, the numbers of lesions did not increase. Lesion size, however, increased to approximately 2-3 mm by day 25 and then stayed constant up to the time of leaf senescence, around 40-45 days after inoculation. ELISA detected a small amount of virus (0.07 μg PVX/gm leaf tissue) in the inoculated leaf of one plant 19 days after inoculation (Fig. 3.2) showing that limited viral replication may have occurred. However, since these low levels are very near the limit of detection with this ELISA method, this conclusion must be tentative.

Fig. 3.2. The time course showing the appearance of viral coat protein in leaves taken from Pentland Ivory and King Edward plants (grown at 10°C) inoculated with Group 2 PVX-KEBRS.

The viral content of each leaf sample is expressed as absorbance at 405 nm from a Titertek Multiskan MC plate ELISA reader. The absorbance of the standards, shown as horizontal lines on each graph, are given as ng of PVX in a 200 µl sample. **Viral levels have been converted from these absorbances to µg virus/gram leaf tissue in the text.** The sensitivity of the ELISA method is ~1 to 10 ng/ml. Leaf samples of 50 mg, consisting of 5 leaf discs cut using a No.5 cork borer, were homogenised in 5 ml of sample buffer giving a dilution of 10^{-2}. The absorbance figures on the graph represent the average absorbance value of two replica 200 µl ELISA samples for each leaf sample time point. Samples were analysed from the inoculated leaf (■) and the uninoculated apical leaf (●). The data at each time point came from a separate plant.

Days = Days after inoculation on a lower leaf with PVX-KEBRS.

Absorbance = The absorbance of a 200 µl sample at 405 nm.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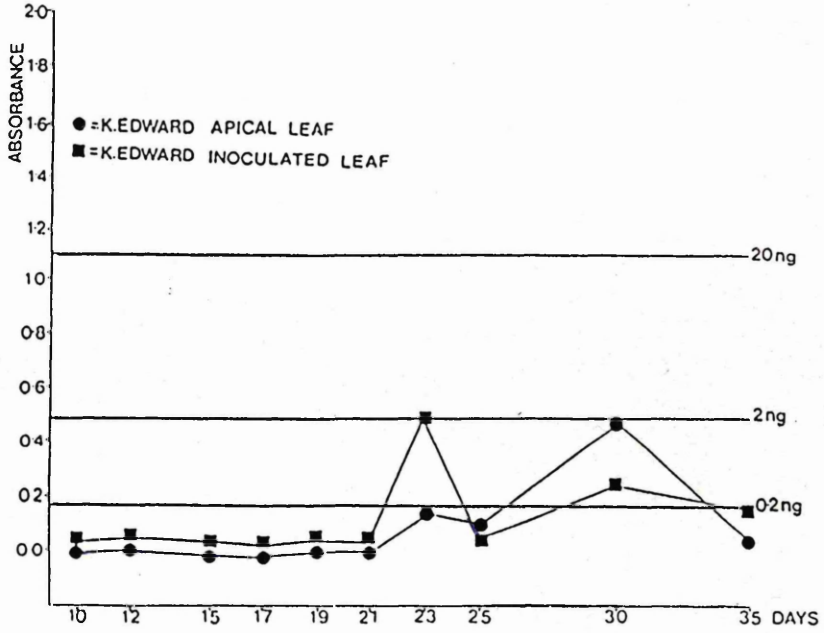
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Fig. 3.3. The effect of the inoculation of King Edward with PVX strain KEBRS.

(a) Two leaves showing systemic mosaic symptoms as a result of KEBRS infection. Note the strong chlorosis on the leaves.

(b) A King Edward potato plant (grown at 10°C) showing systemic infection 30 days after inoculation of a lower leaf with KEBRS.

a

b

3.1.1.2. Strain KEBRS inoculated onto King Edward (*Nx nb*).

King Edward plants inoculated with KEBRS developed a slight necrosis on their inoculated leaves 2-3 days after inoculation, as a result of carborundum damage. The necrosis due to inoculation damage was more severe than that which occurred in Pentland Ivory plants as a consequence of inoculation. In extreme cases the inoculated leaf would collapse completely as a result of this damage. (This finding was common to all later experiments). By day 7, chlorotic patches had developed and they persisted for the duration of the experiment. Ten days after inoculation the first light mosaic symptoms developed in apical leaves. By day 20 these mosaic symptoms had become more intense, had spread over the whole plant and they persisted for the duration of the experiment (Fig. 3.3). Local lesions were never observed on either inoculated or non-inoculated leaves. ELISA showed that systemic spread had occurred (Fig. 3.2). Detectable increases in viral titre, both in the inoculated and apical leaves, were preceded by symptom development. Very low, but detectable, levels of virus were identified in the inoculated leaf from day 12, the level remained low until day 23 then increased significantly to 1μg PVX/gm leaf tissue. From day 23, virus levels remained high for the remainder of the experiment. The apical leaves also showed detectable levels of virus from day 23 (though virus was not detectable before this time). The virus level in the apical leaves increased from day 23 thereafter until the end of the experiment, achieving a maximal level of 1 μg PVX/gm leaf tissue. During this period the severity of the visual mosaic symptoms increased. Mosaic symptoms appeared to precede the detection of virus in apical leaves by ELISA.

3.1.1.3. Strain KEBX inoculated onto Pentland Ivory (*nx Nb*).

Pentland Ivory reacted to inoculation with KEBX by developing necrotic local lesions 13 days after inoculation, each inoculated leaf showing an average of 10 lesions. The lesions ranged in size from a few cells to a maximum of 0.5 mm in diameter, with a chlorotic halo around the necrotic centre. The lesion size had increased to around 2 mm in diameter within 10 days, and continued to enlarge to a maximum diameter of about 4 mm by day 37 after inoculation. Virus was detected by

ELISA in excised PVX lesions from inoculated leaves at 22 days after inoculation, but levels decreased from 250 ng to 50 ng PVX/gm leaf tissue by day 27, and remained at this lower level in later samples (data not shown). Green leaf tissue taken from around the lesions showed no detectable virus content. The site of viral replication appears to be restricted to the lesion.

Leaves in the apical region of the plant appeared completely healthy throughout the experiment with no mosaic symptoms developing. No virus accumulation was detected by ELISA in these apical leaves (Fig. 3.4). Occasional local lesions did, however, develop in leaves above the inoculated leaf 37-42 days after inoculation. A small amount of virus could be detected by ELISA in some of these lesions with a maximum of 0.5 µg PVX/gm leaf tissue (data not shown). This suggests that the localisation of this viral strain to inoculated leaves was not 100%, and that small amounts of this strain could apparently spread systemically and undergo replication.

3.1.1.4. Strain KEBX inoculated onto King Edward (*Nx nb*).

The inoculated leaves of these plants developed severe necrosis 3-5 days after inoculation, making them impossible to use for ELISA. This was a response to abrasion of the leaves, as a similar response was also seen in the mock-inoculated control plants. It appeared that King Edward plants were more susceptible to mechanical damage than Pentland Ivory plants. The plants became systemically infected with KEBX and by day 37 had developed light mosaic symptoms over the whole plant. No necrotic lesions were observed at any time on any leaves. ELISA detected 5 µg PVX/gm leaf tissue in apical leaves 22 days after inoculation reaching a plateau of around 6 µg PVX/gm leaf tissue by 37 days after inoculation (Fig. 3.4). With this strain of PVX, symptoms of systemic infection were light and developed slowly. However, the virus rapidly reached high levels in infected tissue.

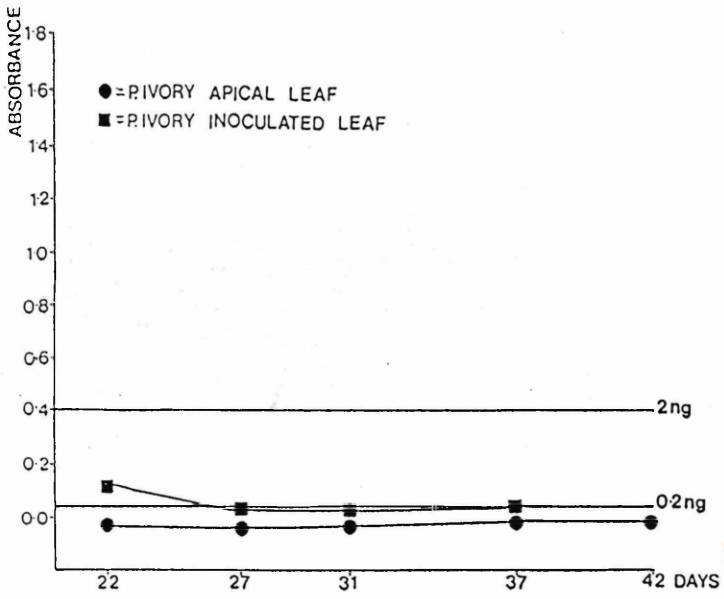
Comparing the two Group 2 strains, KEBRS and KEBX, (which were both named KEB when supplied) has demonstrated that these two strains are quite clearly different. When they were tested on the susceptible potato cultivar King Edward the symptoms and the rate of viral accumulation was quite different for these two viral

Fig. 3.4. The time course showing the appearance of viral coat protein in leaves taken from Pentland Ivory and King Edward plants (grown at 10°C) inoculated with Group 2 KEBX.

The viral content of each leaf sample is expressed as absorbance at 405 nm from a Titertek Multiskan MC plate ELISA reader. The absorbance of the standards, shown as horizontal lines on each graph, are given as ng of PVX in a 200 µl sample. **Viral levels have been converted from these absorbances to µg virus/gram leaf tissue in the text.** The sensitivity of the ELISA method is ~1 to 10 ng/ml. Leaf samples of 50 mg, consisting of 5 leaf discs cut using a No.5 cork borer, were homogenised in 5 ml of sample buffer giving a dilution of 10^{-2}. The absorbance figures on the graph represent the average absorbance value of two replica 200 µl ELISA samples for each leaf sample time point. Samples were analysed from the inoculated leaf (■) and the uninoculated apical leaf (●). The data at each time point came from a separate plant.

Days = Days after inoculation on a lower leaf with PVX-KEBX.

Absorbance = The absorbance of a 200 µl sample at 405 nm.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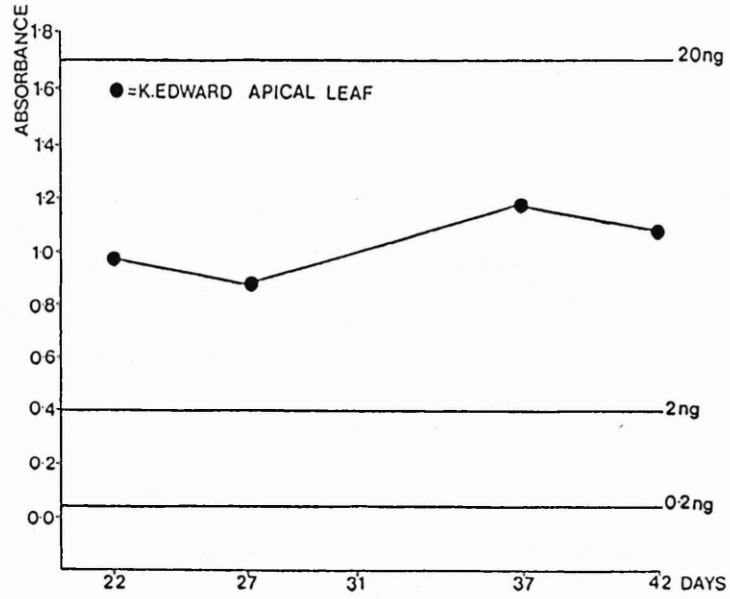
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Fig.3.5. The effect of the inoculation of Pentland Ivory with PVX strain 2PBI.

(a) A non-inoculated leaf, 28 days after the plant was inoculated with PVX strain 2PBI. The lesions average 1 mm in diameter, as indicated by arrows.

(b) A non-inoculated leaf, 33 days after the plant was infected with PVX strain 2PBI. The lesions average 2-3 mm in diameter and some lesions have coalesced, as indicated by arrows.

a

b

isolates. King Edward plants infected with KEBRS developed mosaic symptoms in apical leaves 10 days after inoculation. These symptoms developed with time into a strong mosaic. King Edward plants inoculated with strain KEBX only developed light mosaic symptoms in apical leaves some 37 days after inoculation. The time course showing the accumulation of viral coat protein, determined by ELISA, was also quite different between these PVX strains. Strain KEBX accumulated 6 μg PVX/gm of apical leaves by day 37 after inoculation, while strain KEBRS only achieved a maximum PVX coat protein concentration of 1 μg PVX/gm of apical leaf. The maximal accumulation of KEBRS was seen at 30 days after inoculation. These results highlight the need for the rigorous testing of each viral isolate even though they may be said to be identical.

3.1.1.5. Strain 2PBI inoculated onto Pentland Ivory (*nx Nb*).

Pentland Ivory plants only showed limited inoculation damage following inoculation with strain 2PBI. Fourteen days after this inoculation the first local lesions developed. They were of variable size, the largest being approximately 1 mm in diameter. These lesions gradually increased in size to approximately 3 mm in diameter by day 25, but did not increase thereafter. Fourteen days after the first lesions developed, systemic necrosis appeared on upper leaves (Fig. 3.5). Initially this consisted of many (in some cases up to 80 per leaf) new lesions of approximately 1 mm diameter. Over a period of 5 days these grew in size to 2-3 mm in diameter, some lesions coalescing to form patches of necrosis 1-2 cm in size. In severe cases up to 80% of the leaf area was covered by necrosis (Fig. 3.5). No mosaic symptoms were observed at any time during the 42-day period of the experiment. In this case, since the viral infection was so marked, ELISA measurements were not considered to be necessary.

3.1.1.6. Strain 2PBI inoculated onto King Edward (*Nx nb*).

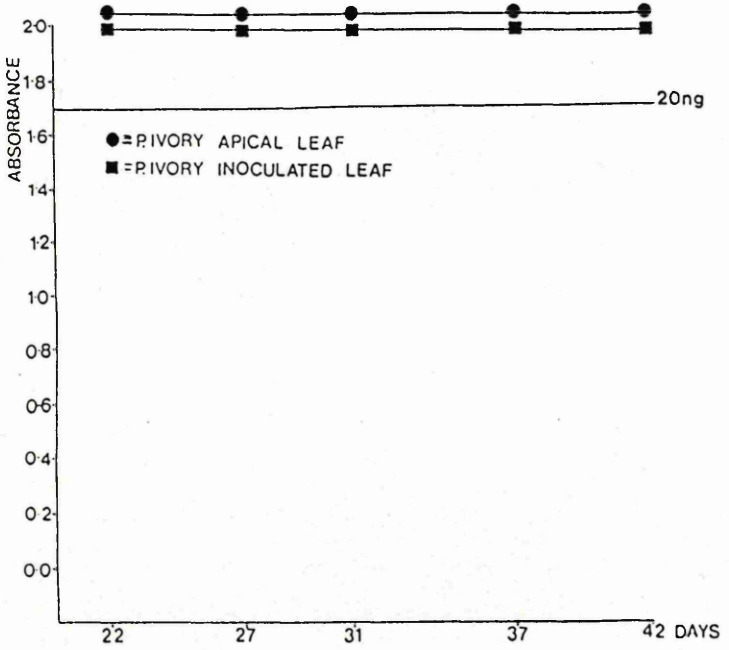
Following inoculation of King Edward with strain 2 PBI, slight damage developed on the inoculated leaves. Light systemic mosaic symptoms were first observed 14 days after inoculation, developing in isolated patches around the plant. The symptoms became progressively more severe and had spread to the whole plant

Fig. 3.6. The time course showing the appearance of viral coat protein in leaves taken from Pentland Ivory and King Edward plants (grown at 10°C) inoculated with Group 3 Gp3RS.

The viral content of each leaf sample is expressed as absorbance at 405 nm from a Titertek Multiskan MC plate ELISA reader. The absorbance of the standards, shown as horizontal lines on each graph, are given as ng of PVX in a 200 µl sample. **Viral levels have been converted from these absorbances to µg virus/gram leaf tissue in the text.** The sensitivity of the ELISA method is ~1 to 10 ng/ml. Leaf samples of 50 mg, consisting of 5 leaf discs cut using a No.5 cork borer, were homogenised in 5 ml of sample buffer giving a dilution of 10^{-2}. The absorbance of x.xxx is a figure above 2.000. Unfortunately, all Pentland Ivory samples read above 2.000. Samples were analysed from the inoculated leaf (■) and the uninoculated apical leaf (●). The data at each time point came from a separate plant.

Days = Days after inoculation on a lower leaf with PVX strain Gp3RS.

Absorbance = The absorbance of a 200 µl sample at 405 nm.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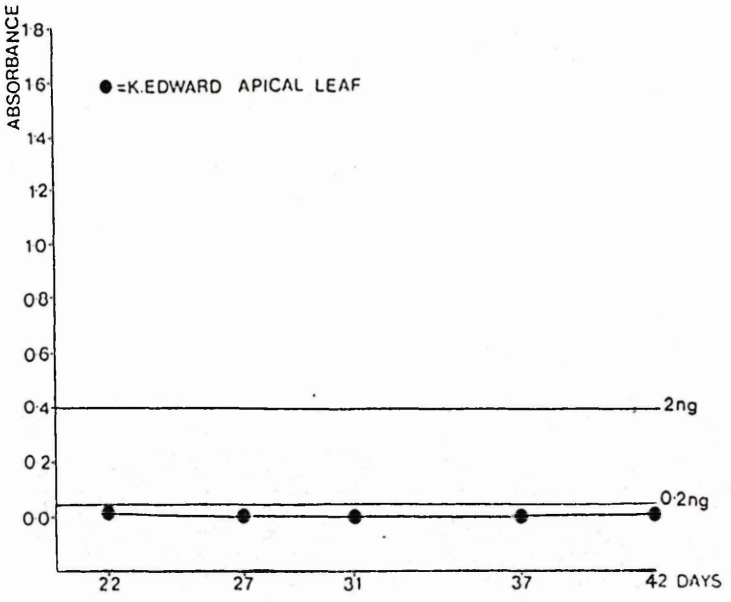
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Fig. 3.7. The effect of the inoculation of Pentland Ivory with PVX strain Gp3RS. A Pentland Ivory plant, grown at 10°C, 30 days after inoculation with Gp3RS. Note the quite obvious mosaic symptoms, compared to the control healthy plant.

CONTROL Gp3RS

by 25 days after inoculation. These symptoms persisted for the 42-day duration of the experiment, though at no time were necrotic local lesions observed. Again the strength of the response indicated that ELISA was not required to confirm the presence of the virus.

3.1.2. The Infectivity of Group 3 strains.

3.1.2.1. Strain Gp3RS inoculated onto King Edward (*Nx nb*).

Following inoculation of King Edward with strain Gp3RS severe damage developed on the inoculated leaves. As discussed in section 3.1.1.2, this cultivar is very susceptible to inoculation damage. During the 42-day period following inoculation with Gp3RS no local lesions formed, and no mosaic symptoms were observed. The plants, in fact, looked completely healthy. This observation was supported by the ELISA data, which did not detect any virus accumulation in the plant (Fig. 3.6).

3.1.2.2. Strain Gp3RS inoculated onto Pentland Ivory (*nx Nb*).

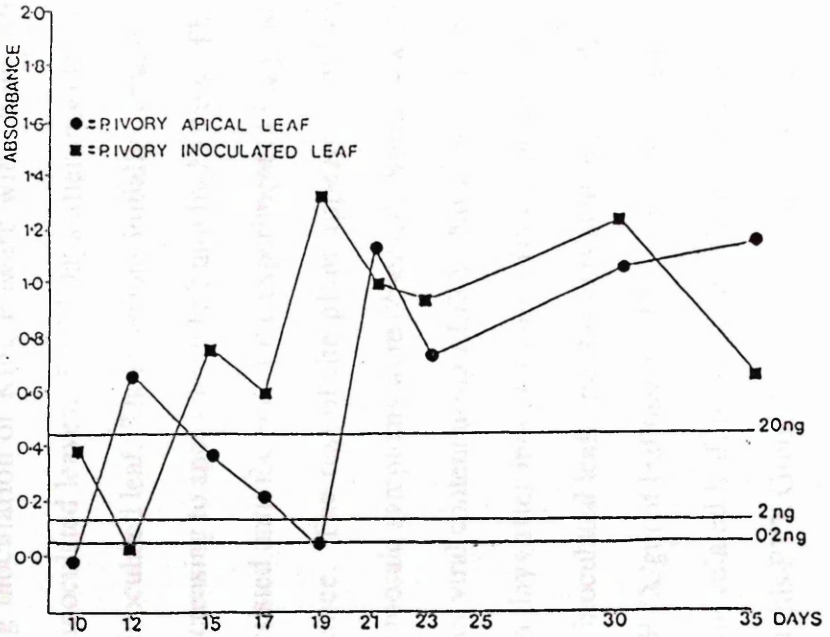
Limited damage developed on the inoculated leaves 2-3 days after Pentland Ivory plants were inoculated with strain Gp3RS. Both inoculated and uninoculated leaves showed light mosaic symptoms 22 days after inoculation. At this time, virus coat protein levels were measured using ELISA. The results showed a high level of virus in both inoculated and non-inoculated leaves (above 10 μg PVX/gm of leaf tissue) (Fig. 3.6). By day 27, the symptoms had developed into a strong systemic mosaic (Fig. 3.7) which remained for the rest of the experiment. The level of viral coat protein detected by ELISA also remained high (above 10 μg PVX/gm of leaf tissue) throughout the plant for the rest of the experiment (Fig. 3.6). Around 13 days after inoculation a few necrotic lesions of approximately 0.25 mm diameter developed on the inoculated leaf of a number of plants. These lesions gradually enlarged over the next 18 days, achieving a maximum diameter of approximately 1.5 mm. This group of plants appeared to be responding like resistant cultivars, developing a number of necrotic local lesions. However, despite some localised necrosis, the virus was obviously able to spread systemically.

Fig. 3.8. The time course showing the appearance of viral coat protein in leaves taken from Pentland Ivory and King Edward plants (grown at 10°C) inoculated with Group 3 3PBI.

The viral content of each leaf sample is expressed as absorbance at 405 nm from a Titertek Multiskan MC plate ELISA reader. The absorbance of the standards, shown as horizontal lines on each graph, are given as ng of PVX in a 200 µl sample. **Viral levels have been converted from these absorbances to µg virus/gram leaf tissue in the text.** The sensitivity of the ELISA method is ~1 to 10 ng/ml. Leaf samples of 50 mg, consisting of 5 leaf discs cut using a No.5 cork borer, were homogenised in 5 ml of sample buffer giving a dilution of 10^{-2}. The absorbance figures on the graph represent the average absorbance value of two replica 200 µl ELISA samples for each leaf sample time point. Samples were analysed from the inoculated leaf (■) and the uninoculated apical leaf (●). The data at each time point came from a separate plant.

Days = Days after inoculation on a lower leaf with PVX strain 3PBI.

Absorbance = The absorbance of a 200 µl sample at 405 nm.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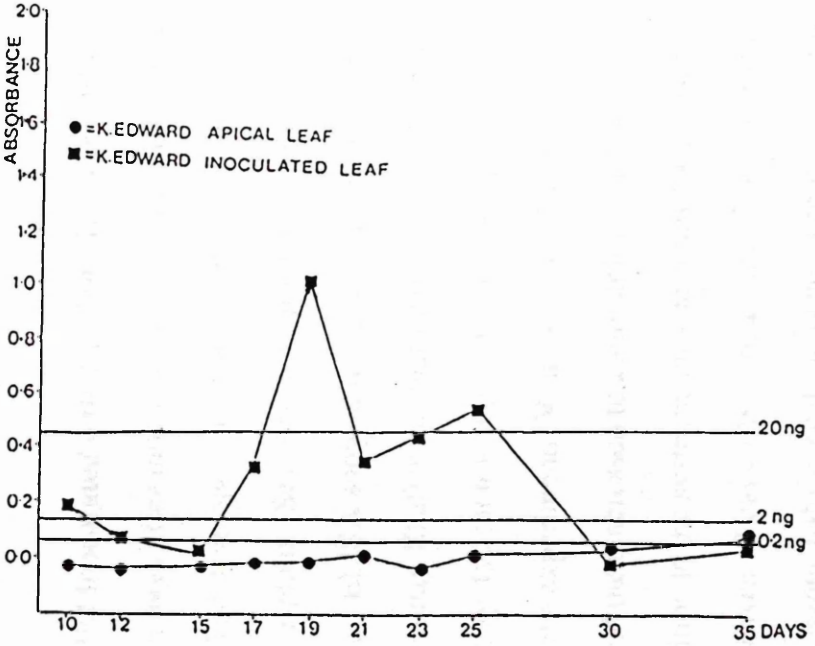
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3.1.2.3. Strain 3PBI inoculated onto King Edward (*Nx nb*).

Following inoculation of King Edward with strain 3PBI slight damage developed on the inoculated leaves. By 17 days after inoculation 1-3 lesions were observed on each inoculated leaf. These lesions initially averaged approximately 0.5 mm in diameter increasing to approximately 2 mm by 25 days. The lesions stabilised at this size and persisted until the end of the experiment (35 days after inoculation) or until leaf senescence. The rest of the plant appeared healthy throughout the experiment and no mosaic symptoms were observed. Samples were taken from apical leaves and tested for viral content using ELISA. No virus was detectable (Fig. 3.8).

Seventeen days after inoculation virus coat protein was detectable using ELISA within the inoculated leaf, and this persisted until 25 days after inoculation (averaging 0.5 µg PVX/gm of leaf tissue). Thus the virus had clearly undergone some replication in the inoculated leaf, presumably within the lesion. King Edward was thus able to restrict this PVX Group 3 strain to the inoculated leaf, although there was a short lag period before the replication and spread of the virus was restricted in the inoculated leaf.

3.1.2.4. Strain 3PBI inoculated onto Pentland Ivory (*nx Nb*).

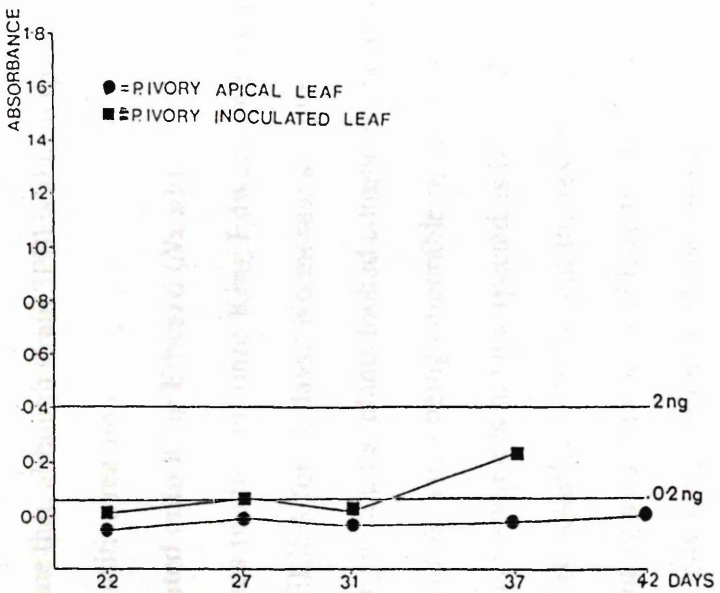
Seventeen days after inoculation of Pentland Ivory with strain 3PBI mild mosaic symptoms had developed in patches, both on inoculated leaves and around the apical region of the plant. Severity of symptoms increased up to day 23 and then remained constant. ELISA showed detectable levels of virus accumulating in the inoculated leaf from day 10 after inoculation (0.45 µg PVX/gm leaf tissue), gradually increasing up to day 19, then staying around 1.4 µg PVX/gm leaf tissue for the remaining time of the experiment. Virus was first detectable in apical leaves 12 days after inoculation and then increased to a similar level to that seen in the inoculated leaf (Fig. 3.8). In addition to the systemic mosaic, a few local lesions developed on some of the inoculated leaves 10 days after inoculation. These lesions were restricted to a few cells with a chlorotic halo around each one. The lesions continued to grow, with the largest being approximately 2 mm in diameter 20 days after inoculation. Each

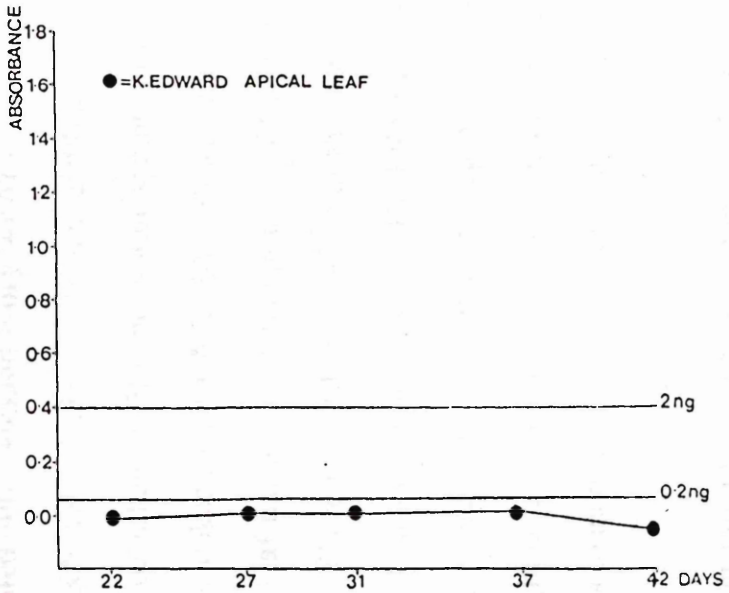
Fig. 3.9. The time course showing the appearance of viral coat protein in leaves taken from Pentland Ivory and King Edward plants (grown at 10°C) inoculated with Group 3 XN.

The viral content of each leaf sample is expressed as absorbance at 405 nm from a Titertek Multiskan MC plate ELISA reader. The absorbance of the standards, shown as horizontal lines on each graph, are given as ng of PVX in a 200 µl sample. **Viral levels have been converted from these absorbances to µg virus/gram leaf tissue in the text.** The sensitivity of the ELISA method is ~1 to 10 ng/ml. Leaf samples of 50 mg, consisting of 5 leaf discs cut using a No.5 cork borer, were homogenised in 5 ml of sample buffer giving a dilution of 10^{-2}. The absorbance figures on the graph represent the average absorbance value of two replica 200 µl ELISA samples for each leaf sample time point. Samples were analysed from the inoculated leaf (■) and the uninoculated apical leaf (●). The data at each time point came from a separate plant. There is no absorbance figure for the Pentland Ivory inoculated leaf 42 days after inoculation as by this time the leaf had senesced and fallen off the plant.

Days = Days after inoculation on a lower leaf with PVX strain XN

Absorbance = The absorbance of a 200 µl sample at 405 nm.


ABSORBANCE
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
●=P.IVORY APICAL LEAF
■=P.IVORY INOCULATED LEAF
2ng
0·2ng
22
27
31
37
42 DAYS


ABSORBANCE
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
●=K.EDWARD APICAL LEAF
2ng
0·2ng
22
27
31
37
42 DAYS

lesion remained constant in size after this point, although there was considerable variation in the size of individual lesions (from 0.5 to 2.0 mm).

These results indicate that, although strain 3PBI is able to spread systemically it can also trigger a hypersensitive reaction.

3.1.2.5. Strain XN inoculated onto King Edward (*Nx nb*).

PVX strain XN was inoculated onto King Edward and the plants were monitored visually and by ELISA for 42 days. No mosaic symptoms were observed and no local lesions formed; in fact, the plants looked completely healthy. This was confirmed using ELISA, with no virus being detectable in either the inoculated or apical leaves (Fig. 3.9). This response is not unexpected as King Edward is resistant to Group 3 strains of PVX (Cockerham, 1970) and the resistance response is not always accompanied by visually recognisable local lesions. Lesions may have been restricted to single necrotic cells and therefore not have been visible to the naked eye.

3.1.2.6. Strain XN inoculated onto Pentland Ivory (*nx Nb*).

When PVX strain XN was inoculated onto Pentland Ivory, a systemic host for Group 3 PVX strains (Cockerham, 1955), no mosaic symptoms or local lesions were observed and the plants looked completely healthy. However, on day 37, a virus level of 500 ng PVX/gm leaf tissue was detected using ELISA, i.e. from the inoculated leaf (Fig. 3.9). There is no ELISA data after this time point as leaf senescence had occurred by 42 days after inoculation. The PVX strain XN, used as inoculum in this experiment, had been maintained by repeated passage through tobacco for several years (Ireland & Pierpoint, 1980). This may have affected its ability to infect potato and/or to induce symptoms in this host.

3.1.3. Conclusions.

In all cases the non-inoculated leaves of control plants, either mock-inoculated or untreated, remained healthy in appearance throughout the experiment, never showing any symptoms of viral infection. The inoculated leaves of control plants in some cases developed necrotic areas as a result of inoculation damage. However, the necrosis induced by wounding was light brown in appearance, quite different to the dark brown almost black necrosis associated with PVX local lesion

development. The leaves of King Edward plants were more susceptible to inoculation damage than the leaves of Pentland Ivory plants; a result of this was that some King Edward plants suffered severe inoculation damage even though great care was taken when inoculating this cultivar.

From the ELISA data it is apparent that when several plants of a single potato cultivar are inoculated simultaneously with a particular PVX strain, parameters such as the timing of symptom expression are rather variable. Different plants develop symptoms and accumulate virus at different rates. As each sample for ELISA was generated from an individual plant, time courses of infection showed considerable scatter. However, this was not important for the purposes of these experiments as all that was required was to determine whether the virus was restricted to the inoculated leaf in the particular viral-host interaction.

Both symptom expression and ELISA data showed that PVX Group 2 strains systemically infected King Edward, and were to a greater or lesser extent localised within Pentland Ivory. The converse is true for the Group 3 strains of PVX. It is clear though, that there was considerable variation between different isolates within the same virus group; some caused severe symptoms, while others gave mild symptoms on susceptible plants. The accumulation of viral coat protein locally and systemically to the point of infection also varied between the different PVX strains with time. Moreover the extent to which the virus remained localised in inoculated leaves of resistant cultivars varied between isolates. However, even given this degree of variability these experiments did enable the selection of the best PVX:potato cultivar combinations for the subsequent experiments described in this thesis to be made.

Of the Group 2 strains, KEBRS (Table 3.1) most effectively fulfilled the criteria previously set out (section 3.1) and was therefore selected for use in the subsequent study. It was restricted to the inoculated leaf in the resistant cv. Pentland Ivory, causing obvious necrotic local lesions, and systemically infected the susceptible cv. King Edward, with the production of obvious mosaic symptoms. The other five strains were less suitable. The other Group 2 strains, KEBX and 2PBI, were both able to evade localisation to some extent in Pentland Ivory plants, moving out of the

Table 3.1. The infection characteristics of the six PVX strains studied with Pentland Ivory and King Edward plants as host.

PVX strain	Group	Locally inoculated Pentland Ivory Plants	Locally inoculated King Edward Plants
KEBRS	2	Necrotic local lesions on inoculated leaf	Mosaic systemic infection
KEBX	2	Necrotic local lesions on inoculated leaf and later a few necrotic lesions developed on upper non-inoculated leaves	Mosaic systemic infection
2PB1	2	Necrotic local lesions on inoculated leaf followed by systemic necrosis of apical leaves	Mosaic systemic infection
Gp3RS	3	Mosaic systemic infection plus a few local lesions on inoculated leaf	No symptoms
3PB1	3	Mosaic systemic infection plus a few local lesions on inoculated leaf	Necrotic local lesions on inoculated leaf
XN	3	No symptoms though a low level of virus was recorded in inoculated leaf	No symptoms

inoculated leaf and forming secondary lesions on uninoculated leaves. All Group 3 strains of virus, although restricted to the inoculated leaf in the resistant cv. King Edward, produced very variable numbers of local lesions (none, in some cases). This might have been due to the formation of local lesions which were restricted to a single cell, and would therefore have been invisible to the naked eye. As the number of local lesions has been reported (Kuc & Richmond, 1977; McIntyre & Dodds, 1979) to be related to the level of induced systemic resistance, this makes Group 3 strains potentially less suitable for use in this study. Furthermore, two of the Group 3 strains, 3PBI and Gp3RS, developed local lesions on the inoculated leaf of *Nb* hosts, although plants later became systemically infected (Table 3.1). The absence of a clear relationship between the presence of *Nx Nb* genes and localised necrosis was considered to be a potential complicating factor.

To confirm that the PVX strain KEBRS was restricted to the necrotic local lesions, electron and light microscopic studies of lesion development were undertaken.

3.2. MICROSCOPIC STUDIES OF LESION DEVELOPMENT FOLLOWING INOCULATION OF PVX-KEBRS ONTO PENTLAND IVORY.

To confirm that the PVX strain KEBRS was indeed restricted to the necrotic local lesion as indicated by ELISA, an electron microscope study of lesion development was undertaken. A record of lesion development by light microscopy was also carried out in parallel (Fig. 3.10) to the electron microscope work, to allow the comparison between ultrastructural changes and the visual observation of lesion development.

