Aphid-Borne Viruses of Potato: Investigations into Virus/Host/Vector Interactions, Serological Detection Using Recombinant Antibodies and Control Strategies

By

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Ahmad

DECLARATIONS

I, Ahmad Al-Mrabeh, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I can confirm that this has been indicated in the thesis.

Ahmad Al-Mrabeh

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This is to certify that this copy of the thesis was checked and approved by the examiners.

Dr Anne Borland

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ABSTRACT

Potato is one of the most important food crops in the world, and viruses are largely responsible for the degeneration of this vegetatively propagated crop. At least 35 viruses have been reported to infect potato naturally. The majority and the most economically important ones are vectored by aphids. The objective of this study was to conduct molecular and biological investigations into virus transmission mechanisms, including developing diagnostic methods to help to control the spread of aphid-borne potato viruses, and disrupting the vectoring ability of their aphid vectors by insecticide spray.

One way to control the spread of aphid-borne viruses is to control their aphid vector, but this is not always feasible as the majority of aphid-borne potato viruses, including the most important ones, are transmitted non-persistently, being acquired within a very short time before agrochemicals can act. Thus an alternative approach to controlling this class of viruses is through a full understanding of the interaction between the virus, the host plant and the aphid vector, which was the first objective of this project. In this respect, some aphid cuticle proteins were identified to interact with potato virus Y helper component (HC-Pro) through screening of an aphid cDNA expression library, and their potential role in virus transmission was discussed. Moreover, the concept of short retention of non-persistent viruses inside their aphid vectors was challenged; the results show that PVY can be retained inside its aphid vector for a long time but it is not transmissible. This novel finding together with binding to aphid cuticle proteins, generated some new ideas about transmission mechanisms that were proposed and discussed. In addition, the effect on aphid vectoring ability of the plants used to rear aphid colonies, as a virus source, and as a virus recipient was investigated. From laboratory studies of aphid transmission, it was concluded that the transmission efficiency of PVY was significantly affected by the host plant species used to rear *M. persicae*, or that used as a virus recipient plant.

The availability of sensitive and cheap virus detection methods is critical for early detection and control of potato viruses. In this project a sensitive fully recombinant ELISA was developed and validated for routine testing of potato leafroll virus. This technology can be applied to detect other potato viruses and has the potential to replace the commonly used immune reagent antisera.

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LIST OF ABBREVIATIONS

AAP: Acquisition access period

AMP: Ampicillin

α: anti

AP: Alkaline phospatase

Approx. Approximately

APs: Ammonium Persulphate

BAD: Biotin acceptor domain

BCCP: Biotin carboxyl-carrier protein

BCIP: 5-bromo-4-chloro-3-indolyl phosphate

BioSS: Biomathematics and Statistics Scotland Research Institution

BLAST: Basic Local Alignment Search Tool

bp: base pair

BSA: Bovine serum albumin, Bovine Albumin

^{o}C: Degree Celsius

CDRs: Complementarity Determining Regions of antibody

CI: Cylindrical inclusion protein

Cam: Chloramphinicol

cDNA: Complementary DNA strain

Chap. Chapter

CP: Coat protein

C-terminus carboxy-terminus

CUPs : Cuticule protein

cv: Cultivar

Deica: diethyldithiocarbamate

DEPC: Diethylpyrocarbonate

DNA: Deoxyribonucleic acid

DTT: DL-Dithiothreitol

DAG: Asp-Ala-Gly triplets

DNAse: desoxiribonuclease

dNTP: Deoxyribonucleotide triphosphate

DMF: Dimethylformamide

dpi: Days post inoculation

E.coli: Escherichia coli

EdBr: Ethidium bromide

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EM: Electron microscopy

EMBL: European Molecular Biology Laboratory

EPG: Electrical penetration graph technique

ESTs: Expression sequence tags

et al. et alii in Latin means and others

EtOH: Ethanol

Fab: Immunoglobulin fragement

FERA: Food and Environment Research Agency

F: Forward

Fig. Figure

GFP: Green fluorescent protein

HC-Pro: Helper component protein

His: Histidine

H: Hour

IAP: Inoculation access period

IgG: Immunoglobulin

IB: Inclusion bodies

IPTG: Isopropyl β-D-1-thiogalactopyranoside

KAN: Kanamycine

kb: Kilobase

kDa: Kilodalton

KITC: Conserved Lys-Ile-Thr-Cys sequence in the HC-Pro

L: Liter

LBAmp: Luria-Bertani Media + Ampicillin

LBAIX: Luria-Bertani Media + AMP + IPTG +X-Gal

M: Molar

Mab: Monoclonal antibody

M: Molecular size marker

mg: milligram

µg: Microgram

M-MLV: Moloney Murine Leukemia Virus

M&M: Material and methods

M.P: *Myzus persicae*

Min: Minute

Ml: Milliliter

µl: Microliter

NBT: Nitro blue tetrazolium

NCBI: National Center for Biotechnology Information

NHS = N-Hydroxysuccinimide ester

NIa: Nuclear inclusion a

NIb: Nuclear inclusion b

Ni-NTA: nickel-nitrilotriacetic acid

N-RT-PCR: Nested RT-PCR

N-terminus: Amino-terminus

OD: Optical density

O/N: Overnight

Pc: Polyclonal antibody

PAGE: Polyacrylamide gel electrophoresis

PCR: Polymerase chain reaction

PE: Periplasmic extract

pmol: Picomole

PEG: Polyethylene glycol

PBS: Phosphate buffered saline

PHT: Post Harvest Test

PTA: Plate-trapped antigen

PTGS: Post transcriptional gene sielecing

PTNRD: Ptato tuber necrotic ringspot disease

PVP: Polyvinylpyrrolidone

R: Reverse

Rev: Reverse

REF: Relative efficiency factor

RNA: Ribonucleic acid

RNase: Ribonuclease

rpm: Revelution per minute

RT-PCR: reverse transcriptase PCR

RT: Room temperature approx. 20-22 ^{o}C

S-AP: Streptavidin Alkaline Phosphatase

SASA: Science and Advice for Scottish Agriculture

scFv: Single chain antibody fragement

SCRI: Scottish Crop Research Institute

Sec: Second

SDS: Sodium dodecyl sulfate

SDW: Sterile double-distilled water

SOC: Super Optimal broth with Catabolite repression

PAGE: Polyacrylamide gel electrophoresis

Tab: Table

Taq: Thermus aquaticus

TBE: Tris/Borate/EDTA

TEMED: Tetramethylethylenediamine

T: Temperature

U: Emzyme unit

UK: United Kingdom

UV: Ultraviolet

W/W: Weight/weight

W/V: Weight/volume

VPg: Viral genome-linked protein

V3: V3HCL

V3-B: V3HCL-Biotin

V/V: Volume /volume

X-Gal: 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

LIST OF VIRUSES AND ACRONYMS

Alfalfa mosaic virus: AMV

Beet necrotic yellow vein virus: BNYVV

Beet yellows virus: BYV

Bean yellow mosaic virus: BYMV

Blackcurrant reversion association virus: BRAV

Cauliflower mosaic virus: CaMV

Lettuce mosaic virus: LMV

Cucumber mosaic virus: CMV

Maize dwarf mosaic potyvirus: MDMV

Pea seed-borne mosaic virus: PSbMV

Peanut mottle virus: PeMoV

Potato aucuba mosaic virus: PAMV

Potato yellow mosaic virus: PYMV

Potato leafroll virus: PLRV

Potato mop-top virus: PMTV

Potato virus A: PVA

Potato virus M: PVM

Potato virus S: PVS

Potato virus V: PVV

Potato virus X: PVX

Potato virus Y: PVY

Potato yellow vein virus: PYVV

Tobacco etch virus: TEV

Tobacco mosaic virus: TMV

Tobacco rattle virus*:* TRV

Tobacco vein mottling virus: TVMV

Tomato spotted wilt virus*:* TSWV

Turnip mosaic virus: TuMV

Wheat streak mosaic virus: WSMV

Zucchini yellow mosaic virus: ZYMV

Chapter 1. Literature review

1.1. General background

Potato (*Solanum tuberosum*) is the third staple food crop in the world (Visser *et al.*, 2009), and the fourth most cultivated crop in the world after wheat, maize and rice (Milbourne *et al*., 2007). Potatoes originated in South America and were first domesticated in Peru approximately 10 thousand years ago. The world's annual production of potatoes has increased remarkably during the past few decades, particularly in developing countries due to regional and international organizations established to help people in these countries. Because of the substantial importance of potatoes in providing food and a source of income to many people around the world, the United Nations announced the year 2008 as the international year of the potato (Stapleton, 2008). Seed potato production is an important industry in the UK, and British potato seed exports are worth about £18 million annually. The importance of this industry stems from the need to plant disease-free seed potatoes to avoid yield losses.

Viruses are considered among the most economically important pathogens which threaten the production of this crop. "Potato degeneration" was the term used in the 1800s to early 1900s to describe poor yields obtained from using potato tubers from plants infected with virus in the previous season. It was speculated that this fatigue or deterioration happened as a result of continuous propagation or unfavourable weather or soil conditions (Van der Want, 1987). It was discovered later that potato viruses including potato leafroll virus (PLRV) and potato virus Y (PVY) were the cause of this degeneration, and these viruses are still major problems for potato production all over the world. The symptomless nature of infection on some potato cultivars (Singh and Singh, 1994; Baldauf *et al.*, 2006; Hamm *et al.*, 2010) and under some weather circumstances (De Bokx, 1977; Marco, 1981) make the control of such diseases very difficult. In addition, there is no drug or chemical which has any effect on plant viruses. *Myzus persicae*, the most naturally efficient vector of aphid-borne potato viruses, plays an important role in spreading infection between plants; considerable amounts of insecticides are being used regularly to control this vector (Parker *et al*., 2006).

Application of insecticides is effective in controlling the spread of PLRV, which is transmitted persistently (Section 1.5.1). But no insecticides are known to date to efficiently control non-persistent viruses (Section 1.5.1) such as PVY and potato virus A (PVA). The limited impact on PVY and PVA spread has resulted in these viruses becoming more dominant in potato production areas. The reasons for this difficulty come from the non-persistent mechanism of transmission which is characterized by brief probing; also a wide range of aphid species can transmit these viruses. Moreover, there is a lack of information about the exact mechanism involved in transmission of these viruses; such knowledge is needed to design methods to block or decrease transmission efficiency by aphid vectors.

In order to obtain durable control of plant viral diseases in general and non-persistent ones in particular, it is important to understand virus – insect – plant interactions on a molecular level. This will help in the discovery of resistance genes to breed resistant cultivars and will provide better understanding of the vector –virus relationship so as to reduce or block virus transmission. In addition, another important component of disease control is to develop more sensitive, robust, and cheap assays for virus detection. Moreover, a goal of sustainable production systems is to optimize agrochemical applications to control the vector aphids. My research is directed towards control of virus spread by aphids in potato crops.

1.2. Viruses which naturally infect potato

There are more than 37 viruses which are reported to naturally infect potato (Beemster and de Bokx, 1987; Jeffries, 1998). However, only about one third of them are reported to commonly occur in the potato crop. Table 1.1 presents a list of the most important viruses which naturally infect potato crops; there are other viruses which naturally infect potato, but they are of limited incidence. The majority of the important potato viruses (more than 70%) are transmitted by insects, and more than 80% of the most common ones are vectored by aphids (Table 1.1). All important potato viruses except PLRV can also be transmitted mechanically. In nature, however, the aphid transmission route is the most important way to spread infection of most of these viruses except potato virus X (PVX) and potato virus S (PVS), which are transmitted by mechanical contact. A few potato viruses are reported to be spread by vectors other than insects, including potato mop-top virus (PMTV) which is vectored in soil by zoospores of the

plasmodiophoromycete fungus *Spongospora subterranea*, and tobacco rattle virus (TRV) which is transmitted by nematodes. Some potato viruses such as potato yellow mosaic virus (PYMV), potato yellow vein virus (PYVV), and tomato spotted wilt virus (TSWV) are of significant importance in some parts of the world on some cultivars and under particular conditions (Jeffries, 1998). Spread of these less economically important viruses should be monitored and controlled as they may emerge as serious pathogens if they spread to different parts of the world or if new crops are introduced in the areas where they exist.

Virus	Genus/Family	Transmission in nature by vectores	Yield decrease
Potato leafroll virus (PLRV)	*Polerovirus/ Luteoviridae*	Aphids persistent	Up to 90% (Jeffries, 1998)
Potato virus Y (PVY)* PVY^{O} PVY^{N} PVY^{C}	*Potyvirus/ Potyviridae*	Aphids non-persistent	Up to 40 –70% (Kerlan, 2006)
Potato virus A (PVA)	*Potyvirus/ Potyviridae*	Aphids non-persistent	Up to 40% (Kerlan, 2006)
Potato virus V (PVV)	*Potyvirus/ Potyviridae*	Aphids non-persistent	Up to 40% (Salazar, 2003)
Potato virus S (PVS)	*Carlavirus/ Flexiviridae*	Some isolates are aphid transmissible	10–20% (Jeffries, 1998)
Potato virus M (PVM)	*Carlavirus/ Flexiviridae*	Aphids non-persistent	15–45% (Jeffries, 1998)
Alfalfa mosaic virus (AMV)	*Alfamovirus/ Bromoviridae*	Aphids non-persistent	20% (Salazar, 2003)
Cucumber mosaic virus (CMV)	*Cucumovirus/ Bromoviridae*	Aphids non-persistent	10% (Salazar, 2003)
Potato mop-top virus (PMTV)	*Pomovirus*	Plasmodiophoromycete fungus *Spongospora subterranea*	40% (Salazar, 2003)
Tobacco rattle virus (TRV)	*Tobravirus*	No aphid vectors Nematodes	Cause spraing (Jeffries,1998)
Potato virus X (PVX)	*Potexvirus/ Comoviridae*	No known vector	15–20% (Jeffries, 1998)
Potato aucuba mosaic virus (PAMV)	*Potexvirus/ Flexiviridae*	No known vector	Little economic importance (Kerlan, 2006)

Table 1.1 Commonly occurring viruses which naturally infect potato.

All viruses can be transmitted through infected tubers; all viruses can be transmitted by mechanical inoculation except PLRV. Virus names and classification are according to the International Committee on the Taxonomy of Viruses (ICTV) http://www.ictvonline.org/.*: PVY^{NTN} , PVY^{NW}, and $PVY^{N:O}$ are new recombinant strains of PVY.

1.3. Brief description of the most important aphid-borne potato viruses

At least 14 potato viruses are transmitted by aphids. Some characteristics of the most important ones are presented below. In addition, a focus of this thesis, PVY, will be discussed further in section 1.4.