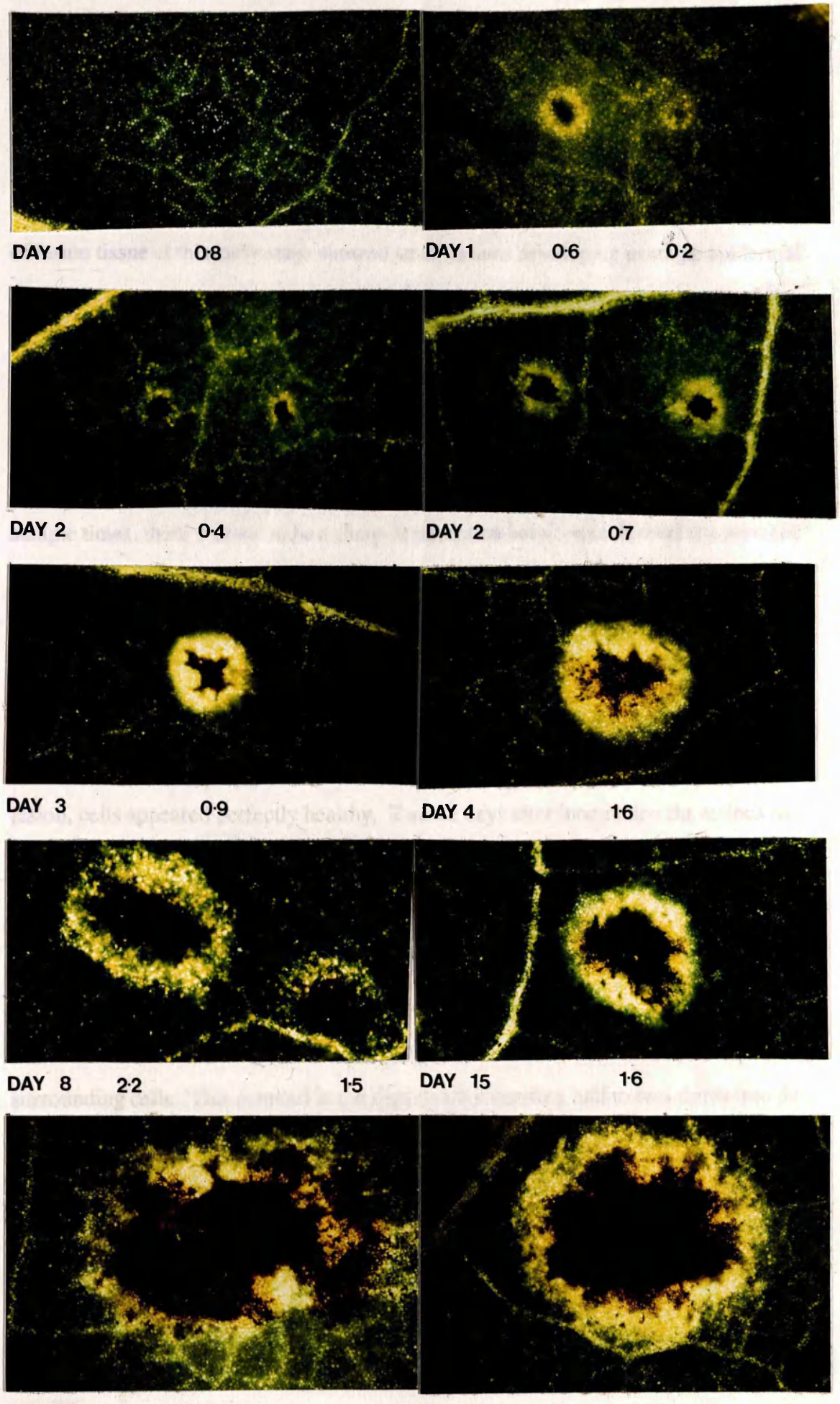
Pentland Ivory plants were grown as described in Materials and Methods (section 2.2) until they attained a height of 15 cm, at which time they were inoculated with 1:10 (w/v) dilution of *N. edwardsonii* systemically infected with KEBRS. The first lesions developed 7 days after inoculation. Lesions were collected 7, 8, 9, 10, 11, 12, 13, 14, 16, 18, 24 and 27 days after inoculation, which covered the time period from the initial collapse and necrosis of a single epidermal cell to the period when the lesion had spread into surrounding cells and become stationary, no longer enlarging. As a control, to confirm that the virus was capable of systemic infection, King Edward plants, which are susceptible to KEBRS, were inoculated in parallel.

Light microscopy identified the lesion first developing as a group of necrotic cells. Within 1-2 days a chlorotic halo of cells had developed around each lesion, and by 18-23 days after inoculation the lesion gradually enlarged to approximately 3-4 mm in diameter. At this stage the lesion stopped enlarging and remained constant in size until the leaf senesced. Throughout the experiment lesions varied in size, however the overall trend was the gradual enlargement of lesions with time up to a maximum diameter of 3-4 mm (Fig. 3.10).

For the electron microscope examination two lesions were excised and prepared at each time point. For each lesion four sections were observed. The excised regions included both zones of the lesion, the necrotic tissue and the chlorotic halo of tissue surrounding it. Healthy green leaf tissue surrounding the lesion was also included in the sample. At no time were PVX virions ever observed inside or outside the necrotic lesion. Presumably some PVX replication must have occurred within the

Fig. 3.10. Light microscopy of lesions developing on Pentland Ivory plants after KEBRS inoculation.

The lesion development on Pentland Ivory inoculated with PVX-KEBRS was followed from the earliest visible lesion until the lesions stopped enlarging. The time in days after the first visible observation of the lesion, and the lesion's diameter in mm are given under each photograph.


DAY 1 0·8
DAY 1 0·6 0·2
DAY 2 0·4
DAY 2 0·7
DAY 3 0·9
DAY 4 1·6
DAY 8 2·2 1·5
DAY 15 1·6
DAY 18 4·0
DAY 23 3·2
1mm

lesion since this could be detected using ELISA (Fig. 3.2). However, the extensive necrosis made the tissue difficult to examine under the electron microscope and no viral particles were to be seen.

Visible lesions developed 7-9 days after inoculation. Electron micrographs of lesion tissue at this early stage showed small lesions developing in single epidermal cells which had collapsed and become necrotic (Fig. 3.11). The palisade cells below the collapsed epidermal cell appeared normal with intact organelles (Fig. 3.12). Although organelles were observed when the cytoplasm first became granular in appearance (Fig. 3.13), as the lesion developed the cells became completely opaque to the electron microscope and no internal structure could be seen (Fig. 3.14). At early sample times, there seemed to be a sharp demarcation between each severely damaged opaque cell and the surrounding, apparently normal, cells. By 11 days after infection the severely damaged cells within the lesion were surrounded by a zone 2-3 cells deep, which appeared rather abnormal, with disrupted cytoplasm and organelles (Fig. 3.13). This could also be seen under the light microscope where, immediately after the appearance of the lesion, a chlorotic halo developed. Further away from the lesion, cells appeared perfectly healthy. Twelve days after inoculation the lesions had spread further into the adaxial surface, encompassing adjacent epidermal and palisade cells. In these cells the cytoplasm became disrupted and granular in appearance, with the eventual breakdown of organelles and the collapse of the cell itself (Fig. 3.13). The collapse of necrotic cells caused the formation of a depression in the adaxial surface of the leaf, starting with a single epidermal cell and eventually extending into surrounding cells. This resulted in the depression extending half to two-thirds into the leaf by the time the lesion had fully developed. The progression from the granular opaque cytoplasm of cells in the centre of the lesion (Fig. 3.14) through a layer of 2-3 abnormal cells with disrupted cytoplasm (Fig. 3.15) to healthy cells (Fig. 3.16) was maintained as the lesion developed, enlarging and spreading into healthy tissue. Lesion enlargement eventually ceased approximately 18 days after its first appearance.

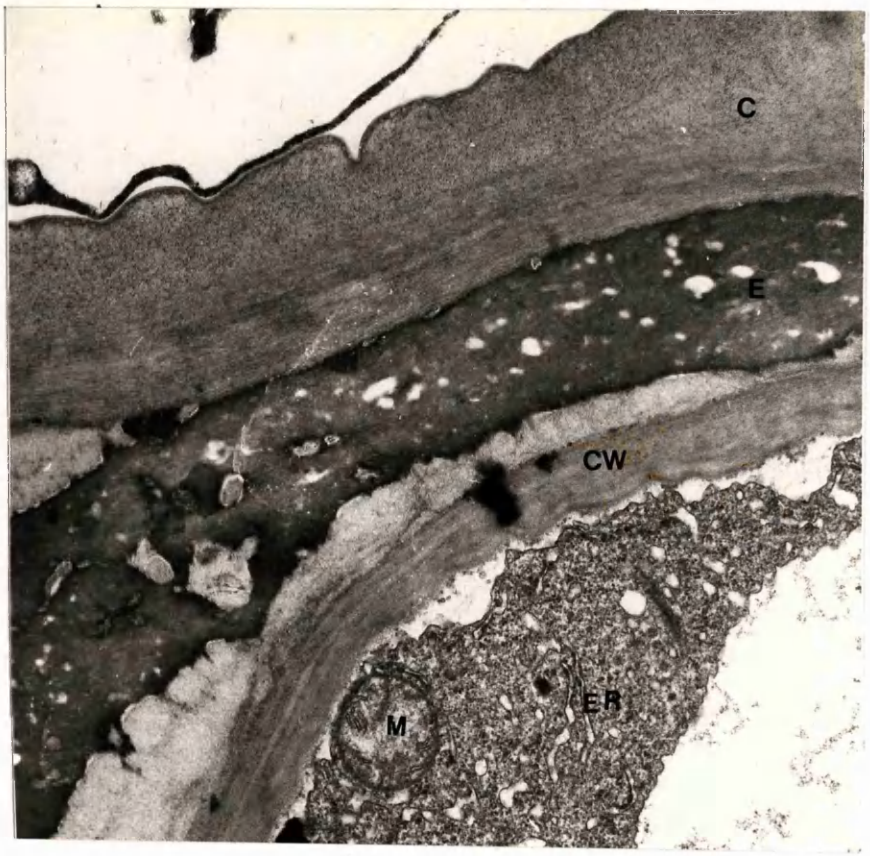
It was noted in this experiment that lesions developed rather earlier than in the previous experiments, and that there was an increase in the number of lesions.

Fig. 3.11. An electron micrograph of a section of a local lesion taken from a leaf of Pentland Ivory 7 days after KEBRS inoculation.

The first signs of lesion development consist of an epidermal cell (E) which has collapsed. The interior of the cell has become necrotic and granular with no visible organelles. The cell immediately adjacent to the necrotic cell looks healthy with an active cytoplasm consisting of mitochondria (M) and endoplasmic reticulum (ER). The cell wall (CW) and cuticle (C) are clearly visible. Magnification X 13,000

Fig. 3.12. An electron micrograph of a section of a local lesion taken from a leaf of Pentland Ivory 8 days after KEBRS inoculation.

The initially infected epidermal cell (E) has become necrotic and collapsed. The cell to the left has a disrupted cytoplasm (DC), though the cell to the right appears to be morphologically unaffected (HC) with mitochondria (M) and endoplasmic reticulum (ER) present. The cuticle (C) and cell walls (CW) are clearly visible. Magnification X 8,000


C
E
CW
M
ER

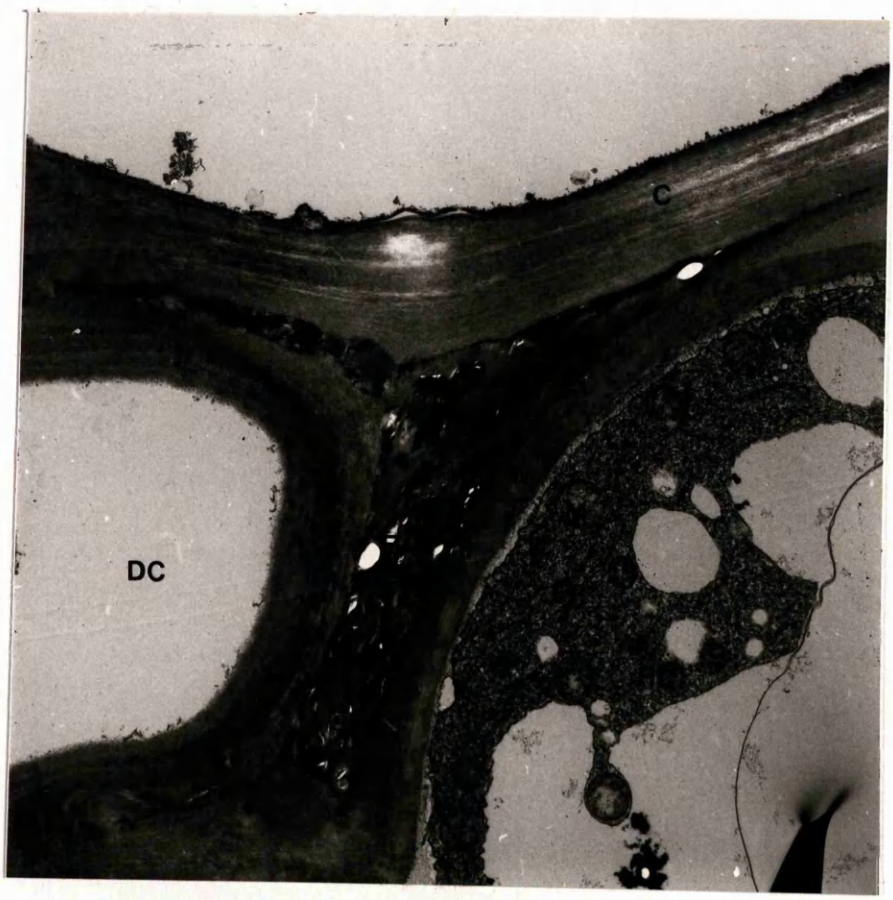
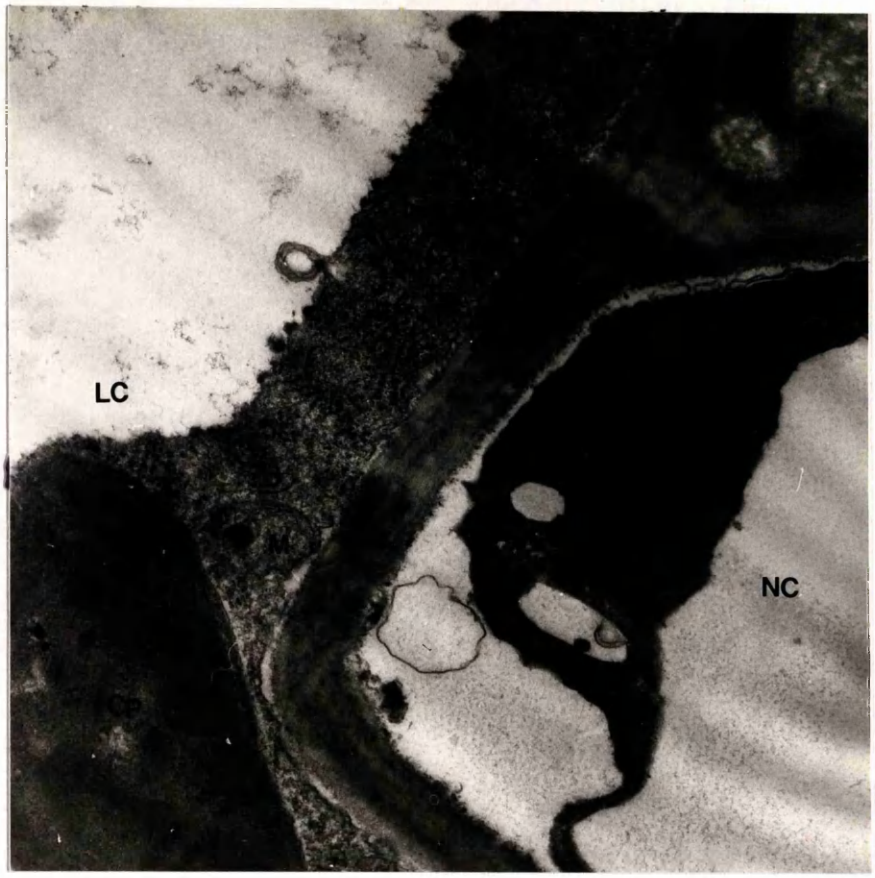

C
DC

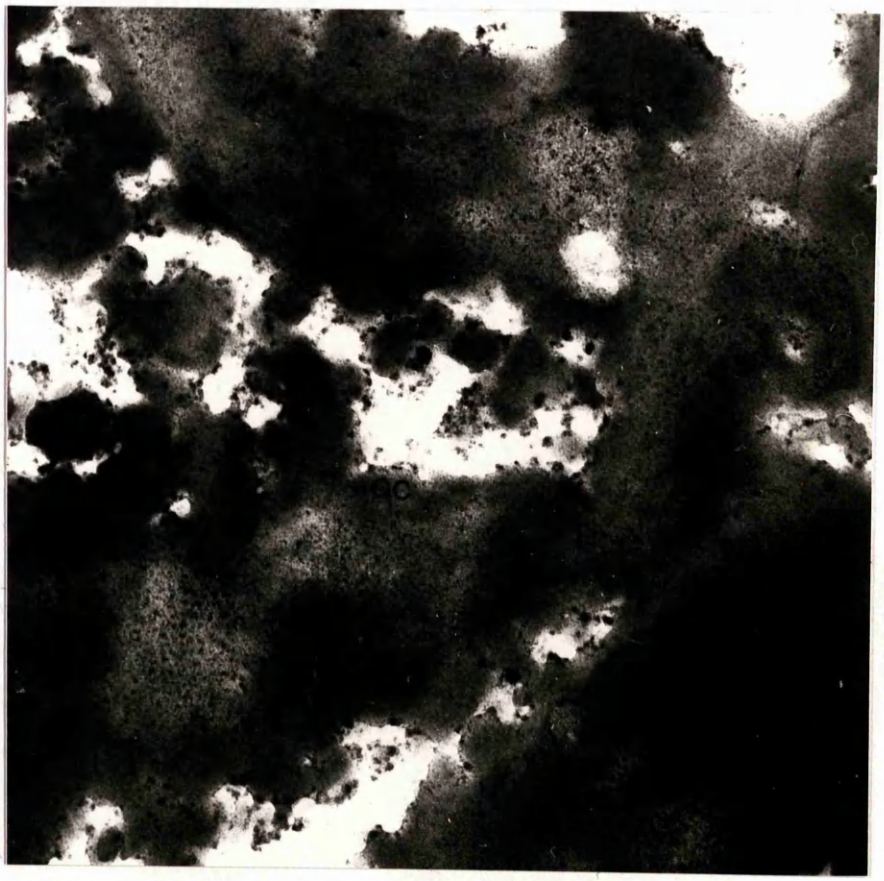
Fig. 3.13. An electron micrograph of a section of a local lesion taken from a leaf of Pentland Ivory 14 days after KEBRS inoculation.

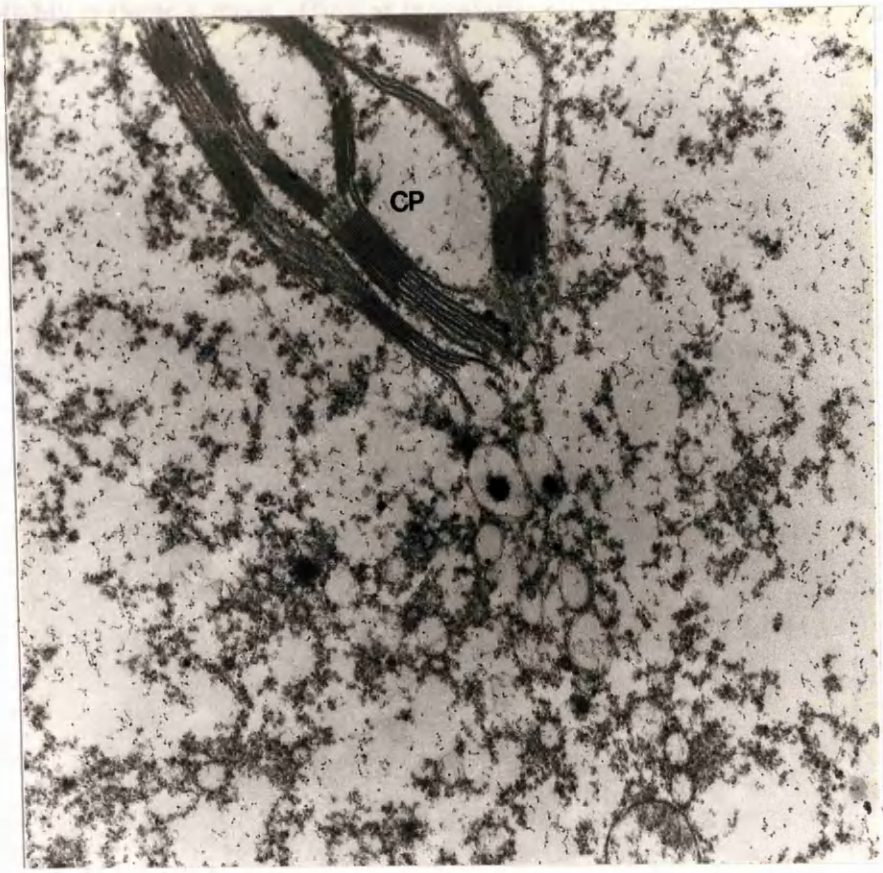
Seven days after the first signs of lesion development were seen, the affected tissue includes several epidermal, palisade and mesophyll cells. A palisade cell is clearly seen at the boundary of the necrotic lesion, with the cell (NC) becoming necrotic and disrupted, though a chloroplast (CP) and mitochondrion (M) are still visible. Vesicles (V) are also seen in the cytoplasm of the dying cell. The cell (LC) outside the necrotic lesion, however, is becoming disrupted with the endoplasmic reticulum (ER) and the mitochondrion (M) starting to break down in the cytoplasm. Magnification X 16,000

Fig. 3.14. An electron micrograph of a section of a local lesion taken from a leaf of Pentland Ivory 14 days after KEBRS inoculation.

Seven days after the first signs of lesion development, the centre of the lesion consists of granular cytoplasm (GC) with no internal structure. No viral particles were observed. Magnification X 54,000


LC
NC




CP


ER
CW
M

This probably reflects a more efficient inoculation as the experimenter became more adept. It may also have been due to passaging the virus strain several times through King Edward plants.

3.3. INDUCED SYSTEMIC RESISTANCE

The object of the experiments described in the following section was to investigate the phenomenon of induced systemic resistance (ISR) in potatoes. Pentland Ivory plants were tested to determine whether pre-inoculation with KEBRS induced an increase in resistance in non-infected parts of the plant to a subsequent challenge with either the same strain of virus or with a systemically infecting PVX strain. Plants were inoculated on their first fully expanded leaf either with KEBRS or mock-inoculated with water. A third group of plants were left undisturbed (referred to subsequently as healthy plants). Twenty days after the first 'inducing' inoculation an upper leaf, two above the inoculated leaf (referred to subsequently as leaf position three), was challenged with either the same strain of PVX (KEBRS) or a systemically infecting strain, PVX Gp3RS. The response to this second inoculation was compared for the three treatment groups both in terms of lesion number and using ELISA.

3.3.1. Analysis of induced systemic resistance using Pentland Ivory and PVX-KEBRS.

Plants were grown as described in Materials and Methods (section 2.2) and were either inoculated with KEBRS, mock-inoculated or left alone as healthy plants. In the PVX inoculated group, slight inoculation damage appeared 2-3 days after inoculation and PVX lesions developed 8-14 days later, ranging from 3 to 26 lesions per inoculated leaf. The lesions grew to approximately 3 mm in diameter (Fig. 3.17) within 16 days, and then remained stable in size and appearance. Twenty days after the initial inoculation procedure all treatment groups were then "challenge inoculated" on leaf position three with a 1:5 (w/v) inoculum of tobacco leaves systemically infected with KEBRS and homogenised in water. Eight days later, lesions started to develop on all three treatment groups on leaf position three. There was no significant difference between the different plant treatment groups in terms of lesion size, or their time of appearance. However, lesion size was quite variable between lesions on the same leaf, for all three treatment groups (Fig. 3.10). This variability was seen in all experiments where PVX lesions developed on potato leaves. After the initial 3-4 day period during which lesions appeared, lesion number on any individual leaf tended to

Fig. 3.17. Lesion development on Pentland Ivory inoculated with KEBRS following different pre-inoculation treatments.

(a) The first fully expanded leaf (leaf position one), 38 days after inoculation with 1:10 w/v of KEBRS, showing five lesions of approximately 3 mm diameter (indicated by arrows). Note the slight inoculation damage resulting from the abrasion of the leaf with carborundum.

(b) Leaf three of a healthy control plant, 18 days after a "challenge inoculation" with 1:5 w/v of KEBRS, showing 26 PVX lesions (indicated by arrows).

(c) Leaf three of a plant previously inoculated with 1:10 w/v of KEBRS on leaf one and subsequently challenged with a 1:5 w/v KEBRS inoculation on leaf three. Only seven PVX lesions were observed 18 days after the "challenge inoculation" on leaf three (indicated by arrows).

a

b

c

TABLE 3.2. The development of PVX-KEBRS lesions on Pentland Ivory plants which had previously been inoculated with KEBRS, compared with the two controls.

	Plants pre-inoculated on leaf position one with KEBRS			Plants pre-inoculated on leaf position one with water		Plants not pre-inoculated on leaf position one	
Plant No.	Lesion number 15 days after KEBRS inoc.on leaf position one	Lesion number 8 days after KEBRS inoc.on leaf position three	Lesion number 14 days after KEBRS inoc.on leaf position three	Lesion number 8 days after KEBRS inoc.on leaf position three	Lesion number 14 days after KEBRS inoc.on leaf position three	Lesion number 8 days after KEBRS inoc.on leaf position three	Lesion number 14 days after KEBRS inoc.on leaf position three
1	72	29	56	0	0	249	326
2	45	40	69	49	109	170	259
3	53	20	60	46	65	381	381
4	39	71	123	57	58	224	241
5	24	46	138	55	74	201	224
6	40	59	253	144	237	183	275
7	33	74	213	43	328	65	132
8	38	24	69	73	127	200	264
9	54	43	146	148	198	78	157
10	33	157	355	76	152	41	47
11	45	63	226	74	130	75	120
12	46	35	100	57	140	103	199
13	54	88	188	1	122	36	72
14	67	112	306	77	109	112	279
15	26	8	80	109	180	95	236
16	101	17	96	71	107	74	187
17	48	27	99	66	144	102	246
18	45	30	74	58	107	-	-
mean±SD	48 ± 18	52 ± 37	147 ± 90	67 ± 38	133 ± 70	140 ± 90	214 ± 88

The data show the number of lesions developing on leaf position three for individual plants of each treatment group, 8 and 14 days after "challenge inoculation" with KEBRS (1:5 w/v). The lesion number for individual plants 15 days after inoculation on leaf one with KEBRS is also shown.

Fig. 3.18. Pentland Ivory plants 14 days after the KEBRS "challenge inoculation" on leaf position three. Plants were either pre-inoculated with KEBRS, mock-inoculated on leaf one or left as healthy controls.

(a) On each plant the arrows indicate leaf position one (L1) and leaf position three (L3). H = a healthy plant, W = a plant mock-inoculated with water on L1 and PVX = a plant inoculated with KEBRS on L1. Plants all appear healthy and uniform apart from the lesions developing on KEBRS inoculated leaves.

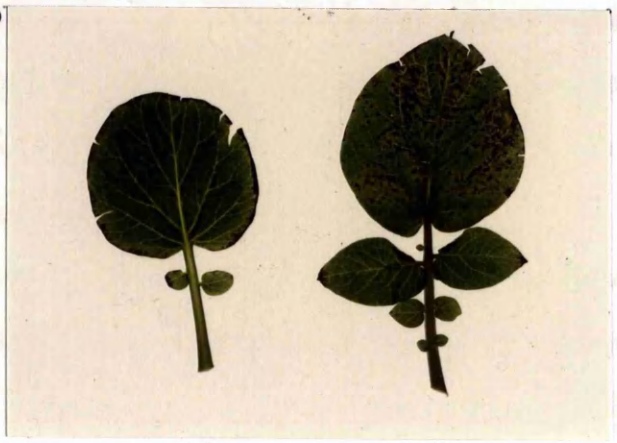
(b) A leaf from position one (L1) and a leaf from position three (L3) of a plant which had not been pre-inoculated. Leaf one was untreated while leaf three was challenged with KEBRS inoculum and has developed many local lesions.

(c) A leaf from position one (L1) and a leaf from position three (L3) of a plant mock-inoculated with water on leaf one. Note the slight inoculation damage on leaf one. Leaf three, which was challenged with KEBRS inoculum, has developed many local lesions.

(d) A leaf from position one (L1) and a leaf from position three (L3) of a plant initially inoculated on leaf one with KEBRS inoculum (1:5 w/v) and later challenged with KEBRS inoculum (1:5 w/v). Note the local lesions on both leaves.

a

b



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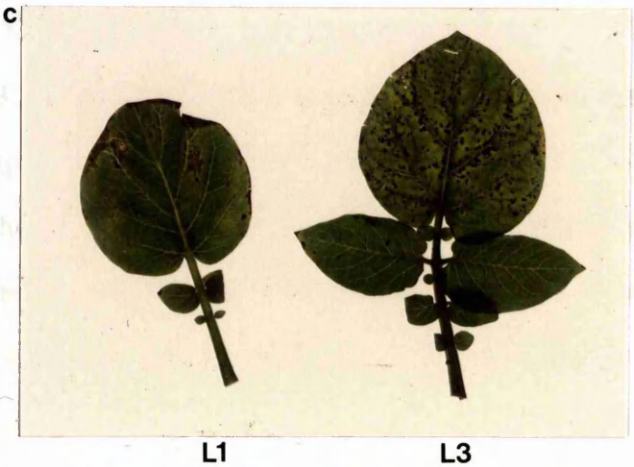
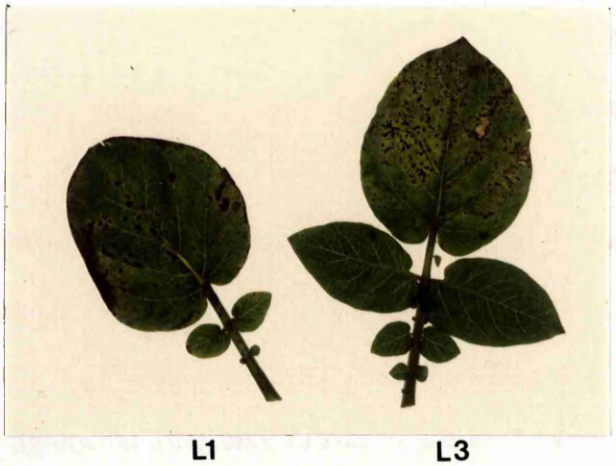


d



remain constant; only lesion size increased, to approximately 2-3 mm in diameter, over a further 16 days. Within all three treatment groups, lesion numbers between different plants in the same treatment group varied considerably. An average of 4.0 ± 2.1 SD lesions developed on leaf position three in plants which had been earlier inoculated with PVX-KEBRS while mock-inoculated plants averaged 7.0 ± 4.0 SD lesions and healthy control plants averaged 10.0 ± 5.1 SD lesions (Fig. 3.17). Therefore, pre-inoculation with KEBRS on leaf position one appeared to reduce subsequent lesion development by ~50% compared to control treatments. However, the small number of plants in this initial experiment (5 to 10 per treatment group) and the large variability of the results made statistical analysis difficult. Therefore, the experiment was repeated with a larger number of plants.

Each treatment group consisted of 18 plants. Six days after PVX-KEBRS inoculation the first lesions (tiny necrotic spots) developed on the inoculated leaves. The other two treatment groups appeared healthy although the mock-inoculated plants did show a little inoculation damage on their inoculated leaves. The number of lesions on the PVX-KEBRS inoculated leaves was recorded fifteen days after inoculation (Table 3.2). At this time, the mean number of lesions was 48 ± 18 SD. As with all previous experiments, there was a sizable variation in lesion number between different plants in this group. This may be due either to individual plants expressing different levels of resistance or to the fact that the inoculation procedure varied in effectiveness between plants. The lesion diameters ranged from 1.0 - 1.5 mm at this time. Twenty days after the first inoculation, the three treatment groups were challenged on leaf position three with a 1:5 (w/v) inoculum of tobacco leaves systemically infected with KEBRS and homogenised in water. By this time plants in all treatment groups showed some necrosis around the edges of the older leaves, possibly as a result of being grown at 10°C. After a further 7 days, all the plants began to show lesions on leaf three. The number of lesions for each treatment was recorded 8 and 14 days after the "challenge inoculation" (Table 3.2, Fig. 3.18). At day 8, lesion size varied between lesions on the same leaf, from 0.25 to 0.5 mm, though there was no significant difference in size of lesions between treatments. The

Fig. 3.19. Statistical analysis, using least significant difference (LSD), of the development of PVX-KEBRS lesion number on leaf position three of Pentland Ivory plants which had previously been inoculated with either PVX-KEBRS, mock-inoculated on leaf position one or left as healthy control plants.

INOC = Plants inoculated on leaf position one with PVX-KEBRS.

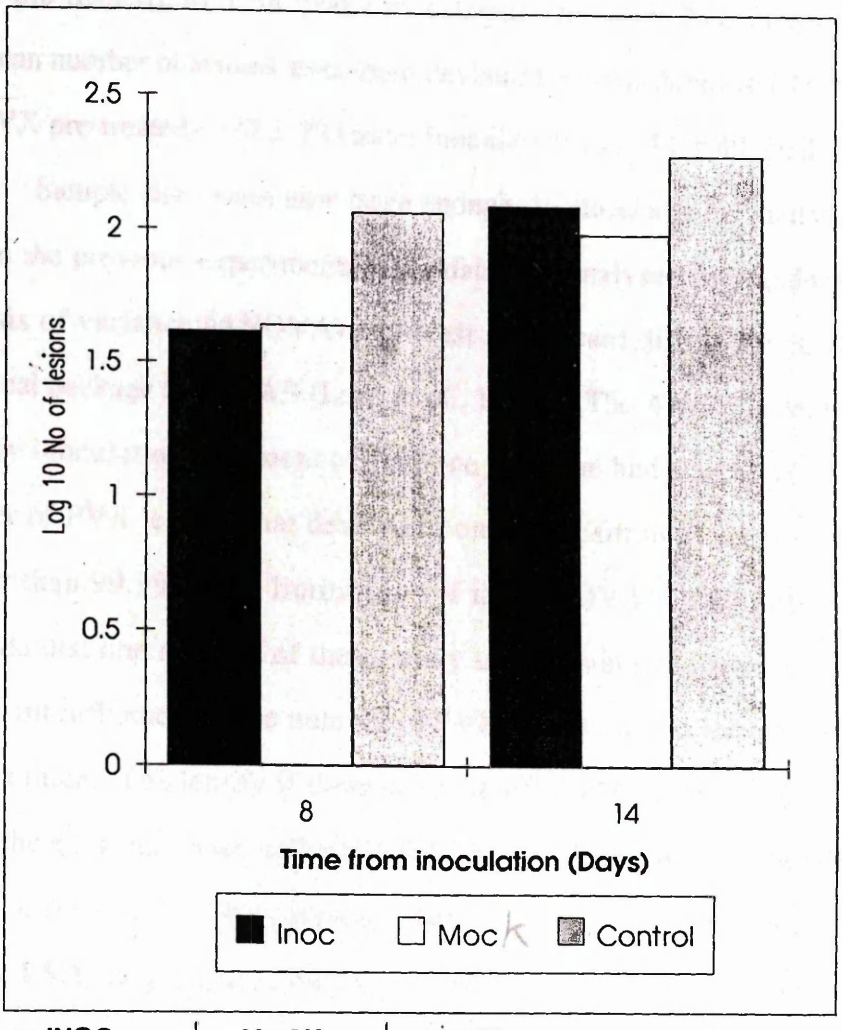
MOCK= Plants mock-inoculated on leaf position one.

CONT = Plants uninoculated on leaf position one.

8 = 8 days after inoculation on leaf position three with PVX-KEBRS.

14 = 14 days after inoculation on leaf position three with PVX-KEBRS.

Number of lesions has been log transformed.



	INOC	MOCK	CONT
8–14	0·4798	0·3486	0·2166

TIME	INOC–MOCK	INOC–CONT	MOCK–CONT
8	0·0239	0·4516	0·4277
14	0·1073	0·1884	0·2957

LSD@ p=0·05 = 0·0876
LSD@ p=0·01 = 0·1157

numbers of lesions on different plants within any one treatment group were quite variable; however, in all cases the lesion number increased up to 14 days. After 8 days, the mean number of lesions ± standard deviation on leaf three was 52 ± 37 (PVX pre-treated), 67 ± 38 (water inoculated) and 140 ± 90 (healthy control), while the mean number of lesions ± standard deviation on leaf three after 14 days was 147 ± 90 (PVX pre-treated), 133 ± 70 (water inoculated) and 214 ± 88 (healthy control).

Sample sizes were now large enough to allow a better statistical evaluation than in the previous experiments. The data was analysed by standard multivariate analysis of variance (ANOVA) and least significant difference (LSD) using the statistical package MINITAB (Lane *et al.*, 1987). The ANOVA identified that both primary inoculation treatment on leaf one and time had a significant effect on the number of PVX lesions that developed on leaf position three, to a probability of greater than 99.9%. The limitations of the ANOVA model only allows one to conclude that one or more of the primary inoculation treatments on leaf one has a significant influence on the number of PVX lesions which later developed on leaf position three. To identify if there were significant differences between treatment groups the least significant difference (LSD) was calculated for probabilities of ⩾95% and ⩾99% (Fig. 3.19). The LSD states that if the difference between groups is greater than one LSD, the groups are different to the level of probability used to generate the LSD. LSD identified that for all three treatment groups lesion number at 8 and at 14 days after challenge inoculation was significantly different to a probability of ⩾95%. Using LSD, to interpret the lesion number data for each treatment, identified that there was no significant difference in the number of lesions that developed on leaf three between PVX pre-treated and water-inoculated plants. When comparing the PVX pre-treated and water-inoculated groups to the healthy control group they were significantly different to a probability of ⩾99% for both time points (Fig. 3.19). A great deal of the apparent induced systemic resistance (if not all of it) appeared to be the result of wounding in potato. In fact, prior infection with PVX might restrict the plant's ability to induce a higher state of resistance compared to water-inoculation alone.

3.3.2. The effect of pre-inoculation with a localised strain of PVX on a subsequent challenge with a systemic strain of the virus.

Pentland Ivory plants were grown as described in Materials and Methods (section 2.2). Two to three days after inoculation (1:5 w/v PVX-KEBRS), slight damage resulting from the carborundum abrasion was seen on both the PVX-KEBRS and mock-inoculated leaves. Other than this the plants all appeared healthy. Eleven days after this inoculation minute necrotic lesions started to develop on the PVX inoculated leaves. After 22 days, half of the plants initially inoculated with KEBRS had developed 1-6 lesions on their inoculated leaf, the other half had not developed visible lesions. The reason for some inoculated plants not developing visible lesions was not clear. Possibly, the lesions may have been too small to be detected by the naked eye. Whatever the explanation, the KEBRS inoculated plants were split into two groups; those with and those without PVX lesions.

At this point, twenty-two days after the initial inoculation, all the plants underwent one of two treatments on leaf position three. The first treatment involved a "challenge inoculation" with the same virus strain (KEBRS) to support the results described in Section 3.3.1. The second treatment involved a "challenge inoculation" with a systemically infecting strain of PVX (Gp3RS). The inocula consisted of a 1:1 (w/v) dilution of ground tobacco leaf systemically infected with KEBRS and a 1:5 (w/v) dilution of ground tobacco leaf systemically infected with PVX Gp3RS, respectively.

All plants inoculated on leaf position three with KEBRS developed lesions after 12 days irrespective of earlier pre-treatment. These lesions first appeared as a pin-prick and then increased in size to approximately 2-3 mm in diameter. There was, however, a large variation in lesion size even on a single leaf. There was no significant difference in average lesion size between any of the different treatment groups.

The average lesion number developing on leaf three of KEBRS inoculated plants was recorded 12, 15, 18, 21 and 24 days after the challenge, and gave a general indication of how the plants responded to their earlier treatment. The plants initially

inoculated with KEBRS on leaf one developed ~50% fewer lesions than in the mock-inoculated treatment group and ~70% fewer lesions than plants which were not treated at all on leaf position one. Once again, the range in lesion number on leaf position three was large within a single treatment group and the ranges in lesion number between treatments overlapped. Unfortunately, because each group contained only 4 to 9 plants (depending on group) accurate interpretation on the statistical analysis of the data is difficult due to variability.

The general conclusion may be drawn that prior inoculation with KEBRS, irrespective or not of lesion formation, seems to reduce the number of viral lesions forming from a second inoculation on leaf position three, compared to control treatments. Mock-inoculation alone, however, also seems to reduce lesion number from a later inoculation with KEBRS. This reduction is however smaller than the effect of KEBRS inoculation. The general trend from this experiment agrees with experiment 3.3.1, that pre-inoculation with KEBRS, or mock-inoculation, increases the plant's resistance to a second infection with KEBRS compared to healthy untreated control plants, indicating that the increased level of resistance in the plant to a later challenge with PVX may be induced as a result of wounding as well as PVX infection.

3.3.2.1. Plants challenged on leaf three with Gp3RS.

The Pentland Ivory plants challenged on leaf three with the systemically infecting PVX strain, Gp3RS, were observed visually and virus concentration was measured using ELISA. Samples for ELISA testing were generated using a No.5 cork borer to cut a disc from inoculated leaf three of each plant and from apical leaves. There were twelve plants in each treatment group; each group was divided into 3 groups of 4 plants and each group of 4 plants was sampled in turn. The samples from each treatment group were homogenised in the appropriate amount of sample buffer (depending on the weight of the leaf sample) to give a 10^2-fold dilution and were assayed using ELISA to determine the concentration of virus coat protein. Each sample was generated from four plants in an attempt to try and reduce the effect of variation between plants, since earlier experiments had shown that potato plants tend

TABLE 3.3. The development of Gp3RS systemic infection in Pentland Ivory plants which had previously been inoculated with KEBRS, or mock-inoculated, or left healthy.

Days after inoc. with	Leaf position three inoculated with 1.5 w/v of inoculum Gp3RS				New expanded apical leaves from plants inoculated with Gp3RS on leaf three			
Gp3RS	PVXL	PVX	Mock	Healthy	PVXL	PVX	Mock	Healthy
3	2	3	3	3	0.5	0.5	0.5	0.5
6	9	15	3	2	0.5	0.5	1.0	0.5
9	280	800	340	450	0.5	2.0	1.0	0.5
12	660	550	720	520	0.5	0.5	1.0	0.5
15	1030	1100	320	600	3.0	1.0	0.5	2.0
18	930	1120	760	1010	0.5	1.0	0.5	10.0
21	600	1140	850	1070	0.5	3.0	1.0	3.0
24	1150	1150	-	1150	4.0	0.5	1.0	740.0
33					0.5	0.5	0.5	1110.0

Each individual reading comprises the viral content of leaf tissue expressed as ng PVX/gm leaf tissue and was generated from a sample from four individual plants. In apical leaves only plants which were not previously inoculated with KEBRS or mock-inoculated show any viral accumulation. These healthy plants start to become systemically infected 24 days after inoculation with Gp3RS suggesting early inoculation with a local lesion forming PVX strain, or mock inoculation alone, prevents or at least delays the systemic viral spread of a later inoculation with a systemically infecting PVX strain.

PVXL = Plants pre-treated on leaf one with KEBRS and local lesions formed.
PVX = Plants pre-treated on leaf one with KEBRS and no lesions formed.
Mock = Plants pre-treated with water on leaf one.
Healthy = Plants with no treatment on leaf one.

to become infected by systemic PVX infection at quite different rates. Samples for ELISA were taken 3, 6, 9, 12, 15, 18, 21, 24 and 33 days after the "challenge inoculation" with Gp3RS and the viral coat protein content at each sample time and treatment was expressed as ng PVX protein/gm leaf tissue (Table 3.3).

All four treatment groups developed slight inoculation damage on leaf position three, 3 days after inoculation with Gp3RS. Eighteen days after the inoculation the first light mosaic symptoms were observed on the upper leaves of a few plants, 24 days after inoculation all plants had developed light mosaic symptoms on upper leaves irrespective of treatment group. The symptoms gradually became more severe for all treatment groups.

The data (Table 3.3) shows that viral accumulation in the inoculated leaf (Leaf 3) of all treatment groups was detectable from around 6 days after inoculation, gradually increasing to reach a plateau of 1 μg PVX/gm leaf tissue between 15-18 days. The level then remained constant to 24 days. Viral accumulation in the apical leaves was *only* seen in the healthy (not pre-inoculated) plant group, first becoming apparent 24 days after inoculation with Gp3RS and increasing until the end of the experiment at day 33. The other two treatment groups were free of virus in their apical leaves. The experiment was terminated 33 days after leaf three was "challenge inoculated", at which time senescence had resulted in the abscission of leaf 3.

Thus, although Gp3RS infected the inoculated leaf of all four treatments, systemic spread was inhibited or at least delayed in plants previously inoculated with KEBRS or mock-inoculated, compared to healthy control plants. What cannot be concluded is whether PVX local lesion formation or inoculation damage alone induces this increased level of resistance. Further experiments are needed to investigate this point.

One feature of all these experiments was that when potato cv. Pentland Ivory was inoculated with a standard inoculum the number of lesions which developed on individual plants in the same treatment group varied quite considerably. Thus, the generation of statistical significantly different data is restricted to experiments with a large sample size. It was also a typically observed finding that the numbers of lesions

per inoculated leaf varied dramatically from experiment to experiment. Though the inoculum was generated from *N. edwardsonii* plants harvested 14 days after inoculation there may have been a large variation in the titre of virus between individual plants within an apparently homogenous group. This phenomenon may have contributed to the variation seen in these experiments as similar variation in viral accumulation in systemically infected tobacco plants has been reported by Aw (1987). The time taken for lesions to develop following inoculation with KEBRS also varied between experiments. In the initial experiments lesions took up to 17 days to develop, while in later experiments lesions developed 6 days after inoculation. Possibly the virus used as inoculum had undergone genetic drift as a result of successive passages through King Edward potato plants. Alternatively these differences might reflect physiological differences within the group of test plants.

3.3.3. Conclusions.

The experiments above were initially undertaken to test whether, in potato, localised PVX infection induced systemic resistance to a subsequent challenge. Previous work, for example investigating the response of tobacco to localised TMV infection, had demonstrated ISR (McIntyre & Dodds, 1979; McIntyre *et al.*, 1981). There are no earlier reports of mock-inoculation alone inducing a higher level of protection (Ross, 1961; McIntyre & Dodds, 1979; McIntyre *et al.*, 1981; Dean and Kuc, 1986a).

In this study both localised PVX infection and mock-inoculation induced a higher level of resistance in the potato plant, to both localised and systemic strains of PVX. The level of protection induced by pre-inoculation with virus or mock-inoculation was not significantly different and may suggest that wounding is the major cause of this increased resistance.

3.4. THE EFFECT OF AGING ON HEALTHY PENTLAND IVORY AND KING EDWARD PLANTS AT DIFFERENT LEAF POSITIONS.

Before attempting to identify changes in gene expression resulting directly from PVX infection, a series of control experiments were carried out to investigate changes in gene expression in healthy plants over the time course used in subsequent experiments. The aim was to see how the mRNA population varied with time and with leaf position, as healthy Pentland Ivory and King Edward plants aged.

The Pentland Ivory and King Edward plants were grown as described in Materials and Methods (section 2.2). Six days after reducing the temperature to 10°C the first leaf sample was taken. A further 9 samples were taken on later dates. The first sample was classified arbitrarily as day zero; this was the time the plants would have been inoculated with PVX in an experiment comparing mRNA expression levels between infected and non-infected plants. Samples were collected on days zero, 2, 4, 6, 8, 10, 12, 14, 16, and 18; they were always taken at 10.00 am to minimise photoregulated mRNA changes between samples. Each sample was generated from four individual plants which grew uniformly throughout the experiment. The leaf samples were numbered 1, 2, and 3, corresponding to leaf number. Leaf one was the first true leaf above the soil, leaf two the second and leaf three the third. RNA was extracted from each leaf position for each sample time and then translated *in vitro* using the wheat germ system. The resulting polypeptides were analysed using SDS-PAGE. The separated polypeptides were detected by fluorography. The translational efficiency of mRNA from different samples of each potato cultivar varied. Using 5 µg of total RNA between 10% and 50% of the ^{35}S methionine was incorporated into TCA-precipitable material. These large differences in translational efficiency between mRNA samples caused some problems in loading equal amounts of radioactivity onto different lanes of the same gel. The reasons for the differences was not clear since all RNA was extracted from leaf tissue under identical conditions. However, irrespective of the translational efficiency of the RNA, the polypeptides synthesised *in vitro* gave a pattern of similar sized products. The presence of high molecular weight products (~100 KDa) indicates that the *in vitro* translation system was capable of producing

Fig. 3.20. One-dimensional SDS-PAGE of *in vitro* translated polypeptides.

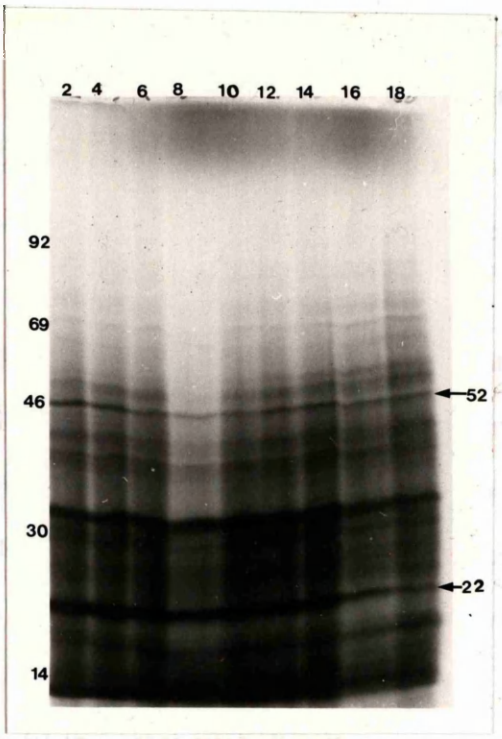
Electrophoresis, in a 10-15% gradient polyacrylamide gel, of *in vitro* translation products from mRNA extracted from Pentland Ivory leaf position two. Samples were taken at days zero, 2, 4, 6, 8, 10, 12, 14, 16 and 18. Each lane was loaded with a sample containing 200,000 d.p.m. of ^{35}S.

The arrows and numbers indicate the position and size in KDa of proteins which change in intensity over the time course.

Fig. 3.21. One-dimensional SDS-PAGE of *in vitro* translated polypeptides.

Electrophoresis, in a 12.5% polyacrylamide gel, of *in vitro* translation products from mRNA extracted from King Edward leaf position two. Samples were taken at days zero, 2, 4, 8, 12, 14, 16 and 18. Each lane was loaded with a sample containing 100,000 d.p.m of ^{35}S.

The arrows and numbers indicate the position and size in KDa of proteins which change in intensity over the time course.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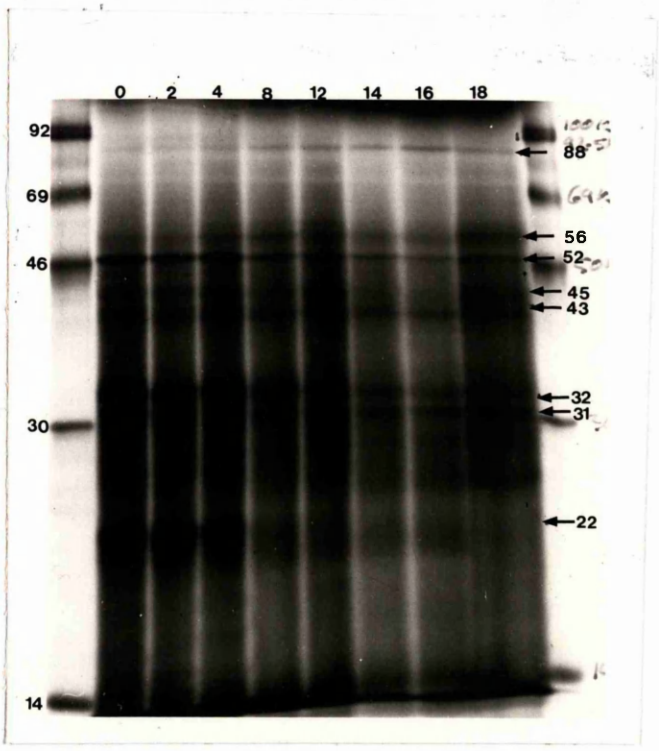
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large transcripts, suggesting that premature termination was not a major problem.

In vitro translated total RNA from each of the three leaf positions in Pentland Ivory gave near-identical polypeptide patterns with little variation over time. Polypeptides of 52 and 22 KDa, however, appeared to decrease slightly in intensity with time. A typical gel showing the pattern of polypeptides seen in Pentland Ivory is presented in Fig. 3.20.

In the case of King Edward, the patterns of *in vitro* synthesised polypeptides between different leaf positions at the same time point appear very similar. Over time, the patterns were also rather similar, although at least 8 polypeptides showed some changes in intensity, with three bands (88, 56 and 31 KDa) increasing and five (52, 45, 43, 32 and 22 KDa) decreasing. A typical gel showing the changes in polypeptides with time in King Edward is shown in Fig. 3.21.

Having shown by ELISA that PVX-KEBRS is restricted to the inoculated leaf of Pentland Ivory, and that healthy Pentland Ivory plants show little apparent change in mRNA population over time and between leaf position, the combination of Pentland Ivory and PVX-KEBRS was the choice for further study.

3.5. ANALYSIS OF CHANGES IN mRNA POPULATIONS BY *IN VITRO* PROTEIN SYNTHESIS IN PENTLAND IVORY PLANTS INOCULATED WITH PVX STRAIN KEBRS OR MOCK-INOCULATED WITH WATER.

The aims of the experiments described below were to determine whether significant changes occurred in the mRNA populations of the inoculated and upper leaves of Pentland Ivory plants which had been either inoculated with PVX-KEBRS on leaf position one, mock-inoculated on leaf position one or left uninoculated as healthy controls, and if so, at what time after inoculation these changes occurred. It was important to have some real data on the time course of any such changes, before the construction of a cDNA library. Pentland Ivory and PVX strain KEBRS were selected as the most suitable combination for such a study.

Changes in mRNA synthesis following PVX-KEBRS inoculation of Pentland Ivory plants were investigated by isolating and translating, *in vitro*, mRNA from PVX-KEBRS-inoculated, mock-inoculated and healthy plants. The translated polypeptides were analysed using one and two-dimensional polyacrylamide gel electrophoresis (PAGE). In the initial experiments one-dimensional SDS-PAGE was used to analyse the polypeptides. Later, two-dimensional gels, with their much greater ability to resolve polypeptides, were used to investigate in more detail specific differences between PVX inoculated, mock-inoculated and healthy plants. Two replica experiments were constructed (Table 3.4) to identify consistent changes in the mRNA populations which resulted from infection of Pentland Ivory with PVX-KEBRS. The first experiment was used to identify the time course of changes in the mRNA populations and the second to further characterise these changes.

The plants used in the two experiments were grown as described in Materials and Methods (section 2.2). The plants in experiment one were grown in the greenhouse at 20°C under natural light, during the month of July. When these plants averaged 10 cm in height they were placed at 10°C in a growth room. Leaf samples, in all treatments in this and subsequent experiments, were taken at 10.00 am in order to minimise any differences between mRNA populations resulting from the activity of photoregulated genes. The leaves from two plants were combined for each sample.

Samples were taken from both the inoculated leaf (leaf one) and leaf three. Samples were taken 24 h before inoculation and then 6 h, 1, 3, 5, 7 and 9 days after inoculation. Over the period of the experiment no plants showed any visible symptoms of systemic PVX infection. ELISA was carried out and confirmed that PVX-KEBRS coat protein could not be detected in inoculated or apical leaves up to nine days after the plants were inoculated. The leaves inoculated with PVX-KEBRS started to develop local lesions 7 days after inoculation. These lesions had developed to a maximal size of approximately 0.5 mm diameter by day 9 and ranged in number from 5 to 10 per inoculated leaf.

The RNA samples were translated *in vitro* and the resulting polypeptides separated on a 12.5%-22.5% gradient SDS-PAGE. Preliminary experiments had shown that these conditions gave good resolution of the polypeptides. Each lane contained 100,000 d.p.m. of TCA precipitable material. Samples from healthy uninoculated and mock-inoculated plants were run in adjacent lanes, as were samples from mock and PVX-inoculated plants. This enabled direct comparison of the different treatments on the same gel.

The patterns of polypeptide bands for each sample time between healthy and mock-inoculated plants were virtually identical (Figs. 3.22 and 3.24). This was true for both the inoculated leaf and leaf three. The only difference seen was an increase in intensity of a 24 KDa band in the mock-inoculated leaf, 6 h and 1 day after inoculation, compared to the healthy control samples (Fig. 3.22). In view of the reported induction of several genes by wounding (Collinge & Milligan, 1987; Logemann *et al.*, 1988) the apparent lack of difference was unexpected; perhaps these types of changes are too subtle to be detected in the relatively insensitive one-dimensional gel system.

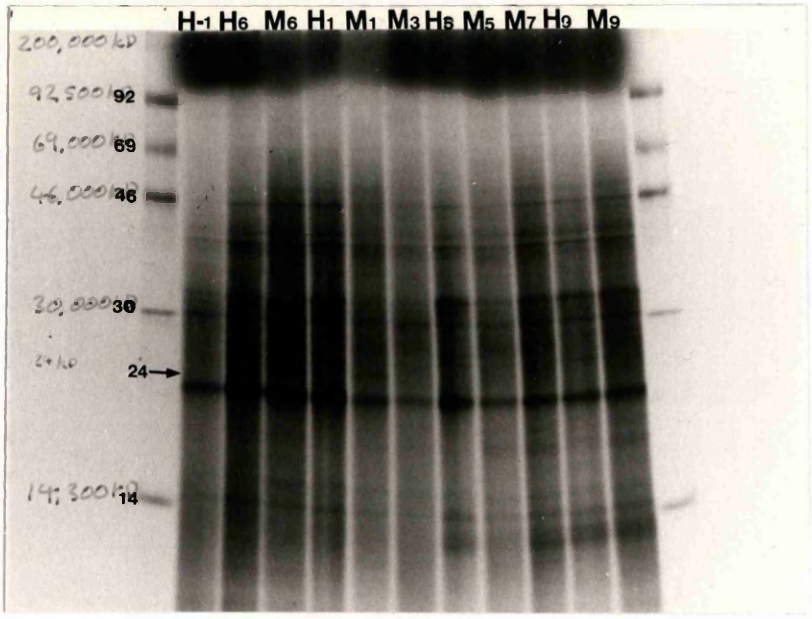
Several differences were apparent between the mock and PVX-inoculated plants. In the leaf inoculated with PVX-KEBRS, a band of 46 KDa increased in relative intensity at 7 and 9 days after inoculation compared to mock-inoculated leaves (Fig. 3.23). Prior to day 7, this band showed the same relative intensity in both sets of plants. Two other bands, of 22 KDa and 15 KDa, also increased in intensity in

Fig. 3.22. Messenger RNA differences in leaf position one between healthy and mock-inoculated plants.

Fluorograph showing SDS-PAGE of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 µg of total RNA from cv. Pentland Ivory leaf position one, 1 day before and 6 h, 1, 3, 5, 7 and 9 days after after the particular treatment. Mock-inoculated plants are indicated by (M) and healthy control plants by (H). The lefthand lane shows the position of labelled molecular weight standards and their sizes in KDa. The arrow marks the polypeptide band which differs between the two treatments (For discussion see text).

Fig. 3.23. Messenger RNA differences in leaf position one between mock-inoculated and PVX-inoculated plants.

Fluorograph showing SDS-PAGE of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 µg of total RNA from cv. Pentland Ivory leaf position one, 1 day before and 6 h, 1, 3, 5, 7 and 9 days after the particular treatment. Mock-inoculated plants are indicated by (M) and PVX strain KEBRS-inoculated plants by (P). The lefthand lane shows the position of labelled molecular weight standards and their size in KDa. Arrows mark the position of the most prominent polypeptides which differ between the two treatments (For discussion see text). The size of each polypeptide change is given in KDa on the right side of the gel.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14,300
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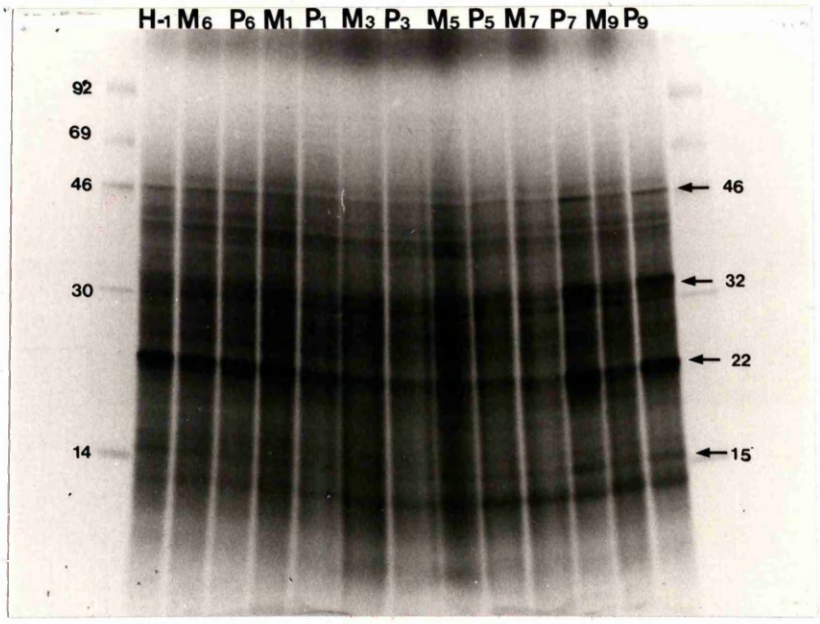
H-1 M6 P6 M1 P1 M3 P3 M5 P5 M7 P7 M9 P9
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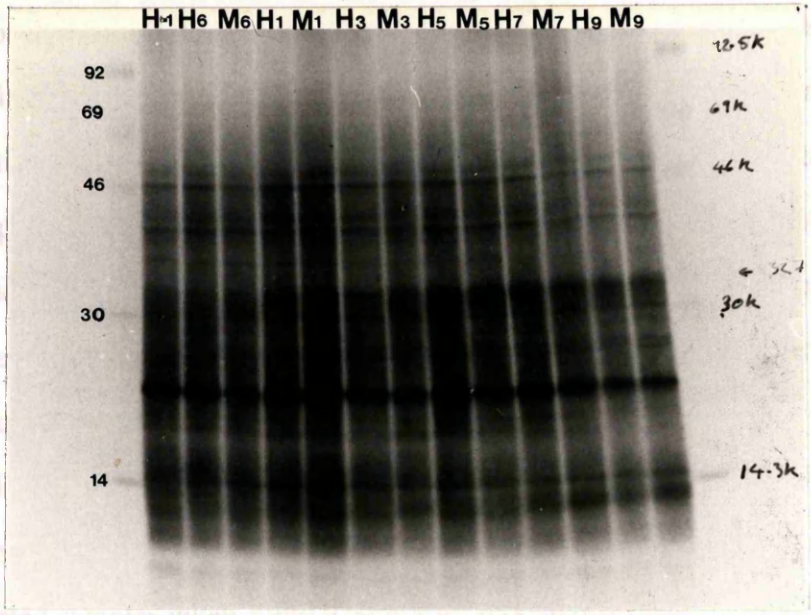
22
15

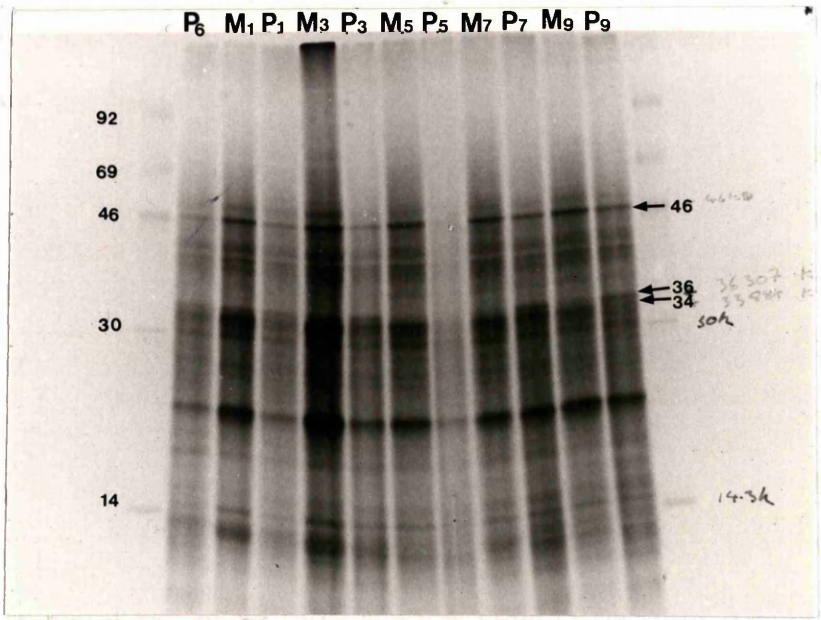
Fig. 3.24. Messenger RNA differences in leaf position three between healthy and mock-inoculated plants on leaf position one.

Fluorograph showing SDS-PAGE of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 μg of total RNA from cv. Pentland Ivory leaf position three, 1 day before and 6 h, 1, 3, 5, 7 and 9 days after the particular treatment. Mock-inoculated plants are indicated by (M) and healthy control plants by (H). The lefthand lane shows the position of labelled molecular weight standards and their size in KDa.

Fig. 3.25. Messenger RNA differences in leaf position three between mock-inoculated and PVX-inoculated plants on leaf position one.

Fluorograph showing SDS-PAGE of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 μg of total RNA from cv. Pentland Ivory leaf position three 6 h, 1, 3, 5, 7 and 9 days after the particular treatment. Mock-inoculated plants are indicated by (M) and PVX strain KEBRS-inoculated plants by (P). The lefthand lane shows the position of labelled molecular weight standards and their size in KDa. Arrows mark the position of the most prominent polypeptides which differ between the two treatments (For discussion see text). The size of each polypeptide change is given in KDa on the right side of the gel.





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samples from PVX-inoculated leaves compared to mock-inoculated leaves at 7 and 9 days after inoculation. A novel band of 32 KDa developed in PVX-inoculated leaves, appearing faintly 6 h after inoculation, 5 days after inoculation appearing again and becoming quite intense by 7 and 9 days after inoculation. A comparison of *in vitro* synthesised polypeptides from PVX and mock-inoculated plants from leaf position three revealed two bands of 36 KDa and 34 KDa which changed in relative intensity between treatments (Fig. 3.25). Nine days after inoculation the lower molecular weight band was more intense in mock-inoculated plants compared to PVX-inoculated. In the sample taken 7 days after inoculation the 34 KDa band appeared equally intense between the two treatments. A band of 36 KDa was more intense in PVX-inoculated samples taken 7 and 9 days after inoculation than in mock-inoculated plants. In mock-inoculated plants a band of 46 KDa was more intense 7 and 9 days after inoculation due to increased expression in these plants, compared to PVX-inoculated plants. The intensity of the 46 KDa band in leaf position three is contrary to that seen in the inoculated leaves of these two treatments (Figs. 3.23 and 3.25).

Comparing the pattern of *in vitro* synthesised polypeptides of mock and PVX-inoculated plants for leaf positions one and three, the band of approximately 36 KDa from leaf three and the band of approximately 32 KDa from leaf one (both show increased relative intensity in PVX-inoculated plants compared to mock-inoculated plants) probably represent the same polypeptide since the positions of both bands relative to the other bands in the same lane are identical (Figs. 3.23 and 3.25). Any differences in the apparent molecular weight are presumably due to errors in measurement or distortion of the gel, i.e. shrinkage. These changes in *in vitro* translation products between PVX and mock-inoculated plants coincided with the first appearance of symptoms, in the form of a few pinprick, necrotic lesions on PVX-KEBRS-inoculated leaves, which occurred 7 days after inoculation. Since the period 7-9 days after inoculation seems to be the time at which the most obvious changes in mRNA populations are detectable, *in vitro* translated polypeptides of samples taken 7 and 9 days after inoculation were analysed further using two-dimensional gel electrophoresis. This permitted a much more detailed analysis of any differences

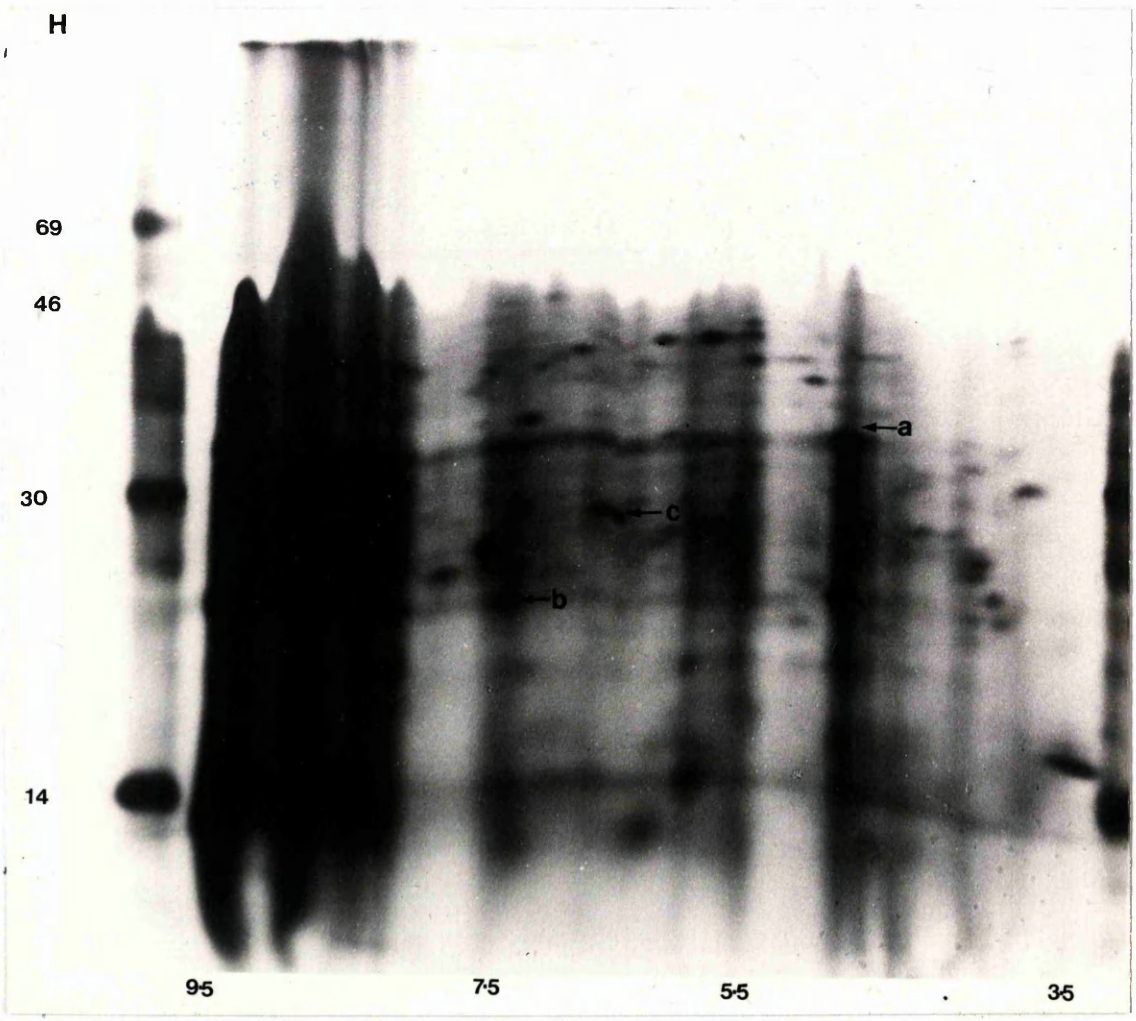
between PVX, mock-inoculated and healthy uninoculated plants. For these gels, samples contained 200,000 d.p.m. of TCA-precipitable material. Polypeptides were separated in the first dimension by IEF, as described in Materials and Methods (section 2.15). For the second dimension, the IEF gel was laid over a 14% SDS-PAGE, which separated the polypeptides on the basis of size.

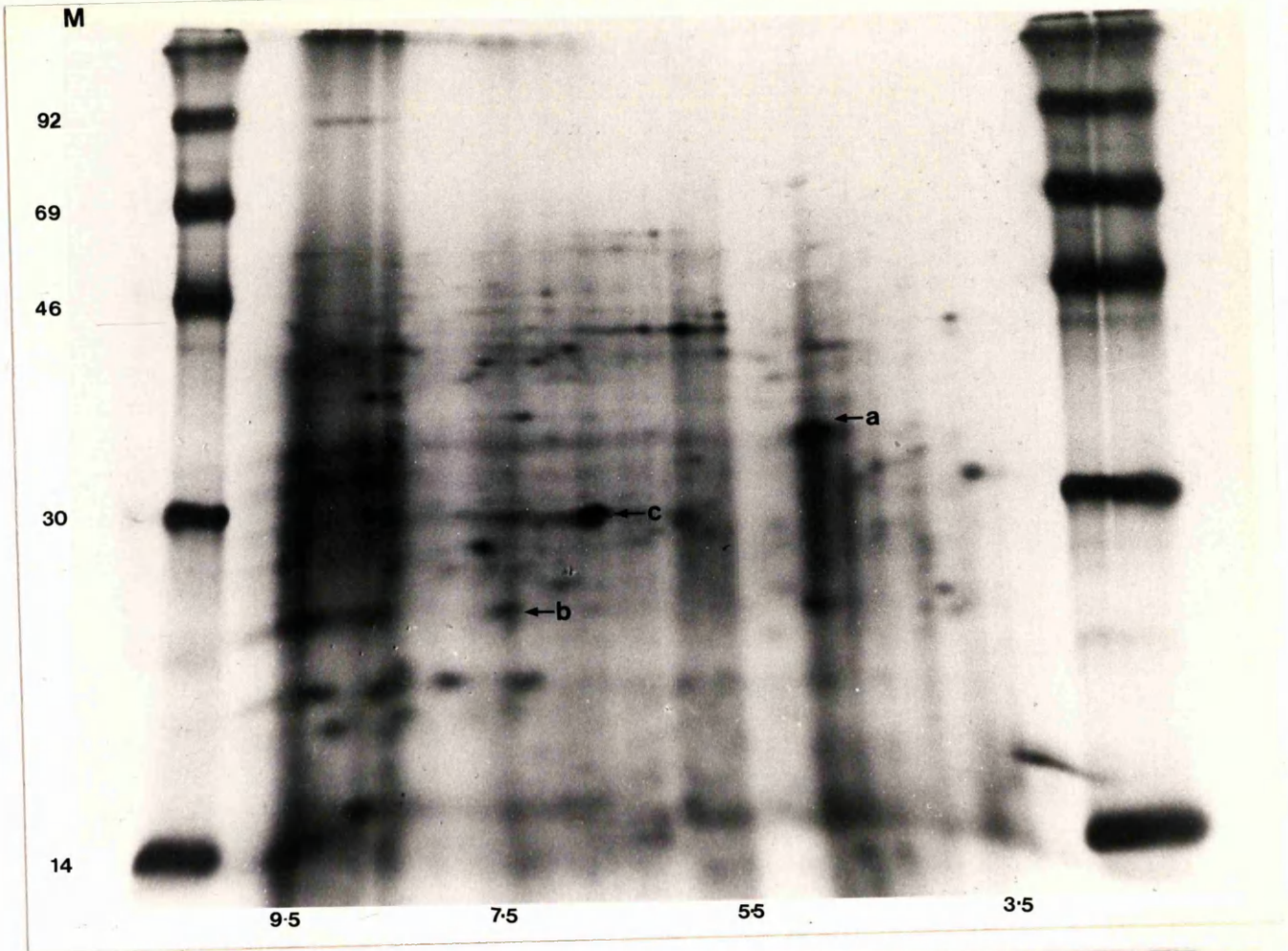
Analysis, using two-dimensional gels, of the polypeptides from the three different treatments on leaf position one, using mRNA extracted 7 days after inoculation, revealed several differences (Fig. 3.26, Table 3.4). A polypeptide (a) of 36 KDa was more intense in PVX-inoculated leaves; this may correspond to the band of 32 KDa seen in the one-dimensional gels (Fig. 3.23). A polypeptide (b) of 22 KDa increased significantly in PVX-inoculated leaves compared to the two controls, while a polypeptide (c) of 30 KDa was more intense in mock-inoculated plants compared to PVX-inoculated or healthy plants. In leaf position three, using RNA extracted 7 days after inoculation, a polypeptide (d) of 33 KDa was more intense in PVX-inoculated plants compared to the mock-inoculated treatment. The *in vitro* translated polypeptides from healthy plants appeared to have been over loaded as all polypeptides seemed more abundant than in the other treatments (Fig. 3.27). Polypeptide (d) may correspond to the polypeptide of approximately 36 KDa identified as being up-regulated in the one-dimensional gels (Fig. 3.25) and also to the polypeptide (a) of Fig. 3.26, although this cannot be confirmed. A polypeptide (e) of 42 KDa was more intense in PVX-inoculated plants, while a polypeptide (f) of 24 KDa, which was present in healthy plants, was very faint in both mock and PVX-inoculated plants (Fig. 3.27).

mRNA samples extracted from plants 9 days after inoculation were translated *in vitro* and the polypeptides analysed by electrophoresis on two-dimensional gels (Fig. 3.28). In samples from leaf position one, two polypeptides (g) and (h) of 42 KDa and 21 KDa respectively were more intense in PVX-inoculated leaves compared to both control treatments. The polypeptide of 21 KDa may correspond to the 22

Fig. 3.26. Messenger RNA differences in leaf position one between PVX-inoculated, mock-inoculated and healthy plants seven days after inoculation on leaf one.

Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 µg total RNA from potato cv. Pentland Ivory leaf position one 7 days after inoculation on leaf one with either PVX strain KEBRS (P) mock-inoculated with water (M) or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups, (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.

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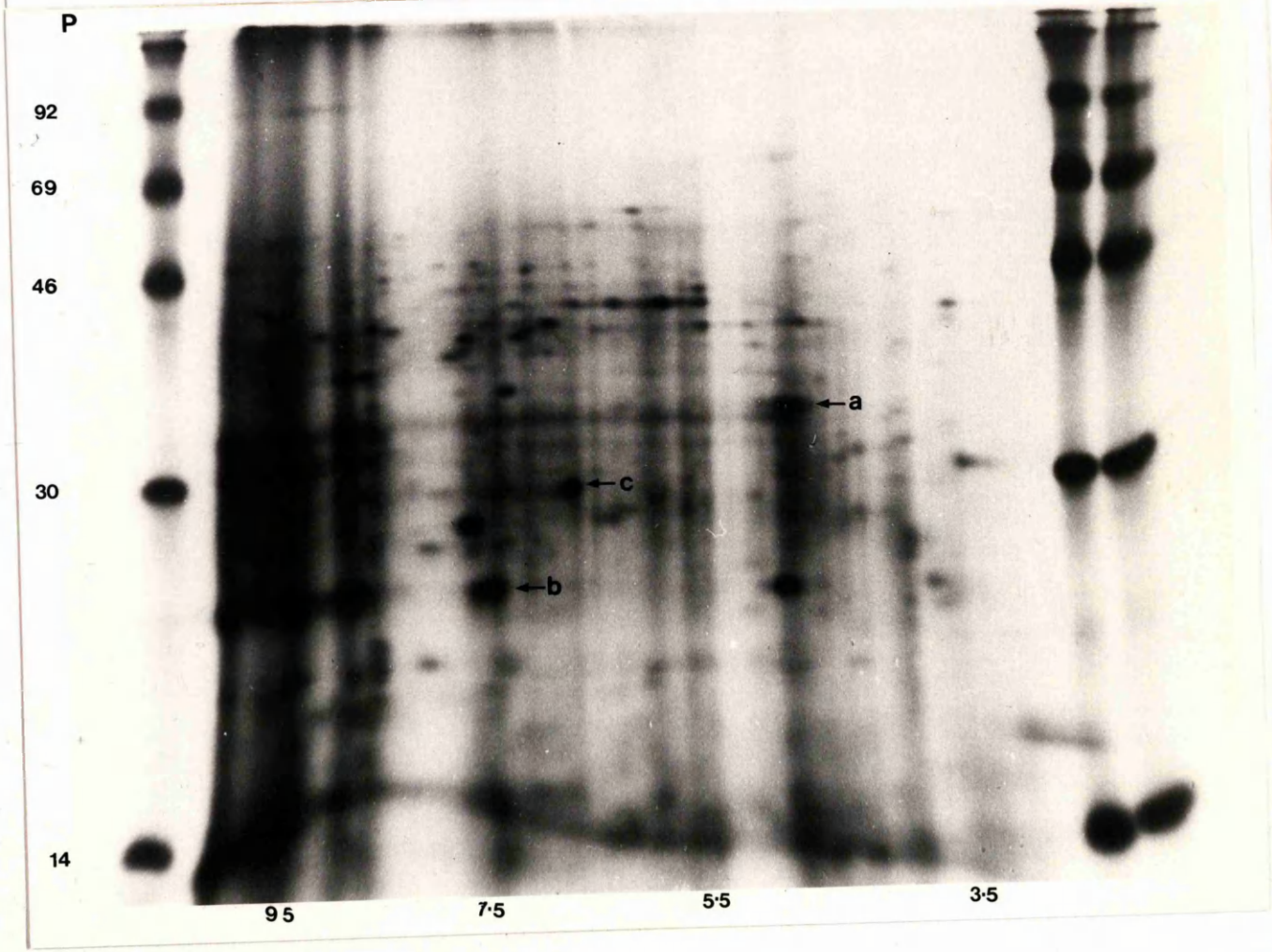
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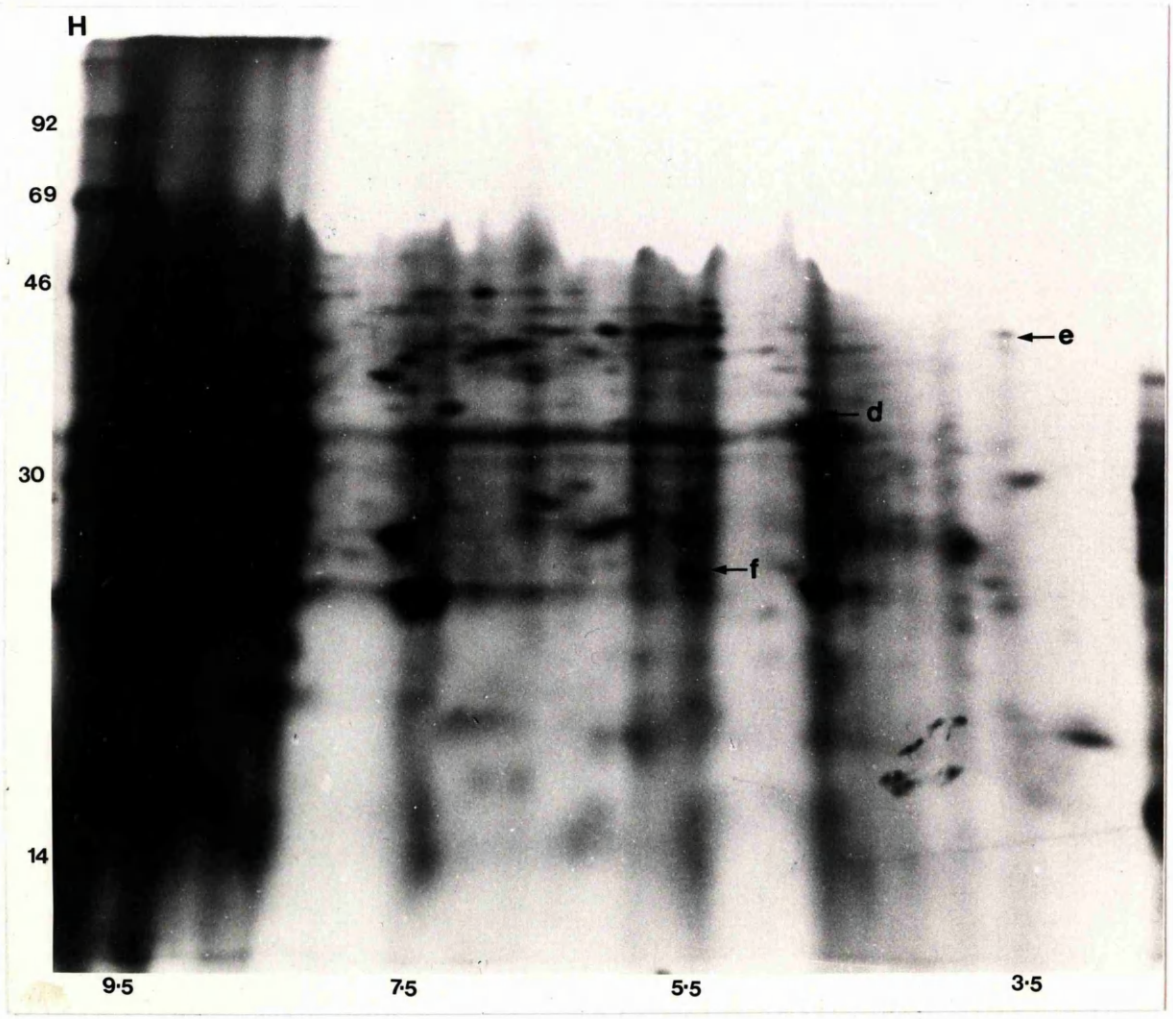
Fig. 3.27. Messenger RNA differences in leaf position three between PVX-inoculated, mock-inoculated and healthy plants, seven days after inoculation on leaf one.

Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 µg of total RNA from potato cv. Pentland Ivory leaf position three 7 days after inoculation on leaf one with PVX strain KEBRS (P), mock-inoculated with water (M), or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.



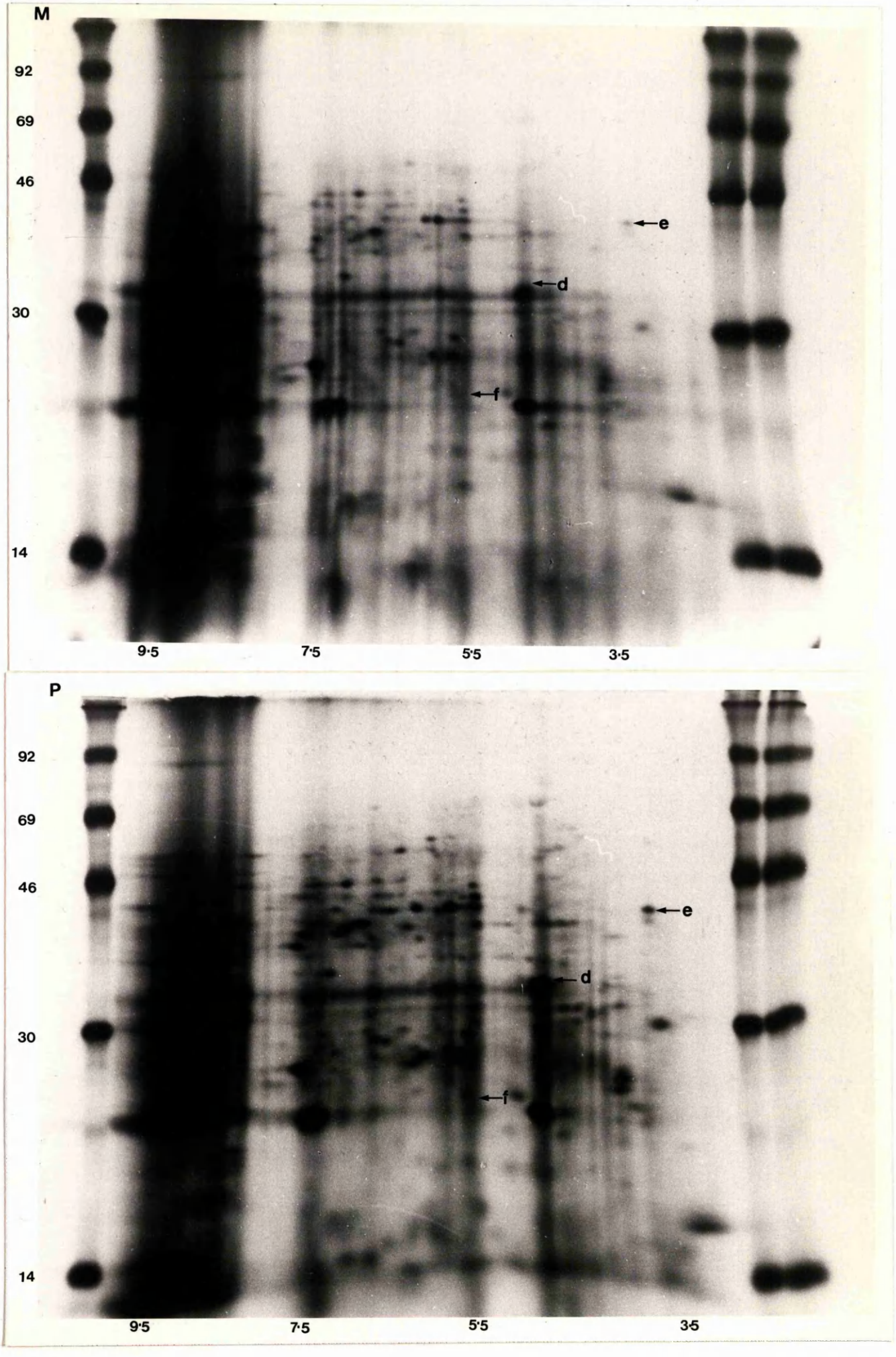

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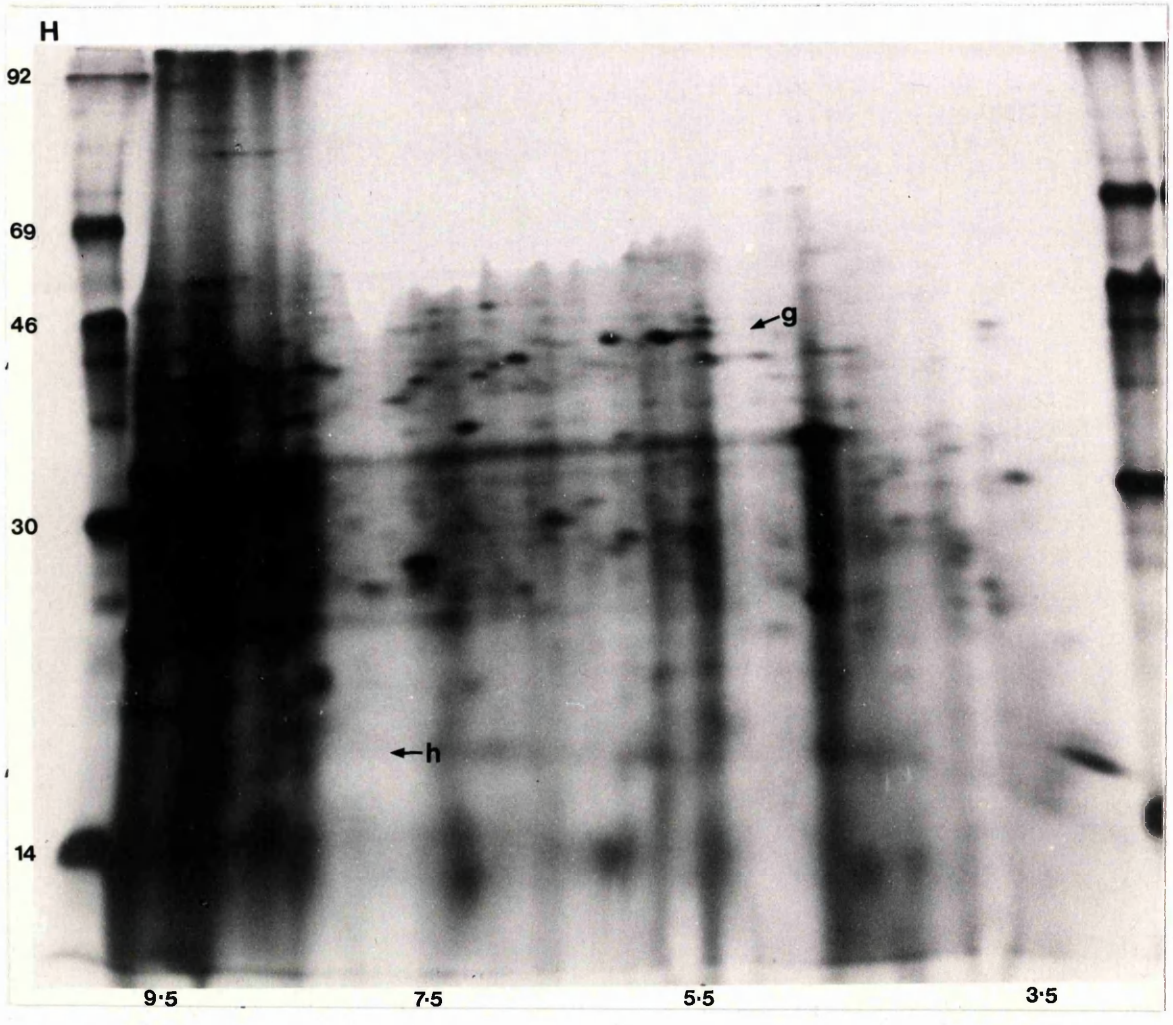
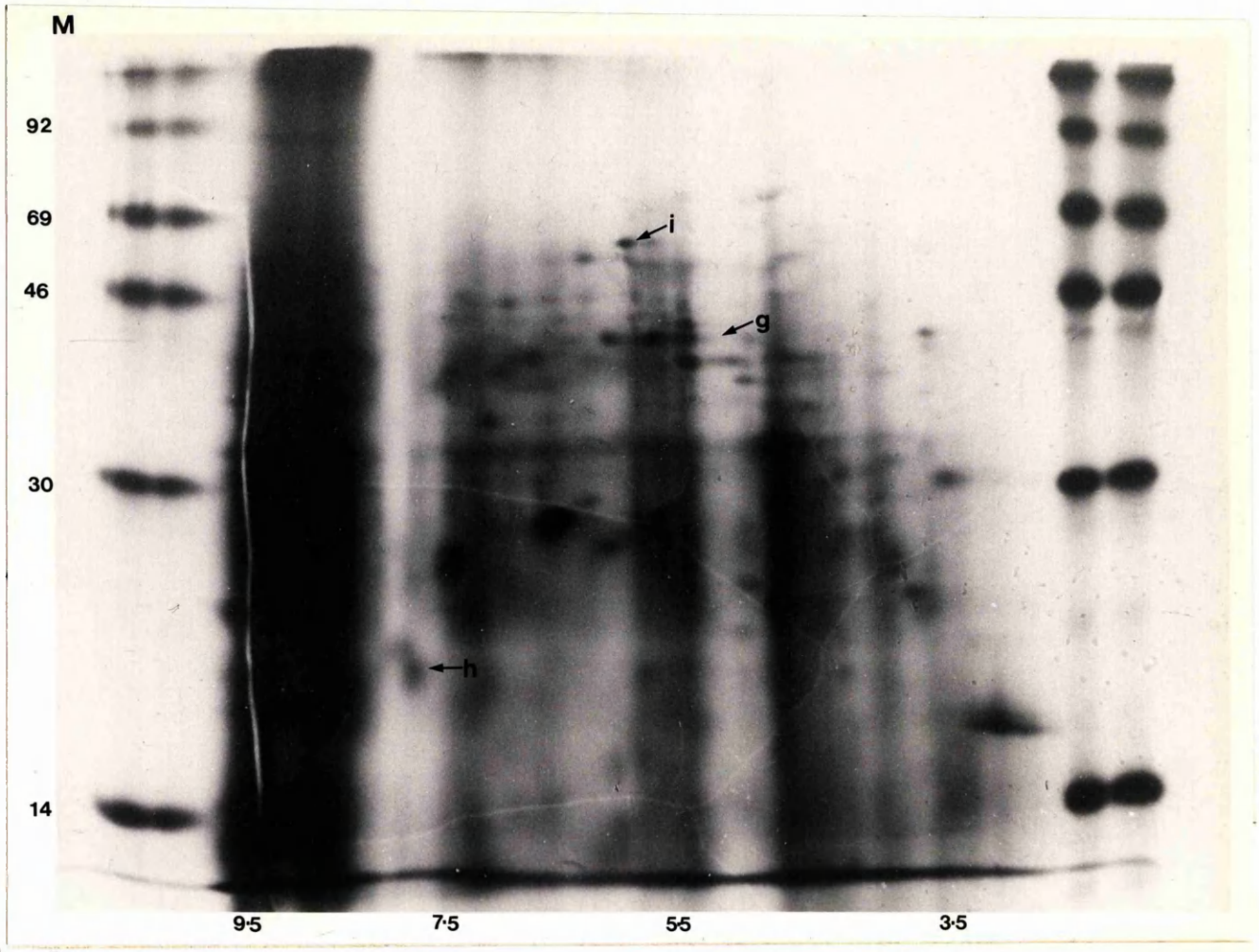
Fig. 3.28. Messenger RNA differences in leaf position one between PVX-inoculated, mock-inoculated and healthy plants, nine days after inoculation on leaf position one. Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 µg total RNA from potato cv. Pentland Ivory leaf position one 9 days after inoculation on leaf one with PVX strain KEBRS (P), mock-inoculated with water (M), or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.

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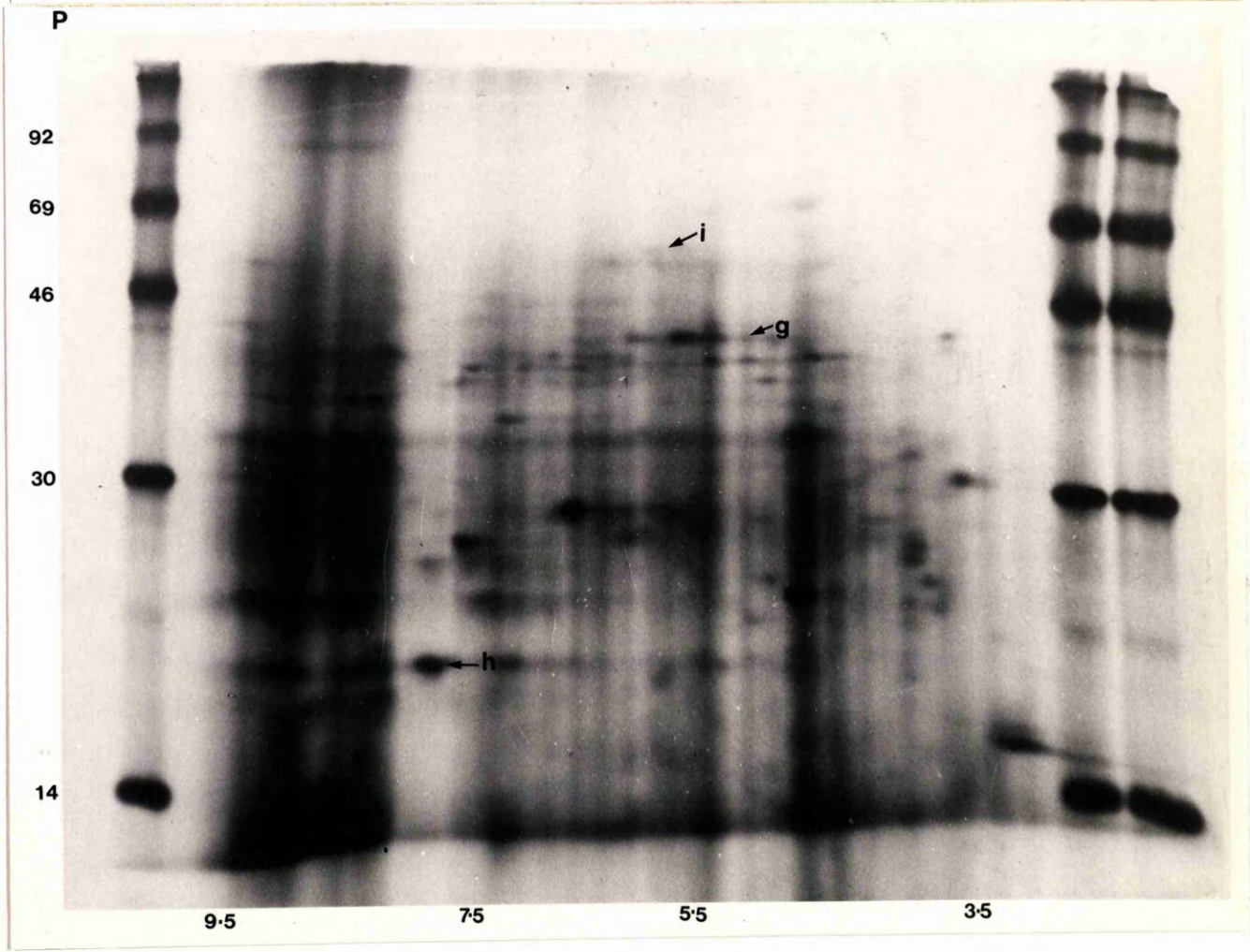
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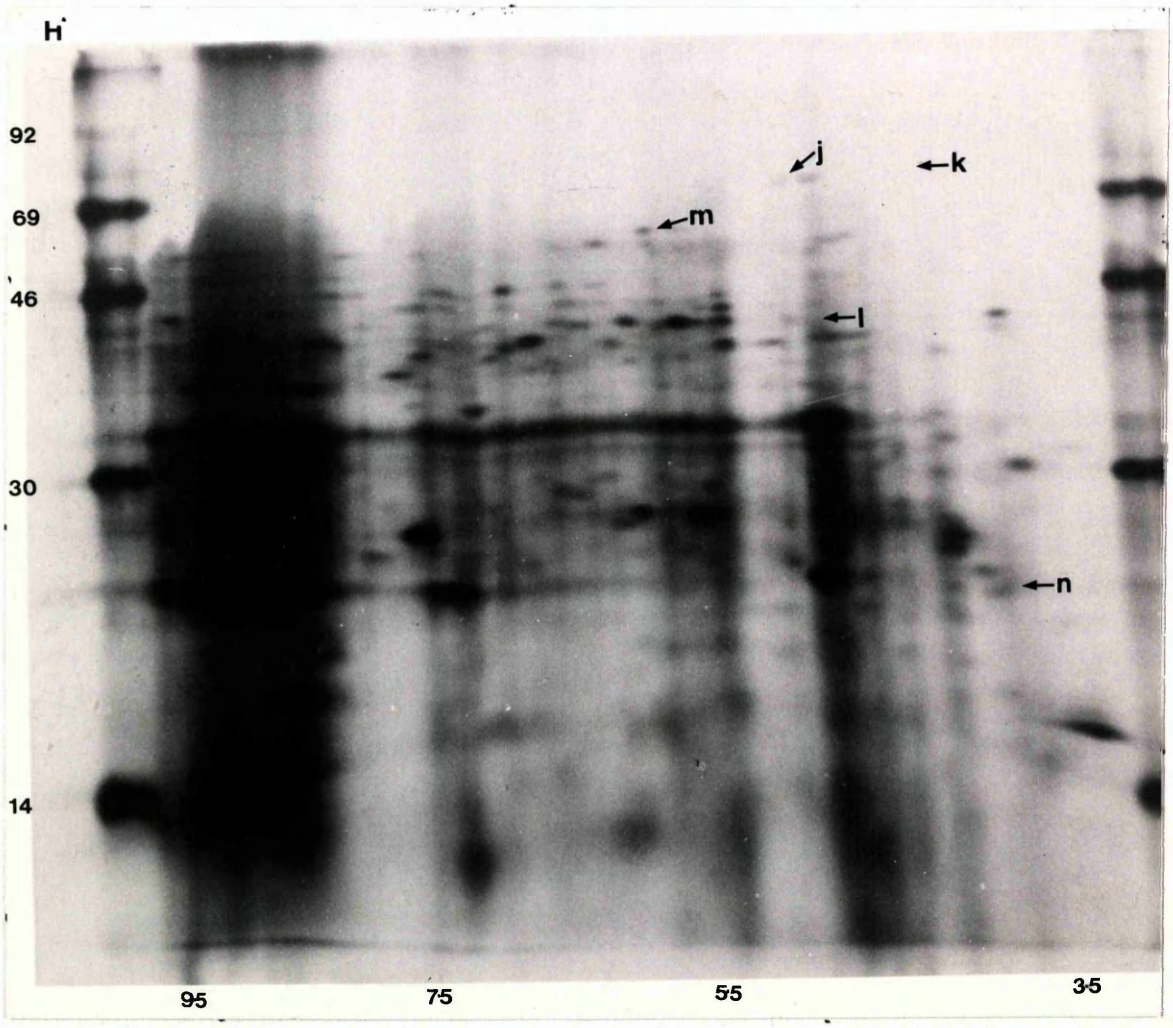
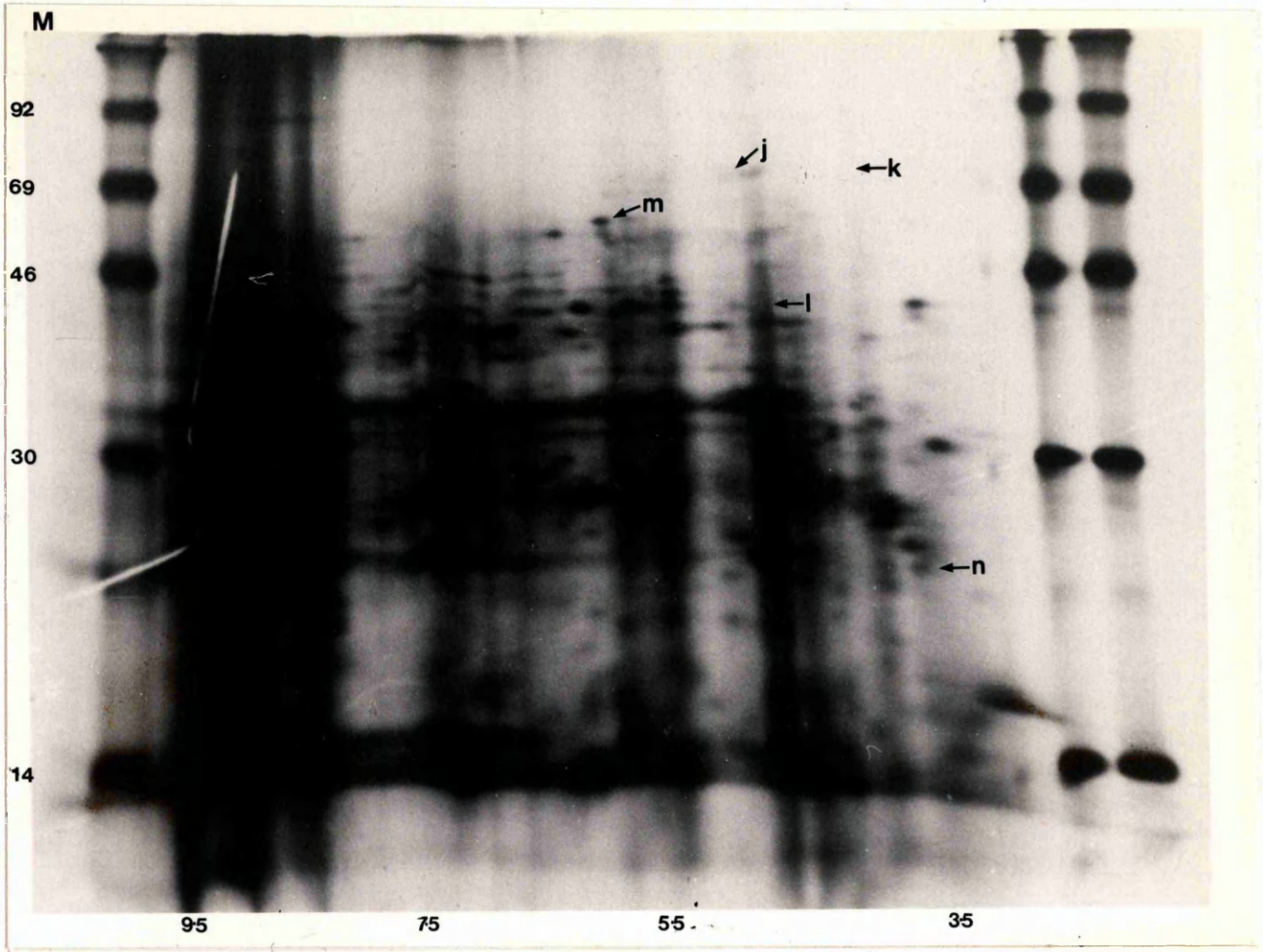
Fig. 3.29. Messenger RNA differences in leaf position three between PVX-inoculated, mock-inoculated and healthy plants, nine days after inoculation on leaf position one. Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 5 µg of total RNA from potato cv. Pentland Ivory leaf position three 9 days after inoculation on leaf one with PVX strain KEBRS (P), mock-inoculated with water (M), or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.

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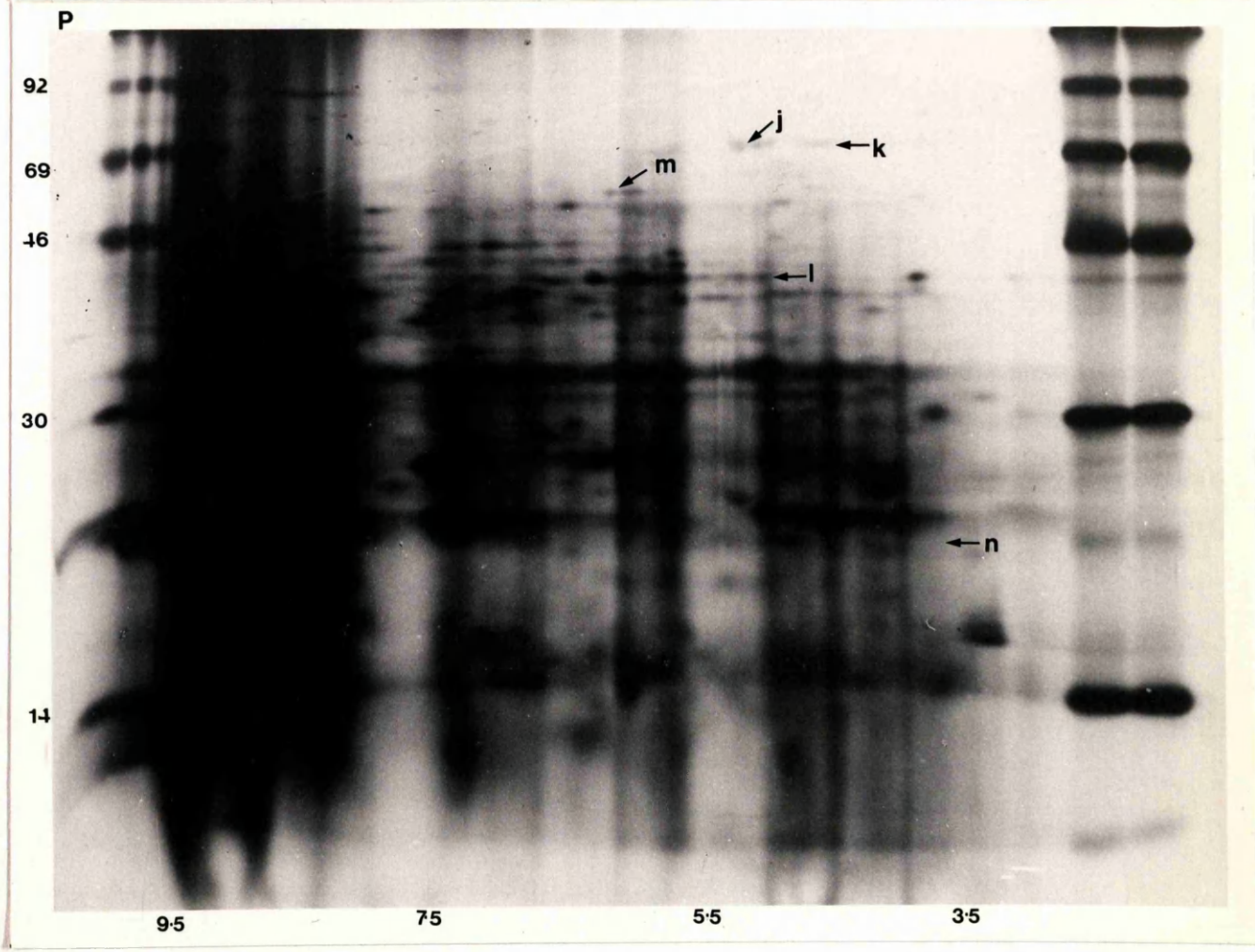
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Table 3.4. Changes in intensity of *in vitro* translated polypeptides between PVX-KEBRS-inoculated, mock-inoculated and healthy control plants for leaf position one (inoculated leaf) and leaf position three (uninoculated leaf) in Pentland Ivory.

		Healthy	Mock-inoculated	PVX-KEBRS inoculated	Size KDa	PI
Leaf One	a	+	+	+++	36	5.0
Day seven	b	+	+	+++	22	7.5
	c	+	+++	++	30	6.8
Leaf Three	d	++	++	+++	33	5.0
Day seven	e	+	+	+++	42	3.5
	f	+++	+	+	24	5.5
Leaf One	g	-	-	++	42	5.0
Day nine	h	-	+	+++	21	8.0
	i	-	+++	+	60	5.8
Leaf Three	j	+	+	++	68	4.0
Day nine	k	-	-	+	68	4.5
	l	-	-	+	40	4.5
	m	++	+++	+	56	6.0
	n	++	++	-	22	3.8
Leaf One	o	++	++	+++	70	4.5-5.0
Day eight	p	-	-	++	92	7.5
	q	+	+	+++	91	8.0-9.0
	r	+	+	+++	36	4.0
	s	+++	++	+	44	7.3
	t	++	-	+	70	7.1
	u	+++	+	++	56	6.8
Leaf Three	v	+	+	+++	34	6.0
Day eight	w	+	+++	+++	35	4.5
	x	+	+++	+++	16	4.2
	y	+++	++	++	32	4.8
Leaf One						
Day twelve	z		-	++	39	5.5
Leaf Three	A	+	++	+++	70	4.5-5.0
Day twelve	B	+	-	+++	52	7.5
	C	+	+	+++	52	6.1
	D	-	-	+++	52	5.5
	E	+	++	+++	45	3.5
	F	+	-	++	35	8.5
	G	-	+	++	28	6.0
	H	++	++	+	40	8.2
	I	++	++	-	35	8.2
	J	+++	+++	+	22	9.5
	K	-	++	-	57	7.3
	L	+	+++	+	50	7.0
	M	+++	+	+++	16	4.8

Intensity: greater +++ —> ++ —> + —> - absent

The table summarises the major polypeptides which appear to change in abundance as a result of the three experimental treatments.
Healthy = Healthy Pentland Ivory plants not inoculated on leaf position one.
Mock-inoculated = Pentland Ivory plants mock-inoculated on leaf position one.
PVX-KEBRS-inoculated = Pentland Ivory plants inoculated with PVX-KEBRS on leaf position one.
Each polypeptide is allocated a letter which relates to the appropriate two-dimensional gel figure it was identified on (see left margin). The pI and molecular weight in KDa are indicated for each polypeptide at the right margin of the table. The polypeptides which appeared to differ in intensity between treatments are allocated an arbitrary value of abundance for each treatment. The gradings are +++>++>+>-, +++ being the most abundant, with progressively reducing values to - which equals the absence of the polypeptide in a particular treatment.