1.3.1. Potato leafroll virus (PLRV)

PLRV is the type member of the genus *Polerovirus* (*Luteoviridae*). It has small isometric particles (24–25 nm in diameter), and a single-stranded positive sense (5880–5990 nucleotides long) RNA. PLRV was one of the first viruses discovered to cause potato "degeneration", and is one of the most studied potato viruses. It is transmitted mainly by *M. persicae* in a persistent non-propagative manner. Aphids can transmit acquired virus after a latent period of 1–2 days, and carry the virus for the rest of their life. Symptoms of primary infection (Fig. 1.1) consist typically of paleness and reddening of the tip leaves, which may become rolled. Secondary symptoms in plants grown from infected tubers include stunting, upward rolling of leaflets and marginal necrosis (Harrison, 1984).

Figure 1.1. Typical PLRV secondary infection symptoms of leaf rolling (SCRI).

1.3.2. Potato virus Y (PVY)

PVY is the type member of the *Potyvirus* genus (family *Potyviridae*), which is the largest plant virus family. PVY is a single-stranded RNA virus with flexuous filamentous particles (730 x 11 nm). It has a positive sense RNA about 9,700 nucleotides in length (Shukla *et al.*, 1994).

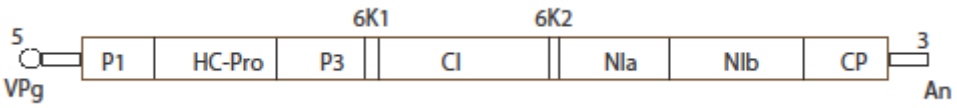


Figure 1.2. Schematic representation of the potyvirus genome map.

VPg: 5' terminal linked protein, HC-Pro helper component CI: cylindrical inclusion, NIa: Nuclear inclusion A, NIb nuclear inclusion B, CP capsid protein. An: 3' poly-A tail

There are three main strains of PVY that infect potato, PVY^N, PVY^O, and PVY^C, differentiated according to the symptoms they induce on tobacco indicator plants. PVY was first recognized to be a problem on potato by Smith in 1931. The disease caused by the virus is known as vein banding, severe mosaic, leaf drop streak, or rugose mosaic. It is transmitted in a non-persistent manner by more than 40 aphid species with variable efficiency (Table 1.2), but *M. persicae* is the most efficient one under laboratory conditions. Symptoms induced by PVY on potato are different depending on the virus isolate, potato cultivar, climatic conditions, and type of infection (primary or secondary) (Draper *et al*., 2002). However, symptoms of the PVY^O (Fig. 1.4) and PVY^C (Fig. 1.3) strains are normally mild to severe mosaic, rugosity, and crinkling, severe systemic necrosis, dropping of leaves (leaf drop streak) and dwarfing (Barker *et al*., 2009). PVY^N is normally symptomless but occasionally induces mild leaf symptoms and tuber necrosis. On the other hand, PVY^{NTN} induces severe leaf symptoms and tuber necrosis.

Figure 1.3. PVY^{C} symptoms on potato Phureja (SCRI).

Figure 1.4. PVY^{O} symptoms on potato cv. Shula (SCRI).

1.3.3. Potato virus A (PVA)

PVA is a commonly occurring potato potyvirus. It is similar to PVY in genome and morphological properties. These viruses cannot be distinguished by electron microscopy. However, they can be differentiated by serology and indicator plants. Symptoms of PVA on potato are mild mosaic, roughness of the surface, and waviness of the leaf margin. Some varieties develop a hypersensitive reaction producing top necrosis. However, symptoms are less on some potato cultivars and under some weather conditions: mixed infection with PVX or PVY produces crinkle symptoms on potato (Bartels, 1971). PVA is transmitted by aphids in the non-persistent manner; however, fewer aphid species were reported to transmit this virus compared with PVY and there is a lack of information about aphid species transmitting this virus and their relative efficiency values (Pickup *et al.*, 2008).

1.3.4. Potato virus V (PVV)

PVV is also a potyvirus. It has filamentous particles of 760 nm in length. It has a positive-sense, single-stranded RNA of 9,851 nucleotides (Oruetxebarria *et al*., 2000). It is transmitted non-persistently by several aphid species including *M*. *persicae*. PVV is less common in potato growing areas than PVY and PVA. PVV is symptomless in the majority of potato cultivars; some cultivars show mosaic and necrotic spots on the lower leaves while severe systemic necrosis and leaf drop may occur on other cultivars (Jeffries, 1998).

1.3.5. Potato virus S (PVS)

PVS is a *Carlavirus* (*Flexiviridae*). It has a straight or slightly curved filamentous particle with dimensions 650 x 12 nm. The RNA is single-stranded of 8,485 nucleotides. Some virus isolates are reported to be transmissible by several aphid species such as *Aphis fabae*, *Aphis nasturtii*, *M*. *persicae* and *Rhopalosiphum padi* in a non-persistent manner (Lin *et al*., 2009). However, it is reported to be transmitted mainly mechanically in Australia where it is considered a problem (Lambert *et al.*, 2007). PVS may cause mild symptoms on some cultivars or remain symptomless on

others. It is widespread and can cause up to a 20% decrease in potato crop yield (Jeffries, 1998).

1.3.6. Potato virus M (PVM)

PVM is a *Carlavirus* (*Flexiviridae*) with straight to slightly curved filamentous particles (650 x 12 nm). PVM has a single-stranded positive-sense RNA of about 8.5 kb (Zavriev *et al*., 1991). Most isolates are transmitted by aphids in a non-persistent manner. It is found worldwide where potatoes are grown, and often found to be accompanied by PVS on potato. Depending on the virus strain and potato variety, PVM symptoms vary from mild to very severe (Jeffries, 1998).

1.4. PVY characteristics

1.4.1. Emergence of PVY as the main potato virus

The relative importance of potato viruses is different over time and region. Historically, PLRV was the most important virus affecting potato, and PVY was thought to be the second most important potato virus. However, all current reports indicate that PVY is the most important potato virus at present in many countries across the world where potatoes are grown (Kerlan, 2006), although it is not considered to be a problem in some countries, such as Australia (Lambert *et al.*, 2007). The reasons for this change in the relative importance of these two viruses can be summarized as follows. (1) Successful chemical control of PLRV transmission with the introduction of new systemic insecticides. (2) The capacity of PVY for recombination between strains and subsequent emergence of new PVY isolates having different biological properties. These novel properties include improved virus transmission efficiency by aphids (Verbeek *et al*., 2010) and more severe symptoms or mild symptoms that mask the infection completely in some potato cultivars (Hamm *et al*., 2010; Whitworth *et al.*, 2008, 2010). (3) The production of new potato cultivars which exhibit very weak or sometimes symptomless infection has led to infection being missed during field inspections. (4) The increase in global trade, even though seed tubers are inspected and certified disease-free by certification schemes, there is a risk of spreading new aggressive recombinant virus isolates between countries.

1.4.2. Host range of PVY

Wide host plant range is a characteristic of viruses belonging to the *Potyviridae.* 495 species in 72 genera of 31 families were reported to be infected by PVY in the laboratory (Kerlan, 2006). PVY has a wide range of natural host species, some of which are edible crops like potato, tobacco, pepper, and tomato, while others are ornamental plants (*Dahlia* and *Petunia* spp.) or weeds such as *Datura* spp., *Physalis* spp., *Solanum dulcamara* and *S. nigrum* (Jeffries, 1998). There are recent reports of such new hosts. For example, Fletcher (2001) reported that *Cotula australis* and *Capsella bursa-pastoris* can be infected naturally by PVY in New Zealand. It has been reported that the weed *Solanum elaeagnifolium*, which is abundant in potato fields in Tunisia and other Mediterranean countries, is infected up to 32% by PVY^N which can provide a source for virus infection of potatoes (Boukhris-Bouhachem *et al*., 2007). Chikh Ali *et al*. (2008) reported that PVY is very common in *S. nigrum* and *Physalis* spp. which are abundant at the field margins in potato crops in Syria. Chikh Ali *et al*. (2008) speculated that these weeds may serve as a virus source for aphids alighting on potato crops. In Europe, Kaliciak and Syller (2009) reported other new wild hosts for PVY including *Erodium cicutarium, Geranium pusillum*, *Lactuca serriola* and *Lamium purpureum*. This growing list of alternative host species is very challenging when designing virus control strategies, particularly because of the important role of these hosts in providing virus sources for aphid vectors.

1.4.3. PVY: Genetic diversity and strains

The PVY genome encodes a large polypeptide which is processed into smaller proteins by virus encoded proteases: P1, HC-Pro, P3, 6K1, CI. 6K2, VPg, NIa, NIb and CP (Fig. 1.2). Before the 1980s, three strains of PVY were known, the ordinary PVY^O strain, the stipple streak PVY^C strain and the veinal necrosis strain (PVY^N). This classification was based mainly on the biological reaction of tobacco plants to these strains; PVY^N is the only strain which induces necrosis on tobacco. However, after the emergence of the new recombinant strains, this old classification depending solely on biological properties became less useful. A new classification of PVY isolates was therefore suggested. This

takes into consideration the biological reaction on different host plants, genome sequence, and serological properties (Singh *et al*., 2008). Natural recombination between PVY^{O} and PVY^{N} has led to the emergence of new aggressive PVY isolates named PVY^{NTN} and PVY^{NW}. Although both recombinant isolates cause necrosis on tobacco, PVY^{NTN} has the PVY^{N} serotype and PVY^{NW} has the PVY^{O} serotype. Recently, a new recombinant PVY isolate designated PVY^{NTN-NW} was reported by Chikh Ali *et al.* (2010) to occur in Syria. This novel isolate shares properties with the two already reported recombinant isolates PVY^{NTN} and PVY^{NW}.

Currently necrotic and recombinant strains are the most dominant PVY isolates in Europe and have largely replaced the ordinary strain population (Verbeek *et al.*, 2010, Karasev *et al.*, 2010). The potato tuber necrosis ring spot disease (PTNR) induced by infection with the PVY^{NTN} strain is a very important factor in decreasing crop yield and quality of infected tubers. Moreover, disease symptoms normally develop on tubers during storage. The increasing numbers of recombinant PVY isolates will require a more comprehensive way to classify them, especially as it has been reported that some isolates show properties outside the known categories. It has been reported recently that some PVY^{NW}, PVY^{N}, and PVY^{NTN} isolates do not induce necrosis in tobacco although they have the K/E amino acid motifs in their HC-Pro (Section 1.5.2.2.2) which is believed to be responsible for inducing such necrosis (Schubert *et al.*, 2007).

1.4.4. Indicator plants for differentiation and propagation of PVY

Strains of the PVY^{N} group are normally differentiated from the other strains by symptoms on *N. tabacum* cultivars White Burley, Samsun NN and Xanthi. The typical reaction to PVY^{N} strains is veinal necrosis, which is generally a unique biological characteristic of this group on tobacco. *Physalis floridana* is a typical indicator plant for PVY^{O} and PVY^{C}, which induce local and systemic necrosis in young plants and severe stunting. On the other hand, *P. floridana* develops mosaic symptoms after infection by PVY^{N}. Local lesions are induced on *Chenopodium amaranticolor* after infection with PVY^{O} and PVY^{C}, but not PVY^{N} (Barker *et al*., 2009).

Differentiation between PVY and PVA is not possible by electron microscopy and indicator plants or serological tests are normally used. Mixed infections of PVY and

PVX are very common in potato. To separate PVY from the mixture, inoculation to potato cultivars resistant to PVX, such as cv. Saco, is used. In contrast, for PVY elimination *D. stramonium*, which is completely immune to PVY, is used. For PVY purification *Nicotiana tabacum* cv Xanthi, cv White Burley, or cv. Samsun are routinely used, and virus is normally purified from leaves 3–4 weeks post inoculation (Kerlan, 2006).

1.5. Transmission of potato viruses by aphids

There are different mechanisms for virus transmission by insects which are highly specific and complex. The terminology used to describe such mechanisms has been changed several times (reviewed by Ng and Falk, 2006).

1.5.1. Virus – vector relationship

Plant viruses have been divided into two main groups according to their relation with their insect vectors. The term persistent is used to describe viruses that can persist in their insect vector for a long time, and non-persistent to describe viruses which do not survive for long inside their vectors. Sylvester (1956) first introduced the term semi-persistent to discriminate between the viruses which can be acquired and transmitted briefly by their vectors (non-persistent) and the viruses that require a longer time than the non-persistent viruses to be acquired and transmitted. Subsequently, people have used different terminologies to describe plant–virus–vector relationships. Circulative and non–circulative viruses are the most comprehensive terms adopted by Ng and Perry (2004). The circulative viruses were divided into circulative propagative viruses and circulative non-propagative viruses. The non-circulative virus does not enter the insect blood system, however; it remains attached to the receptors in the insect mouth parts, and viruses belonging to this category were divided into non-circulative semi-persistent viruses and non–circulative non-persistent viruses (for review see Ng and Falk, 2006).

In the circulative mode of virus transmission (reviewed by Sylvester, 1980; Gray and Gildow, 2003), the insect must feed on the phloem in order to acquire virus from the plant vascular system. The plant therefore must be a natural host for the insect vector in order to transmit these kinds of viruses. Aphids are unable to transmit persistent viruses immediately after feeding on the infected plant: a transit period inside the insect body is

required (hours to days depending on the virus). This period is known as the latent period and has a particular importance in the chemical control of these viruses as aphid vector can be targeted before it becomes cabable to transmit virus. PLRV is one of the most studied potato viruses with a circulative non-propagative mode of transmission and its spread is now efficiently controlled by insecticides.

The term non-circulative is split into non-circulative semi-persistent and non-circulative non-persistent. There are no economically important potato viruses that are transmitted by the non-circulative semi-persistent mode. The majority of potato viruses, including the most important viruses PVY and PVA, are non-circulative non-persistently transmitted. It has been shown experimentally that aphids can acquire the virus during very short probes (5 seconds to a few minutes) provided they have fasted for some time before acquisition. Similarly, aphids can inoculate virus within a very short time and they lose the ability to transmit virus after a maximum of a few hours (Bradley, 1959). For simplicity, the terms persistent, semi-persistent and non-persistent will be used throughout this thesis.