KDa polypeptide seen in one-dimensional gels (Fig. 3.23). A polypeptide (i) of 60 KDa was more intense in mock-inoculated plants compared to the other two treatments (Fig. 3.28).

Samples of mRNA, taken 9 days after inoculation from leaf position three and translated *in vitro*, showed three polypeptides (j, k, and l) that were more intense in PVX-inoculated plants compared to both controls (Fig. 3.29). Their respective sizes were 68, 68, and 40 KDa. Two polypeptides (m and n) of 56 KDa and 22 KDa respectively were reduced in intensity in PVX-inoculated plants compared to mock-inoculated and healthy plants (Fig. 3.29).

Several differences were observed between inoculated and non-inoculated leaves of PVX-inoculated plants, compared with mock-inoculated and healthy plants. Thus several significant changes in gene expression apparently occurred by day 7 after inoculation and by day 9 even more were obvious. The experiment was therefore repeated to confirm that the changes seen were genuine, and also to investigate the mRNA population later, at 12 days after inoculation.

In this second experiment the plants were grown in a growth room as described in Materials and Methods (section 2.2). Samples from PVX-KEBRS inoculated plants were generated from 6 plants for each sample time, while mock-inoculated and healthy samples were generated from 12 plants for each sample time. Samples from leaves one and three were collected 8 days after inoculation, to confirm the changes detected 7 and 9 days after inoculation in the first experiment, and also 12 days after inoculation, to extend the time period of observation. The PVX-KEBRS inoculated plants first developed lesions 7 days after inoculation, at 8 days after inoculation there was an average of 20 lesions per inoculated leaf and by 12 days the lesion number per leaf had further increased, averaging 40, with the average lesion diameter having increased from approximately 0.5 to approximately 1.0 mm. Two µg of poly A^+ RNA from each sample was translated and 5,000,000 d.p.m of TCA precipitable material from each translation was analysed by two-dimensional PAGE (Table 3.4).

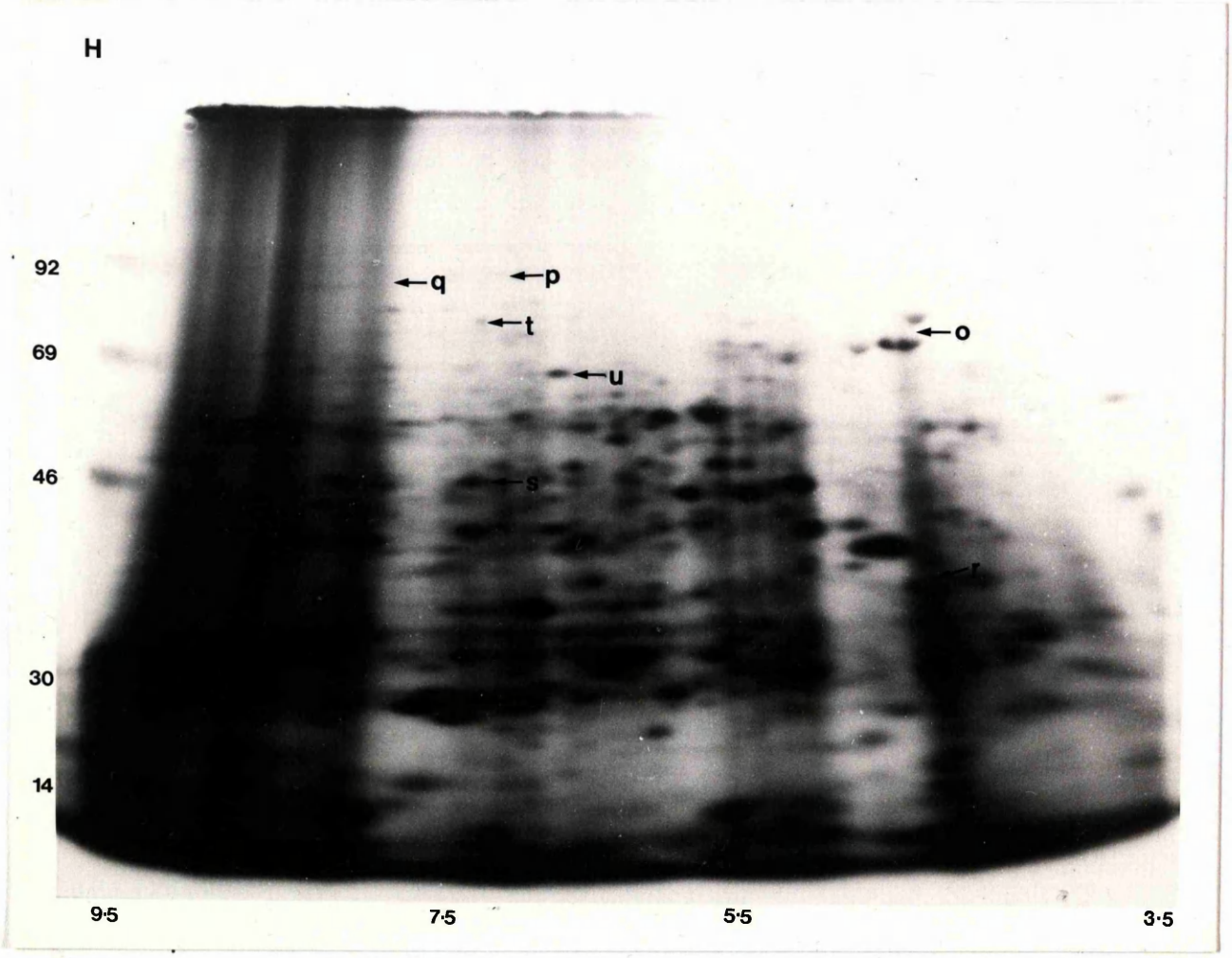
Eight days after inoculation, samples from leaf position one showed several changes (Fig. 3.30). Eleven polypeptides increased and three polypeptides decreased in intensity in PVX-inoculated leaves compared to controls. The polypeptides which increased in intensity consisted of a cluster of four polypeptides (o) of size 70 KDa, a second cluster of three polypeptides (p) of size 92 KDa, a third cluster of three polypeptides (q) of size 91 KDa, and a single polypeptide (r) of 36 KDa. The 36 KDa polypeptide corresponded in size and charge to the polypeptide (a), seen in leaf position one 7 days after inoculation with PVX in the previous experiment, and may therefore be the same (Fig. 3.26). This polypeptide might be associated with PVX resistance and lesion formation. A polypeptide (s) of 44 KDa was reduced in intensity in PVX-inoculated leaves compared to both control treatments. Polypeptides (t) of 70 KDa and (u) of 56 KDa were less intense in PVX and mock-inoculated leaves compared to healthy plants. These changes suggest that both PVX and mock-inoculation, as well as up-regulating several genes, also suppress the expression of a number of genes compared to healthy plants.

Differences between samples from leaf position three, 8 days after inoculation (Fig. 3.31), were limited to a polypeptide (v) of 34 KDa which was most intense in PVX-inoculated plants, less so in mock-inoculated plants, and at the lowest levels of expression in healthy control plants. Two polypeptides (w) and (x), of 35 KDa and 16 KDa respectively, were more intense in both PVX and mock-inoculated plants compared to healthy control plants, showing that inoculation damage alone can induce systemic expression of certain genes. A polypeptide (y) of 32 KDa was expressed at higher levels in healthy plants compared to both mock and PVX-inoculated plants.

By day 12, fewer differences were seen in the inoculated leaves. The only possible difference was a polypeptide (z) of 39 KDa which was more intense in PVX-inoculated leaves compared to control plants (data not shown).

In contrast, 12 days after inoculation, leaf position three showed greater differences compared to controls. Ten polypeptides, consisting of a group of four polypeptides (A) of 70 KDa and six others (B), (C), (D), (E), (F) and (G) of 52, 52,

Fig. 3.30. Messenger RNA differences in leaf position one between PVX-inoculated, mock-inoculated and healthy plants, eight days after inoculation on leaf position one. Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 2 µg of poly A^+ RNA from potato cv. Pentland Ivory leaf position one, 8 days after inoculation on leaf one with PVX strain KEBRS (P), mock-inoculated with water (M), or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.



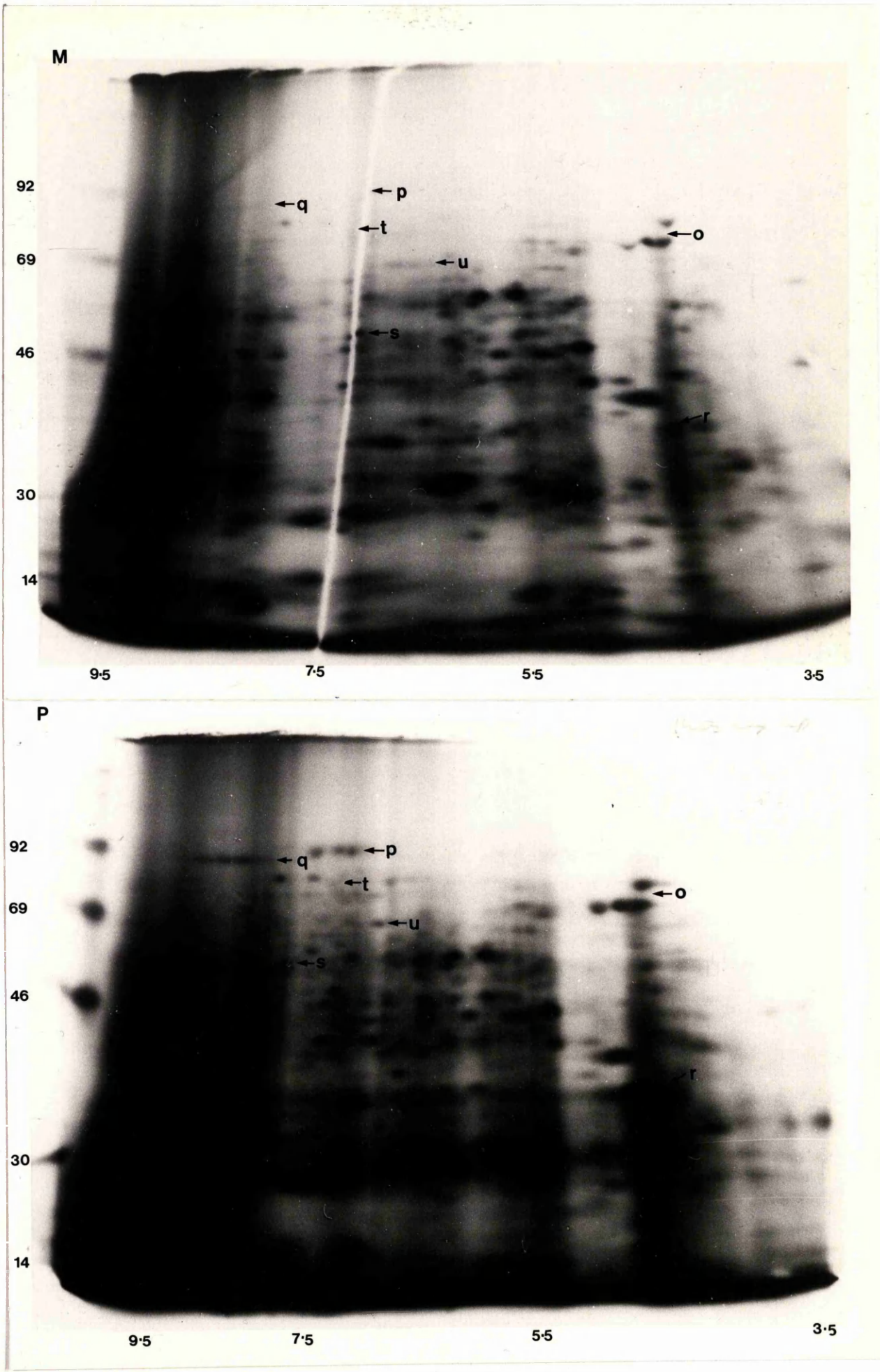

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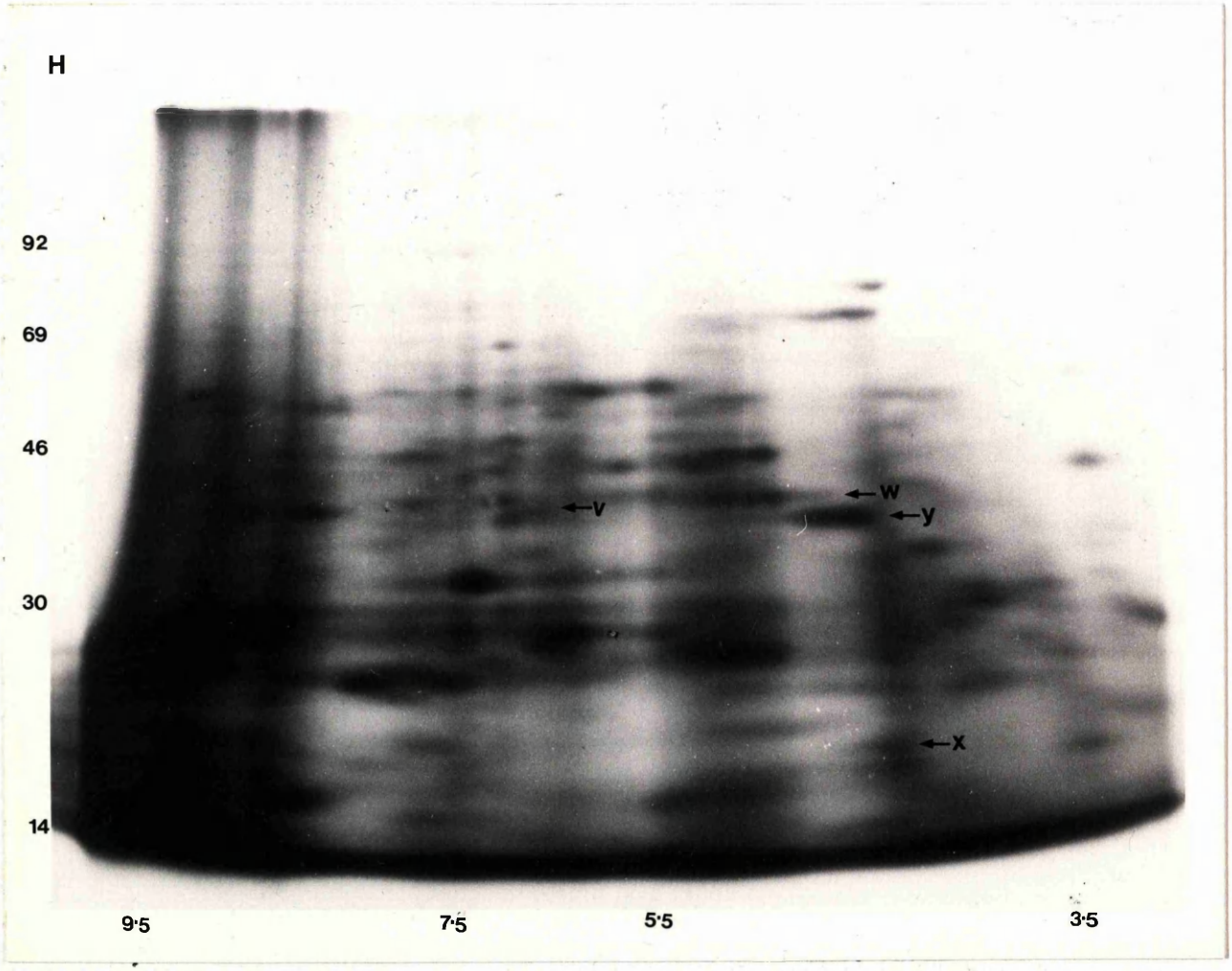
Fig. 3.31. Messenger RNA differences in leaf position three between PVX-inoculated, mock-inoculated and healthy plants, eight days after inoculation on leaf one.
Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 2 µg of poly A^+ RNA from potato cv. Pentland Ivory leaf position three 8 days after inoculation on leaf one with PVX strain KEBRS (P), mock-inoculated with water (M), or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.



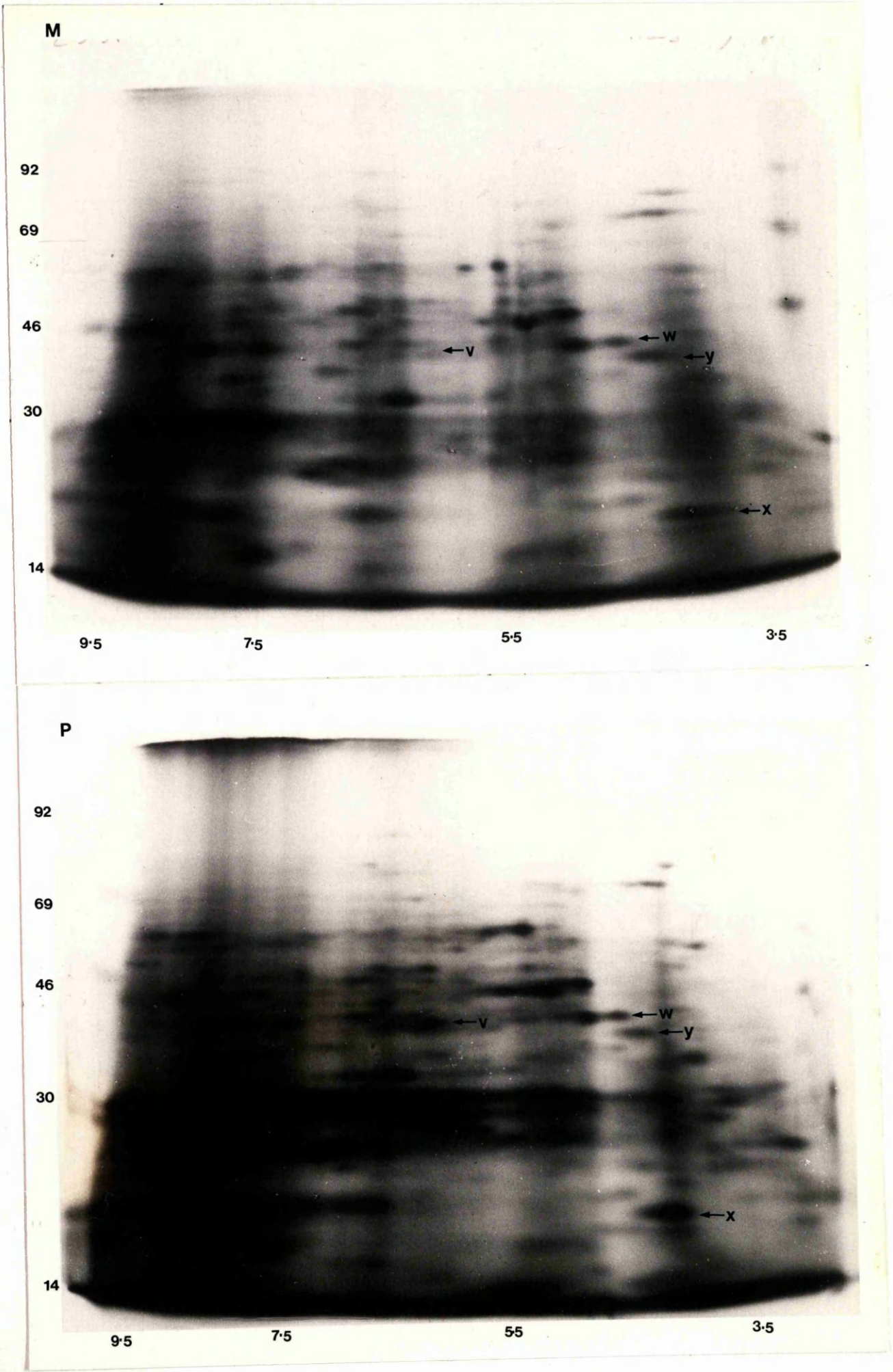

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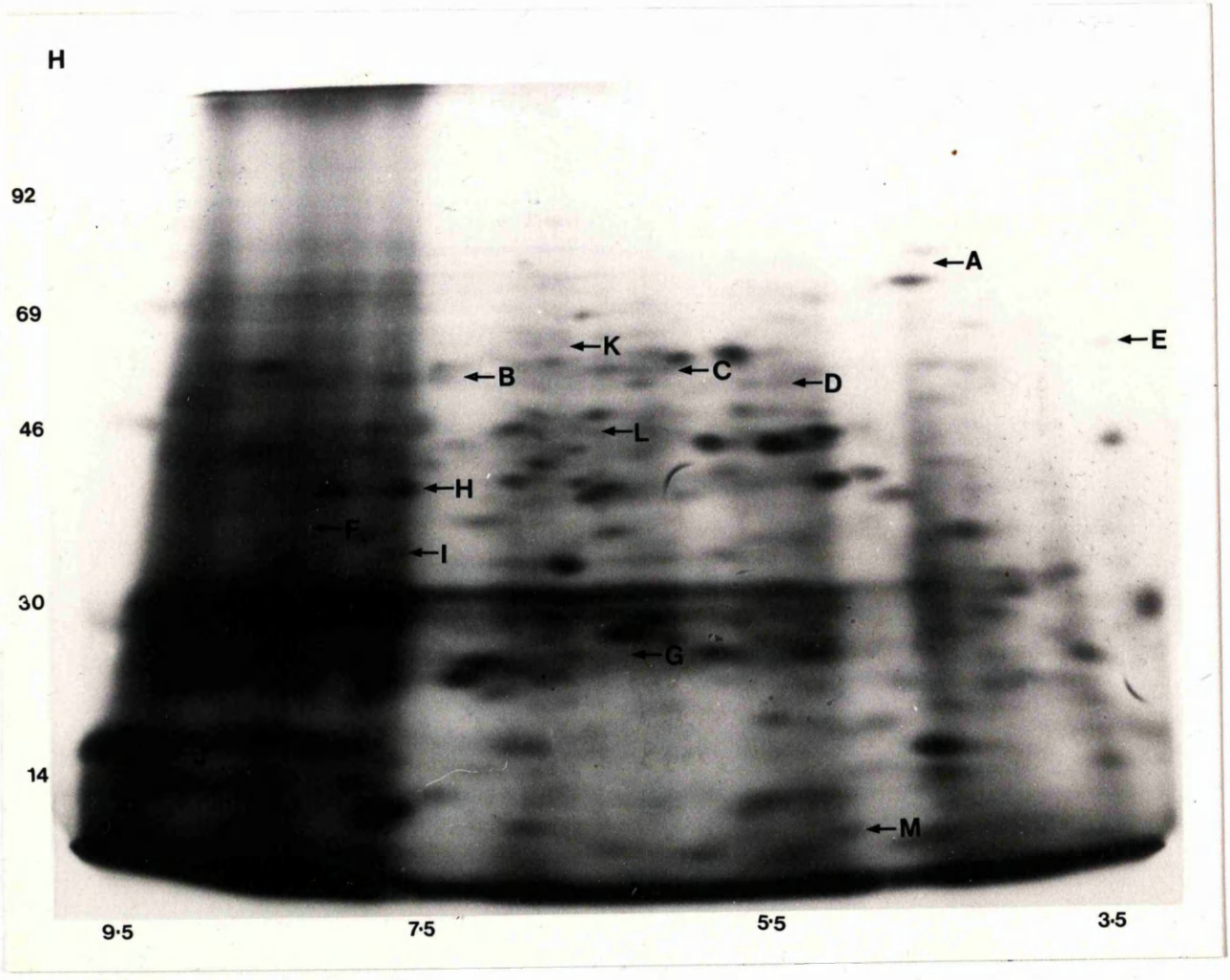
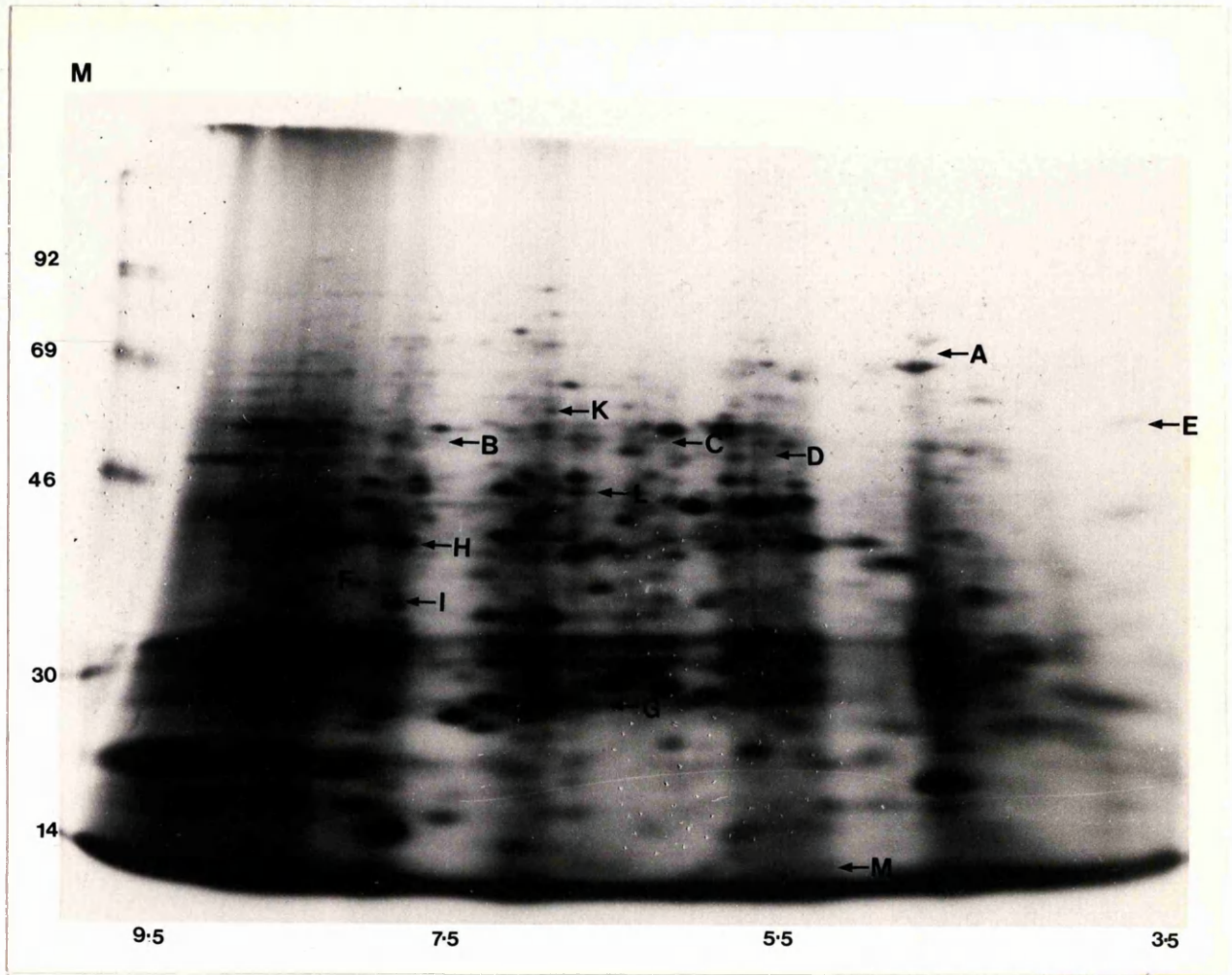
Fig. 3.32. Messenger RNA differences in leaf position three between PVX-inoculated, mock-inoculated and healthy plants, twelve days after inoculation on leaf one.

Two-dimensional fluorographs of ^{35}S methionine labelled polypeptides synthesised *in vitro* using 2 µg of poly A^+ RNA from potato cv. Pentland Ivory leaf position three 12 days after inoculation on leaf one with PVX strain KEBRS (P), mock-inoculated with water (M), or left alone as healthy controls (H). Arrows mark the position of the most prominent polypeptides which differ between treatment groups (For discussion see text). The size and pI of each polypeptide change is given in Table 3.4. The lefthand lane shows the position of labelled molecular weight standards and their size in KDa; pI is indicated along the bottom.

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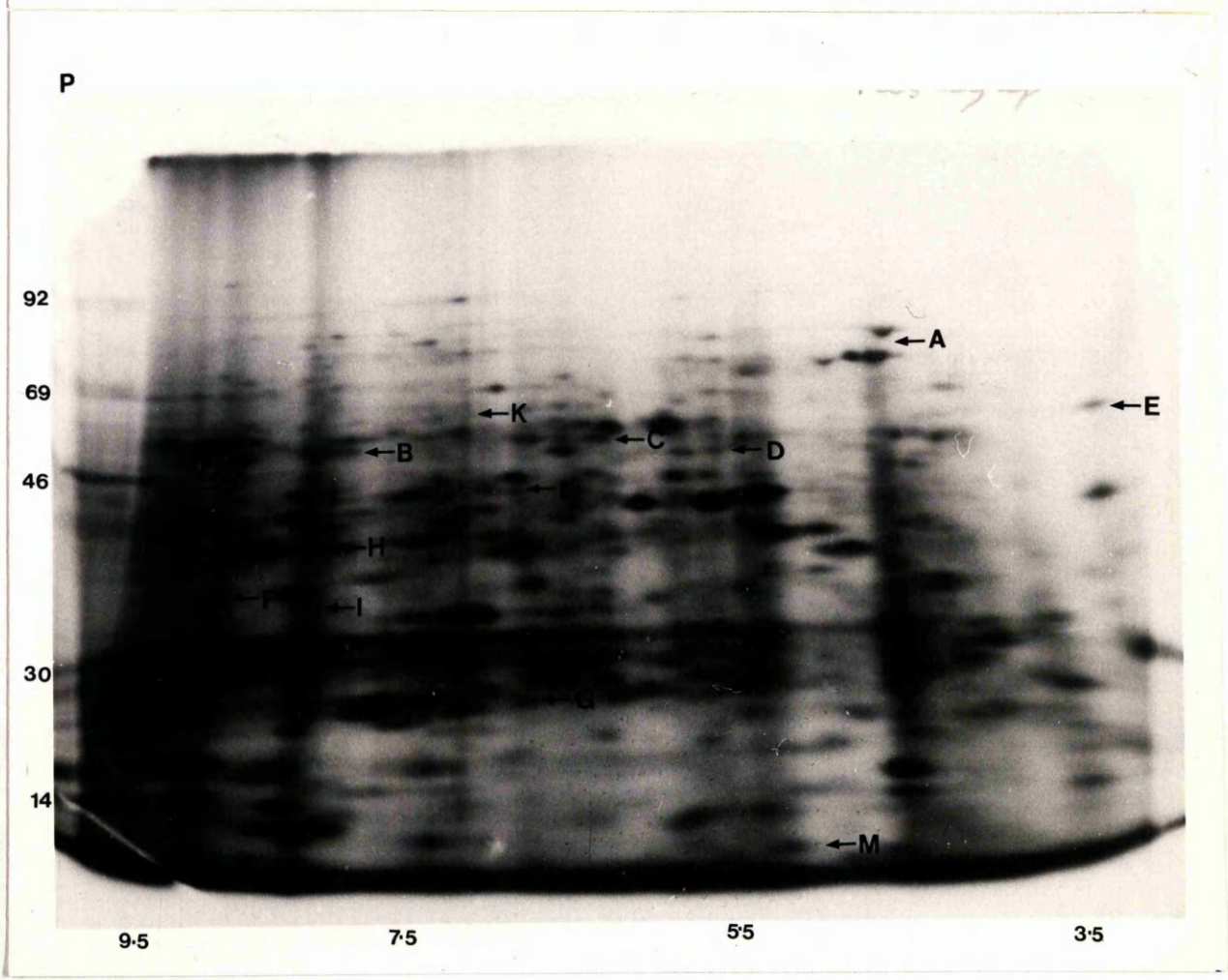
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52, 45, 35 and 28 KDa respectively (Fig. 3.32), were more intense in samples from PVX-KEBRS inoculated plants compared to control plants. The four polypeptides (A) of 70 KDa corresponded in size and charge to the cluster of four polypeptides (o) of size 70 KDa seen on leaf one 8 days after inoculation and the polypeptide (j) of 68 KDa seen in leaf three 9 days after inoculation in the first experiment (Figs. 3.29 and 3.30). Three other polypeptides (H), (I) and (J), of 40, 35 and 22 KDa respectively, were more intensely expressed in control plants compared to PVX inoculated plants. There were also polypeptides (K) and (L), of 57 and 50 KDa respectively, which were more strongly expressed in mock-inoculated plants compared to the other two treatments, while the polypeptide (M) of 16 KDa was expressed at a lower level in mock-inoculated plants compared to the other two treatments (Fig. 3.32).

Comparing the two experiments, many of the differences between PVX, mock-inoculated and control plants were not observed consistently. Some changes were consistent between experiments and these are highlighted in the description of protein changes between treatments. However, two-dimensional gels are notorious for generating variable results due to the use of carrier ampholytes during the IEF step, as prolonged focusing time results in pH gradient instability. Secondly, electroendosmotic effects result in the pH gradient moving to the cathode (cathodic drift) and flattening in the centre (plateau phenomenon); consequently time-dependent, not stationary, protein patterns are obtained. In addition, the reproducibility of pH gradient profiles is limited by the batch-to-batch variability of carrier ampholyte preparations (Görg, 1991). Although these inconsistencies make absolute interpretation of the changes difficult, they indicate that 7-9 days after inoculation obvious changes in gene expression as a consequence of PVX-inoculation can be detected in the inoculated leaf and that 9-12 days is the corresponding time in upper leaves. Some of the differences between experiments may be due to physiological differences between the plants in each experiment. Although in both experiments plants were maintained in a growth room under identical conditions for the duration of the experiment, plants used for the first experiment were raised in the glasshouse under a long-day. Also, the plants for each experiment were grown from

tubers which differed in age. These physiological differences may be responsible for the difference in the pattern of gene expression in the plant. Differences were apparent in general patterns of polypeptides on the two-dimensional gels between the two experiments. Different batches of wheatgerm were used to translate the mRNA, possibly this may have influenced the translation products. Translational differences of the same mRNA, occurring due to the use of different commercial batches of wheat germ, have been shown by other workers (Dominy & Thomas, Personal communication, 1991).

However, despite these inconsistencies, a visual examination of the overall pattern of polypeptides between the different treatments in the *same* experiment showed the patterns to be virtually identical in both intensity and position. Thus, differences between treatments within the same experiment can be attributed to genuine effects of virus or mock-inoculation.

Analysis of the two-dimensional gels showed both local and systemic changes resulting from PVX-KEBRS inoculation starting 7 days after inoculation, with the maximum number of systemic changes seen at day 12. The cDNA library was therefore generated using mRNA extracted from leaf position three 12 days after inoculation with PVX-KEBRS, since plants clearly showed changes in systemically expressed mRNAs at this time. The length of time taken for systemic changes in mRNA populations to peak compared to the time taken from changes in gene expression in the inoculated leaves are in accordance with the results of Cornelissen *et al.* (1986) in tobacco.

3.6. CONSTRUCTION OF THE POTATO cDNA LIBRARY.

A cDNA library was constructed from mRNA isolated from non-inoculated leaves (above the inoculated leaf) from Pentland Ivory plants pre-inoculated with PVX-KEBRS. By isolating mRNA from a non-inoculated leaf, it was hoped that changes in the mRNA population due to wounding, and resulting from the hypersensitive necrosis, would be minimised. Moreover, by isolating RNA from upper leaves, the possibility of cloning cDNAs from viral mRNAs could be avoided.

The cDNA library was generated from poly A^+ RNA extracted from leaf position three from Pentland Ivory plants, 12 days after inoculation of leaf one with PVX-KEBRS (Table 3.5). This particular time after inoculation was chosen because earlier two-dimensional gel analysis of mRNA changes after PVX-KEBRS inoculation had identified 9-12 days as the time at which the most obvious changes in mRNA populations could be detected (section 3.5).

The Pentland Ivory plants inoculated with PVX-KEBRS developed, on average, 20 lesions on their inoculated leaves 8 days after inoculation. The average lesion diameter was approximately 0.5 mm. The lesions gradually increased in number to average 35 lesions per leaf 20 days after inoculation, and with a maximum diameter of approximately 2 mm by day 18. Mock-inoculated plants only showed slight inoculation damage on their inoculated leaf. With the exception of the inoculated leaf, plants in all three treatment groups appeared healthy throughout the experiment. A control group of King Edward plants developed a light systemic mosaic 14 days after inoculation, increasing in intensity up to 20 days. This confirmed that the PVX-KEBRS inoculum used was still capable of causing systemic infection in a susceptible cultivar.

The poly A^+ RNA extracted from leaf position three leaves 12 days after PVX-KEBRS-inoculation on leaf one was analysed by gel electrophoresis on a 1.5% non-denaturing agarose gel (Fig. 3.33). Since the poly A^+ RNA appeared to be undegraded it was used to generate a cDNA library as described in Materials and Methods. One µg of poly A^+ RNA was converted into double stranded cDNA. The first strand cDNA reaction transcribed 50% of the available poly A^+ RNA yielding

TABLE 3.5. Characterisics of the Pentland Ivory plants used to generate and screen the potato cDNA library.