1.5.2. Mechanism of non-persistent virus transmission

1.5.2.1. Background

The non-persistent mode of virus transmission is a unique characteristic of aphids, which have piercing-sucking mouth parts (reviewed by Pirone and Harris, 1977; Pirone and Blanc, 1996; Ng and Falk, 2006). As mentioned above this kind of transmission is characterized by short acquisition and inoculation times. The virus can be acquired within very short probes. Unlike persistent viruses, acquisition of non-persistent viruses is normally decreased by increasing the acquisition period and enhanced by starving aphids for some time before they acquire the virus (Watson and Roberts, 1939). Once virus is acquired, the aphid vector can make up to 10 infectious probes (Hashiba and Misawa, 1969a). This means that one winged viruliferous aphid can spread a virus infection to 10 healthy plants if it performs a single probe on each one. Alternatively, many infection sites could be initiated if the aphid continued to probe on the same plant. In both situations, this probing behaviour will be harmful by spreading infection to many new healthy plants and increasing the probability of infection. Moreover, the majority of the aphid vectors of non-persistent transmitted viruses are non-colonizing

species, which means that the aphid will be likely to perform single probes on many plants while searching for a suitable host.

It was first thought that non-persistent transmission was a purely mechanical process, and the needle-like hypothesis was proposed by Doolittle and Walker (1928). This hypothesis was challenged as early as the 1930s when Watson (1936) indicated that non-persistent transmission is a more complicated process and hypothesised that a vector substance is involved in the transmission process (Watson and Roberts, 1939). Many other studies criticized the needle-like hypothesis and suggested more complicated transmission mechanisms.

The ingestion-egestion theory (Harris, 1977) proposed that non-persistent virus was acquired by ingestion and carried at the lining of the alimentary canal until inoculated by saliva egestion. This theory was extensively investigated but its importance declined after the emergence of new hypotheses, namely the helper virus strategy (Pirone and Blanc, 1996)**,** and the conformational change hypothesis (Salomon and Bernardi, 1995). These hypotheses propose roles for virus-encoded helper component and the coat protein.

1.5.2.2. Helper component (HC-Pro)

1.5.2.2.1. Discovery and properties

The concept of helper component (HC-Pro) was first introduced by Govier and Kassanis (1974 a,b) without exact characterization of its nature. They speculated that this helper component could be either a virus-coded or virus-induced sap component or a virus protein subunit that assisted aphid transmission. Studies on partially purified infected leaf tissues containing HC-Pro revealed that this helper factor is of a protein nature distinct from PVY coat protein or inclusion protein (Govier *et al.*, 1977). HC-Pro was purified and characterized (Govier *et al.*, 1977; Thornbury *et al*., 1985), and it was concluded that it is a virus-encoded non-structural protein of molecular mass between 50 and 58 kDa depending on the potyvirus (Thornbury *et al*., 1985). However, the biologically active HC-Pro was between 100 and 150 kDa, which indicated a polymeric structure. Further structural characterization studies confirmed that HC-Pro is a dimer in

solution (Plisson *et al*., 2003). HC-Pro mediates the transmission of non-persistent viruses by a mechanism not fully understood.

In addition, HC-Pro is a multifunctional protein (Reviewed by Maia and Bernardi, 1996; Syller, 2005) which has been reported to have a role in many other functions, including proteinase activity (Carrington *et al.*, 1990), virus movement (Rojas *et al.*, 1997; Saenz *et al.*, 2002), virus synergy (Pruss *et al*., 1997), and suppression of gene silencing (Kasschau and Carrington, 1998). Furthermore, HC-Pro is suspected to be responsible for the necrotic symptoms induced by some PVY isolates on different hosts (Tribodet *et al*., 2005, Rolland *et al.*, 2009). HC-Pro is able to interact with itself (Urcuqui-Inchima *et al*., 2001) and with some other viral gene products, including coat protein (Atreya *et al*., 1990; Roudet-Tavert *et al*., 2002), P1 protein (Merits *et al*., 1999), and VPg (Yambao *et al*., 2003). Moreover, HC-Pro is reported to interact with many host proteins (Guo *et al*., 2003).

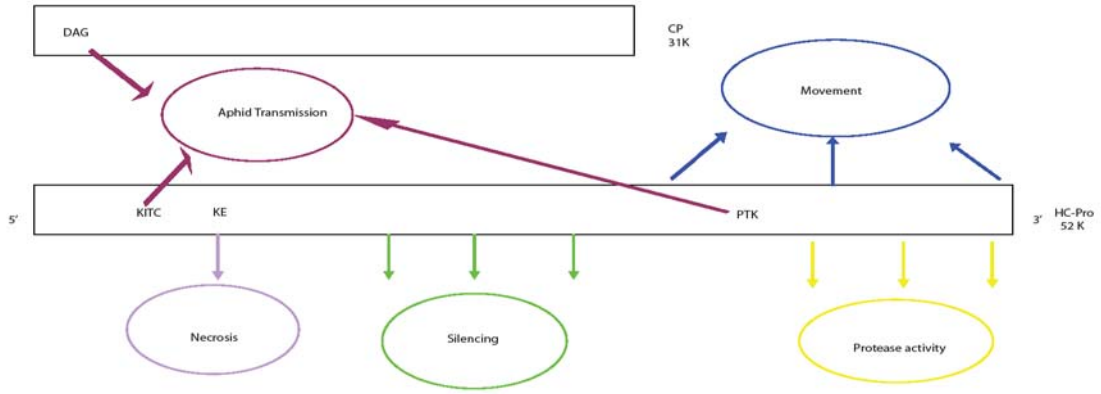


Figure 1.5. Schematic representation of PVY HC-Pro showing the different molecular determinants and their biological roles.

CP: Coat protein, DAG: motif at the N-terminus of CP involved in aphid transmission, KITC: higily conserved region at the N-terminus of the HC-Pro involved in transmission by aphids, PTK: conserved motif in the HC-Pro interact with virus particles, KE, amino acides at specific location at the N-terminus of HC-Pro reported to be responsible for inducing necrosis.

1.5.2.2.2. HC-Pro function in virus transmission

It was speculated at an early stage of HC-Pro discovery that this protein controls aphid transmission by regulating virus uptake, binding virus to receptor sites in the aphid food canal to be eluted later, or protecting virus particles in the alimentary tract (Govier and

Kassanis, 1974; Lobez-Abella *et al*., 1981; Raccah and Pirone, 1984). Naturally occurring aphid non-transmissible isolates of PVY (Thornbury *et al*, 1990; Canto *et al*., 1995) produce transmission-defective HC-Pro due to the substitution of the amino acid lysine (K) with glutamic acid (E) in a conserved motif (KITC) at the N-terminus (Fig. 1.5) (Thornbury *et al.*, 1990). Similarly, TVMV HC-Pro lost its function when the corresponding positively charged lysine was substituted with glutamic acid (Atreya *et al*., 1992; Atreya and Pirone, 1993). Moreover, Blanc *et al.* (1998) found that the K to E mutation in the KITC motif of an aphid non-transmissible isolate of PVY (PVY^C) and in the mutant TEV isolate resulted in loss of aphid transmissibility. In addition, they found that this K to E mutation does not affect binding of HC-Pro to virus capsid protein but does prevent HC-Pro binding to aphid stylet. Additionally, the N-terminus of the HC-Pro was confirmed to be involved with other functions including virulence, genome multiplication, and virus accumulation (Atreya and Pirone, 1992; Atreya *et al*., 1992; Kasschau and Carrington, 1998).

Another conserved area containing proline-threonine-lysine (PTK) in the C-terminal part of HC-Pro (Fig. 1.5) was identified (Granier *et al*., 1993) and proved to be involved in binding to virions (Peng *et al*., 1998). The C-terminal region is also involved in the cell to cell movement and protease activity functions. The central area between the N and C terminal of HC-Pro was also reported to mediate long-distance movement and virus replication (Cronin *et al*., 1995; Kasschau *et al.*, 1997; Klein *et al*., 1994). Furthermore, this central region of the HC-Pro molecule is responsible for suppression of gene silencing in the host's defence response. It was reported that HC-Pro belonging to one potyvirus may assist transmission of another potyvirus; however, this is not a general rule (Harrison and Robinson, 1988; Lopez-Moya *et al.*, 1995). All the reports of HC-Pro mediated transmission of potyviruses are for aphid vectors except for the semi-persistent WSMV by the eriophyid mite *Aceria tosichella* (Stenger *et al*., 2005).

1.5.2.3. Coat protein (CP)

Molecular characterization of the coat protein of PVY started in the 1970s (Makkouk and Gumpf, 1975, 1976). Regarding aphid transmission, at least two viral proteins have been confirmed to be involved in the transmission of non-persistent viruses, the HC-Pro and the coat protein (CP) (Pirone, 1991; Pirone and Blanc, 1996). Shukla and Ward (1989) divided the CP into three main regions: an N-terminal surface-exposed region of

30 amino acids which is different in length and sequence in different potyviruses, a core region of 218 amino acids which is highly conserved among potyviruses, and a region of 19 amino acids which is surface-located at the C-terminus. Harrison and Robinson (1988) predicted that a highly conserved DAG motif located on the surface-exposed N-terminus of potyvirus coat protein is involved in aphid transmission (Fig. 1.5). The DAG triplet was reported to exist in all PVY aphid transmissible isolates (Shukla *et al.*, 1991; Galon *et al.*, 1992). However, this is not consistent between different potyviruses. While some potyviruses like PVY contain one DAG triplet, others like TEV contain two consecutive DAG motifs separated by the amino acid alanine. In contrast, some potyviruses such as PSbMV have a DAS motif. Mutation of the DAG motif to DAS in TVMV (Lopez- Moya *et al*., 1999) or to DAA or DAL in TVMV (Atreya *et al*., 1995; Lopez-Moya *et al*., 1999) decreased aphid transmission significantly, confirming that this motif plays an important role in aphid transmission but the exact motif depends on the potyvirus. For example, PVY with the motif DAGE was aphid transmissible but the same motif did not reflect aphid transmissibility in TVMV (Shukla *et al.*, 1994). In addition, although the DAG region is highly conserved between aphid transmissible potyviruses (Atreya *et al.,* 1995), there are some exceptions where it is found in viruses outside this category, for example the British isolate of PAMV, *Potexvirus* (Baulcombe *et al*., 1993). Other variants include a DAA motif in PeMoV (Flasinsky and Cassidy, 1998) and DDG in PVY (Rosner and Raccah, 1988). Moreover, introducing a DAG motif in the N-terminus of the coat protein of DAS or DAL mutants of TVMV, to become DAS-DAG or DAL-DAG respectively, was not sufficient to restore aphid transmissibility. This finding suggests that the context in which the DAG motif exists plays a role in aphid transmission (Lopez-Moya *et al*., 1999). It seems likely that the third position in the DAG triplet is the most critical determinant for aphid transmission, and substituting the glycine residue in this position with glutamate, serine, asparagine, asparatate, or leucine always disabled aphid transmissibility (Atreya *et al.*, 1991). Furthermore, aphid transmissibility is adversely affected by amino acid substitutions in the region directly after the DAG motif (Atreya *et al.*, 1991; Lopez-Moya *et al*., 1999). Mutagenesis of this region of TVMV coat protein resulted in loss of binding between CP and HC-Pro (Blanc *et al*., 1997). In addition to binding to HC-Pro, the DAG motif was reported to be involved in potyvirus movement (Lopez-Moya and Pirone, 1998).

1.5.2.4. Hypotheses proposed for molecular mechanisms of potyvirus transmission

There are two different models for non-persistent virus transmission, termed capsid strategy and helper strategy (reviewed by Pirone and Blanc, 1996). Direct interaction between the virion and the vector is the basis of the capsid strategy, which is the case for transmission of CMV, whereas a helper factor is required to mediate transmission in the helper strategy (Pirone and Blanc, 1996).

The bridge hypothesis, introduced by Pirone and Blanc (1996), is a refinement of the early bridging concept suggested by Govier and Kassanis (1974), who suggested that HC-Pro works as a bifunctional molecule by joining the virus particles with putative virus receptors on the aphid's stylet. This proposal followed their observations which demonstrated that aphid non-transmissible viruses (PVC, PAMV) were transmitted if aphids were fed first on a PVY infected source, whereas the transmission was inhibited if the feeding sequence was reversed (Kassanis and Govier, 1971a,b). Much evidence supports the bridge hypothesis (Taylor and Robertson, 1974; Pirone and Thornbury, 1984; Berger and Pirone, 1986; Ammar *et al.*, 1994; Wang *et al.*, 1996; Blanc *et al.*, 1997, 1998; Uzest *et al.*, 2007).

The conformational change hypothesis suggests the indirect involvement of HC-Pro through causing conformational changes in the N-terminal part of the CP that eventually lead to direct attachment between the virus particles and the aphid's receptors (Salomon and Bernardi, 1995). However, it was reported that intitation of the conformational change on the coat protein may be related to the DAG motif which highly conseved among potyviruses on the N-terminus of the coat protein (Jayaram, 1998).

Although most lines of evidence support the bridge hypothesis, the exact mechanism behind non-persistent virus transmission is still unclear and further work is needed to investigate both hypotheses.

1.6. Aphid species transmitting potato viruses

There are many aphid species which colonize potato crops. Colonizing aphids damage potato by depleting nutrients through feeding as well as by transmitting plant viruses. In addition, there are many aphid species that do not colonize potato but can transmit viruses. Table 1.2 presents a list of the aphid vectors that transmit PVY together with their reported efficiencies, and other potato viruses they transmit. The most important vector is *M. persicae* (Figs. 1.6, 1.7).

Aphid species	Reference	Virus isolate	Transmission efficiency*	Other potato viruses transmitted
Acyrthosiphon pisum	Van Hoof (1980)	PVY^N	14%	PLRV, PVS
A. primulae	Ragsdale *et al*. (2001)	PVY^N	15%	NA
Aphis citricola	Raccah *et al.* (1985)	PVY in pepper	6.2%	CMV
Aphis craccivora	Fereres *et al*. (1993)	PVY in pepper	4%	NA
Aphis fabae	Van Hoof (1980)	PVY^N $PVY^{O'}$	24%	PLRV TEV
Aphis frangulae	Verbeek *et al*. (2008)	PVY	42%	PVA
Aphis glycines	Davis *et al.* (2005); Davis and Radcliffe (2008)	PVY^O, PVY^N, PVP^{NTN}	14% to 75% depending on strain	CMV, AMV, PLRV
Aphis gossypi	Raccah *et al.* (1985)	PVY^O	31%	PLRV, PVA
Aphis nasturtii	Sigvald (1984)	PVY^O	7.1%	PLRV, PVA, PVS
Aphis pomi	Van Hoof (1980)	PVY^N	9%	CMV
	Harrington and Gibson (1989)	PVY^O	2%	
Aphis spp.	Harrington *et al.* (1986)	PVY^O, PVY^N	6%	CMV
Aphis sambuci	Harrington and Gibson (1989)	PVY^O	4.3%	NA
	De Bokx and Piron (1990)	PVY^N	12%	
Aulacorthum solani	Van Hoof (1980)	PVY^N PVY^O	5%	PLRV
Brachycaudus helechrysi	Piron (1986)	PVY^N	12.5%	PVA

Brachycaudus helechrysi				
	Harrington *et al.*(1986)	PVY^{O} PVY^{N},	7.2% PVY^{O} 0.9% PVY^{N}	NA
	Harrington and Gibson, (1989)	PVY^{N}	5.9%	
Brachycaudus spp.	Piron , (1986)	PVY^{N}	14.7%	NA
B. cardui *B. amygdalinus* *B. rumexicolens*	Perez *et al.* (1995)	PVY pepper	NA	NA
Capitophorus hippophoes	Van Hoof, (1980)	PVY^{N}	3%	NA
Capitophorus eleagni	Halbert *et al*. (2003)	PVY^{O}	2%	NA
Capitophorus spp.	Perez *et al.* (1995)	PVY pepper	NA	NA
Cavariella aegopodii	Piron , (1986)	PVY^{N}	0.4%	
	Harrington and Gibson (1989)	PVY	0.2%	NA
Cavariella pastinaca	Salazar (1996)	PVY^{N}	NA	NA
Cryptomyzus ballotae	Harrington *et al.* (1986)	PVY	100%	NA
Cryptomyzus galeopsidis	Piron (1986)	PVY^{N}	17.4%	NA
Cryptomyzus ribis	Piron (1986)	PVY^{N}	15.4%	NA
Diuraphis noxia	Perez *et al.* (1995)	PVY-Pepper	4-7%	NA
Dysaphis spp	Harrington and Gibson (1989)	PVY^{O}	1.8%	NA
Drepanosiphum platanoidis	Powell *et al*. (1995)	PVY^{N}	0.6%	NA
Hyadaphis foeniculi	Piron (1986)	PVY^{N}	14.7%	NA
Hyalopterus pruni	Piron (1986)	PVY^{N}	13.9%	NA

Hyperomyzus lactucae	Piron (1986)	PVY^{N}	17.4%	NA
Macrosiphum euphorbiae	Van Hoof (1980)	PVY^{N}	29%	PLRV , TEV
Metopolophium dirhodum	Van Hoof (1980)	PVY^{N}	3%	NA
Metopolophium albidum	Van Hoof, (1980)	PVY^{N}	11%	NA
Metopolophium festucae	Harrington *et al.* (1986)	PVY^{O}	0.5%	NA
Myzaphis rosarum	Harrington *et al*. (1986)	PVY^{O}	10%	NA
Neomyzus circumflexus	Salazar (1996)	PVY^{O} , PVY^{N}	NA	PLRV
Myzus ascalonicus	Verbeek *et al*. (2010)	PVY^{N} ,PVY^{NTN}, PVY^{NW},	NA	PLRV
Myzus cerasi	Harrington and Gibson (1989)	$PVY^{O,}$ PVY^{N}	3.2%	NA
Myzus certus	Van Hoof (1980)	PVY^{N}	71%	NA
Myzus ligustri	Harrington and Gibson (1989)	PVY^{O}	50%	NA
Myzus myosotidis	Harrington *et al.* (1986)	PVY^{O}	100%	NA
M. persice nicotianae	Kanavaki *et al.* (2006)	PVY^{N}	15.3%	PLRV
	Halbert *et al.* (1995)	PVY	NA	
Myzus persicae	Van Hoof (1980)	PVY^{N}	50%	PLRV, PVA, PVS, TEV
	Harrington and Gibson (1989)	PVY^{O} , PVY^{N}	8.4%	
	Piron (1986)	PVY^{N}	71.1%	
Phorodon humuli	Van Hoof, (1980)	PVY^{N}	35%	PLRV
Rhopalosiphum insertum	Van Hoof, (1980)	PVY^{N}	50%	NA

Rhopalosiphum maidis	Helbert *et al*. (2003)	PVY^{O}	1.5%	NA
Rhopalosiphum padi	Kostiw (1979)	PVY^{O}	2.7%	NA
	Van Hoof (1980)	PVY^{N}	2%	
	Piron (1986)	PVY^{N}	11.5%	
	Harrington and Gibson (1989)	PVY^{O}	2.4%	
Rhopalosiphum pseudobrassicae	Ragsdale *et al*. (2001)	PVY	N/A	NA
Schizaphis graminum	Perez *et al.* (1995)	PVY pepper	NA	NA
Sitobion avenae	Harrington and Gibson (1989)	PVY^{O}	0.1%	NA
	Piron (1986)	PVY^{N}	1.8%	
Sitobion fragariae	Harrington and Gibson (1989)	PVY^{O}	0.5%	NA
	Piron (1986)	PVY^{N}	10.1%	
Sitobion graminum	Verbeek *et al*. (2010)	PVY^{NTN} PVY^{NW},	NA	NA
Staphylea tulipaellus	Salazar (1996)	PVY^{N}	NA	PLRV
Therioaphis trifolii *Therioaphis* sp.	Perez *et al.* (1995)	PVY pepper	NA	NA
Uroleucon spp.	Harrington and Gibson (1989)	PVY^{O}	0.5%	NA
	Piron (1986)	PVY^{N}	8.3%	

Table 1.2. Aphid species which are reported to transmit PVY.

* Transmission efficiency was determined differently depending on the methods used by the author and the variable experimental conditions.

Figure 1.6. *M. persicae* (winged adult), the main vector aphid species of PVY, SCRI.

Figure 1.7. Colony of apterous *M. persicae* red colour lineage (SCRI).

1.7. Controlling the spread of aphid transmitted viruses on potato crops

Virus incidence in seed tubers is controlled by seed classification schemes, which are supported by monitoring of aphid activity and by using sensitive virus detection techniques. A second, long-term alternative is to breed virus or aphid resistant potato cultivars which can limit virus multiplication and spread. The third option is to control aphids using insecticides, but this is not effective for controlling the majority of non-persistently transmitted viruses.

1.7.1. Seed classification schemes

Potatoes are vegetatively propagated and thus susceptible to build-up of disease when grown over successive generations. Seed classification schemes are employed to control the spread of disease in such vegetatively propagated crops. There are two main elements to these schemes. 1. Inspections of growing crops, which is the main element used in the UK. This depends on regular field inspections (normally two per crop) by qualified inspectors to check plants for disease symptoms and trueness-to-type. 2. Post harvesting testing (PHT), which is more expensive but routinely used in the Netherlands and in France.

1.7.1.1. British seed classification schemes

70% of British potato seeds are produced in Scotland, and Science and Advice for Scottish Agriculture (SASA) is responsible for advising on all the seed certification activities. The remaining 30% of British potato seeds are produced in England, and the Food and Environment Research Agency (FERA) is responsible for this programme. Seed classification programmes in the UK are generally based on visual inspection of infected plants, which are carried out at different occasions during the growing season by experienced inspectors, and the crop is assigned a score reflecting its virus incidence before harvesting. Field inspection has some advantages over PHT, as a wide range of diseases can be inspected at the same time as well as trueness to type. Field inspection is normally repeated at least twice, which allows for development of disease symptoms.

Roguing, known as negative selection, is a disease control method used to identify and eliminate diseased and undesired plants from the crop according to their visual symptoms (Cortbaoui, 1984). Roguing is a common practice in potato crops to reduce virus spread by removing virus sources (Cadman and Chambers, 1960). Radcliffe (2006) reported that roguing is effective when the infection by virus is at low levels (<1-2%), and if the field is small to enable inspection of all plants in the field. Roguing virus infected plants is normally performed before crop flowering, in order to be able to identify atypical plants because cultivars are more easily identified at the flowering stage, and during cloudy weather, as mosaic symptoms are more recognizable under uniform light and direct sunlight prevents identification of changes in plant leaf color (Cortbaoui, 1984). It is recommended to perform roguing before the closure of the plant canopy, and not late in the season as the symptoms become less visible (Woodford and Gordon, 1990).

1.7.1.2. Aphid monitoring and forecasting

In addition to roguing, controlling virus spread on potato is enhanced by aphid monitoring programmes. Aphid forecasting to control virus spread in potato crops started with monitoring incidence of the most efficient vector, *M. persicae*. However, as information about the role of non-colonizing aphid species in transmission of non-persistent viruses emerged, the importance of monitoring a greater number of aphid species has increased. Aphid monitoring can be performed on a regional basis using suction traps (Pickup and Brewer, 1994). Aphid forecasting networks were established as early as 1965. A network was initiated in the UK by installing one 12.2 m suction trap, and nowadays the network has expanded to comprise 73 traps in 19 European countries (Radcliffe, 2006). Rothamsted Research in collaboration with SASA runs a forecasting system based on a network of 16 12.2 m suction traps (Fig. 1.8) in different sites in the UK. Information about aphid forecasting can be found on these two websites: http://www.rothamsted.ac.uk/insect-survey/ and http://www.sasa.gov.uk/seed_potatoes/aphids/bulletins/index.cfm.
Aphid activity on crops can not be monitored using suction traps, and different kinds of traps are used for this pupose.

Figure 1.8. 12.2 m Rothamsted type suction trap, used to monitor aphids in the UK (SCRI).

The most widely commonly used type is the yellow water trap (Fig. 1.9). FERA is currently running an aphid monitoring and forecasting programme in England. Information is obtained from about 100 water traps in the main potato production areas. Information about aphid flights is presented on this website: http://aphmon.fera.defra.gov.uk, or on the potato council website: http://www.potato.org.uk/aphids. Information at the website is updated twice a day, and it can provide up-to-date information for seed potato producers about aphid flights. In

addition, farmers can sign up to receive weekly information, and email alerts when the number of aphids requires chemical spray in a certain region.

Figure 1.9. Yellow water trap used to monitor aphids in potato crops in the UK (SCRI).

1.7.1.3. Interpreting aphid monitoring data

Capturing aphids does not provide a direct estimate of their potential risk in virus transmission. Raw data obtained from traps are interpreted in different ways. Relative efficiency factor (REF) values are commonly applied to estimate vector pressure; the bait plant system is another method which is used to interpret aphid efficiency in virus transmission.

1.7.1.3.1. Relative efficiency factor (REF)

REF is a score given to a particular vector to represent its efficacy in virus transmission compared with the most efficient vector. Van Harten (1983) suggested assigning a value of 1 to the most efficient vector of PVY (*M. persicae*), and values were assigned for

other vectors depending on their efficiency in PVY transmission compared with *M. persicae*. This method emerged in the 1980s, and was used commonly in epidemiological studies of PVY in order to estimate the best timing of chemical application or haulm destruction according to the abundance of the virus vectors caught in traps (Van Harten, 1983; Sigvald, 1987). Nowadays REFs are available for the most efficient PVY vectors (Table 1.3). However, there is some variation in these values (De Bokx and Piron, 1990). For example, the cereal aphid *M. dirhodum* was assigned the value of 0.3 although many reports in the literature confirm that the efficiency of this vector is only about 0.1-1%, which suggests the need to revise the assigned values. In addition, these values were calculated according to the efficiency of aphid vectors in transmitting the PVY^O and PVY^N stains only. The recent emergence of new recombinant isolates of PVY will necessitate some revision (Verbeek *et al*., 2010).

Aphid species	**Common name**	**REF FERA**	**REF Netherlands**
Myzus persicae	Peach-Potato Aphid	1	1
****Acyrthosiphon pisum***	**Pea Aphid**	**0.7**	**0.05**
Myzus certus	Black Peach aphid	NA	0.44
Aphis frangulae	NA	NA	0.42
Aphis nasturtii	Buckthorn-Potato Aphid	0.4	0.42
Rhopalosiphum padi	Bird Cherry-Oat Aphid	0.4	0.03
****Metopolophium dirhodum***	**Rose-Grain Aphid**	**0.3**	**0.01**
Brachycaudus helichrysi	Leaf-Curling Plum Aphid	0.21	0.01
Aulacorthum solani	Glasshouse and Potato Aphid	0.2	NA
Myzus ascolonicus	Shallot Aphid	0.2	0 - 0.01
Macrosiphum euphorbiae	Potato Aphid	0.2	0.1
Myzus ornatus	Violet Aphid	0.2	NA
Rhopalosiphoninus latysiphon	Bulb and Potato Aphid	0.2	NA
Hyperomyzus lactucae	Currant-Sowthistle Aphid	0.16	NA
Phordon humuli	Damson-hop aphid	NA	0.15
Aphis fabae	Black-Bean Aphid	0.1	0.1
Brevicoryne brassicae	Cabbage Aphid	0.01	NA
Sitobion avenae	Grain Aphid	0.01	0 - 0.05

Table 1.3. REF values for PVY aphid vectors reflecting difference in values between British and Dutch systems for some aphid vectors.

*Aphid species which show big difference were presented in bold.

1.7.1.3.2. How REF is assigned

REFs have been assigned to PVY vectors based on different experimental criteria, which give different values (Table 1.3). In the UK, Harrington *et al.* (1986) used indicator tobacco plants to assess if live-captured alate aphids were viruliferous or not. In the Netherlands, REFs were normally assigned based on laboratory and greenhouse experiments on apterous aphid transmission to indicator plants (Van Hoof, 1980). Live-caught winged aphids were released in cages containing infected and healthy potato plants, and transmission efficiency was estimated based on transmission from potato to potato. Recently, Verbeek *et al.* (2010) in the Netherlands introduced another system to assess the REF for the new recombinant PVY isolates. In their new system they used laboratory maintained aphids to acquire PVY from potato and transmit to *P. floridana* indicator plants. The values determined in this system and the REF values determined in 1980s were comparable for most PVY vectors. However, the results indicated that efficiency of some aphid species were underestimated (Verbeek *et al*., 2010).

1.7.1.3.3. Bait plant system

Bait plants are used in monitoring aphid activity on potato crops. Bait plants such as tobacco or potato plants are distributed in the field for a certain time and then transferred to a glasshouse to monitor symptom development. They are normally used to identify the time of virus spread in the field, determine the aphid vector species pressure, and to reveal the role of alate and apterous aphids in virus transmission (Peters, 1987). The bait plant system in the Netherlands was based on distribution of healthy tobacco at weekly intervals and assessing the effect of alate aphids carrying infection from potato virus sources distributed at the field borders (Van Hoof, 1977). Such tests revealed that there are aphid species other than *M. persicae* participating in the PVY transmission process (Van Hoof, 1977; Ryden *et al.,* 1983).