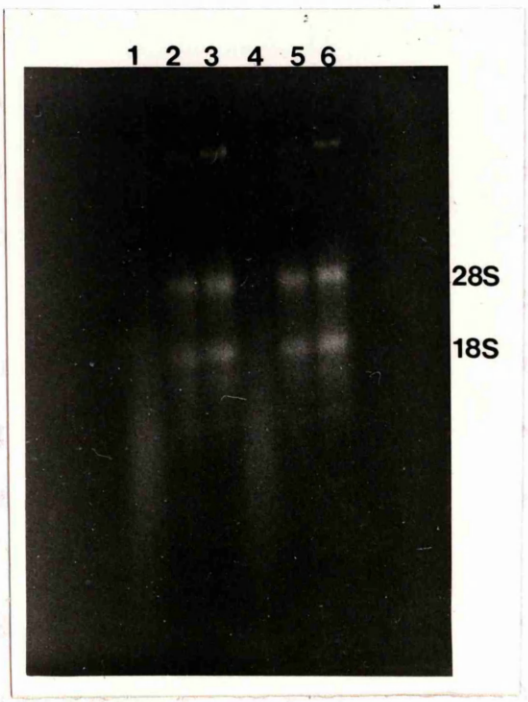
Experiment 1

Days after inoc.	Average height in cm	Average number leaves	L1 gm	L3 gm	RNA L1 µg	RNA L3 µg
KEBRS inoculated plants on leaf one						
6	10.2	10.5	4.5	3.7	1960	3480
8	13.0	12.0	6.3	4.3	1920	2580
10	11.5	11.0	4.3	4.8	2040	2160
12	13.7	12.0	5.3	5.2	1200	2560
14	13.5	12.0	5.0	5.5	1360	2960
16	14.5	13.0	5.7	4.1	2080	1720
18	17.0	13.0	4.4	5.4	1280	3400
20	16.0	13.0	6.0	6.3	1720	2700
Mock-inoculated plants on leaf one						
6	9.5	10.2	5.3	3.4	3200	3440
8	11.0	11.0	5.1	3.5	2280	5820
10	12.5	12.0	4.7	5.1	3660	2460
12	14.5	12.5	4.2	5.2	1560	2200
14	12.0	12.5	4.2	4.3	2120	2360
16	13.0	12.0	5.3	5.3	2520	2720
18	13.5	13.0	5.2	5.1	2400	1260
20	14.0	13.0	5.2	5.7	2080	3160
Healthy plants						
6	9.8	10.5	4.3	3.3	1840	2880
8	13.0	12.0	5.5	3.9	2820	2820
10	13.5	12.5	4.5	3.7	1800	3540
12	15.7	13.0	5.2	4.8	2640	2960
14	17.0	13.2	3.9	4.5	1800	2800
16	16.0	12.0	5.2	4.5	2040	2760
18	16.2	12.0	5.7	4.8	1860	2940
20	15.0	11.0	4.8	3.3	3040	3000

Each sample consisted of 4 individual plants collected 6, 8, 10, 12, 14, 16, 18 and 20 days after the plants were either inoculated on leaf position one with KEBRS of 1:5 (w/v), or mock-inoculated, or left alone as healthy plants. At each sample time the plant height was recorded and presented as an average height in centimetres. The number of leaves on each plant were also recorded and presented as an average number per plant. These data show that the plants are highly uniform between treatment groups. For each sample time leaf position one (L1) and leaf three (L3) were sampled and the total weight of leaves for each sample is given in grams. The total RNA from each leaf sample was extracted and is presented as the total RNA extracted from leaf one (RNA L1) and leaf three (RNA L3) as µg RNA. The poly A^+ RNA was extracted from only certain RNA samples and used for construction and screening the cDNA library, these samples are referred to in the text.

Fig. 3.33. The poly A^+ RNA used to generate the cDNA library.

Poly A^+ RNA from Pentland Ivory (lanes 1 and 4) resolved on a 1.5% non-denaturing agarose gel. The Poly A^+ RNA was undegraded and of typical size to that seen for potato Poly A^+ RNA. Total Pentland Ivory RNA (lanes 2, 3, 5 and 6) shows the position of the 28S and 18S ribosomal bands.


1 2 3 4 5 6
28S
18S

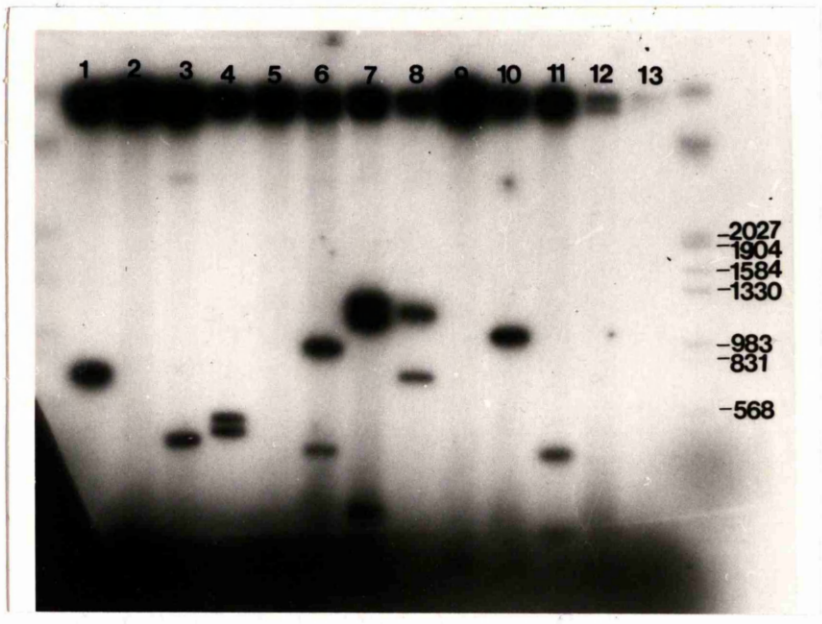
500 ng of first strand cDNA. The second strand cDNA reaction converted 90% of the first strand cDNA into double stranded cDNA giving a final yield of 900 ng of double stranded cDNA. Analysis of the first and second strand reactions on a 1.5% alkaline agarose gel (Sambrook *et al.*, 1989) showed the ss cDNA and ds cDNA samples to be of equal size ranging from 0.5-3.5 Kbp and indicated a low level of hairpin structures (Watson & Jackson, 1986). These data are not shown. The cDNA was methylated, linkers added (see Materials and Methods) and then ligated with arms from the lambda vector NM1149. Two libraries were generated, the larger using 1 μg of lambda arms ligated to 50 ng of cDNA and the smaller using 0.5 μg lambda arms ligated to 25 ng of cDNA. Each library was packaged using Giga Pack Gold Plus packaging extracts (Stratagene). A control of 1 μg of vector arms alone were ligated and on plating 3,500 plaques were seen. Both potato cDNA libraries were titred and the smaller, containing a total of 2.5×10^5 p.f.u., was used for subtractive and differential screening. The larger library, containing 5×10^5 p.f.u., was amplified (Sambrook *et al.*, 1989) giving a total of 1.56×10^9 p.f.u. Both the amplified and unamplified libraries were stored at 4°C.

To determine the proportion of recombinants (phage) containing cDNA inserts, DNA was prepared from twenty plaques chosen at random from the unamplified library. The phage DNA was digested with *Eco*R1 and *Hin*dIII to release the cDNA insert. The restriction fragments were then end-labelled with α^{32}P dATP, using the Klenow fragment (Sambrook *et al.*, 1989). The labelled phage DNA preparations were then analysed by electrophoresis on a 1.5% agarose gel and the bands visualised by autoradiography. A cDNA insert was detectable in 16 of the plaques. The inserts ranged in size from 2.7 Kbp to 0.1 Kbp (Fig. 3.34).

To check that the library was representative of the mRNA population in potato, it was screened with a Rubisco small subunit cDNA clone pPVSS 1672 from *P. vulgaris* which contained a full length cDNA insert cloned in pUC 18. (Knight, 1989). The plasmid was digested with *Eco*RI to cut out the Rubisco insert and this was then purified using 'Gene Clean' (Materials and Methods, section 2.28). The insert was labelled and then used to screen 9,000 plaques. A total of 234 plaques

Fig. 3.34. Electrophoresis of NM1149 recombinant phage.

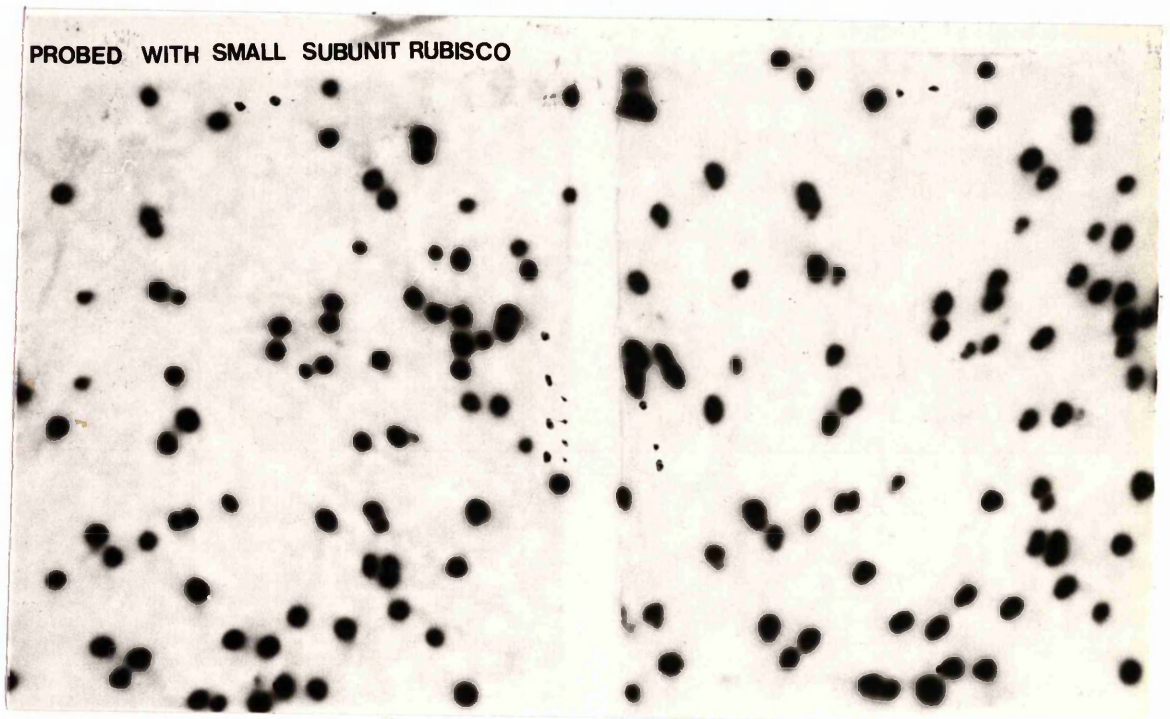
Twenty plaques were picked randomly (13 are shown here) and the cDNA insert labelled with ^{32}P. The cDNA inserts were electrophoresed on a 1.5% non-denaturing agarose gel and are numbered 1-13. 80% of the plaques picked had a detectable cDNA insert within them, the size of cDNA insert ranged from 2.7-0.1 Kbp. Size standards, in base pairs, are indicated on the right side of the gel.


1
2
3
4
5
6
7
8
9
10
11
12
13
2027
1904
1584
1330
983
831
568

(2.6% of the total number of recombinants) hybridised to the probe (Fig 3.35). This suggests that the cDNA library contained a representative sample of a potato mRNA population since the amount of Rubisco small subunit mRNA as a proportion of the total mRNA in light grown plants is 3%-5% (Jenkins, G.I. personal communication). To confirm that no contaminating plasmid DNA had been cloned, 3,000 plaques were screened with a pBR322 recombinant containing a cDNA Drosophila insert (Russell, personal communication). No clones hybridised.

Fig. 3.35. Potato cDNA library screened with a probe for the small subunit of Rubisco.

Nine thousand plaques (3,000 plaques per plate) were screened with a ^{32}P labelled random primed *P. vulgaris* small subunit Rubisco cDNA clone. Duplicate filters from each plate were hybridised with the probe and washed with 1X SET at 68°C. The probe hybridised to 234 plaques, representing that a frequency of 2.6% of the clones in the library were small subunit Rubisco cDNA clones. The photograph shows duplicate filters from a single plate.


PROBED WITH SMALL SUBUNIT RUBISCO

3.7. DIFFERENTIAL SCREENING OF THE UNAMPLIFIED LIBRARY.

The library was screened differentially with ss cDNA probes generated from poly A^+ RNA extracted from leaf position three of PVX-KEBRS-inoculated (on leaf position one), mock-inoculated and healthy Pentland Ivory plants. The results were analysed in order to identify plaques hybridising differentially to one or two of the three probes. This enabled the identification of differentially expressed cDNA clones, either those induced as a result of inoculation damage, or those induced by PVX-KEBRS localisation on Pentland Ivory.

Two methods were used to screen a total of 28,000 plaques. For the first, duplicate plaque lifts were hybridised individually to either cDNA probes from PVX-KEBRS-inoculated or mock-inoculated plants, which had been subtracted with mock-inoculated poly A^+ RNA (for subtractive probes see Materials & Methods, Section 2.24). For the second screen, triplicate plaque lifts were taken and each filter was hybridised to a cDNA probe from one of the three treatment groups (differential probes).

The library was screened several times with RNA from the same group of plants used to produce the cDNA library (experiment 1, Table 3.5) and also with RNA generated from plants grown under identical conditions to those used to make the library but at a different time (experiments 2 and 3, Tables 3.6 and 3.7 respectively). This was required as there was not sufficient mRNA generated in experiment 1 to complete the screening of the library.

Plants in the second group (experiment 2, Table 3.6) developed lesions on inoculated leaves 7 days after inoculation; these were approximately 0.5 mm in diameter by day 8, and approximately 1 mm in diameter, with an average of 40 lesions per inoculated leaf, by day 12. Mock-inoculated plants only showed slight inoculation damage, and plants from all three treatment groups, with the exception of the inoculated leaves, looked healthy throughout the experiment.

The Pentland Ivory plants from experiment 3 developed their first lesions 6 days after inoculation. Eight days after inoculation the lesions averaged 0.5 mm in diameter with 5-10 lesions per inoculated leaf. The lesion size and number gradually

TABLE 3.6. Characteristics of the Pentland Ivory plants used to screen the potato cDNA library.

Experiment 2

Days after inoc.	Average height in cm	Average number leaves	L1 gm	L3 gm	RNA L1 µg	RNA L3 µg	L1 Poly A^+ RNA µg	L1 % yield	L3 Poly A^+ RNA µg	L3 % yield
KEBRS inoculated plants										
8	10	12	6.6	5.6	2460	3130	76	3.1	48	1.5
12	10	13	5.9	7.6	4560	5930	Zero		61	1.0
Mock-inoculated plants										
8	10	12	13.3	9.9	5880	6140	192	3.3	250	4.0
12	10	12	14.5	11.0	8011	5400	186	2.3	102	1.8
Healthy plants										
8	10	12	13.6	12.4	7950	6960	64	0.8	260	3.7
12	10	12	11.3	10.3	4430	4290	141	3.2	128	2.9

The plants inoculated on leaf one with PVX-KEBRS (1:5 w/v) inoculum consisted of 6 plants per sample group. The mock inoculated and healthy control plants consisted of 12 plants per sample group. Samples were collected 8 and 12 days after inoculation treatment. At each sample time the height of the plant was recorded and presented as an average height in centimetres. The number of leaves on each plant was also recorded and presented as an average number per plant. For each sample time, leaf position one (L1) and leaf three (L3) were sampled and the total weight of leaves for each sample is given in grams. The total RNA from each leaf sample was extracted and is presented as the total RNA extracted from leaf one (RNA L1) and leaf three (RNA L3) as µg RNA.

TABLE 3.7. Characteristics of the Pentland Ivory plants used to screen the cDNA library and to investigate the differential expression of cDNA clones with time.

Experiment 3

Days after inoc.	Average height in cm	Average number leaves	RNA L1 µg	RNA L3 µg	L1 Poly A$^+$ RNA µg	L1 % yield	L3 Poly A$^+$ RNA µg	L3 % yield
KEBRS inoculated plants on leaf one								
8	10.0	10.0	1513	1561	27	1.8	27	1.8
10	11.0	11.0	1519	1575	22	1.5	28	1.9
12	12.0	12.0	1024	1506	19	1.9	11	0.7
14	13.5	12.5	1135	2010	16	1.6	32	1.6
Mock-inoculated plants on leaf one								
8	10.0	10.0	1542	2164	30	2.0	30	1.5
10	11.5	12.0	1510	2046	22	1.4	35	1.7
12	12.5	12.5	2115	2125	32	1.6	14	0.7
14	14.0	13.0	1508	2031	22	1.4	28	1.4
Healthy plants								
8	10.0	10.0	1023	1054	40	4.0	33	3.3
10	11.0	12.0	2204	1507	41	2.0	35	2.3
12	12.5	12.0	2119	2057	35	1.7	18	0.9
14	14.0	12.5	2009	2210	29	1.4	32	1.6

Each sample consisted of 5 individual plants collected 8, 10, 12 and 14 days after the plants were either inoculated on leaf position one with KEBRS 1:5 (w/v) inoculum, or mock-inoculated, or left alone as healthy plants. At each sample time the height of the plant was recorded and presented as an average height in centimetres. The number of leaves on each plant was also recorded and presented as an average number per plant. For each sample time, leaf position one (L1) and leaf three (L3) were sampled and the total weight of leaves for each sample is given in grams. The total RNA from each leaf sample was extracted and is presented as the total RNA extracted from leaf one (RNA L1) and leaf three (RNA L3) as µg RNA.

increased to 1.0-1.5 mm in diameter with 10-20 lesions per inoculated leaf 14 days after inoculation. Plants from all three treatment groups, with the exception of the inoculated leaves, looked perfectly healthy throughout the duration of the experiment (Table 3.7).

3.7.1. Subtractive probes used to screen the cDNA library.

Single stranded cDNA probes were generated from the poly A^+ RNA isolated from leaf position three of PVX-KEBRS-inoculated and from mock-inoculated plants 12 days after inoculation. The poly A^+ RNA from the PVX-KEBRS inoculated plants was part of that used to generate the library. The mock-inoculated plants were grown in parallel. The cDNA probes were generated from 1 µg poly A^+ RNA; about 80 µCi α^{32}P dCTP was incorporated into cDNA. In the first round of subtraction, each cDNA probe was incubated for 24 h with 13 µg of pooled driver poly A^+ RNA generated from leaf position three of mock-inoculated plants 8, 10, 14 and 16 days after inoculation. As the subtraction of common cDNA between the two probes is not 100% efficient, the subtraction of mock-inoculated cDNA probe with mock-inoculated mRNA acts as a control. Although, kinetically, a larger amount of driver poly A^+ RNA (about 40 µg) would have been optimal, this amount of poly A^+ RNA was too great to produce from the small amount of leaf tissue available in this experiment. Therefore, the amount of driver mRNA used was sub-optimal The RNA:cDNA duplexes which formed represent the common messages between PVX-KEBRS-inoculated and mock-inoculated plants. They were separated from ss cDNA messages by passage over hydroxylapatite, which binds ds RNA/cDNA more strongly than ss cDNA. The unhybridised ss cDNA was eluted from the column. The first round of hybridisation removed about 90% of the labelled cDNA. A further round of subtraction was carried out on each probe, using 10 µg of mock-inoculated mRNA as driver. This removed a further 5% of the total radioactivity of each ss cDNA probe. This implies that the greatest majority of hybridisable sequences were removed during the first round of hybridisation. The remaining subtractive probes were then used to differentially screen 12,000 plaques plated at a density of 3,000 per 10 x 10 cm petri-dish. Duplicate filters, lifted from the plaque arrays, were hybridised with the two

subtractive probes for 36 h and then washed at 68° in 0.2 X SET. These washing conditions are highly stringent.

Autoradiographs of these filters showed that five plaques hybridised preferentially to cDNA from PVX-KEBRS inoculated plants and one to cDNA from mock-inoculated plants (Fig. 3.36). Theses six plaques and their surrounding areas were picked using the wide end of a Pasteur pipette, replated to a final density of 300 plaques per 10 x 10 cm petri dish and rescreened with ss cDNA probes. The plaque PIX 2 (No. 2, Fig. 3.36) which showed the strongest differential expression with subtractive probes was plated out in a grid pattern of 840 plaques, and rescreened along with the other five clones. From this grid pattern three plaques, designated PIX 21, 24 and 26, were identified (Fig. 3.37). Of the remaining five potential differential clones, only one hybridised strongly. This was picked off the plate and designated PIX 6 (Fig 3.38). Therefore, as a result of screening with subtractive probes, four clones from an initial total of 12,000 recombinants were subjected to further analysis.

3.7.2. Differential screening of the library using three ss cDNA probes.

Single stranded cDNA probes, produced from poly A^+ RNA isolated from leaf position three of PVX-KEBRS-inoculated, mock-inoculated and untreated healthy plants 12 days after inoculation, were used to screen 28,000 plaques. In this case, the poly A^+ RNA was from the same batch of plants as that used to generate the cDNA library. The 12,000 plaques screened with subtractive probes were also rescreened differentially. Fourteen plaques preferentially hybridised to the probe from PVX-KEBRS-inoculated plants (potential positives), but only one seemed to be the same as one of those previously identified with the subtractive probes. However, on rescreening with new probes generated from the same batch of poly A^+ RNA, no differences were seen, suggesting that the results of the initial differential screen were false positives.

A further 16,000 plaques, plated at a density of 1,000 plaques per 10 x 10 cm petri dish, were screened. From this screen, 14 separate plaques hybridised preferentially to a probe from PVX-KEBRS-inoculated plants; 16 plaques hybridised

Fig. 3.36. Screening the potato cDNA library with subtractive probes.

Single stranded cDNA probes were generated from mRNA extracted from leaf position three, 12 days after PVX-KEBRS inoculation or mock-inoculation on leaf position one. Each ss cDNA probe was subtractively hybridised with mRNA from leaf position three, pooled from mRNA samples taken 8, 10, 14 and 16 days after mock-inoculation on leaf one. The autoradiograph, generated from screening 3,000 plaques with either PVX-KEBRS (PVX) or mock subtractive probes (MOCK), identified three plaques (1, 2 and 3), which represented potentially systemically up-regulated mRNAs in PVX-inoculated plants, and one plaque (4) which represented a potentially systemically up-regulated mRNA in mock-inoculated plants. The different specific activity of the two cDNA probes made interpretation of the plaque lifts difficult.

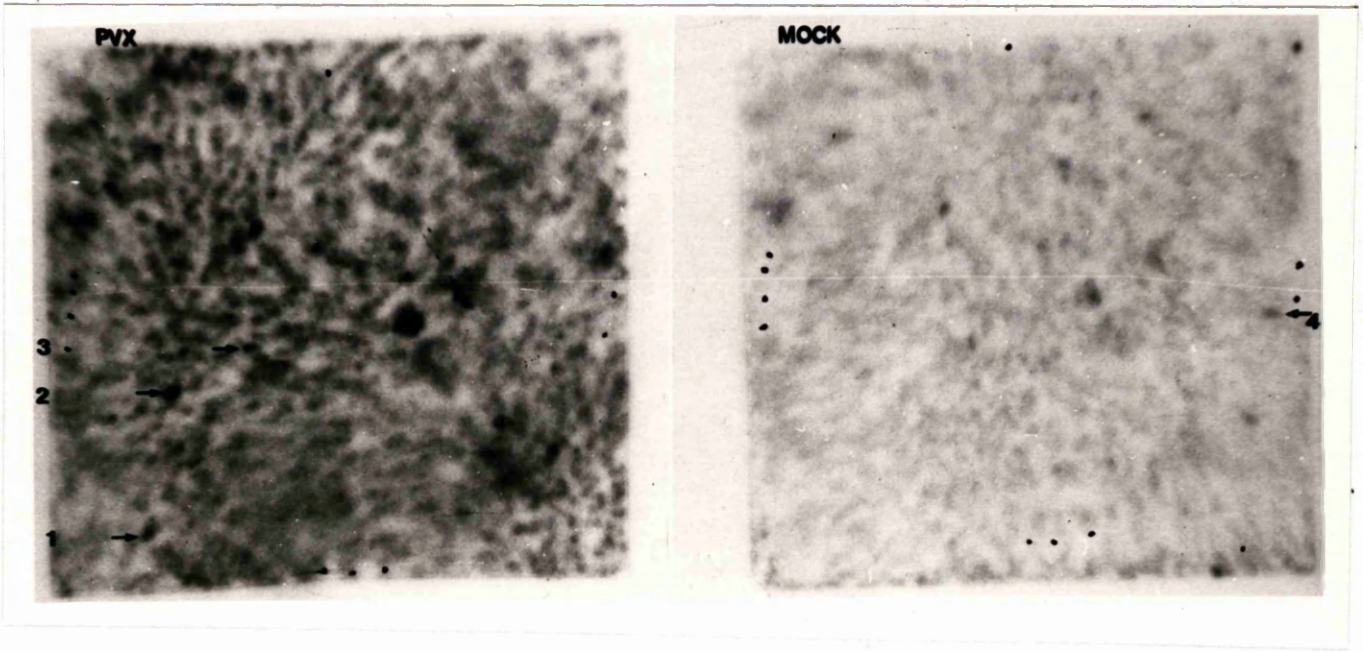

PVX
MOCK
3
2
1
4

Fig. 3.37. Rescreen of PIX 2 identified using subtractive probes.

The area of the plate containing the plaque which hybridised differentially to subtractive probes was picked and re-plated in a grid pattern of 840 plaques. Duplicate filters were screened with ss cDNA probes generated from leaf position three, 12 days after inoculation with either PVX-KEBRS (PVX) or mock-inoculation with water (MOCK) on leaf position one. Three plaques, PIX 21, 24 and 26, preferentially hybridised to the ss cDNA probe generated from PVX induced plants.

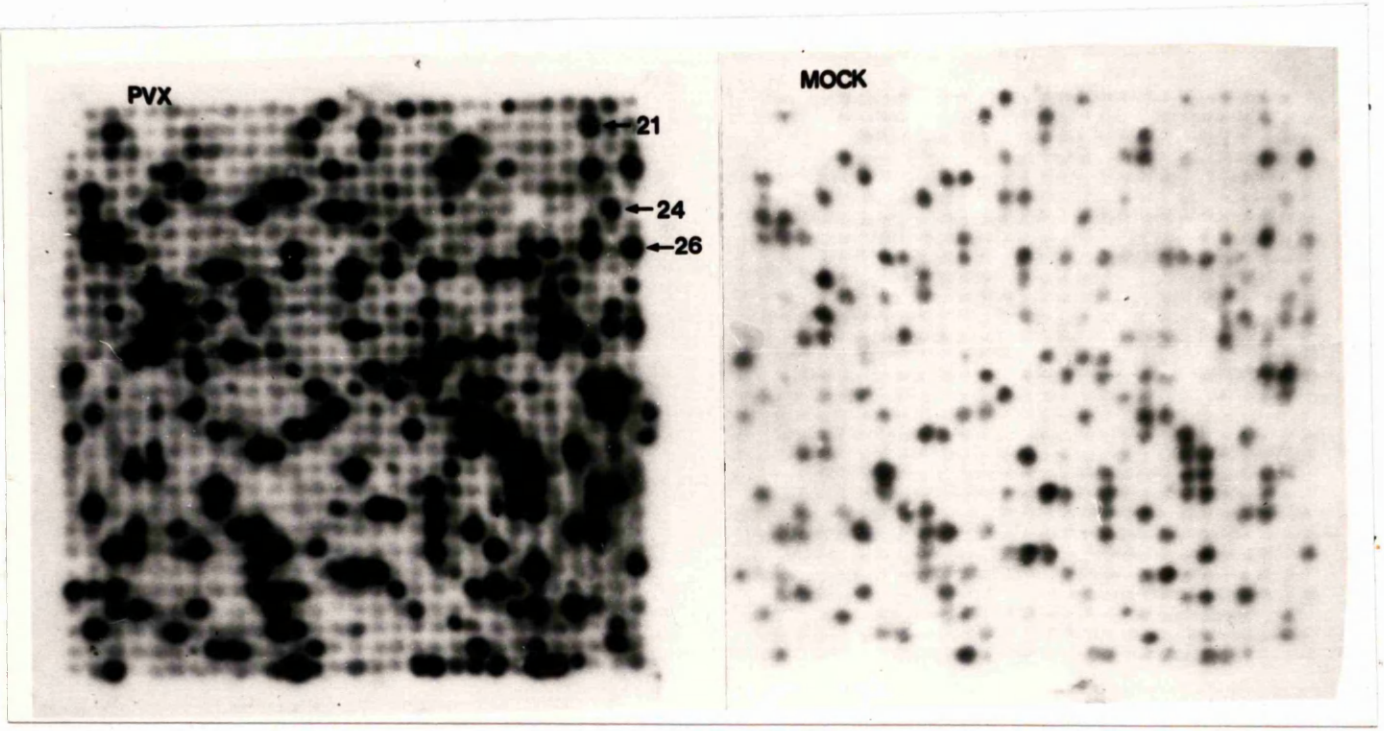
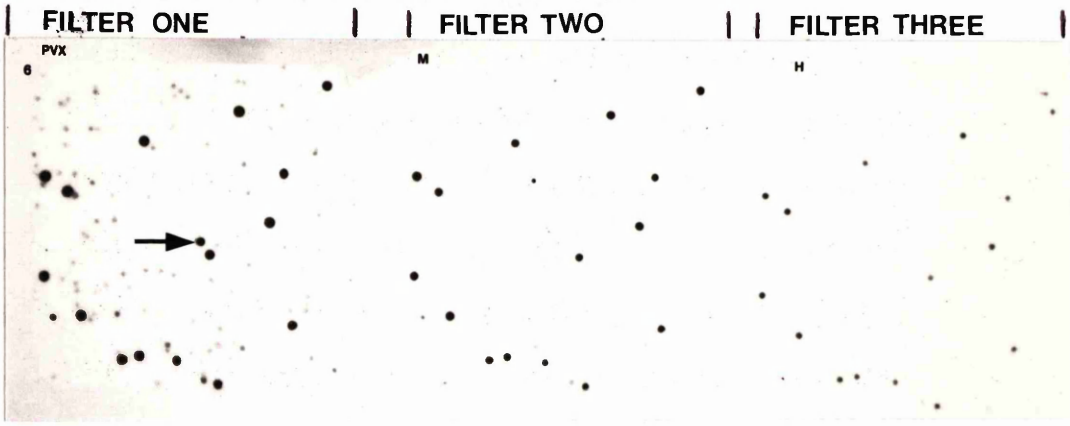

PVX
21
24
26
MOCK

Fig. 3.38. Rescreen of PIX 6 identified using subtractive probes.

The area containing the plaque which hybridised differentially to subtractive probes was picked and re-plated. Triplicate filters were screened with ss cDNA probes generated from leaf position three, 12 days after inoculation on leaf position one with PVX strain KEBRS (PVX, filter one), mock-inoculation with water (M, filter two), or from plants left as healthy controls (H, filter three). A single plaque strongly hybridised preferentially to the ss cDNA probe generated from PVX induced plants. The plaque, indicated by the arrow, was picked and named PIX 6.


FILTER ONE
FILTER TWO
FILTER THREE
PVX
6
M
H

preferentially to probe from mock-inoculated plants; and 6 plaques hybridised preferentially to probe from healthy plants. The plaques which hybridised most strongly for the mock-inoculated and the healthy plants were not analysed further. The plaques which hybridised preferentially to ss cDNA probes generated from PVX-KEBRS-inoculated plants were rescreened as above. Of the original fourteen, three again hybridised preferentially to the probe generated from PVX-KEBRS-inoculated plants and these were then subjected to further study.

3.7.3. Rescreening.

In order to eliminate the possibility that the differential hybridisation was a result of differences in the mRNA population caused by factors other than virus inoculation the clones identified above were rescreened with probes generated from 2 independent batches of plants. A total of seven clones, four clones identified by screening with subtractive probes and three clones identified from differential screening with ss cDNA probes, were each plated out at a density of 200 plaques per plate. They were rescreened differentially using ss cDNA, generated from PVX-KEBRS-inoculated, mock-inoculated and healthy plants. Each cDNA clone was screened using poly A^+ RNA from experiments 2 and 3 (described above).

During the differential screening of the library it became apparent that variations in the specific activity of ss cDNA probes were causing difficulties in interpreting differences between treatments. This was not too much of a problem when initially screening the library as the majority of plaques represented mRNAs which were expressed at a constant level in each of the plant treatment groups. These constitutively expressed probes were used as internal standards, allowing any discrepancies in the specific activities of the different cDNA probes to be eliminated.

In all subsequent rescreening experiments a second recombinant plaque, representing a sequence expressed equally between the different treatment groups, was introduced into the screen as an internal control. This was spotted on each plaque array, to allow correction for differences in plaque intensity (for the seven potential differentially expressed clones) resulting solely from differences in the specific activities of the different probes.

Fig. 3.39. Differential rescreen of PIX 6, 11, 12, 24, 26 and 30.

Each cDNA clone was differentially screened with ss cDNA probes generated from leaf position three, 12 days after inoculation on leaf position one with PVX strain KEBRS (PVX, filter one), mock-inoculation (M, filter 2), or from plants left as healthy controls (H, filter three). Each cDNA clone showed increased mRNA expression in PVX treated plants compared to the other two treatments. The rescreen of clones 6, 12, 24, 26 and 30 contained control plaques (C) which hybridised to a mRNA that did not change in level of expression between treatments. This enabled direct comparison between the same cDNA clone, probed with different ss cDNA probes.

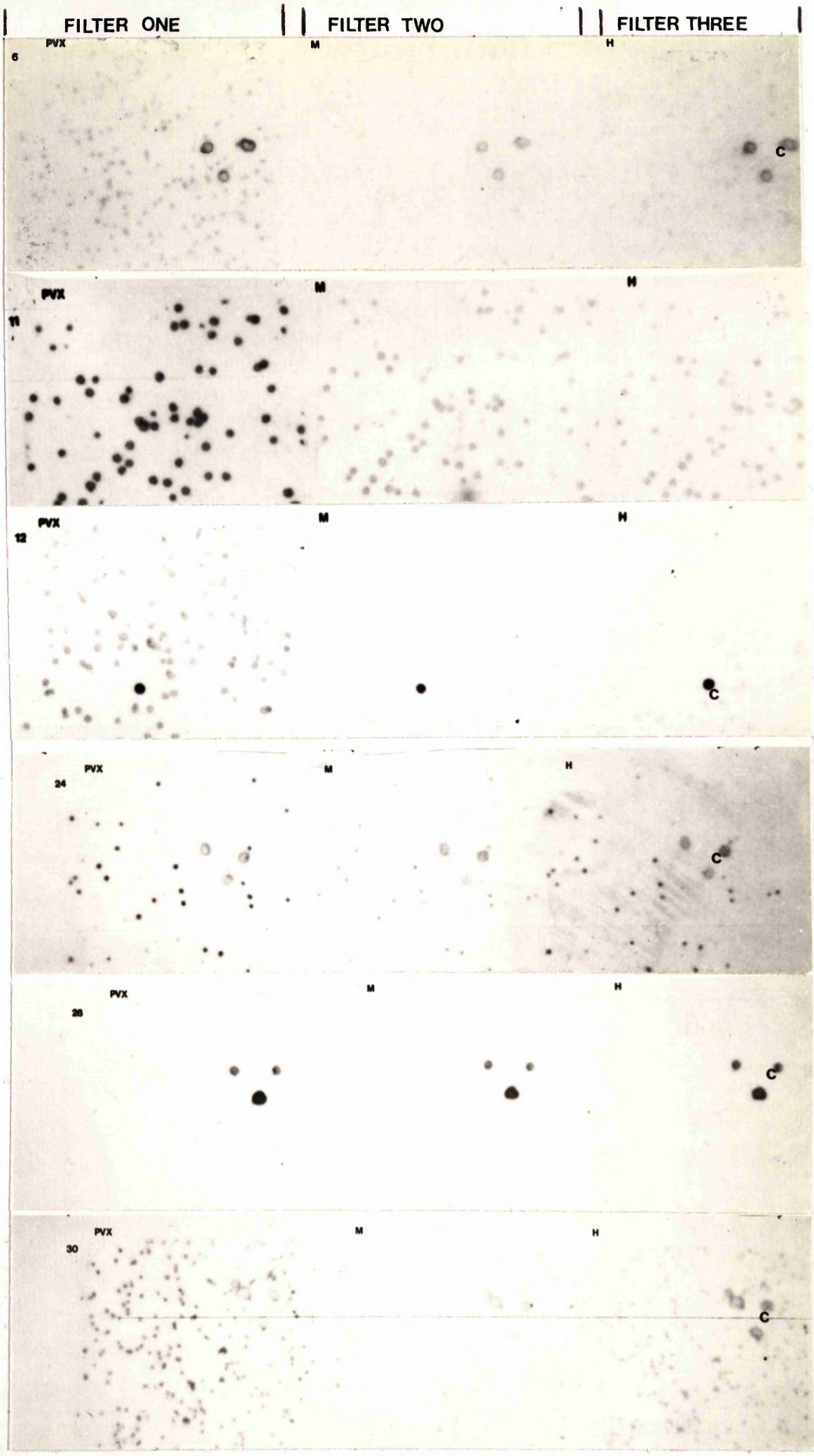
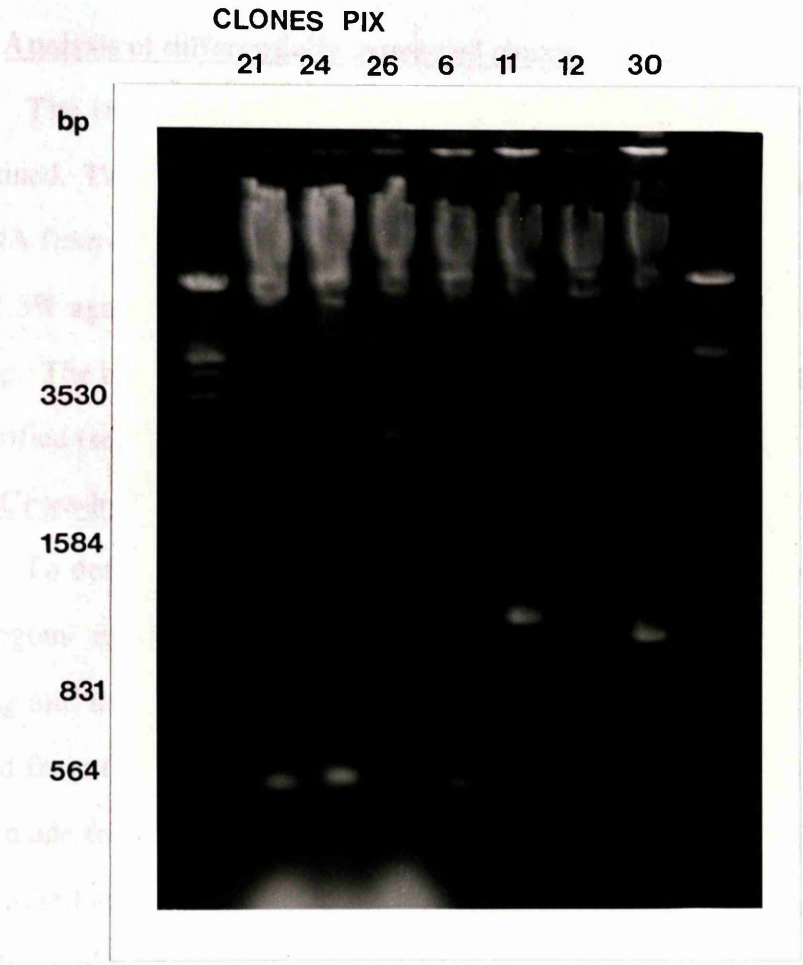

FILTER ONE
FILTER TWO
FILTER THREE
PVX
M
H
6
C
PVX
M
H
11
PVX
M
H
12
C
PVX
M
H
24
C
PVX
M
H
28
C
PVX
M
H
30
C

Fig. 3.40. The size of each insert sequence for the PVX-KEBRS induced clones.