Peters (1987) reported that the bait plant method is of limited use in evaluating the magnitude of virus transmission in the field because of the potential higher transmission to these plants compared with the crop plants. Moreover, there are other factors which affect the results obtained from the bait plant method, including the distance of the bait plants from the crop, the virus source used, physical properties of the bait (colour and

size), and the location of the bait plants in the field, in particular proximity to the field borders.

1.7.1.4. Aphid thresholds

Control strategies should be started after the number of captured winged aphids reaches a certain threshold which is in the range of 20-100 aphids/100 leaves (Radcliffe and Ragsdale, 2002). However, advice differs depending on factors such as the kind of potato crop (ware or seed), the kind of virus (persistent or non-persistent), the virus inoculum source (e.g. virus-free seed or virus-contaminated seed), and the geographical isolation of the potato production area. In the UK, for example, it is recommended to start chemical applications in seed crops when aphids start to appear in the region, and to repeat the treatment every 7-10 days. On ware potato crops, chemical treatment is recommended at the start of aphid activity if the ware crops are in a close proximity to seed crops. But if ware crops are isolated then chemical treatment should be started only when the number of aphids exceeds 5-6 winged aphids/plant (Pickup and Evans, 2008).

In Minnesota and North Dakota, USA, and in Canada, they recommend starting chemical applications when the number of aphids exceeds the 20-100 aphids/100 leaves, but this threshold should be decreased to 1-10 aphids per 100 leaves when potato cultivars are susceptible to net necrosis caused by PLRV or if the crop is grown for seed production (Mowry, 2001). In the Netherlands, capturing more than two *M. persicae* in a yellow water trap was the threshold to start haulm destruction (Van Harten, 1983). PVY is more dangerous than PLRV in seed potato production in the Netherlands (De Bokx and Piron, 1990). The importance of PVY was particularly noticeable after invasion of the new PVY^N isolates, which induce very mild symptoms that are difficult to recognize during roguing (Van Harten, 1983). Currently haulm destruction dates in the Netherlands are based upon aphid monitoring of the 11 most efficient aphid species in PVY transmission (Verbeek *et al*., 2010). In New Zealand, van Toor *et al.* (2009) reported that the threshold of 10 winged aphids/150 potato leaves is the start point for chemical control of aphid vectors to control aphid-borne viruses in potato fields.

1.7.1.5. Post-harvest testing/ Enzyme-linked immunosorbent assay (ELISA)

Post-harvest testing is widely applied in the Netherlands and some European countries and involves ELISA testing samples of all lots of potato seed tubers. It is also used occasionally in some countries like Canada and Northern USA where inspection of growing crops is less reliable. In the post-harvest test, a sample of several hundred tubers is required for basic seeds, and one hundred tubers are enough for lower grade certified seeds (Oosterveld, 1987). In the UK, the scheme is used only to test cv. Estima, which induces indistinguishable symptoms when infected with PVA (E. Anderson, Scottish Agronomy, personal communication 2009).
If the seed lot is given a score higher than a particular threshold (different for each virus), then the lot will be downgraded or even rejected. In the latter case, the potato lot will be used for human consumption or for starch production. Post-harvest testing is essential to detect late virus infection, which normally remains symptomless.

ELISA is routinely used for the PHT. In the Netherlands for example, about 1.8 million ELISA tests are conducted annually for detection of potato viruses during the seed certification programme (Bergervoet *et al.*, 2008). Nucleic-acid-based techniques such as RT-PCR are not yet suited to such high throughput, and so cannot replace ELISA when screening many samples. In addition, ELISA is a valuable tool during breeding programmes for virus resistance, when screening a large number of samples for the presence of the virus is needed.

1.7.2. Breeding for virus and vector resistance

Developing disease-resistant varieties by conventional breeding is difficult because the process is extremely long in potato. Moreover, virus resistance was not previously considered a priority in some breeding programmes, for example the breeding programmes in the UK. Thus most commercial potato cultivars lack virus resistance genes. The success of conventional breeding strategies is based on the fact that dominant monogenic resistance (R) genes confer good protection against plant viruses (Fraser, 1990). However, this approach is limited because it relies on the allele specific genetic interaction between a host R-gene and a pathogen avirulence (*avr*) gene. There are many review papers that discuss virus resistance in potato (Valkonen, 1994; Valkonen *et al.*, 1996; Solomon-Blackburn and Barker, 2001a,b; Gebhardt and

Valkonen, 2001). Resistance to virus infection was reported to exist in some potato species such as *S. phureja* and *S. brevidens* (Gibson *et al.*, 1990). Valkonen (1994) divided natural resistance genes in potato towards viruses into R type, which confer complete immunity or extreme resistance (ER), and N type, which confer a hypersensitive response (HR). In the ER resistance, plants stay symptomless or develop limited necrosis when inoculated with virus. On the other hand, the HR infected plant develops local lesions or systemic necrosis. The ER type resistance can sometimes confer broad spectrum resistance to more than one virus. For example, the Rysto gene confers resistance to PVY, PVA and PVV. In contrast, the HR type resistance is specific and is liable to breakdown in some environmental conditions (Solomon-Blackburn and Barker, 2001, b).

The mechanism by which R and *avr* gene products trigger signal transduction that leads to a hypersensitive response and the arrest of pathogen invasion is still an open question. There is currently a list of resistance genes for the main viruses infecting potato with their locations (Solomon-Blackburn and Barker, 2001a).

Given the increase in the incidence of aphid-borne viruses, I believe that genes conferring resistance to aphid attack should also be incorporated in breeding programmes. However, controlling virus spread through breeding for vector resistance is more likely to provide partial control which can be useful in integrated pest management if combined with other control measures, and vector resistance will be less effective in controlling non-persistent viruses compared with the semi-persistent and persistently transmitted viruses (Mutschler and Wintermante, 2006).There are many wild potato lines which have genes for resistance to aphids. For example, 36 potato species were identified which have high resistance towards *M. persicae* and 24 species which have resistance to *M. euphorbiae* (Ragsdale *et al.*,, 2001). Only limited efforts have been made to introduce these resistance genes into commercial potato cultivars (Flanders *et al*., 1999). Effects of glandular trichomes on non-persistent virus transmission by *M. persicae* were reported in some wild potato species (Gunenc and Gibson, 1980; Lapointe *et al*., 1987). However, recent investigations have focused on resistance to phloem feeding by aphids (Wilson and Jones, 1992; Alvarez *et al*., 2006; Le Roux *et al*., 2010), which has little impact on the transmission of non-persistent viruses. Alvarez *et al*. (2006) reported that combining aphid resistance at the early stage of crop growth with mature plant resistance (Beemster, 1987) to virus infection at the late stage can be very useful in controlling virus spread by aphids in potato crops. In

addition to natural resistance to aphids, Moran and Thompson (2001) demonstrated that phloem-feeding by the aphid *M. persicae* induced expression of genes in *Arabidopsis thaliana* associated with responses to pathogens as well as a gene involved in the wounding response pathway. Similar genes may be found to be expressed after virus infection or insect infestation on potato, which could be useful if exploited in virus and aphid resistance programmes. For example, different genes were induced in potato after mechanical inoculation of the PVY^{NTN} strain (Pompe-Novak *et al*., 2005; Baebler *et al*., 2009).

As conventional potato breeding programmes are time consuming, "pathogen-derived resistance" has been investigated as a way to create virus resistant cultivars by introducing into plants viral components. These can be used as a plant defence against many viruses. Pathogen-derived resistance (Hull and Davies, 1992; Carr *et al*., 1992; Barker *et al*., 1992; Barker *et al*., 1998; Thomas *et al*., 2000) has been successfully applied to offer protection against plant viruses. For example, potato plants expressing the coat protein gene of PLRV have been developed (Barker *et al.,* 1992), and several transgenic potato lines tested under greenhouse conditions show high levels of resistance to aphid inoculated PLRV. During infection, the host plant is often able to destroy the viral RNAs naturally by means of a gene silencing system (English *et al*., 1996; Guo and García, 1997; Baulcombe, 1999). On the other hand, plant viruses can overcome this defense by encoding proteins responsible for suppressing this natural gene silencing (Kasschau and Carrington, 1998; Baulcombe, 2002; Simón-Mateo *et al.*, 2003; Chen *et al*., 2004; Qu and Morris, 2005). Gene silencing through genetic modification can be exploited to inhibit viral gene function, and fragments of viral RNA can be used to induce this mechanism in the plant (Ratcliff *et al*., 1997; Waterhouse *et al*., 1999). For example, Rovere *et al.* (2001) produced plants resistant to PLRV by introduction of the replicase coding sequence into potatoes. In addition, a successful silencing-based resistance to PVY was reported by Barker and McGeachy (2003). However, such genetic modification of crop plants (GM crops) has met with strong consumer resistance (Solomon-Blackburn and Barker, 2001a).

1.7.3. Chemical strategies for controlling potato viruses

Methods used to target aphid vectors of potato viruses are divided into chemical methods and non-chemical methods. Chemical control of potato viruses started before the vectors responsible for virus transmission were known (Broadbent, 1957). Subsequent reviews have dealt with this topic (Perring *et al.,* 1999; Robert *et al.*, 2000; Radcliffe and Ragsdale, 2002). Chemical methods used in controlling potato virus spread belong to two main groups: mineral oils and synthetic insecticides.

1.7.3.1. Mineral oils

The earliest report about inhibition of non-persistent viruses by oil came from Bradley *et al*. (1962); there have been many subsequent reviews (Vanderveken, 1977; Loebenstein and Raccah, 1980; Raccah *et al*., 1980; Simons and Zitter, 1980; Sharma and Varma, 1982; Simons, 1982; Raccah, 1986). The clearest conclusion that can be drawn from all of this information is that mineral oils do protect crops from non-persistent viruses but have no effect on the spread of persistent viruses. Virus control by mineral oils requires very frequent application because of the contact mode of action. However, plant phytotoxicity and yield loss were reported with oil concentrations of more than 1-2%. In order to be effective paraffin oils should have certain characteristics, the most important being a viscosity range of 66-150 SUS (Saybolt Universal Seconds; De Wijs *et al.*, 1979). In addition, the application frequency should provide continuous coverage to new plant growth. In some reports a few applications (3-8) during the growing season were enough to offer reasonable protection (Shands, 1977; Martin-Lopez *et al,* 2006), whereas others found that applications at weekly intervals were required (Boiteau *et al.*, 2009; Groves *et al*., 2009).

1.7.3.1.1. Mode of action of mineral oil in inhibiting virus transmission

Considerable work has been carried out to investigate the mode of action of oils in inhibiting virus transmission and many hypotheses have been proposed (Bradley *et al.*, 1963; Vanderveken, 1977; Simons *et al*., 1977; Qui and Pirone, 1989; Powell, 1992; Wang and Pirone, 1996). However, the exact mode of action of mineral oils in inhibition of non-persistent virus transmission is still unknown. It seems that oil most likely interferes with the retention and inoculation of virus by somehow disrupting

attachment and/or release of viruses from their receptors on the aphid mouthparts (Qui and Pirone 1989; Wang and Pirone, 1996; Powell *et al.*, 1998). This is supported by the fact that oil inhibits transmission of non-persistent and semi-persistent viruses but not persistent viruses. Further investigations should be done in order to understand the virus-vector relationship more clearly.

1.7.3.1.2. Mixing oils with pyrethroids

Gibson and Cayley (1984) reported that application of a mixture of mineral oil (Sunoco 7E) and a pyrethroid insecticide (Cypermethrin) provided better inhibition of virus transmission than the application of each component separately. In addition to a decrease in PVY incidence they also found that colonizing aphid density was reduced compared to the control. The knock-down effect of pyrethroids was not increased by mixing with oil. However, it was speculated that toxicity was enhanced by mixing with oils (Gibson and Cayley, 1984). Electrostatic spraying of a mixture of cypermethrin and paraffin oil enhanced the deposition of the chemical on the plant and decreased the spray volume required. Efficiency of two mineral oils, Bayol 52 and SC811, in decreasing PVY spread on tobacco in the laboratory was greatly enhanced when they were mixed with low doses of WL85871, which is an enriched form of cypermethrin (Gibson and Rice, 1986). Similarly, Bell (1989) reported a 54% reduction in PVY transmission in a potato crop in Ireland when the crop was sprayed with a mixture of mineral oil SC811 and Cypermethrin.

1.7.3.1.3. Synthetic insecticides

Direct damage from insect attack on crops can be controlled using insecticides to decrease insect populations. However, the indirect damage caused by vectoring plant viruses is more difficult to prevent. This is because the aphid vectors can come from a variety of sources, both alate and apterous aphids from within and outwith the crop (Matthews, 1991).

1.7.3.1.3.1. Old classes of insecticides

Insecticides have been used extensively to control vector-borne plant viruses (Broadbent, 1957), and the epidemic spread of persistent viruses like PLRV is normally controlled by chemical application (reviewed in Perring *et al*., 1999; Irwin *et al.*, 2000; Robert *et al.*, 2000; Radcliffe and Ragsdale, 2002). Unlike conventional contact insecticides, systemic insecticides provide consistent and long lasting protection from persistent viruses because they are transported throughout the plant in the phloem on which the aphids feed. Contact pesticides such as organophosphates and carbamates are of limited use to control non-persistent viruses because non-colonizing aphids acquire virus from the epidermal cells. Consequently, most attempts to decrease transmission of non-persistent viruses using synthetic insecticides have failed. The only exception was pyrethroids, one of the most important classes of pesticides which act quickly. However, their fast mode of action is still generally insufficient to prevent or decrease transmission of potyviruses alone and their performance is improved considerably when they are mixed with mineral oils as described above. It is argued that the repellent effect of pyrethroids may cause more virus spread by increasing aphid flights and probing activities (Gibson and Rice, 1989), but there is no report or evidence that supports this claim in the literature.

1.7.3.1.3.2. New classes of insecticides

New insecticides belonging to the Neonicotinoid and Pyridine groups are being used widely in potato crops and they were reported to be effective in decreasing PLRV spread (Mowry, 2005). Because some of the neonicotinoid and pyridine insecticides have antifeeding effects, they have a promising future in controlling non-persistent virus transmission. However, more investigation is required to validate such products in non-persistent virus control programmes. Imidacloprid was reported to be effective in decreasing PVY spread when applied alone (Alyokhin *et al*., 2002) or mixed with mineral oils (Martin-Lopez *et al.*, 2006). Harrewijn and Piron (1994) found that pymetrozine reduced PVY acquisition but not transmission. Similar results were recently reported by Davis *et al.* (2009). Margaritopoulos *et al.* (2010) reported that Pymetrozine is effective in decreasing PVY spread in tobacco crops in Greece; they found that pymetrozine can decrease both acquisition and transmission.