Clone	Insert bp
PIX 21	465
PIX 24	465
PIX 26	2,650
PIX 6	465
PIX 11	280 + 1,000 = 1,280
PIX 12	440
PIX 30	950

The seven recombinant clones PIX 21, 24, 26, 6, 11, 12 and 30, double digested with *Eco*RI and *Hin*dIII, were electrophoresed on a 1.5% agarose gel. The left lane contains molecular weight standards (lambda CI 857 DNA digested with *Eco*RI and *Hin*dIII). The sizes of selected fragments are indicated at the left of the photograph.


CLONES PIX
21 24 26 6 11 12 30
bp
3530
1584
831
564

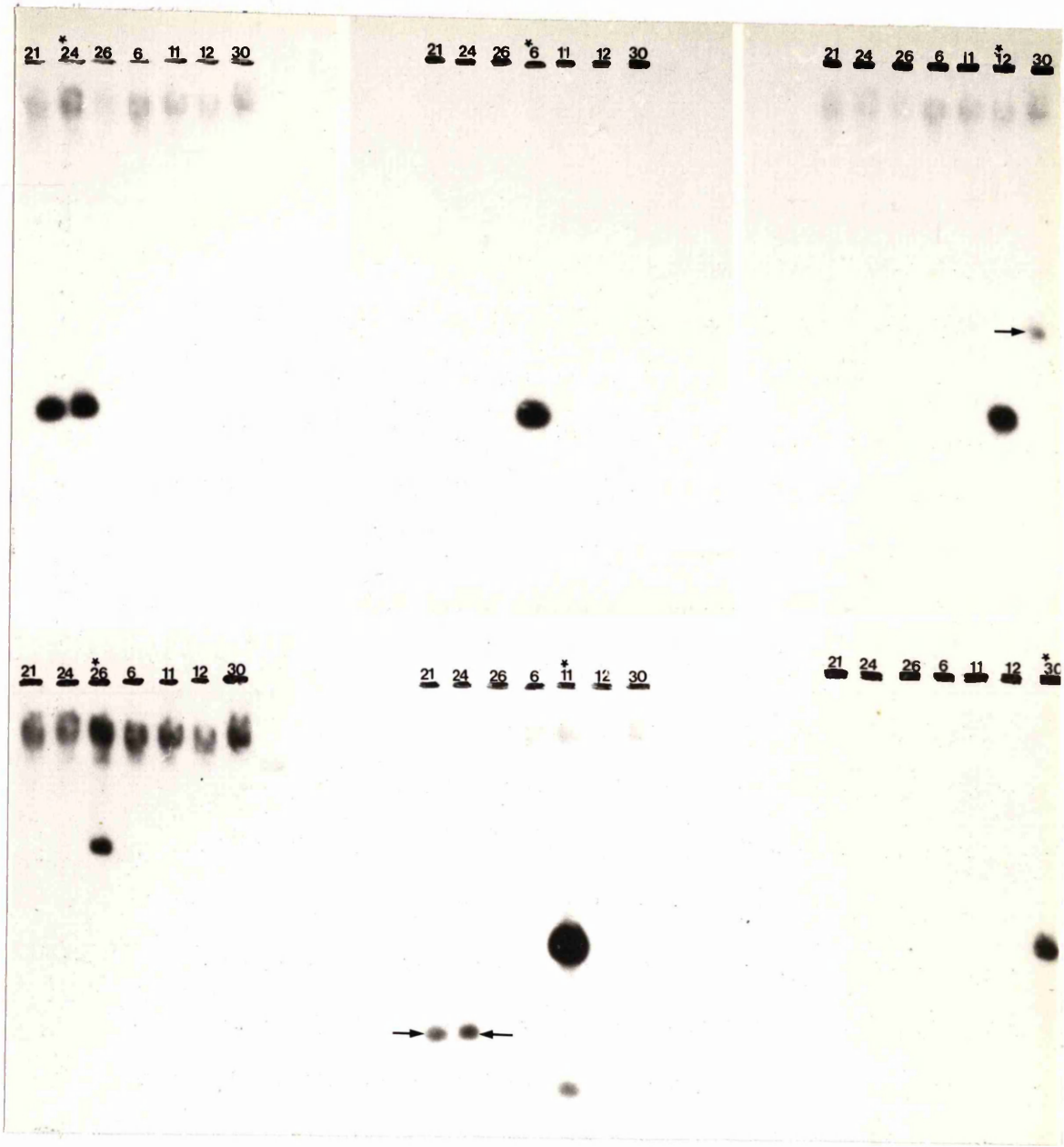
All seven cDNA clones hybridised most strongly to probes from PVX-KEBRS-inoculated plants, although clone PIX 26 was very faint on the filter, indicating a low level of expression in the mRNA population induced by PVX-KEBRS inoculation (Fig. 3.39). This strongly suggests that these clones do indeed represent sequences which are more abundant in PVX-KEBRS-inoculated plants than in control treated plants.

3.7.4. Analysis of differentially expressed clones.

The insert sizes of each differentially expressed cDNA clone were determined. The DNA was digested with *Eco*RI and *Hin*dIII to liberate the insert, and the DNA from each clone was then resolved by gel electrophoresis on a low melting point 1.5% agarose gel (Fig. 3.40). The cDNA inserts ranged in size from 440 to 2650 bp. The bands representing the insert fragments were then excised from the gel and purified (see Materials and Methods, section 2.26).

3.7.5. Cross-hybridisation between cloned inserts.

To determine whether any of the clones were derived from identical or homologous mRNAs, individual cDNA inserts were labelled with ^{32}P by random priming and used to probe a Southern blot of an *Eco*RI-*Hin*dIII digest of DNA isolated from the seven clones. The nylon filter was sequentially screened with probes made from each of the inserts from each clone. After hybridisation, the blots were washed under highly stringent conditions (0.1 X SSC, 68°C) and subjected to autoradiography. After each hybridisation, any previously hybridised cDNA probe was removed from the nylon filter using NaOH (see Materials and Methods, section 2.32) and the blots rescreened. Only clones PIX 21 and PIX 24 cross-hybridised (Fig. 3.41). Therefore, six of the seven clones obtained by differentially screening 28,000 plaques are apparently non-homologous. This is a reasonable assumption as all clones should contain a 3'poly A^+ tail (or a short internal 'A' sequence) as the cloning strategy utilised an oligo $(dT)_{12-18}$ polymer as a primer to initiate first strand cDNA synthesis. The linker used also utilised two bases of the poly A^+ tail to construct the *Hin*dIII restriction site, so any homologous clones should be 3' co-terminal. Clones PIX 21 and 24 may represent a single recombinant since both were isolated from an


21 *24 26 6 11 12 30
21 24 26 *6 11 12 30
21 24 26 6 11 *12 30
21 24 *26 6 11 12 30
21 24 26 6 *11 12 30
21 24 26 6 11 12 *30

area of plaques which only showed one differential signal. Interestingly, PIX 26 originated from the same area as PIX 21 and PIX 24, but neither cross-hybridises nor is the same size as these two clones (section 3.7.1). The apparent cross hybridisation of PIX 11 to PIX 21 and 24, and the apparent cross hybridisation of PIX 12 to PIX 30 (Fig. 3.41), is an artefact. These bands represent radioactive probes from the previous experiment which were not fully stripped from the membrane. The apparent cross hybridisation of the random primed probes PIX 11, 12, 24 and 26 to the lambda arms is also an artefact due to lambda DNA contaminating the insert used to make the probe.

The six remaining clones were then used as probes to determine how their expression of homologous mRNA varied with time in PVX-KEBRS or mock-inoculated plants.

3.8. THE TIME COURSE OF EXPRESSION OF mRNAs COMPLEMENTARY TO DIFFERENTIAL CLONES.

The expression of mRNAs complementary to each of the differential clones was investigated by labelling the inserts with ^{32}P and using them as a probe to screen Northern blots containing total RNA isolated from PVX-KEBRS-inoculated, mock-inoculated, and healthy plants. Total RNA (20 µg per lane) was isolated from leaf position one (the inoculated leaf) and leaf position three, 8, 10, 12 and 14 days after inoculation (RNA from experiment 3, section 3.7). Although poly A^+ RNA gave a stronger signal, the yields of poly A^+ RNA from different batches of total RNA were quite variable (0.7% to 3.3% of total RNA, Table 3.7). This made it very difficult to quantify accurately the amount of RNA loaded from each sample. Therefore, in order to avoid possible errors resulting from these differences in yield, total RNA rather than poly A^+ RNA was used. The autoradiographs were examined visually and the sizes and intensities of any hybridising bands were compared. Three clones, PIX 30, 24, and 6, gave detectable signals on the Northern blots. In order to obtain a more quantitative estimate of the amount of hybridising mRNA the autoradiographs were scanned with a Biorad 620 video densitometer.

3.8.1. Analysis of the mRNA homologous to PIX 24.

The labelled PIX 24 insert DNA hybridised to a single RNA band of an approximate molecular size of 750 nt. Levels of expression were quantified visually (Fig. 3.42) and by densitometric scanning (Table 3.8). In leaf position one, the level of expression of this mRNA was greatest for the PVX-KEBRS-inoculated leaves, less for mock-inoculated leaves and less again for healthy leaves at 8, 10 and 12 days after inoculation (Fig. 3.42). The greatest difference between expression was seen 8 days after inoculation, being expressed 2-fold greater in the PVX-KEBRS-inoculated leaf compared to the mock-inoculated leaf and 10 times greater in PVX-KEBRS-inoculated leaves than seen in healthy plants (Table 3.8). No difference in the level of expression was seen 14 days after inoculation in any of the treatment groups.

For RNA from leaf position three, the level of expression of mRNA was approximately half that seen in the inoculated leaves (Fig. 3.42). Little difference in

expression was seen between the three treatment groups 8, 10 and 14 days after inoculation. The RNA samples from day 12 after inoculation appeared to have transferred poorly from the gel to the nylon filter. Therefore, no comment may be made for this time point.

These results show that PIX 24 appears to be derived from mRNA which is predominantly up-regulated in PVX-KEBRS-inoculated and mock-inoculated leaves. The differences in expression are greater 8 to 10 days after inoculation.

3.8.2. Analysis of the mRNA homologous to PIX 30.

Labelled PIX 30 insert DNA hybridised to a single RNA band, of an approximate molecular size of 1,100 nt. Levels of expression were quantified visually (Fig. 3.43) and by densitometric scanning (Table 3.8).

In leaf position three, the level of expression of the mRNA was greatest in PVX-KEBRS-inoculated plants 8, 10 and 12 days after inoculation compared to the other two treatment groups although samples taken 12 days after inoculation appeared to have again transferred poorly to the nylon filter (Fig. 3.43). The highest level of expression was 8 and 10 days after inoculation with PVX-KEBRS. Fourteen days after inoculation the level of mRNA was similar in all treatment groups. In leaf position one (the inoculated leaf) the level of expression in the leaf varied between sample times but little differential expression was seen between treatments (Fig. 3.43). Thus PIX 30 appears to be derived from a mRNA which is up-regulated, by up to 3-fold, by PVX-KEBRS-inoculation compared to control plants (Table 3.8). This up-regulation is observed systemically but is not detected in the inoculated leaf. The greatest differences in expression are seen about 10 days after inoculation.

3.8.3. Analysis of the mRNA homologous to PIX 6.

The labelled PIX 6 insert DNA hybridised to a single RNA band of an approximate molecular size of 850 nt. Levels of expression were quantified visually (Fig. 3.44) and by densitometric scanning (Table 3.8).

The levels of expression of the mRNA in leaf positions one and three in PVX-KEBRS-inoculated plants were only slightly higher than in healthy plants 10

Fig. 3.42. Time course expression of mRNA homologous to PIX 24 following inoculation of Pentland Ivory with PVX strain KEBRS.

Samples (20 μg) of total RNA taken from leaf one (inoculated leaf) and leaf three of Pentland Ivory plants, 8, 10, 12 and 14 days after inoculation with PVX strain KEBRS, mock-inoculation with water, or from plants left as healthy controls, were electrophoresed through a MOPS-formaldehyde-agarose gel, transferred to a nylon filter and hybridised to ^{32}P labelled PIX 24 insert DNA. The figures to the left of the gel show the position of RNA molecular weight standards (3,580 nt and 1,920 nt, stained with 0.2% Toluidene blue).

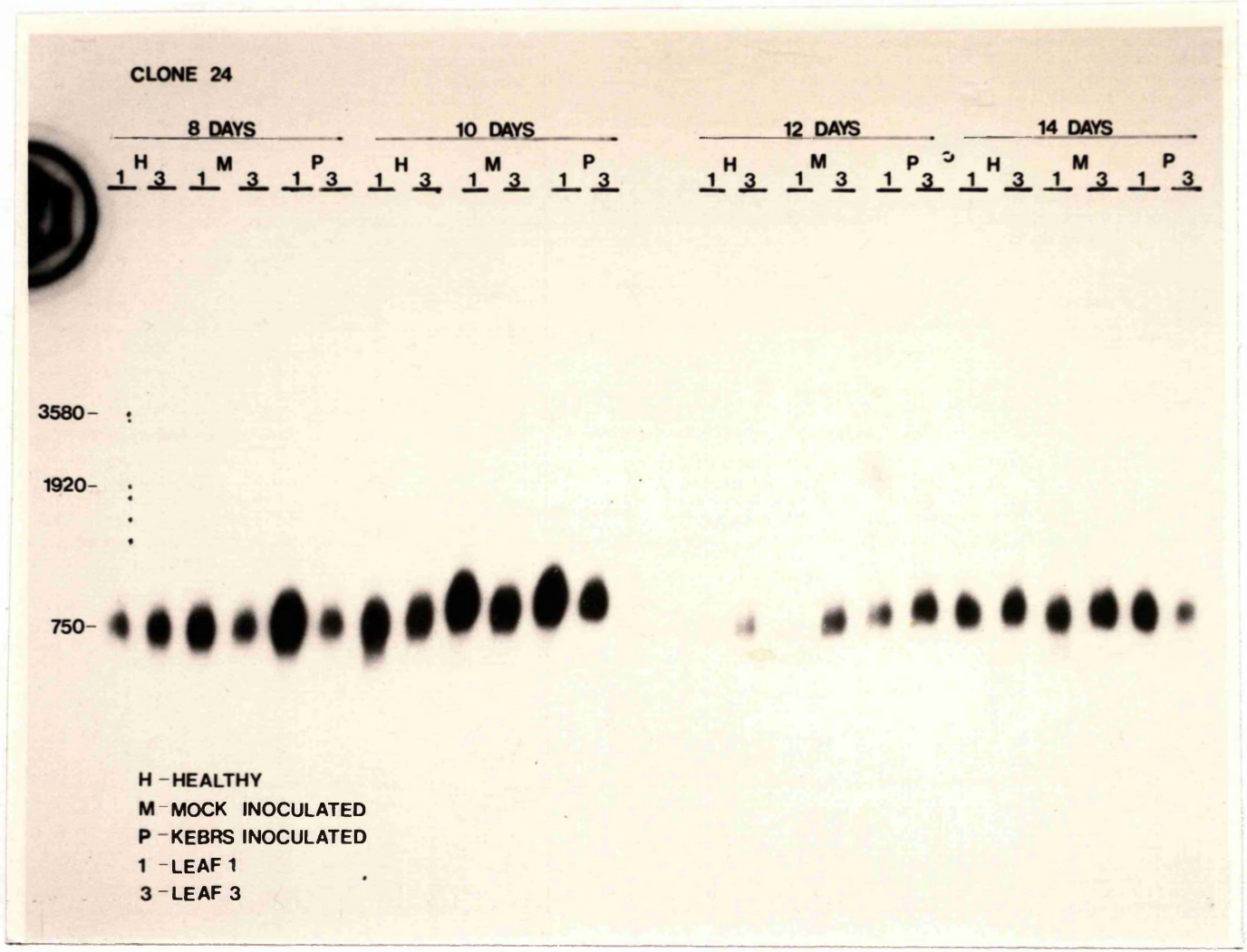

CLONE 24
8 DAYS
10 DAYS
12 DAYS
14 DAYS
H 1 3
M 1 3
P 1 3
3580-
1920-
750-
H - HEALTHY
M - MOCK INOCULATED
P - KEBRS INOCULATED
1 - LEAF 1
3 - LEAF 3

Fig. 3.43. Time course expression of mRNA homologous to PIX 30 following inoculation of Pentland Ivory with PVX strain KEBRS.

Samples (20 μg) of total RNA taken from leaf one (inoculated leaf) and leaf three of Pentland Ivory plants, 8, 10, 12 and 14 days after inoculation with PVX strain KEBRS, or mock-inoculation with water, or from plants left as healthy controls, were electrophoresed through a MOPS-formaldehyde-agarose gel, transferred to a nylon filter and hybridised to ^{32}P labelled PIX 30 insert DNA. The figures to the left of the gel show the position of RNA molecular weight standards (2,400nt, 1,400nt and 240nt, stained with 0.2% Toluidene blue).

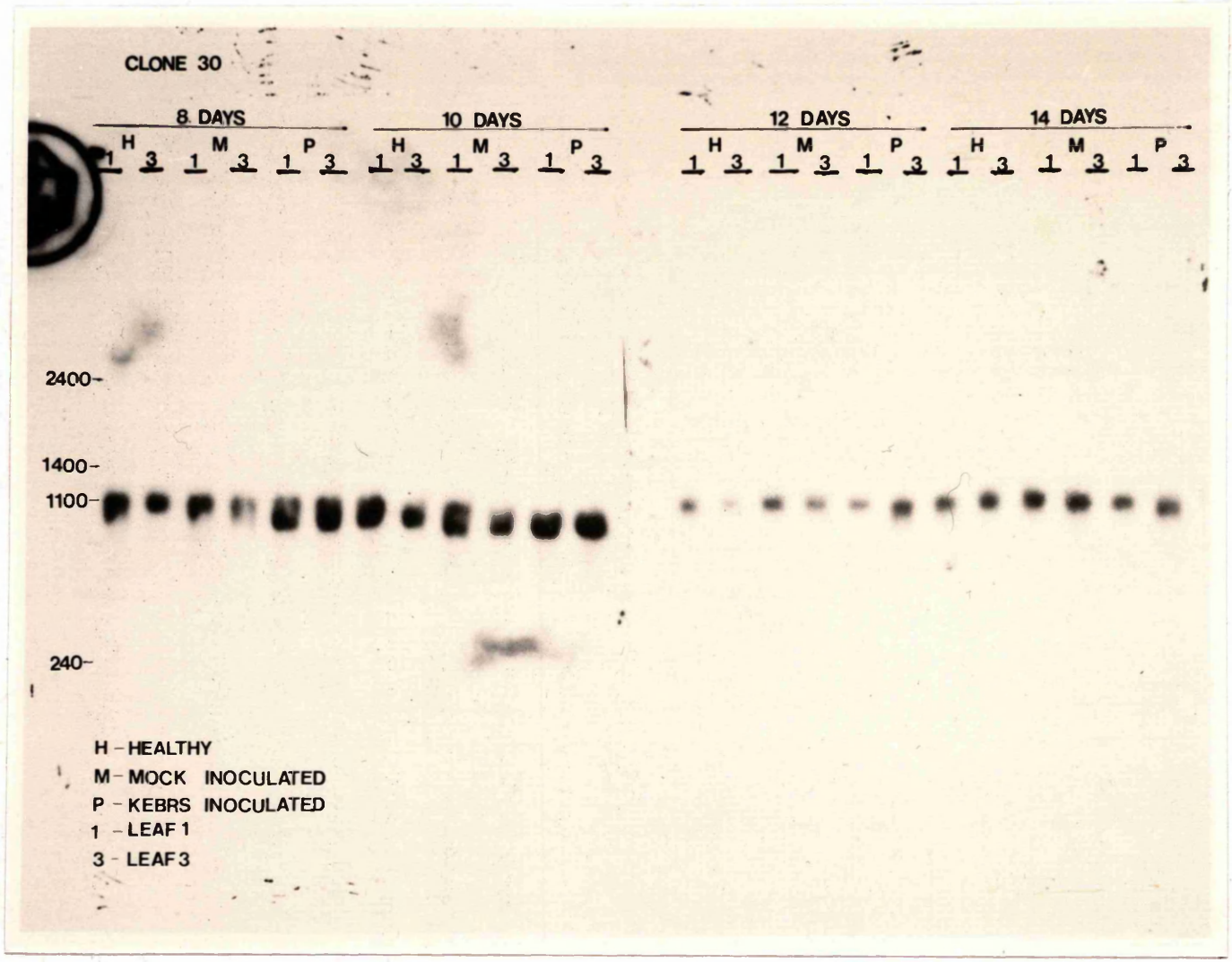

CLONE 30
8 DAYS
10 DAYS
12 DAYS
14 DAYS
H 1 3 M 1 3 P 1 3
2400-
1400-
1100-
240-
H - HEALTHY
M - MOCK INOCULATED
P - KEBRS INOCULATED
1 - LEAF 1
3 - LEAF 3

Fig. 3.44. Time course expression of mRNA homologous to PIX 6 following inoculation of Pentland Ivory with PVX strain KEBRS.

Samples (20 µg) of total RNA taken from leaf one (inoculated leaf) and leaf three of Pentland Ivory plants, 8, 10, 12 and 14 days after inoculation with PVX strain KEBRS, or mock-inoculation with water, or from plants left as healthy controls, were electrophoresed through a MOPS-formaldehyde-agarose gel, transferred to a nylon filter and hybridised to ^{32}P labelled PIX 6 insert DNA. The figures to the left of the gel show the position of RNA molecular weight standards (2,400nt, 1,400nt and 240nt, stained with 0.2% Toluidene blue).

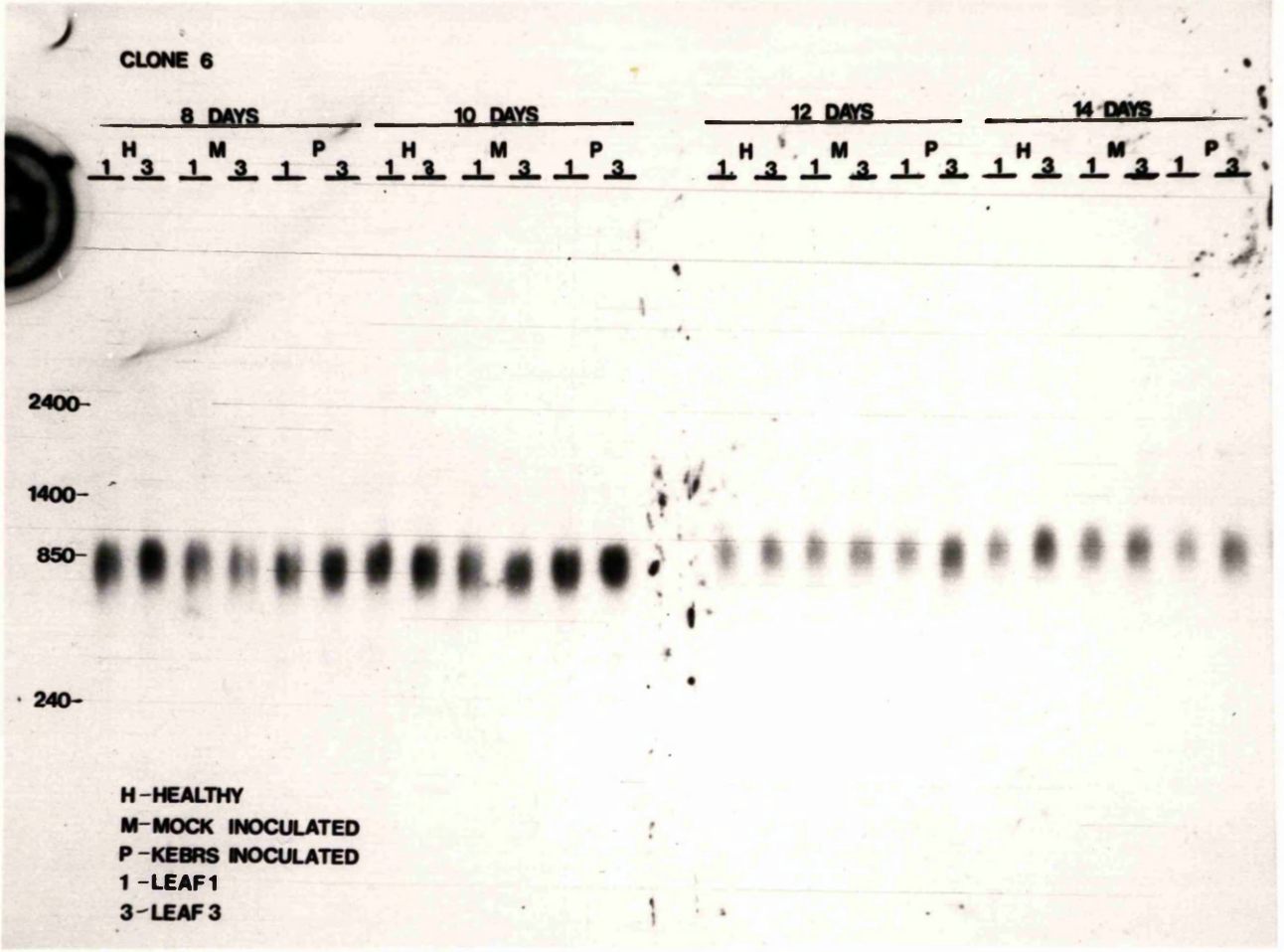

CLONE 6
8 DAYS
10 DAYS
12 DAYS
14 DAYS
H M P
1 3
2400-
1400-
850-
240-
H-HEALTHY
M-MOCK INOCULATED
P-KEBRS INOCULATED
1-LEAF 1
3-LEAF 3

TABLE 3.8. Densitometric quantification of expression of mRNAs complementary to PIX 6, 24 and 30 in PVX-inoculated, mock-inoculated and healthy uninoculated plants. The data represent the quantification of expression of mRNAs in the Northern blots shown in Figs. 3.42, 3.43 and 3.44.

	8 days						10 days						12 days						14 days					
	HL1	HL3	ML1	ML3	PL1	PL3	HL1	HL3	ML1	ML3	PL1	PL3	HL1	HL3	ML1	ML3	PL1	PL3	HL1	HL3	ML1	ML3	PL1	PL3
PIX 30	8.2	7.7	8.1	4.5	6.5	9.8	11.4	8.1	6.8	5.4	9.4	11.7	3.6	1.3	5.8	3.9	3.1	5.0	5.6	7.2	8.7	9.5	5.9	4.2
PIX 24	**1.4**	4.6	**7.7**	3.4	**17.0**	2.7	**5.7**	4.9	**17.0**	9.1	**16.0**	7.4	0.0	0.3	0.0	2.9	2.8	5.2	7.5	6.6	5.7	10.4	12.1	1.9
PIX 6	6.9	10.4	4.3	2.6	4.4	8.0	8.4	10.2	4.6	6.7	11.0	12.4	3.2	4.2	3.2	3.8	3.5	5.4	3.2	5.8	3.4	4.2	2.1	4.1

HL1 = Healthy leaf one; HL3 = Healthy leaf three; ML1 = Mock-inoculated leaf one; ML3 = Leaf three from a plant mock-inoculated on leaf one; PL1 = KEBRS inoculated leaf one; PL3 = Leaf three from a plant inoculated on leaf one with KEBRS
The absorbance figures presented here were generated by a Biorad 620 video densitometer which scanned a narrow straight band across the autogradiograph of each Northern blot. This scanning mode was prone to error as it appeared that the RNA lanes on the gel had not run equally for each sample, and that the cross-hybridising RNA band for each cDNA probe on the Northern blot was rather diffuse for certain samples. This resulted in the video densitometer's scanning band not always going through the point of maximum exposure on the autoradiograph. Apart from this problem, the video densitometer, used in conjunction with visual observation, was helpful in quantifying differences between samples. The most significant absorbance values are highlighted in Bold type.

and 12 days after the inoculation (Fig. 3.44). No differences in expression were observed 8 and 14 days after inoculation.

Thus, clone PIX 6 appears to be derived from mRNA which is slightly up-regulated in PVX-KEBRS-inoculated plants compared to control plants (Table 3.8). However, this small difference may be due to experimental variation.

3.8.4. Clones PIX 11, 12 and 26.

The Northern blots were also screened for mRNAs homologous to PIX 11, 12 and 26 using random primed probes. Despite the use of probes of high specific activity (6.6×10^6 d.p.m./ng) neither PIX 11 or 26 generated a signal on the Northern blot, irrespective of the length of exposure time to X-ray film; PIX 12 showed some non-specific hybridisation to the 18S and 16S ribosomal RNA bands on longer exposure. Presumably mRNAs complementary to these clones are much less abundant than those complementary to PIX 6, 24 and 30. This is always a problem with low abundance mRNAs.

In conclusion, the screening of the Northern blots initially identified three of the possible six differentially expressed clones. Of these three none showed strong, sustained, differential expression. However, the mRNA represented by clone PIX 30 did apparently show distinct systemic up-regulation in PVX-KEBRS-inoculated plants compared to the other two treatments at the 8 and 10-day time points, although not at the 12 and 14-day time points. Interestingly, this clone did not seem to be expressed differentially in the inoculated leaf. The mRNA represented by PIX 24 seems to increase in abundance following PVX-KEBRS-inoculation in the inoculated leaf and also, albeit at a slightly lower level, following mock-inoculation. This mRNA did not show any significant differential expression in leaf position three. PIX 6 showed a little increased expression in PVX-KEBRS-inoculated plants in both leaf positions but only for the time points 10 and 12 days after inoculation. It is not clear if this is significant. The results from the Northern blots show that plaques which appear to be strongly differentially expressed when screening with subtractive or differential probes may in fact show little or no difference in expression. Thus any plaque screening strategy that uses only one time point is likely to be inadequate.

The cDNA clones which were not detected on the Northern blots (perhaps due to their low level of expression) may yield interesting findings. However, as with many resistance responses, it is not the absence or presence of a gene product but rather the rate of expression that confers resistance. The clones isolated may therefore be involved in the more rapid triggering of the plant's resistance responses so inducing a higher level of resistance to a second pathogen attack. This speculation requires further Northern blot analysis to identify the mRNAs and further characterise their expression. The isolation of full length transcripts of such clones from the library and the sequencing of them would enable them to be compared with other genes which have been identified as being induced by pathogen attack.

Clearly this thesis represents the start of this project and it will require further work to really characterise these clones and to critically assess their roles (if any) in the resistance mechanisms.

3.9. ANALYSIS OF POTATO cDNA CLONES WHICH SHOW SIGNIFICANT HOMOLOGY TO THE TOBACCO PR-1A AND THAUMATIN-LIKE PROTEINS.

The potato cDNA library was screened with clones for cDNAs of two tobacco PR proteins, PR-1a and the Thaumatin-like protein (Cornelissen *et al.*, 1986). These cDNA clones had inserts of 2,000 bp and 564 bp respectively and were a gift from Dr J.F. Bol. It was of interest to see whether mRNAs related to these inserts were expressed in potato leaves distant from the point of localised PVX infection, and, if so, at what frequency they were expressed (Cornelissen *et al.*, 1986). Pierpoint *et al.* (1990) have identified a thaumatin-like PR protein induced in potato leaves sprayed with salicylate. The tobacco PR-1a protein has also been shown to be serologically homologous to proteins induced in other species (e.g. tomato, cowpea, potato, maize, barley, etc.) by pathogen attack (Bol, 1988).

The two PR protein cDNA inserts, cloned in pUC 18, were transformed into *E. coli* strain XL1 blue. A plasmid miniprep was carried out (Sambrook *et al.*, 1989); the plasmid DNA was digested with *Eco*RI and *Hin*dIII and the insert fragment purified on a 1.5% agarose gel. The fragment was excised from the gel, purified and used to generate random primed probes. Each PR protein probe incorporated ~55 μCi of ^{32}P in 20 ng of DNA, and was used to screen a total of 18,000 plaques at a density of 3,000 plaques per 10 x 10 cm petri-dish.

Filters were washed under stringent conditions of 0.2 X SET at 50°C, and were then exposed to preflashed X-ray film.

The tobacco PR-1a cDNA hybridised to 18 plaques, while the thaumatin-like PR clone hybridised to 9 plaques out of a total of 18,000. Thus PR protein sequences homologous to the tobacco PR proteins were present at ~0.1% and ~0.05% frequencies respectively, in the potato cDNA library. These are relatively high frequencies for PR protein mRNAs and indicate a high abundance of respective mRNA in the potato leaf distant from PVX localised infection. The identification of PR protein mRNAs occurring systemically in potato cv. Pentland Ivory infected with PVX-KEBRS is consistent with previous findings that PR proteins are induced

systemically in tobacco following localised infection (Bol & van Kan, 1988; Collinge & Slusarenko, 1987). However, in the absence of a control library produced from healthy plants, or similar further data, this must remain speculation. The homology, at a nucleotide level, between these two tobacco and potato PR proteins must be considerable, as the hybridisation and wash conditions were performed at high stringency (0.2X SET, 50°C).

The abundance of PR protein cDNA clones in the potato library (~0.1% + ~0.05%) is much greater than the apparent abundance of differentially expressed clones as identified by differential screening of the cDNA library. Why this should be is not clear. Perhaps the PR proteins are not differentially expressed between PVX-KEBRS-inoculated, mock-inoculated or healthy potato plants. Alternatively, the differential screening technique might be failing to identify the majority of differentially expressed clones. Many PR proteins are induced by wounding (Bol, 1988); cDNA clones representing such species might be expected to be present in both PVX-KEBRS-inoculated and mock-inoculated plants. Since only clones specifically hybridising to probes from PVX-KEBRS-inoculated plants were selected, such PR clones might have been overlooked. It would have been interesting, if there had been more time, to have probed cDNA libraries constructed from mock-inoculated leaf position three and healthy Pentland Ivory plants, to see if mRNAs for the PR proteins were found in these two treatment groups and at what frequency. Alternatively, the use of time-course Northern blots of mRNA isolated from leaf position one (inoculated leaf) and leaf position three from PVX-KEBRS, mock-inoculated and healthy plants at specific times after inoculation, could have been used to identify in which treatment group(s) mRNAs homologous to the two PR proteins were expressed, their level of expression in inoculated and uninoculated leaves, and at what time they were expressed after inoculation.

One possibility suggested by the results of this experiment is that the high level of systemic expression of these two PR proteins in Pentland Ivory 12 days after PVX-KEBRS-inoculation may indicate that other mRNAs are induced by the PVX-KEBRS-inoculation and are present in the cDNA library at this time point after

inoculation. The library generated may contain cDNA clones which relate to mRNAs induced by PVX and which possess anti-viral properties; to identify these would require more extensive screening of the library.

DISCUSSION

4.0. THE SELECTION OF A POTATO VIRUS X STRAIN AND POTATO CULTIVAR FOR USE IN THIS THESIS.

A first concern (since later work relied on the viral strain not infecting the plant systemically) was to use a host virus combination where the plant was capable of completely restricting the viral infection to the inoculated leaf. Six PVX isolates were obtained and their infection characteristics were investigated in potato cultivars Pentland Ivory (*nx, Nb*) and King Edward (*Nx, nb*). The techniques of ELISA and light and electron microscopy were used to monitor local and systemic spread of virus in the two potato cultivars. ELISA was used to investigate the accumulation of PVX coat protein in the inoculated and apical leaves of susceptible and resistant potato cultivars for each PVX strain. In all these experiments, PVX tended to infect potato plants rather asynchronously. The time at which individual plants in a group developed visual symptoms, and the level of PVX which accumulated following inoculation were also variable. These observations are similar to those of Aw (1987) who described corresponding differences in PVX accumulation in *N. tabacum* grown under similar conditions.

The initial ELISA experiments in which only a single plant was used for each time point, cannot be regarded as highly significant, although the general trend showed PVX titre increasing with time in susceptible potato plants. Obviously, in any comprehensive study of viral titre large numbers of plants are required in order to overcome plant-to-plant variability. In the later ELISA experiments (see 3.3.2), 4 plants per group were used to reduce the influence of asynchronous infection between plants. The resulting ELISA data showed that one of the three Group 3 PVX strains, 3PBI, was able to escape localisation by the *Nx* gene in King Edward to a limited

degree in the inoculated leaf, while the Group 3 strain XN was not even very infectious to susceptible Pentland Ivory plants. The inability of the XN virus to infect potatoes was in contrast to the ready systemic infection achieved by this strain with *N. edwardsonii* as host. This may be the result of the strain having been maintained in tobacco species for many years (Ireland & Pierpoint, 1980). The two PVX strains which systemically infected Pentland Ivory showed different time courses of symptom development; they also showed different levels of viral accumulation. Furthermore, these two viral strains each showed quite different symptom development in systemically infected *N. edwardsonii*. These data show that strains classified as belonging to the same group can be quite different in their infection characteristics. The Group 2 PVX strains also showed differences, both in the time at which symptoms first appeared and in the level of viral accumulation. Two of the strains, 2PBI and KEBX, were to a limited extent able to escape localisation in Pentland Ivory. This was manifested as local-lesion development in leaves distant from the initial point of inoculation. This limited ability to spread systemically might result from viral particles being introduced into the vascular system either by the initial act of inoculation or as virus is transported between cells prior to the full development of the resistant state. The viral particles might only later fully induce the hypersensitive response when they leave the phloem and infect a leaf cell. The third strain, PVX-KEBRS, was not detectable outwith the inoculated leaf of Pentland Ivory. This strain was the only one tested which appeared to be fully localised to the inoculated leaf.