1.7.4. Non-chemical control strategies

Various non-chemical control strategies were used to control virus transmission on potato before the identity of the vectoring agent was known. Farmers used separate areas for production of potato seeds from those used for growing potatoes for consumption. This idea came after they recognized that fields in certain areas were better than those in others for producing healthy potato seed (Van der Want, 1987). Early harvesting of potato tubers was another practice which was applied by farmers. Physical heat treatment of potato tubers before growing was observed to promote sprouting of virus-free tissues. For example, potato tubers were observed to be free from PLRV if stored at 37.5 °C for a few weeks before planting (Van der Want, 1987). These early observations were exploited in seed production and multiplication programmes to produce virus-free potato stocks in the past.

1.7.4.1. Isolation of seed potato fields

It is normal practice to separate growing of seed and ware crops of potato (Radcliffe, 2006). The recommended isolation distance differs according to the transmission mechanism of particular viruses and the type of crop (seed or ware). Persistent viruses normally require more distance than non-persistently transmitted ones. It was suggested that a distance of 400 m to 5 km between seed potato fields and other potato fields is effective in decreasing PVY transmission. However, a space of 30 km or more is required to decrease the spread of PLRV (Halbert *et al*., 1990). In England, it is recommended to leave at least 800 m between potato seed crops and any potential PVY source (Harrington *et al*, 1986). In contrast, in Denmark, leaving only 40 m was enough to decrease PVY spread (Hiddema, 1972).

1.7.4.2. Removing weeds and groundkeepers

Weeds can be an important source of virus for aphids, which then spread the virus to potato crops. As discussed in section 1.4.2 on PVY host range, a large number of weed species can serve as virus reservoirs, particularly in warm regions like the Mediterranean countries where mild weather conditions enable overwintering of

infected plants. This has very important epidemiological implications in field conditions. Groundkeepers, on the other hand, are plants which have grown from potato tubers left in the field after crop harvest. In ware potato, the incidence of PVY infection is normally high, and can be 100% in some fields and on particular cultivars (Barker, 1994). In this situation, groundkeepers from such highly infected fields will serve as an early virus source during the next season. Moreover, if these volunteer plants are present in potato fields, their harmful effect might increase because they will be a source of infection to neighbouring plants as well as producing infected tubers which will contaminate the crop. Potato groundkeepers were reported to be an important source of PVY inoculum in the Netherlands (Van Hoof, 1979) and in the USA (Thomas, 1983). Jones *et al.* (1996) investigated this problem in the ware potato cv. Record grown in different sites in the UK. They found a significant percentage of PVY^N infection among the groundkeeper plants, which ranged between 16.5 and 73.5%. Srinivasan and Alvarez, (2008) reported that the weed hairy nightshade (*Solanum sarrachoides*) is an important virus source in potato fields in the north of the USA. They found that transmission of PLRV from infected weeds to potatoes is four times higher than the transmission efficiency from potato to potato. Moreover, they also found that aphids transmitted PVY^O and PVY^{NTN} more frequently to hairy nightshade than they did to potato, regardless of the virus source used (J. Alvarez, University of Idaho, USA personal communication 2010).

1.7.4.3. Haulm destruction

The practice of haulm destruction started in the Netherlands in the 1940s (Van Harten, 1983), and is now applied in many potato producing countries around the world. Radcliffe (2006) reported that haulm destruction or vine-kill is a widely recognized field practice in production of elite seed tubers. This method is especially effective when the aphid species do not colonize the crop until late in the season, which is the case in PVY transmission by cereal aphids leaving cereals after ripening. In the Netherlands, PVY is controlled by a system based on monitoring virus infection in the field, aphid monitoring by traps, and PHT by ELISA. Haulm destruction is carried out above a certain threshold in order to protect the daughter tubers from virus infection (Verbeek *et al*., 2008). It was reported that vine killing is performed in seed potatoes in Canada by mid August to decrease the risk of virus transmission to seed tubers by late season aphid flight. In addition, vine killing is effective in producing tubers of a specific size by

stopping tuber growth, promoting skin development and preventing bruising, and facilitates harvesting by easy separation of tubers (Lidgett, 2003).

1.7.4.4. Border crops

Border crops have been used in crop protection programmes for many years. The border crop is the first line of contact for any pests coming from other fields, and thus offers protection against infection or insect damage. There are many hypotheses about the mode of action of border crops in limiting spread of non-persistent viruses (Hooks and Fereres, 2006). However, the two most common hypotheses will be outlined briefly.

The physical barrier hypothesis postulates that border crops attract aphids which alight on them and so reduce the aphid density inside the field (Simons, 1957). In experiments on PVY spread on pepper, Simons (1957) found that sunflower borders provided protection from PVY infection by physically trapping the alate aphids. In agreement with the above hypothesis, field studies on potato crops confirmed that *M. persicae* was more abundant in field borders compared to central areas after the aphids alighted on the crop (Suranyi, 1999; Carrol *et al*., 2004).

The sink hypothesis proposes that aphids lose their virus charge when they land on the border crop and probe the leaves while exploring the host suitability (Toba *et al.*, 1977). A correlation was found between using border crops and decreasing PVY spread in potato but there was no difference between plant species used in crop borders (DiFonzo *et al.,* 1996). It was concluded that a border of any unrelated crop could be effective in reducing virus spread. More recently, Fereres (2000) used sorghum and maize borders to protect pepper from PVY infection. In this work barrier plants failed to decrease the number of aphids entering the pepper field but succeeded in limiting virus transmission. Moreover, laboratory experiments confirmed that the aphids lost their virus inoculum when allowed to probe sorghum and maize (Fereres, 2000). A study by Boiteau *et al.* (2009) also supports the sink hypothesis in decreasing PVY transmission in potato fields in Canada. They found a negative correlation between crop border incorporation and PVY incidence.

1.7.4.5. Host masking and reflective mulching

It has been proposed that elimination of the contrast in the background between host plants and the soil is very useful in controlling non-persistent viruses (Jones, 1994). Many synthetic reflective materials have been used to evaluate the effect on transmission of non-persistent viruses on different crops. These include plastic (Loebenstein *et al.*, 1975), aluminium foil (Shands and Simpson, 1972), and polyethylene coated with aluminium (McLean *et al*., 1982; Jones, 1991). In addition to this synthetic mulching, Saucke and Doring (2004) found that straw mulching was highly effective in suppressing PVY transmission. The effect was greater during the early stage of plant growth when aphid activity occurred early in the season, but efficiency declined after the plant canopy covered the ground and when aphid activity started later in the season. The maximum protection was achieved by combining pre-sprouting with straw mulching.

1.7.4.6. Using natural products

Some natural compounds exhibit repellent effects on vector aphids (Hori, 1999; Halbert *et al.*, 2009), and thus can be useful in controlling virus spread when applied on the plant alone or in combination with other synthetic compounds or agricultural practices **(**Radcliffe, 2006). Gibson *et al.* (1982) found that polygodial, which is an aphid antifeedant extracted from *Polygonium hydropiper*, decreased the acquisition of PVY by *M. persicae.* A contradictory result was reported by Powell *et al.* (1996), who also investigated the repellent effects of polygodial on stylet penetration and non-persistent transmission of plant viruses by aphids. They concluded that polygodial had no effect on stylet penetration and subsequent transmission of PVY or CMV. Extracts from the neem tree, *Azadirachta indica*, have been used to protect crops from a wide range of pests. Azadirachtin is a chemical compound extracted from the seed of the neem tree and has used as an antifeeding for many insects. Inhibition of acquisition and inoculation of PLRV by Azadirachtin was reported (Nisbet *et al*., 1996). There is limited information about the effect of such natural products on non-persistent virus transmission by aphids. For example, it was reported that 1.0% or 2.0% neem seed oil has an inhibitory effect on PVY acquisition and transmission to pepper by *M. persicae* (Lowery, 1997).

Vegetable oils were found to decrease PVY transmission in the laboratory (Bradley, 1962). More recently, Martin-Lopez *et al.* (2006) reported that refined rapeseed oil combined with a low dose of imidacloprid decreased PVY infection by 40% in potato crops.

The effect of aphids' alarm pheromones on virus transmission was investigated (Dawson *et al.*, 1982; Rice *et al.*, 1983). It was reported that PVY acquisition and transmission by *M. persicae* was inhibited by aphid alarm pheromones, (E)-β-farnesene and the saturated straight 14-carbon chain dialkyl ester of acetylene dicarboxylic acid (Gibson *et al*., 1984; Gibson, and pickett, 1984).

1.8. Project's aims

This thesis will investigate diverse topics related to the most two important potato viruses (PLRV, PVY), which are of great importance due to their economic impact in potato yield reduction. The ultimate goal is to control the spread of these viruses by aphids, and in order to achieve that, the following sub-topics were investigated:

1- Mechanisms of potyvirus transmission are still uncertain, but many reports support that HC-Pro mediates the aphid transmission of potyviruses by forming bridge-like structures between the virus particles and putative receptors in the aphid stylets. However, limited information is available about the exact location or the nature of these proposed receptors, thus in chapter 3, an aphid cDNA library was screened for some aphid proteins that can interact with HC-Pro *in vitro* and can serve as virus receptors. In addition, sequence analysis was performed to characterize the identified clones and compare their sequence with other published aphid proteins.

2- In order to understand the transmission process of potyviruses, interaction between the three components involved in the transmission is required. The host plant effect on the aphid vectoring ability was investigated in the fourth chapter; in addition, the effect of the host plant when used as a virus source or as a test plant on transmission efficiency was studied. Findings obtained would have two implications, first they can be utilized to understand more clearly the transmission mechanism, second, they can provide valuable information to control virus spread in the fields by aphids.

3- Potyviruses are retained for a short time inside their aphid vectors, and this behaviour was behind the assumption that these viruses are stylet-borne or non-persistent. In chapter 5 of this thesis, the concept of short retention was challenged and the likelihood of longer retention time of the virus inside different parts of the insect body and for a long time after acquisition will be investigated. The obtained results were discussed in the context of virus transmission mechanism by aphids.

4- During certification schemes or screening virus resistant cultivars ELISA is routinely used to perform a large number of tests. In the sixth chapter, the aim was to develop a fully recombinant ELISA assay which can be used routinely to test for PLRV and can be as an alternative to the immune reagent antibodies which are currently being used. This will create a cheap consistent, and unlimited source of testing reagents without the need of immunizing animals.

5- Agrochemicals are currently being used extensively to control the spread of non-persistent potato viruses but with very limited effect. It is arguable that some insecticides can decrease virus transmission by interfering with aphid probing behaviour; this was the rationale for investigating the repellent effect of one insecticide on PVY transmission by aphids.

Chapter 2. General Materials and Methods

2.1. Virus and aphid cultures

Virus isolates used in this project are presented in table 2.1. Potyviruses were propagated by manual inoculation by pulverising 3–4 systemically-infected leaf tips in 5–10 ml of SDW using a mortar and pestle. The extract was rubbed on to leaves dusted with carborundum powder, then the leaves were rinsed with water and plants were placed in a glasshouse at approximately 18 °C with a 16 h: 8 h light: dark cycle.

Virus isolate	Host	Source
PLRV	*Physalis floridana*	SCRI stock isolate
PVY^{O}-L	*Nicotiana tabacum* cv. White Burley	SCRI stock isolate
PVY^{O}-F	Potato cv. Rosetta	Field isolate, SASA
PVY^{C}	*Nicotiana tabacum* cv. White Burley	SCRI stock isolate
PVA	Potato cv. Hermens	Field isolate, SASA
PVX	*Nicotiana benthamiana*	SCRI
TuMV	*Nicotiana benthamiana*	SCRI stock isolate
TEV	*N. tabacum* cv. Xanthi (N/N)	J.J. Lopez-Moya, (Spain)

Table 2.1. Virus isolates used in this project.

M. persicae (genotype E), was obtained from Dr. Brian Fenton, SCRI. *M. persicae* colonies were maintained on *Solanum tuberosum* cv. Desiree, *N. tabacum* cv. White Burley, *Brassica napus* (oilseed rape), and *P. floridana* at 18 °C in a 16 h: 8 h light: dark cycle with plants replaced on a weekly basis. Aphid colonies were reared in clear perspex cages (35x40x45 cm). The front side was surrounded with a thin mesh for ventilation. In addition, the cage has an opening in the back side to pass air inside from a central fan to allow airflow. Whole plants were used for colony rearing, and plants were replaced every week. Sub-colonies were reared on detached leaves in plastic pots with a mesh on the top for ventilation.

To kill aphids on plants, the plant and pot were submerged in a bucket of hot soapy water for 5 min. Plants used for virus culture were disposed of after autoclaving.

2.2. Virus transmission by aphids

For potyviruses transmission wingless individuals (3^{rd}–4^{th} instar) were selected from the stock culture. They were fasted in batches of 1, 2, 3, 4, 5 or 10 in a plastic 1.5-ml micro centrifuge tube for a period of 2–3 h at 18 °C. Routinely, 5 individuals were used for virus transmission unless otherwise mentioned.

Detached PVY-infected potato or tobacco leaves were placed onto a wet sponge within a small plastic box (Blackman box). Fasted aphids were placed on under side of the infected source leaf and allowed to acquire virus for 1, 2, 3, 4, 5, 10 or 20 min. A maximum of 5 individual aphids was used per batch. Aphids were then moved directly to test plants and the plants covered. Tobacco plants were covered with small plastic cages. For potato plants Perspex® tubes (8 cm external diameter, 7 cm internal diameter × 16 cm length; Stockline Plastics, UK) were used. To allow air flow, all tubes were capped with a thin muslin mesh (mesh size 200 micron, John Lewis, UK). In some experiments aphids were confined to one leaf by a clip cage. Aphids were allowed to feed for 12–16 h then killed by a combination of nicotine fumigation and the insecticide Plenum in a controlled access fumigation chamber.

For PLRV transmission by aphids, Wingless aphids (3^{rd}–4^{th} instar) were transferred to PLRV source (detached *P. floridana* leaves) for a 48–72 h acquisition access period (AAP), then transferred to healthy *P. floridana* for a similar transmission access period (TAP). Plants were then transferred to the fumigation chamber and treated as described above.

2.3. Virus detection

ELISA was used routinely for PVY and PLRV detection in plants and occasionally RT-PCR, nested RT-PCR, electron microscopy and Western blot were used. However, for PVY detection inside aphids, nested RT-PCR was the only method used, as ELISA and normal RT-PCR were not sensitive enough to detect low virus quantities**.**

2.3.1. General buffers for virus detection

ELISA coating buffer

0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6

ELISA extraction buffer

PBS containing 0.05% (v/v) Tween 20 and 1% polyvinylpyrrolidone M.W. 44,000, BDH Laboratory Supplies, UK.