The time course and levels of viral antigen in susceptible cv. King Edward also depended upon viral strain. The strain PVX-KEBRS-inoculated onto the susceptible potato cv. King Edward accumulated less virus than strain PVX-KEBX infecting King Edward plants, while mosaic symptoms developed first in King Edward plants infected with KEBRS and became more severe than in KEBX infected plants. The reduced level of PVX-KEBRS in susceptible potato plants compared to the other strains may indicate a less fit PVX strain, poorly adapted for rapid growth in a susceptible host compared to PVX-KEBX. This might be why the *Nb* gene is more effective in restricting PVX-KEBRS (a poorly adapted strain) infection compared to

KEBX or 2 PBI. Consequently, Group 2 strains in general may be localised more effectively by the resistance mechanisms in potato compared to the more virulent Group 3 strains of PVX. Some of the variation seen between the six strains investigated may be a consequence of repeated passage through a particular plant species. These findings, described in section 3.1, show clearly that PVX strains within the same group can show marked differences in the extent to which they are able to infect a given potato cultivar. Baulcombe *et al.* (1984) confirm these findings as they identified Group 3 PVX strains which accumulated in potato plants to varying levels, as measured by the content of viral RNA. General differences between viral groups may reflect general fitness of each group of virus. Aw (1987) has shown that Group 3 PVX strains accumulated to higher concentrations in a susceptible host (*N. tabacum* cv. White Burley) than did Group 2 PVX strains, suggesting that Group 2 strains are less well adapted to rapid replication than Group 3 strains. These results demonstrate that PVX strains which have similar or identical assigned names may have quite different infection characteristics in a given potato cultivar. These differences must reflect differences in the nucleotide sequences of Group 2 and Group 3 PVX strains resulting in differences in the virus-encoded proteins (eg. coat protein, replicase or transport factor), which make the virus more or less able to exploit the host. Therefore one can only be confident that PVX strains are identical if they originated from the same isolate or if the strains in question have been sequenced.

To identify which potato cultivars showed least physiological changes during normal growth, one-dimensional gels were used to analyse polypeptides synthesised *in vitro* from RNA isolated from Pentland Ivory and King Edward plants. The RNA population isolated from healthy Pentland Ivory plants grown over a period of 20 days maintained a basically constant mRNA population although two mRNA changes were observed (Fig. 3.20), as judged by *in vitro* translation of the mRNA. In contrast, over the same time period, King Edward plants showed several changes in the mRNA population, significant enough to be detected as differences in the pattern of the translation products seen on a one-dimensional polyacrylamide gel. This experiment showed that the mRNA populations of apparently identical plants from two different

potato cultivars can vary quite considerably over time. These differences between the two cultivars, Pentland Ivory and King Edward, might be the result of physiological differences. The King Edward plants in many cases had started to show flower bud development whereas Pentland Ivory plants showed no flower bud development over the period of the experiment. The induction of flowering is certainly associated with many changes in gene expression (Fraser, 1981).

In later experiments, it was important that confusion between mRNA changes as a result of PVX localisation or wounding and those due to normal developmental changes were kept to a minimum. Therefore, the cv. Pentland Ivory was chosen for further work. However, even with this cultivar there were some changes, over time, in the mRNA population in healthily grown plants, as detected by one-dimensional gel electrophoresis of *in vitro* translated mRNA products. Possibly many more small changes were also present but beyond the sensitivity of detection of one-dimensional PAGE. Therefore, it was important that control samples were taken from healthy plants at every sample time point so that such background changes could be differentiated from potato gene expression influenced by PVX infection.

4.1. INDUCED SYSTEMIC RESISTANCE.

Many publications have reported the phenomenon of induced systemic resistance, in which a plant responds to pathogen attack by developing a necrotic lesion which in turn appears to lead to the induction of increased resistance to later pathogen attack on hitherto uninfected parts of the plant (Chester, 1933; Ross, 1961; Kuc, 1982). The phenomenon has been reported in many plant species and with several types of pathogen, e.g. fungi (Hecht & Bateman, 1964; McIntyre & Dodds, 1979), bacteria (McIntyre *et al.*, 1981) and viruses (Ross, 1961, 1966; Van Loon & Dijkstra, 1976).

An example of this resistance induction has been extensively studied in tobacco. Plants carrying the *N* gene (eg. *N. tabacum* cv. Samsun *NN*) react hypersensitively to infection by TMV. The high level of resistance, expressed systemically throughout the plant, is manifested as the plant being less susceptible to a

second inoculation with TMV. This increased resistance is manifested as smaller or fewer lesions (or both) (Ross, 1961) resulting from a second inoculation, compared to control plants. This response implies the existence of a messenger, which moves from infected tissue to other parts of the plant and induces a "resistant" state.

These early works do not always state clearly whether the controls were either mock-inoculated plants or untreated healthy plants, but most reports state that there was no increased resistance in the plants purely as a consequence of inoculation damage (Ross, 1961; van Loon & Dijkstra, 1976).

Since induced systemic resistance had not been investigated in resistant potato cultivars inoculated with PVX, an investigation was undertaken.

The experiments clearly showed a reduction in lesion number and restriction of systemic spread that was highly significant for plants initially inoculated with PVX-KEBRS or mock-inoculated plants compared to the healthy uninoculated controls. There was no significant difference between PVX-KEBRS and mock-inoculated plants to the confidence level of 95% while both PVX inoculated and mock-inoculated plants were different to healthy uninoculated plants at a confidence level of 99%.

Earlier studies were confined to species other than potato (Ross & Bozarth, 1960; Ross, 1961; Bozarth, 1962; Loebenstein, 1963; Jenns & Kuc, 1977; McIntyre & Dodds, 1979; McIntyre *et al.*, 1981; Bergstrom *et al.*, 1982; Pennazio & Roggero, 1988). Interestingly, Yarwood (1960) has reported that mock-inoculation alone on *P. vulgaris* cv. Pinto induced localised resistance to subsequent TMV infection. Therefore, at least one previous study has shown that wounding alone can induce resistance, albeit only locally. There may be significantly different responses to wounding and pathogen attack between different plant species; similarly different pathogens may be more or less susceptible to changes induced by wounding and consequently influence their ability to parasitise the host plant. With *N. tabacum*, *C. sativus*, *Datura stramonium* and *G. globosa* wounding may be little or no stimulus whereas in *S. tuberosum* and *P. vulgaris* cv. Pinto wounding may have significant influence in increasing the level of resistance of the plant to subsequent infection. The finding that potatoes show increased levels of resistance to subsequent PVX infection

following wounding is not completely unexpected as several defence genes induced by pathogen attack are also induced by wounding in potato; these include *wun*I, II (Logemann *et al.*, 1988; Matton & Brisson, 1989) and *win*I, II which show striking homologies to several chitin binding proteins (Stanford *et al.*, 1989) and proteinase inhibitors induced both locally and systemically in the foliage as a consequence of wounding (Green & Ryan, 1972; Keil *et al.*, 1990; Peña-Cortés *et al.*, 1991; Suh *et al.*, 1991). Genes induced by wounding also include those involved in increased cell wall thickening (Friend, 1985) and the production of sesquiterpenoid phytoalexins e.g. 3-Hydroxy-3-Methylglutaryl Coenzyme A reductase (Stermer & Bostock, 1987), phenylalanine ammonia-lyase (PAL), HRGP and Peroxidase (Crosby and Vayda, 1991). Several mRNAs induced by wounding and pathogen attack have as yet not been assigned a function, e.g. SAR 8.2 (Ward *et al.*, 1991). Possibly some of the general responses by potato to both virus infection and wounding are similar. This is not necessarily unexpected, because many of the gene products described above,which are induced by wounding, are concerned with wound healing and the prevention of pathogen invasion. Several types of pathogens take advantage of naturally occurring wounds to evade the protective layers normally hindering their penetration. Why some of these gene products are induced systemically and are not restricted to the wound site is less clear.

As well as inducing resistance to a "challenge inoculation" with a localised strain of PVX, prior inoculation of cv. Pentland Ivory with PVX-KEBRS or mock-inoculation also reduced the level of accumulation of a Group 3 PVX strain, RSGp3, following a subsequent challenge. This strain normally spread systemically in Pentland Ivory plants and accumulated to high levels throughout the plant. In the plants which had previously been inoculated with a localised strain of virus, or mock-inoculated, the replication of the systemic strain was restricted to the inoculated leaf for the duration of the experiment, while healthy control plants inoculated in parallel with RSGp3 became systemically infected. This response to a challenge with a systemic virus resembles that reported by Bergstrom *et al.* (1982) who showed that in cucumber plants systemic spread of CMV was delayed by an initial challenge with

TNV (TNV is localised in cucumber). Van Loon and Dijkstra (1976) have also shown that ISR reduces TMV systemic infection in *N. tabacum* cv. Samsun In contrast, Fraser (1979) found in *N. tabacum* cv. White Burley that a systemic infection of a non-localised strain of TMV was not restricted as a result of an earlier localised TMV infection. Again, the restriction of a systemic virus may depend on the particular host-viral combination, although most research supports the finding of Fraser (Pennazio & Roggero, 1988). These results emphasise the differences between particular host-pathogen interactions. From my data it would appear that spread of a systemically infecting strain of PVX is inhibited by prior wounding alone.

The induction of resistance to a systemic viral strain would indicate that whatever product is induced by the first localised PVX infection or by wounding it has a true anti-viral effect, perhaps restricting the viral spread from cell to cell i.e. through plasmadesmata (Deom *et al.*, 1991) or by restricting its ability to replicate (Adams *et al.*, 1986a). In contrast, the results of Fraser (1979) suggest that the ability to restrict a localised virus in tobacco, as opposed to the ability to restrict systemic virus, may be achieved by increased effectiveness of general physical cell wall barriers, but not by the induction of an anti-viral factor.

Many of the early publications on ISR in resistant cultivars of *N tabacum,* following infection by TMV or other necroticising pathogens, reported a reduction in both the number and size of lesions developing on previously uninfected leaves following the second "challenge inoculation" (Fraser, 1979; Roberts, 1983). The extent of reduction in number and size of lesion as a result of ISR varies considerably depending on the particular host-pathogen interactions. In my investigation of Pentland Ivory plants inoculated and later challenged with a Group 2 PVX strain (PVX-KEBRS) only a reduction in lesion number was ever observed. Ross (1961) suggested that the apparent effect on lesion number might be an artefact, as it was not clear what effect ISR could have on the frequency of establishment of initial points of infection. He expected the increased level of resistance to result in a general reduction in lesion size (i.e. restricting the extent of viral replication and cell to cell spread). However, reduced lesion size was not seen in the Pentland Ivory inoculated

with PVX-KEBRS, and the effect of prior inoculation was to reduce lesion number only. I am confident that this reduction in lesion number was not an artefact. Both prior inoculation with PVX-KEBRS and mock-inoculation resulted in a reduction in lesion numbers following a second challenge with PVX-KEBRS, or in reduced systemic spread of a Group 3 PVX strain in Pentland Ivory, compared to uninoculated control plants. This reduction in lesion number might be explained as follows. It is not known whether ISR is uniformly expressed in all cells. In tobacco systemically infected with TMV, Atkinson & Matthews (1970) and Murakishi & Carlson (1976) found that cells in different regions of the leaf showed varying susceptibility to infection. This resulted in green-islands of cells in the leaf which were often free of TMV particles. If prior inoculation of potato with a PVX strain that causes local lesions, or wounding, induces ISR non-uniformly, following the second "challenge inoculation" PVX lesion formation may be inhibited in regions of the leaf expressing high levels of resistance. PVX replication might then be restricted to the single initially infected cell, whereas in areas not manifesting such a high level of resistance lesions might develop normally. The overall result of this would be the reduction in apparent lesion number compared to control plants. Lesion number and size varied quite considerably on Pentland Ivory plants inoculated with PVX-KEBRS, irrespective of any previous treatment. Possibly individual plants, or cells within an individual leaf, might be expressing different levels of resistance to lesion formation.

One possible mechanism of increased resistance to PVX might be that the primary inoculation of Pentland Ivory with a Group 2 PVX strain, e.g. PVX-KEBRS, interacts directly with the *Nb* gene product (Cruz *et al.*, 1990; Baulcombe, personal communication 1990). This might result directly, or indirectly via a cascade, in the induction of "anti-viral genes" whose products might restrict viral replication (Adams *et al.*, 1986a) or viral cell to cell movement (Deom *et al.*, 1991; Adams *et al.*, 1985). This response might be active against both localised and non-localised strains. It would then be expected that the compounds involved in restricting viral replication or spread would be induced both locally at the point of infection and systemically.

The systemic response to a localised infection implies the presence of an intermediate signal compound that is transported throughout the plant. One possible candidate for such an intermediate might be salacylic acid, as its level increases both locally, at the point of localised infection, and systemically in tobacco inoculated with TMV and cucumber inoculated with TNV (Malamy *et al.*, 1990; Metraux *et al.*, 1990). Salacylic acid (SA) has been shown to be associated with ISR and gene products which are assumed to play a role in ISR e.g. proteinase inhibitors, glutinases and chitinases (Yalpani *et al.*, 1991; Ward *et al.*, 1991). Since my results show Pentland Ivory expresses higher levels of resistance to both localised and non-localised strains of PVX, following localised PVX infection or wounding, it would be interesting to see if wounding could elevate the levels of SA. However, no increases in endogenous SA levels were observed in *N. tabacum* cv. Xanthia upon wounding (Malamy *et al.*, 1990). This lack of response might be expected, however, because wounding in tobacco does not induce ISR. The results presented in Section 3.3 suggest that, in contrast to tobacco, wounding *can* induce systemic resistance in potato and might therefore induce SA accumulation. This could act as a signal to activate resistance genes throughout the potato plant against later viral infection. However, in wounded potato plants it has been shown that endogenous levels of abscisic acid (ABA) increase both locally and systemically (Peña-Cortés *et al.*, 1989), and that ABA regulates the expression of several potato genes involved in resistance, e.g. proteinase inhibitors (Hildmann *et al.*, 1992). Ward *et al.* (1991) have proposed that pathogen-induced necrosis is tied to the biosynthesis of SA and the induction of ISR. It is possible that in potato, the pathogen-induced necrosis or wounding-induced necrosis triggers the synthesis of a transduction signal for increased resistance, whereas in other plants such as tobacco pathogen-induced necrosis is much more effective than wounding in inducing such a signal. These findings indicate that although the potato and tobacco systems may share several factors involved in ISR, the induction of such resistance mechanisms may be different.

The work described in this thesis raises the question as to whether induced systemic resistance actually occurs in potato. The results indicate that, in the case of

resistance to PVX, the wounding response alone is able to induce systemic changes in the potato plant which raise its resistance level to subsequent inoculation with either localised or systemic strains of PVX. The level of increase in resistance in PVX-inoculated plants was not significantly different to the mock-inoculated ones. This suggests that potatoes react quite differently compared with the other plants in which ISR has been previously monitored.

An interesting question is whether the ISR response varies from species to species or even cultivar to cultivar. It would be of interest to see if other potato cultivars and different viral strains showed similar responses to both infection and wounding. If future experiments were to confirm that potato plants can show a generalised wounding response which results in increased resistance, in particular to systemic strains of PVX but also to other viruses, this might provide a mechanism to increase the plants' general resistance to viral infection, where specific resistance genes are not available for plant breeding.

It would be of particular interest to repeat some of the work described here, but with the inclusion of a parallel control experiment with a different species which had been specifically reported to show ISR, e.g. cucumber and TNV (Dean & Kuc, 1985). Such an experiment would test whether ISR does, in fact, exist in either species and would provide a good positive control for the experiments presented here.

4.2. GENE EXPRESSION IN INFECTED POTATO PLANTS.

The object of this part of the present study was to attempt to characterise changes in gene expression in Pentland Ivory plants following inoculation with PVX. mRNAs were translated *in vitro* and the polypeptides analysed by one and two-dimensional PAGE. One-dimensional gels were used successfully to identify the initial signs of major changes in the mRNA population. The first clear changes in the mRNA population were seen 7 days after inoculation (Figs. 3.23 and 3.25). The most notable change was the induction of a 36 KDa polypeptide. This is approximately the same size as a basic β-1-3 glucanase which has been reported to be induced following *P. infestans* inoculation of potato (Bol *et al.*, 1990) although there is no direct

evidence to link the two polypeptides. Increases and decreases in the levels of individual mRNAs were seen using one-dimensional SDS PAGE, both locally and systemically in PVX-inoculated plants compared to mock-inoculated plants. This demonstrated that localised PVX infection influences potato mRNA populations both locally at the point of infection and systemically. These results indicate that the changes in mRNA population were not related solely to the wounding response. The subsequent use of two-dimensional gels was intended to analyse these changes in more detail and identify differences between treatment groups in levels of specific moderately abundant mRNAs. One aim was to pinpoint a time when change in the mRNA population in leaves distant from the point of PVX localised infection was greatest, compared to the leaves from mock-inoculated and healthy control plants. One advantage of using *in vitro* translation of mRNA is that it allows observation of the mRNA population at fixed time points whereas analysis of extracted proteins is influenced by differential rates of turnover and does not precisely pinpoint differences in the activity of specific genes.

The variability between experiments in the pattern of polypeptides detected in two-dimensional gels caused difficulty in interpretation. These differences, which may be attributed to differences between plants, occurred despite strenuous efforts to grow all plants uniformly between and within experiments. One possible reason for such variability is the physiological differences between the tubers used for different experiments. The time of year during which the plants were grown, and the age of tubers, were different from experiment to experiment because it was not possible to use tubers of the same age for replica experiments. Such variation might have had a pronounced effect on the physiology of the plants. Another possible reason for differences between plants is reported by Braum and Davis (1990) who showed that calmodulin and related genes in *Arabidopsis thaliana* were up-regulated to significant levels as a result of touch, wind and rain; it is likely that such mechanisms also exist in potato. Watering and movement of the plants might not have been uniform between and throughout the experiments. Induction of heat shock proteins have also been reported (Schoffi *et al.*, 1988) following wounding and water stress. It has also

become apparent that different batches of wheat germ extract used, for *in vitro* translation, seem to favour the translation of different mRNA populations (personal communication, Thomas & Dominy, 1991). This obviously complicates direct comparison between experiments. These effects might also be responsible for some of the observed variability from experiment to experiment.However, despite some variability, the two-dimensional gels *did* identify a number of consistent changes induced both systemically and in inoculated leaves of Pentland Ivory plants which were locally infected with PVX.

Differences in mRNA populations were seen between all three treatment groups, though mock-inoculated plants showed less changes than PVX-inoculated plants when compared to the uninoculated control. Both up and down regulation of gene expression appears to have occurred following both PVX and mock-inoculation. The repression of certain genes at moments of stress is a common plant response. For example, some of the genes involved in photosynthesis are turned off following the severe stress of wounding (Peña-Cortés *et al.*, 1991) while in sugar cane salt stress induces and suppresses gene expression (Ramagopal & Carr, 1991). A polypeptide of 22 KDa (b and h, Figs. 3.26 and 3.28) was upregulated in inoculated leaves and a polypeptide of 70 KDa (o and A, Figs. 3.30 and 3.32) was up-regulated in both inoculated and systemic leaves after PVX inoculation. The identity of the 22 KDa protein induced here is not known. However, a protein of similar size (24 KDa, pI 9.5) is induced in leaves of Samsun *NN* tobacco inoculated with TMV and in the tomato cv. Moneymaker inoculated with *P. infestans* (Woloshuk *et al.*, 1991). This protein shares homology with osmatin and has been shown to inhibit the growth and development of *P. infestans*. Both PVX and mock-inoculation appeared to induce several of the same mRNAs, with PVX-inoculation increasing mRNA expression to a greater extent than mock-inoculation alone. However, many of the changes in the *in vitro* translation products were specific to each treatment group. Previous data (Section 3.3) had indicated that mock-inoculation was no less effective in inducing systemic resistance than was PVX-inoculation. However, the mRNAs up-regulated by PVX and mock-inoculation were by no means identical. Perhaps the initial changes

relating to ISR are only a minor component of the overall changes induced by inoculation. The characterisation of changes specifically related to ISR is probably not possible with experiments of this sort.

Analysis of the two-dimensional gels identified relatively few changes between the three treatment groups. In contrast other workers, using two-dimensional gels to characterise different plant inducible systems, have identified many changes between treatment types (Ramagopal & Carr, 1991; Wagoner *et al.*, 1982). It might be that this reflects the difference between the systems. Moreover, small changes in the intensity of spots on autoradiographs of different treatments are difficult to interpret given the previously mentioned variability between experiments. Clearer differences between plant treatments might have been identifiable if mRNA from run off transcription had been used. It has been shown that mRNAs associated with polysomes are often not translated (Taliercio & Chourey, 1989). During translation *in vitro* many mRNAs are translated which *in vivo* are not translated (Crosby & Vayda, 1991). Analysis of run off transcripts would have allowed translational regulation to be analysed.

The analysis of *in vitro* translation products using two-dimensional PAGE systems has its limitations as regards the detection of mRNA changes between plant treatments. Slighton & Quemada (1988) have suggested that there are 10000 low abundance mRNAs in each cell in tobacco. As these mRNAs represent around 30% of the total mRNA by mass, the number of polypeptide spots visible on a typical two-dimensional gel represents only a fraction of the mRNA population. Low abundance mRNAs, which are potentially of interest, are poorly detectable by such techniques. The choice of time, after inoculation with PVX, at which mRNA was extracted in order to construct a cDNA library rested on the assumption that changes in levels of low abundance mRNAs occur simultaneously with changes in levels of more abundant mRNAs. Whether such an assumption is valid is not clear. Obviously, the use of two-dimensional gels to select a "best" time after inoculation is a compromise. A more rigorous approach would have been to have generated cDNA libraries from a number of time points after PVX inoculation and to have screened each library for

mRNA changes. However, given the constraints of time available to carry out the project, the strategy used represented a reasonable compromise for an initial study.

It is not clear why one-dimensional SDS PAGE did not identify several of the previously reported changes induced by wounding and pathogen attack, in the inoculated leaf in potato, which take place a few hours after inoculation. These include the up-regulation of *win* and *wun* genes, genes for proteinase inhibitors (Stanford *et al.*, 1989; Logemann *et al.*, 1989; Peña-Cortés *et al.*, 1989; Suh *et al.*, 1991), and genes involved in the biosynthesis of phytoalexins (Yang *et al.*, 1991). It is possible that one-dimensional SDS-PAGE, used to screen the samples extracted early after inoculation, is just too insensitive to detect the above-mentioned changes in gene expression, or the potato cultivar in question does not respond to the same extent as the potato cultivar used to characterise the expression of the *win, wun* and proteinase inhibitor genes.

4.3. GENERATION AND SCREENING OF THE cDNA LIBRARY.

The cDNA library was generated using mRNA from Pentland Ivory leaf position three, twelve days after leaf one was inoculated with PVX-KEBRS. Two strategies were used to screen the library; subtracted probes and differential screening. Subtracted ss cDNA probes were generated from PVX and mock-inoculated plants (leaf position three). mRNA from leaf position three of mock-inoculated plants was hybridised to its ss cDNA complementary sequence. This procedure removes sequences from the probe which are common to the two mRNA populations. The second strategy employed ss cDNA probes generated from PVX-inoculated, mock-inoculated and healthy uninoculated plants to screen the library differentially. Both methods showed their own advantages and disadvantages. Subtracted probes gave easily interpretable results as a consequence of common mRNAs between treatments being removed, enabling many plaques to be screened in a single screening. However, the probes were time consuming to generate and required a large amount of driver mRNA to be used in the subtraction procedure. The large amount of driver mRNA is required to remove moderate and low abundance mRNAs and is a

consequence of the kinetics of hybrid formation in a mixed population of varying abundance mRNAs. Failure to drive the hybridisation reaction to completion results in low abundance non-differentially expressed cDNAs remaining in the probe. As the ability to generate such large amounts of driver mRNA from single leaves was limited, this screening procedure had its drawbacks. The advantages of using conventional differential screening were that only small amounts of mRNA were required as templates for generation of probes and that the procedure to generate the probes was relatively quick. However, the disadvantage of differential screening was common mRNAs tended to over expose the autoradiographs so making low abundance mRNAs, which might be expressed differentially, difficult to identify. To overcome the problem recombinants were plated at lower density so allowing clear identification of individual plaques, however this in turn required an increased number of plates to be screened to investigate a relatively small number of recombinant phage in the library.

Having differentially screened 28,000 plaques from the cDNA library, 6 plaques were identified which represent the most clearly up-regulated sequences expressed in PVX-induced plants. This number is small compared to the number reported for other systems. In *N. tabacum* cv. Samsun *NN* differential plant gene expression in response to a localised TMV infection was quite dramatic. Thirty-two different clones were identified when 1400 plaques were differentially screened (Hooft van Huijsduijnen *et al.*, 1986b). However, Hooft van Huijsduijnen and co-workers were looking at the inoculated leaf and not at leaves distant to the point of inoculation as in the work described in this thesis. They also used a healthy plant as control and not a mock-inoculated plant, so many of their reported changes might have been wound-induced rather than solely the result of TMV localisation. Many of the mRNAs they identified were, however, found to be systemically up-regulated throughout Samsun *NN* plants, despite localisation of the virus to the inoculated leaf. The 32 recombinants were sub-divided into six clusters on the basis of cross-hybridisation; four recombinants were identified as coding for proteins with sequences serologically related to PR proteins.

A further possible reason for the low number of differential clones identified might be that the technique used to screen the library differentially was too insensitive to identify systemic changes induced in potato by localised PVX-KEBRS infection. Thus plaques representing sequences which are of moderate abundance can be overlooked even if they are strongly up-regulated. Such genes might be expressed differentially in Pentland Ivory inoculated with PVX-KEBRS but at lower levels than that seen in Samsun *NN* plants inoculated with TMV. The use of subtracted probes represents a more sensitive technique (and appears to be more effective as a means of screening the library). However even this technique involves great practical problems. Recent methods which overcome some of these problems are discussed later. The apparent differences between treatment groups, as identified on PAGE analysis, might have been due in part to limitations of the *in vitro* mRNA translation system: polypeptides which are very intense in gels from one set of plants might be the result of very efficient *in vitro* translation and could in fact be less abundant than apparent. Also, as the intensity of a spot on an autoradiograph depends not only on the amount of polypeptide but also on the number of methionine residues in the polypeptide, intense spots might represent relatively rare mRNAs with a large number of methionine codons.

The small number of recombinants identified by differential or subtractive screening contrasted with the number of clones which hybridised to cDNA clones for the tobacco PR proteins, PR-1a and the thaumatin-like protein. These seemed to be present at an abundance level of ~0.1% and ~0.05% of the library respectively. Assuming there must be additional PR protein clones in the Pentland Ivory cDNA library, many more differentially expressed clones would have been expected. Perhaps these genes are also induced by wounding and were thus overlooked because only clones hybridising specifically to sequences from PVX-inoculated plants were considered. This hypothesis is supported by the analysis of *in vitro* translation products; more differences in mRNA expression were seen between healthy uninoculated and mock-inoculated plants than between mock and PVX-inoculated plants. The first round of screening identified 16 plaques representing sequences

which hybridised most intensely to probes from upper leaves of mock-inoculated plants, similar or slightly less to probes from PVX-inoculated plants and much less (but still present) to probes from uninoculated control plants. Thus, wounding alone appears to induce expression of several genes systemically throughout the plant. Some of these might be involved in the induction of systemic resistance.

Only three of the six clones representing mRNAs which were up-regulated following PVX inoculation were detected on Northern blots. These three clones, PIX 24, 6 and 30, hybridised to mRNAs of 750, 850 and 1100 nt in length respectively. These three clones appear to code for relatively abundant messages which were present in all three treatment groups and both leaf positions. The presence of these three mRNAs in healthy tissue would suggest that they are not PR proteins as these are reported to be only expressed at very low levels in healthy plants, at least in tobacco (Hooft van Huijsduijnen *et al.*, 1986b; Cornelissen *et al.*, 1986). The extent of up-regulation of these mRNAs as assayed on Northern blots was limited. The maximum increase in abundance in PVX-inoculated plants compared to healthy plants was by a factor of approximately ten, and the abundance in mock-inoculated plants was five-fold above healthy plants for PIX 24. The level of expression for PIX 24 varied with time and leaf position for all three treatments. The difference in level of expression of PIX 24 between inoculated and non-inoculated plants can be considered to be highly significant. PIX 6 which appeared to be the most strongly differentially expressed clone on the basis of the screening of the library showed little, if any, differential expression using Northern blot analysis. This problem, in which clones that appeared to be strongly up-regulated turned out not to be so when the abundance of the mRNA was directly measured, has been reported elsewhere (Dunigan *et al.*, 1987). Of the mRNAs identified in PVX induced plants, none was constantly up-regulated above the level of expression seen in healthy control plants.

A sustained increased level of expression over that seen in the healthy controls might be expected for a polypeptide that was directly involved in ISR, i.e. an anti-viral protein. The time after inoculation that changes appeared in the RNA population (as visualised by Northern blot hybridisation) corresponded to the same

time after inoculation that changes were seen in the one and two-dimensional gels and in lesion development on the inoculated plant. However, the time course of up-regulation of the mRNAs homologous to clones PIX 24 and PIX 30 indicated a rather late response time to localised infection compared to responses relating to inoculation damage and viral localisation in the tobacco Samsun *NN*, TMV systems (Ward, *et al.*, 1991) or wound response genes in potato (Yang *et al.*, 1991; Logemann *et al.*, 1988; Stanford *et al.*, 1989; Suh *et al.*, 1991). This makes it difficult to relate these changes to the typical early changes in gene expression following wounding, as described by other workers (Bol & van Kan, 1988; Logemann *et al.*, 1988; Hooft van Huijsduijnen *et al.*, 1986b; Fritig *et al.*, 1987). Though the differentially expressed clones isolated from potato hybridised to mRNAs present at significant levels in healthy plants, this does not preclude them from having a resistance role. For example, mRNA for the protease inhibitor II gene in potato, which is believed to be involved in resistance, is expressed at low levels in healthy plants (Suh *et al.*, 1991).

PIX 30 hybridised to a mRNA which was expressed at a higher level in leaf position three (ie. systemically) in plants locally infected with PVX, compared to the other two controls. The protein might, for example, be involved in a reaction such as increased cell wall thickening, which has been shown to act in restricting pathogen attack and would explain the presence of such mRNA expression in control plants. In contrast, PIX 24 appeared to be up-regulated in the inoculated leaf but showed little differential expression systemically. Possibly the mRNA homologous to PIX 24 might be involved in the synthesis or release of a signal molecule which is systemically transported throughout the plant and involved in ISR. It is believed, from earlier work using leaf abscission, that the signal for ISR is produced in the inoculated leaf (Dean & Kuc, 1986b). This is consistent with the results of my earlier experiments which showed that ISR occurred in plants locally infected with PVX or mock-inoculated. However, it is more likely that a mRNA homologous to PIX 24 is in some way involved in the local wounding response in common with a large number of other gene products (Dixon & Harrison, 1990). Thus, although these two clones

represented mRNAs with a modest degree of up-regulation, a significant positional difference in induction was apparent.

PIX 6, which appeared to be the strongest differentially expressed plaque, hybridised to a mRNA which, on the basis of Northern blots, showed little or no changes in expression after inoculation. Preliminary sequence data has shown this clone to have 75% homology at the DNA level to the gene for the a S11 ribosomal protein from *A. thaliana* (Colthart, personal communication 1992). It is doubtful, therefore, if this clone represents a gene which is up-regulated by PVX infection.

The three clones (PIX 11, 12, and 26) which did not hybridise during Northern blot analysis are presumably homologous to low abundance mRNAs. This does not mean that these clones are of no significance since they might still show significant up-regulation. These clones may be studied further in the future by the amplification of their mRNA by the use of polymerase chain reaction. Various workers have identified, by differential screening, cDNA clones which are expressed both systemically and locally at the point of infection in tobacco resistant to TMV infection, but which are also up-regulated at a lower level by wounding alone (Ward *et al.*, 1991). These include an acid peroxidase (which is active in lignin, suberin, etc. synthesis) and a clone, SAR 8.2, of unknown function. However, in potato, wounding alone seems to induce a number of changes in gene expression e.g. there is an increased level of proteinase inhibitors (Peña-Cortés *et al.*, 1991) which occur both locally and systemically and are connected with functions related to increased resistance. Therefore, in potato, wounding alone appears to induce several different responses.

To date no PR protein has been shown to exhibit specific anti-viral properties and PR protein genes of unknown function (PR-1a and PR-S) when introduced into tobacco plants did not increase resistance to viral infection (Linthorst *et al.*, 1989). Perhaps genes, like those for PR proteins, do not hold the secret to any anti-viral response. This response might, in fact, depend on genes which are expressed in healthy plants and appear to have relatively unimpressive levels of induction following wounding or pathogen attack. Such candidates may be the genes involved

in SA and/or ABA production. SA may act as a signal molecule moving throughout the plant, regulating genes which are involved in systemic resistance. SA shows a 20-fold increase in inoculated leaves and only a 5-fold increase in systemic leaves when TMV is inoculated onto a resistant tobacco variety (Malamy *et al.*, 1990), while a mere 10-fold increase in endogenous SA induces many of the PR proteins and possibly induces systemic resistance in tobacco Samsun *NN* after the TMV hypersensitive response (Yalpani *et al.*, 1991). A mere 3-fold increase in ABA is seen in local and systemic leaves of potato as a consequence of wounding, though many defence genes are induced by ABA (Hildmann *et al.*, 1992). Thus small changes in the levels of enzymes involved in the synthesis of SA or ABA may have a profound effect on the actual outcome of a plant's response to pathogen attack.

Differences between the wound response of potato and tobacco may be significant. In potato many wound-inducible genes have been identified, whereas tobacco has only shown a small number of genes induced by wounding (Ward *et al.*, 1991). Therefore, the regulation of defence genes in potato may be quite different to that in tobacco. The fact that, in potato, wounding alone appears to trigger increased resistance to both localised and systemic strains of PVX, is certainly consistent with this observation.

In summary, six clones which represent potentially differentially expressed sequences were identified as being systemically induced in the potato cv. Pentland Ivory following localised PVX infection. Wounding alone and PVX inoculation seemed to induce a similar number of changes in potato (Yang *et al.*, 1991). The small number of changes in leaves distant from the point of inoculation might therefore have been expected in potato, because those genes which are induced systemically by localised infection alone are probably few in number. Most previous studies have concentrated on the regulation of genes in the locally infected leaves. Screening changes in gene expression in upper leaves, away from the point of infection, should exclude those changes directly related to local responses to PVX infection and to necrosis in the inoculated leaf, leaving the genes directly related to systemic response in the plant, induced by localised pathogen attack, to be identified.

The number of clones showing differential expression was small. Perhaps the use of a subtractive library, with its potential for identifying less abundant mRNA species, might be a useful future strategy. When these experiments were planned, and given the information from the one and two-dimensional gels (which showed a number of significant changes in protein synthesis between the three treatment groups), the construction of a conventional library using differential probes to screen it was the best method available for the identification of up-regulated sequences. The limited sensitivity of this technique and the difficulty in interpreting the plaque hybridisations may have been a contributing factor in the limited number of clones identified. Today more sensitive and convenient procedures are available for identifying up-regulating mRNAs. Subtracted cDNA can be relatively easily generated by biotinylating driver sequences and then removing the hybrids with streptavidin and phenol extraction, a more efficient process than using hydroxapatite (Duguid *et al.*, 1988). The subtracted cDNA sequences remaining can be used to generate a substantial cDNA library (Innis *et al.*, 1990; Timblin *et al.*, 1990). Since it is then possible to amplify the small amount of remaining unhybridised cDNA by the technique of polymerase chain reaction (PCR), one can generate cDNA libraries with a potentially unlimited number of clones from minimal quantities of cDNA. PCR can also be used to amplify control ds cDNA which can then be biotinylated and used to remove common messages from the ds cDNA generated from the induced tissue of choice so enabling a vast excess of driver sequences to be generated, resulting in the kinetic conditions of hybrid formation which enabled the removal of low abundance sequences which are not differentially expressed. Combinations of the available methods briefly described above will enable the generation of subtracted libraries and allow very much easier screening of such libraries. This should result in the identification of differential clones which are expressed at low levels in the induced tissue without the high background of common clones obscuring the clones of choice, as is seen with conventional libraries and differential screening.

APPENDIX

Source of materials:

Enzymes

Enzymes were purchased and used as instructed by the manufacturer

Enzymes purchased from:

BRL	Klenow fragment-DNA, polymerase *E. coli*, T4 DNA polymerase, RNase H, DNase, RNase A, Eco RI, Hind III
New England Biolabs	EcoRI Methylase, Alu I Methylase
Pharmacia	AMV reverse transcriptase
Boehringer	DNA ligase *E. coli* and T4.

Specific reagents purchased from:

BDH	Ampholines pH5-7. pH 3-5-10
Sigma	S-adenosylmethionine (SMA)
Pharmacia	dATP, dTTP, dCTP, dGTP, Hexadeoxynucleotides
Boehringer	oligo dT cellulose
Amersham	Creatine phosphokinase, Phosphocreatine

Source of chemicals:

General laboratory reagents were obtained from various sources and were "Analytical" grades, except where stated.

Buffers and media

Most common buffers and media recipes were obtained from Cold Spring Harbor Molecular Cloning Manual (Sambrook *et al.*, 1989). Buffers for restriction and modifying enzymes were prepared according to the manufacturers recommendations.

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