ELISA colour substrate buffer

9.7 % (V/V) diethanolamine-HCl in SDW, pH 9.8

Blot and ELISA blocking buffer

5% (w/v) non-fat dried milk (Marvel) in PBS

ELISA and Western blot washing buffer (PBST)

1x PBS containing 0.025–0.05% (v/v) Tween 20

PBS

PBS was obtained from the SCRI media kitchen as a 10x stock, and then diluted in SDW to working concentration. 1x stock contains 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4.

SDS-PAGE 4x sample buffer

0.1g bromophenol blue powder, 6.25 ml 1 M Tris-HCl (pH 6.8), 10 ml glycerol, 20 ml 10% (w/v) SDS, 5 ml of β-mercaptoethanol. Then the volume was adjusted to 100 ml by addition of distilled water.

PAGE electrophoresis buffer

0.025 M Tris base, 0.192 M glycine, pH 8.3, 0.1 % (w/v) SDS.

Immunoblot transfer buffer

0.025 M Tris base, 0.192 M glycine, 20 % (v/v) methanol, pH 8.3.

Ponceau S stain

0.5 % (w/v) Ponceau S in 10 % acetic acid

Substrate stop solution

20 mM Tris-HCl, pH 2.9, 1 mM EDTA

2.3.2. ELISA

ELISA was done following the methods described by Torrance (1992). Briefly, the wells of ELISA plates (Nunc, Maxisorp Immunoplate) were coated using 100 µl per well of the working dilution of coating reagents (Table 2.2), and incubated for 2–3 h at 37 °C. Plates were then washed three times with PBST. Extracts of infected or healthy leaf tissues (1 g in 5 ml of extraction buffer) were added to coated wells (100 µl samples were placed in duplicate wells). The plates were incubated overnight at 4 °C, then detecting reagents were added at the working concentration and plates were incubated for 2–3 h at 30 °C. Plates were then washed as before, and conjugates were added at working concentration and incubated for 2–3 h at 30 °C except for the recombinant V3HCL-B (biotin-conjugated V3HCL) reagent, which was incubated together with streptavidin-AP for 2–3 h at 37 °C, and the α-PVA-AP Mab, which is conjugated directly to AP. Plates were washed as before.

To prepare plant leaf extract, samples were pulverised in extraction buffer at 1:10 (w/v) using a mortar and pestle. For large number of samples, a power-driven crusher was used to prepare leaf extract as explained by De Bokx (1987).

Antibody	Working dilution	Source	Purpose
α-PLRV PC	1:1000	SCRI	Trap PLRV
SCR1 Mab	1:2000	SCRI	Detect PLRV
α-PVY PC	1:1000	SCRI	Trap PVY
SCR39 Mab	1:1000	SCRI	Detect PVY
α-PVY Mab	1:1000	Agdia	Detect PVY
V3HCL	10 µg/ml	SCRI	Trap PLRV
V3HCL-B	10 µg/ml	SCRI	Detect PLRV
α-PVY HC-Pro PC	1:2000	SCRI	Detect HC-Pro
α-PVA-AP Mab	1:4000	SCRI	Detect PVA
α-PVA Mab	1:1000	SCRI	Trap PVA
α-TEV HC-Pro PC	1:500	J.J. Lopez-Moya	Detect HC-Pro
α-PVX PC	1:1000	SCRI	Detect PVX
α-PVY CI PC	1:400	Prepared	Detect CI
α-MPCP2D	1:2000	Dombrovsky *et al*. (2007b)	Detect M.P CUPs
α-MPCP2N	1:2000	”	Detect M.P CUPs
α-MPCP5D	1:2000	”	Detect M.P CUPs
α-MPCP2N	1:2000	”	Detect M.P CUPs
α-histidine Mab	1:3000	Sigma	Detect His-tagged protein
α-Myc Mab	1:1000	SCRI	Detect Myc-tagged protein
Streptavidin-AP	1:4000	Zymed	Enzyme conjugate
α-Mouse AP	1:1000	Sigma	Enzyme conjugate
α-Rabbit AP	1:1000	Sigma	Enzyme conjugate

Table 2.2. Antibodies and enzyme conjugates used in this project with their sources and working dilutions.

Mab: Monoclonal, PC: Polyclonal, AP: alkaline phosphatase, His: 6-Histidines tag, V3HCL: α-PLRV single-chain variable fragment (scFv).

The substrate p-nitrophenyl phosphate (Sigma) was added (1 tablet/10 ml 9.7% diethanolamine pH 9.8) to each well and the plate was incubated at room temperature (approx. 22 °C) for 1 and 2 h then overnight (12 h) at 4 °C. Absorbance values (A_{405}) at each time point were recorded using a Titertek Multiskan PLUS Photometer (Titertek, Huntsville, AL). Values were considered positive if they exceeded the mean control values by a factor of two.

2.3.3. RT-PCR for potyvirus detection

2.3.3.1. cDNA synthesis

Total RNA was prepared from leaf tissue using the Qiagen RNeasy Mini kit following the manufacturer's instructions and quantified (Section 2.4.1.4).

1 µg of RNA was mixed with 1 µl reverse primer (Singh AS480; Table 2.4) at 10 pmol/µl and SDW added to a final volume of 11 µl. The solution was incubated at 70 °C for 10 min, then immediately cooled in ice. 4 µl of M-MLV RT buffer (Promega), 1 µl of RNasin ribonuclease inhibitor (Promega), 1 µl of 10 mM dNTPs, and 2 µl of sterile distilled water were added. The mixture was warmed up at 37 °C for 2 min, then 1 µl of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) reverse transcriptase (Promega) was added and the solution incubated at 37 ^{o}C for 1 h.

2.3.3.2. PCR conditions

PCR was performed by mixing 2 µl of cDNA template, 4 µl 25 mM $MgCl_2$, 10 µl of 5x GoTaq® Flexi buffer, 1 µl of 10 mM dNTPs, 2 µl of each forward and reverse primer (Singh S0 and Singh AS480; Table 2.4), 28.5 µl of water and 0.5 µl of Promega GoTaq® DNA polymerase (5 U/ µl). PCR conditions were: 95°C for 5 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min.

2.3.4. Nested RT-PCR

2.3.4.1. RNA extraction from aphids

Individual aphids were collected in 1.5 ml micro centrifuge tubes, deep frozen in liquid nitrogen, then homogenised with a mini-pestle in 100 µl of TriPure isolation reagent (Roche Diagnostics) and incubated at room temperature for 5–10 min. An equal volume of chloroform was added, and then the tube was inverted to mix and centrifuged at 13,000 rpm for 15 min. The aqueous layer was collected, mixed with 125 µl of isopropanol and allowed to precipitate for 10 min, then centrifuged at 14,000 rpm for 10 min. The resulting pellet was washed with 500 µl of 75% (v/v) EtOH, centrifuged at

14,000 rpm for 5 min and air-dried. The pellet was resuspended in 5–10 µl of DEPC-treated or RNase-free water and incubated at 50 °C for 5 min.

2.3.4.2. RNA extraction from plants

RNA was extracted from 50 to 100 mg leaf tissue using TriPure isolation reagent as described in section 2.3.4.1. The Triton method (Singh, 1999) was used as well to extract viral RNA from plant leaf material. Briefly, a sample of the infected plant leaf was placed in a 1.5-ml micro centrifuge tube then 100 µl of 0.5% (v/v) Triton X-450R (Sigma) solution was added to the leaf sample, and leaf tissue was ground with a chloroform- rinsed mini-pestle. The mixture was incubated at 37 °C for 30 min, and then centrifuged at 12,000 rpm for 15 min in a bench top centrifuge. The supernatant was recovered and transferred to a new tube and 1–2 µl was used as a template for cDNA synthesis or for the first round of nested RT-PCR.

2.3.4.3. First round – RT-PCR

Illustra™ Ready-to-Go RT-PCR Beads (GE Healthcare, UK) were used following the manufacturer's instructions with some modifications. PVY primers (Singh *et al.*, 1996; Singh, 1998) (Table 2.4) were used. Briefly, Illustra™ Ready-to-Go RT-PCR beads were dissolved in 48 µl of RNase-free water plus 1 µl each of forward and reverse primers (Singh S0 and Singh AS480; Table 4.2) and incubated on ice for 5 min. Then 24 µl was added to a 0.2 ml PCR tube (Thermo Scientific, UK). To each aliquot was added 3 µl of the template RNA. The solutions were mixed and the tubes were placed in an Eppendorf Mastercycler PCR machine using the following programme: 42°C for 30 min; 95 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and then 72 °C for 10 min.

2.3.4.4. Second round PCR

Briefly, each Illustra™ Ready-to-Go PCR bead was dissolved in 22 µl of RNase-free water plus 1 µl of each nested forward and reverse primer (Malloch F, Malloch R; Table 2.4) and incubated on ice for 5 min. The contents of each PCR bead sample were then split between two 0.2 ml PCR tubes. To each was added 1 µl of the first round PCR

product. Tubes were transferred to a PCR machine programmed as above excluding the initial incubation at 42 °C.

2.3.5. Electron microscopy (EM) negative staining

To extract virus, leaf samples were pulverised in Sorensen's phosphate buffer, pH 7.5–8.0, using a mortar and pestle. A drop of virus extract was placed on a carbon-coated grid for a few seconds and drained with filter paper, and then one drop of phosphotungstic acid (PTA) was added for approximately 20 sec. Grids were drained and allowed to air dry then washed three times and examined under the electron microscope.

2.3.6. SDS-PAGE

SDS gel electrophoresis was performed essentially as described by Laemmli *et al*. (1970) and Sambrook and Russell (2001). The Novex mini-PAGE system (Invitrogen) was used according to the manufacturer's instructions.

2.3.6.1. Resolving gel

Resolving gels were made using Sigma Acrylamide/Bis-acrylamide, 30% solution (Mix Ratio 29:1), following the recipe in Table 2.3. Then, 33 µl of 10% ammonium persulphate (APS) was added to the mixture and the solution was mixed by gentle inversion. 8 ml was pipetted into a Novex gel cassette, and 1–2 ml of water was added gently to the top of the gel, which was left for a minimum of one hour to polymerise.

Resolving gel composition	10% Novex-PAGE	12.5% Novex-PAGE
Acrylamide 30%	3.33 ml	4.16 ml
1 M Tris-HCl (pH 8.8)	3.75 ml	3.75 ml
H_2O	2.66 ml	1.83 ml
10% SDS	100 µl	100 µl
TEMED	5 µl	5 µl
10% APS	33 µl	33 µl

Table 2.3. Resolving gel preparation.

2.3.6.2. Stacking gel

Stacking gels were prepared by mixing 1.5 ml of Acrylamide/Bis-acrylamide, 30% solution (Mix Ratio 29:1), 1.25 ml of 1 M Tris-HCl (pH 6.8), 7 ml of H_2O, 100 µl of 10% SDS, and 5 µl of TEMED. Then 200 µl of 10% ammonium persulphate (APS) was added. The solution was mixed by gentle inversion, and then applied to the top of the polymerised resolving gel and the sample comb inserted.

2.3.6.3. Sample loading

Protein samples were diluted in an equal volume of SDS-PAGE sample buffer (Section 2.8.3) and boiled for 5 min. Gels were run at 140 V (constant current) for 1.5 h.

2.3.6.4. Coomassie Blue gel staining

Following electrophoresis, gels were washed with water then immersed in GelCode® Blue Stain Reagent (Thermo Scientific) and left overnight with gentle agitation. The gel was immersed in water for destaining and photos were taken digitally.

2.3.7. Western blot analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes essentially as described by Towbin *et al*. (1979). Briefly, two pieces of Whatman 3MM filter-paper and Hybond-ECL™ Nitrocellulose membrane (Amersham Bioscience) were cut to the same size as the gel and rinsed in transfer buffer (Section 2.8.3) together with similar sized sponge pads.

A transfer sandwich was made comprising a piece of pre-soaked filter paper, the gel, pre-soaked nitrocellulose membrane, and another piece of pre-soaked filter paper. Transfer was done using an XCell SureLock™ Mini Cell (Invitrogen) for 1.5 h at 125 mA with constant voltage. Following transfer, membranes were stained in Ponceau S stain for 2 min to mark the position of the marker. Stained protein bands were visualized by destaining in 1x PBS; when visible the positions of the molecular weight markers was marked with a pencil. Membranes were then blocked by immersion in 5%

non-fat powdered milk (Marvel) in 1x PBS with gentle agitation for at least 1 h at room temperature.

Primary and secondary antibodies (Table 2.2) were diluted to working concentration in 0.1% milk in PBST, and incubated sequentially with the membrane in a sealed plastic bag for 2–3 hours at room temperature or overnight at 4 °C. After each incubation step, membranes were rinsed with PBST for 10 min with gentle agitation, repeated three times. Membranes were finally incubated with NBT/BCIP substrate solution (Sigma catalogue number 72091) at room temperature for 20–30 min. The reaction was stopped by immersion in stopping solution or SDW.

2. 3.8. Dot blot assay

5 µl of protein sample was spotted on to nitrocellulose membrane (Amersham) and air dried. Blocking, incubation with antibodies, washing, and colour development steps were performed as described above for western blot analysis.

2.4. Molecular biology methods

2.4.1. Nucleic acid methods

General molecular biology methods followed those described by Sambrook and Russell (2001).

2.4.1.1. General media, buffers, and reagents

LB Agar plates

LB plates prepared using the following recipe was provided in-house at SCRI.
2% (w/v) Agar powder, 1% (w/v) Bacto-tryptone, 0.5 % (w/v) Yeast extract, 1% (w/v) sodium chloride, pH adjusted to 7 with NaOH.
LB AIX plates were prepared as for LB plates above with the addition of ampicillin (100 µg/ml), IPTG (32 µg/ml) and X-Gal (32 µg/ml).
LB AMP plates were prepared as for LB plates above with the addition of ampicillin

(100 µg/ml).
LB KAN plates were prepared as for LB plates above with the addition of kanamycin (50 µg/ml).

SOC Medium

2% (w/v) Tryptone, 0.66% (w/v) glucose, 0.5% (w/v) Yeast extract, 0.5 % (w/v) NaCl, 0.25% (w/v) KCl.

Antibiotics and inducers

All antibiotics and inducers were prepared in the laboratory from powder and stored as frozen stocks at -20 °C.

Ampicillin stock

1.0 g in 10 ml SDW, filter/sterilise = 100 mg/ml
Working concentration 100 µg/ml

Kanamycin stock

1.0 g in 10 ml SDW = 100 mg/ml
Working concentration 50 µg/ml

Chloramphenicol stock

1.0 g in 29.4 ml EtOH = 34 mg/ml
Working concentration 34 µg/ml

X-GAL

1 g in 10 ml di-methyl formamide = 100 mg/ml
Working concentration 32 µg/ml

TBE

This buffer is prepared by the SCRI media kitchen as a 10x stock. Then working 1x solution is prepared by 1:10 dilution with SDW.
The recipe for 1x stock is: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA.

6 x DNA Gel loading Buffer

0.25% (w/v) bromophenol blue, 0.25% xylene cyanol, and 40% (w/v) sucrose in dH_2O.

2.4.1.2. Agarose gel electrophoresis of DNA

A Horizon® 11X14 (Life Technologies™) gel apparatus was used; 1–2 % (w/v) agarose (Invitrogen, UK) gels were prepared in 1x TBE, then the mixture was heated in a microwave until the agarose completely melted. Ethidium bromide (EdBr) was added to 0.5 µg/ml and the gel allowed to set at room temperature before use. DNA samples were mixed with 6x DNA loading dye (3–4:1 v/v) , and loaded into sample wells, then gels were run at 100 V. DNA bands were visualized under a UV transilluminator and photos were recorded using the UVP gel documentation system (Ultra-Violet Products Ltd).

2.4.1.3. DNA extraction from agarose gel

After electrophoresis, DNA bands were visualised using UV light and bands of interest excised from the gel using a clean razor blade. Samples were either processed immediately or stored at -20 °C. DNA was recovered from the gel pieces using the QIAquick Gel Extraction Kit (QIAGEN Ltd.).

2.4.1.4. DNA recovery from PCR product and after enzyme digestion

DNA recovery was performed using the Mini Elute PCR purification kit (QIAGEN). Briefly, 5 volumes of the supplied buffer PB was added to 1 volume of PCR reaction and mixed. The mixture was applied to a Mini Elute column and centrifuged at 14,000 rpm for 1 min. Then the column was washed once with supplied buffer PE and eluted with 10 µl of supplied elution buffer (EB) or SDW. For DNA recovery after restriction digestion, at least two volumes of PB buffer was added to each sample and applied to a QIA miniprep column and centrifuged for 1 min at 14,000 rpm. The column was washed twice with QIA washing buffer and eluted with 30–50 µl SDW.

2.4.1.5. DNA and RNA quantification

DNA concentration was estimated by measuring the absorbance at 260 nm (A_{260}), multiplying by the dilution factor, and using the formula A_{260} of 1.0 = 50 µg/ml pure DNA or 40 µg/ml pure RNA.
Concentration (µg/ml) = (A_{260} reading) × dilution factor × 50 µg/ml (or 40 for RNA)

2.4.1.6. DNA ligation

T4 DNA ligase (Promega) was routinely used for all ligation of DNA inserts into cloning vectors. Ligation mixtures were prepared with variable molar ratios of vector and DNA insert according to the formula:

$$\frac{\text{Amount of vector (ng) x size of insert (kbp)}}{\text{Size of vector (kbp)}} \times \text{vector: insert} = \text{amount of insert (ng)}$$

Ligation reactions were continued for a minimum of 1 h at room temperature or overnight at 4^{o}C.
pGEM-T and pGEM-T Easy vectors (Fig. 2.1) were used routinely for cloning procedures. They enabled white–blue colony screening of transformed clones. Other vectors were used occasionally for different purposes, and they are presented whenever they were used.

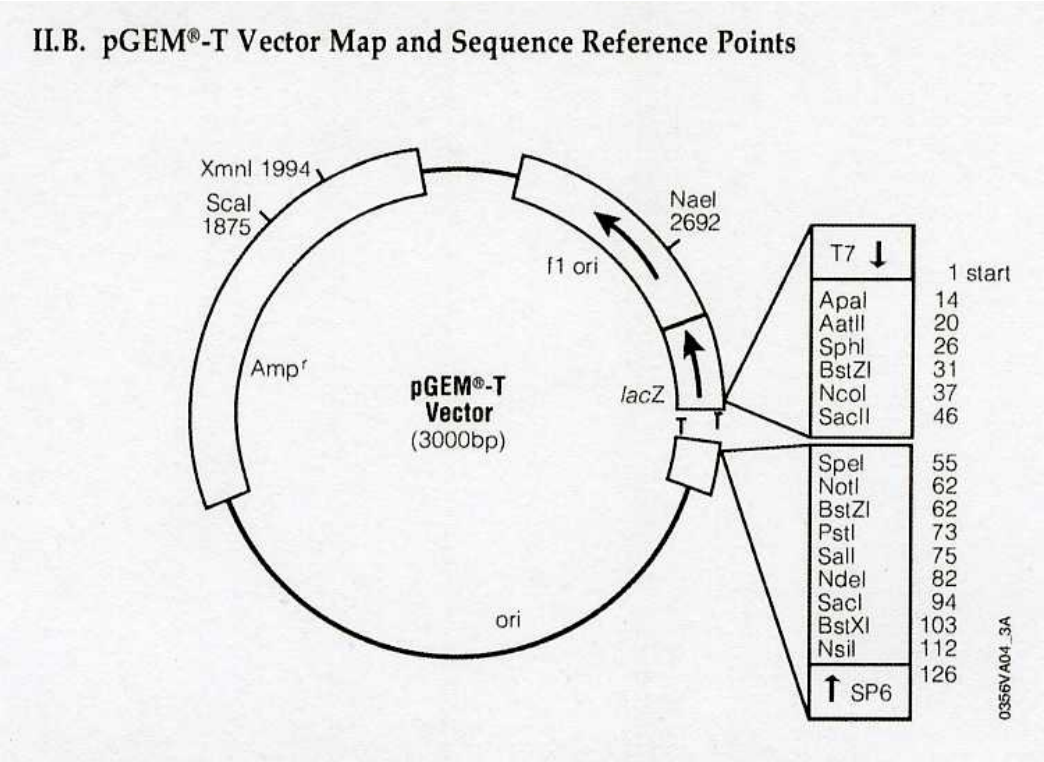


Figure 2.1. pGEM-T Vector Map and Multiple Cloning Site, Promega

2.4.1.7. Chemical transformation of *E. coli*

Transformation was basically performed as described by Sambrook and Russell (2001). An aliquot of competent cells (XL10 Gold cells) (Stratagene) was thawed on ice. 5 µl of β-mercaptoethanol was added and mixed then the solution was kept on ice for 10 min. 1–5 µl of plasmid prep or ligation reaction was mixed with the competent cells. The mixture was kept on ice for 45 min, given a heat shock at 42 °C for 45 sec, then incubated on ice for 2 min. 1 ml of SOC medium was added to the mixture, and the cells incubated with shaking at 37 °C for 40 min. 100 µl of the mixture was spread on to a pre-warmed LB agar plate containing a suitable selective marker and plates were incubated in an inverted position for about 16 h at 37 °C to allow colony growth.

2.4.1.8. Transformation by electroporation

E. coli strain AVB100 (Avidity), which has the *birA* (biotin holoenzyme synthetase) gene incorporated into the chromosome under the control of L-arabinose as inducer, was used for recombinant protein production after transformation by electroporation. 500 µl of SOC medium was added to the electroporated cells, and the mixture was

transferred to a 50 ml tube, incubated for 45 min at 30 °C, then spread on prewarmed selective LB plates and incubated at 30 °C for 16–18 h.

2.4.1.9. Colony pick PCR

Sample colonies were transferred to 1.5 ml tubes containing 100 µl of sterile water, then 10 µl of the mixture was inoculated in 5 ml LB containing selective antibiotic. The remaining test sample was boiled for 5 min then centrifuged for 5 min at 14,000 rpm. To give a quick identification of positive cultures from which to prepare plasmids, 5 µl of the supernatant was used as template for PCR with primers that amplify the insert.

2.4.1.10. PCR primers

The majority of primers used in this project were designed. Primers underlined in Table 2.4 were already designed. Primers were desined manually according to the following general rules:

1- Primer length should be 20–30 bp with melting temperatures (Tm) of 55–65 °C.
2- GC content 50–55%.
3- Long runs of a single base, especially at the 3' end, were avoided to prevent secondary priming.
4- The 3' end always terminated with a G or C.

Primer Name	**Primer Sequence** 5'……3'	**Purpose**
P72F-FOW	GCGGTACCGAACAAAAACTCATCTCAGAAGAG G ATCTGACGCGTCCGTTTCTGTACACAGTCATCG	Amplify P72F
P72F-REV	CGAAGCTTGTATGCTGGTTTGTAAGCTGGC	”
P817P-FOW	GCGGTACCGAACAAAAACTCATCTCAGAAGAG GA TCTGACGCGTCCGACACGTCCG	Amplify P817P
P817P-REV	CGAAGCTTTGGGAGGCGATCAATTCCAAAGAC	”
P820P-FOW	GCGGATCCGAACAAAAACTCATCTCAGAAGAG GATCTGCCCACGCGTCCGGTTGTCAG	Amplify P820P
P820P-REV	CGAAGCTTATGCGTATGTGGTTGGCG	”

PVYFOR	GGCTGAACACAGGCTTGAGGCG	Amplify DAG motif
PVYREV	GCCCATTCATCACAGTTGGCATC	”
HCspeFOR	GACTAGTATGCTGAGAATTTTTGGAAG	Amplify KITC motif
HCrev GFP	CCTCGCCCTTGC TCACCATAACTCTATAGT GTTTTATATCAG	”
HC-seqFow	GGTCTGGATAGTAATTGGGCACG	Sequencing KITC motif
HC-seqRev	GATAACCTGCCCCACCTGTGC	”
M13-F	GTAAAACGACGGCCAGT	sequencing pGEMT
M13-R	GGAAACAGCTATGACCATG	”
Singh S0*	ACGTCCAAAATGAGAATGCC	Detect PVY
SinghAS480*	TGGTGTTCGTGATGTGACCT	Detect PVY
Malloch F^+	GGAGCAGCCGTGCTAAACTTAGAAC	Detection PVY
Malloch R^+	CGCGCTAAACCTACATCCCGCAGA	Detect PVY
V3FWDSf	ATTACTCGCGGCCCAGCCGGCC	Amplify scFv-C_L Insert
V3REVNot	CGTGCGGCCGCGGACTCTCCCCTGT	”
BIOFWD	GTCTCCGCGGCCGCGGGGGCC	Introduce *Not*I site into pAK300Bio
BIOREV	GGCCCCCGCGGCCGCGGAGAC	”
V3NOTMutFwd	GCGGCCGGAGGATCCGC	Mutate *Not*I site in the V3HCL
V3NOTMutRev	GCGGATCCTCCGGCCGC	”
PAK300BioNot-F	ACCGATAGCCGGAGCTCC	Sequencing the *Not*I site of pAK300Bio
PAK300BioNot-R	CGAAGATGTCGTTCAGAC	”
PVYOCIFWD	CGCGAGCTCTCCTTAGACGATG	Amplifying PVY-CI insert
PVYOCIREV	CGCAAGCTTTTGGTGATGAACG	Amplifying PVY-CI insert

Table 2.4. Primers used in the project for cloning, detection, mutagenesis, and sequencing.

Primers with underlined names were already designed. * Are from Singh *et al*. (1996), + are from Malloch *et al*. (unpublished), and the rest of the primers were designed.

Primers were synthesised commercially (Eurofins MWG Operon), and were received lyophilized then reconstituted in either TE buffer or SDW to 100 µM and kept at -20^{o}C.

2.4.1.11. DNA sequencing, editing, and alignment

All DNA sequencing was performed in-house using the sequencing lab facilities at SCRI. For sequencing a gene or part of a gene, inserts were PCR amplified then cloned into pGEM-T, and then plasmid minipreps were prepared and submitted directly to the sequencing lab. Sometimes amplified PCR products were directly sequenced without cloning into the pGEM-T vector. In this case, DNA samples were cleaned up first.

All sequence analysis was done using the BioEdit program (Tom Hall). Sequence comparisons were performed using the BLAST (Blastx or Blastn) program through the NCBI website (http://blast.ncbi.nlm.nih.gov/) or the EMBL website (http://www.ebi.ac.uk/Tools/blast/).

Analysis of aphid protein sequences was done using Blastn and Scaffolds databases available on-line at http://www.aphidbase.com/aphidbase/.

2.4.1.12. Site-Directed Mutagenesis

The Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used either to introduce or to mutate restriction enzyme sites in plasmid DNA following the manufacturer's instructions. The template DNA was digested by the addition of 10 U of *Dpn*I enzyme for 1 h at 37 °C. 2 µl of the reaction mix was used to transform BL21 Gold competent cells (Stratagene) as described in section 2.6.3. Plates were incubated for around 36 h at 30 °C. Colonies were grown overnight in LB agar medium containing selective antibiotic and plasmid minipreps prepared then digested with restriction enzyme. The enzyme reactions and an undigested control sample were analysed on a 1.2% agarose gel to confirm the mutation. Required bands were excised from the gel, and DNA was recovered using a QIAGEN gel purification kit

2.4.2. Proteomic methods

2.4.2.1. General buffers and recipes

Freezing broth

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
K_2HPO_4	6.3 g
Na Citrate	0.45 g
$MgSO_4.7H_2O$	0.09 g
KH_2PO_4	1.8 g
Glycerol	44.0 g

Adjust the pH to 7.2 with NaOH and the volume to 1000 ml with distilled H_2O.

2 x TY

Peptone	16 g/l
Yeast	10 g/l
Sodium Chloride	5 g/l
Agar (Gibco)	15 g/l

L-arabinose

0.15 g in 10 ml SDW, then filter-sterilised = 100 mM

working concentration 1.5 μM

IPTG

0.25 g in 10 ml SDW, then filter-sterilised = 100 mM

working concentration 1 mM

TBS

20 mM Tris-HCl, pH = 7.5

150 mM NaCl

Lysozyme buffer

50 mM Tris-HCl, pH 8.0

150 mM NaCl

5 mM $MgCl_2$

3% (w/v) BSA

Add the following before use:

Lysozyme from chicken egg white (Sigma) to 400 mg/ml

RQ1 RNase-free DNASE (Promega) to 1 U/ml

Sorensen's Phosphate Buffer

1 part of 0.1 M KH_2PO_4 + 4.5 parts of 0.1 M Na_2HPO_4, then adjust pH to 7.5–8.0.

KPB buffer

0.3 M Potassium phosphate, pH 9 containing 1 tablet/50 ml of protease inhibitor cocktail tablets.

TSM buffer

100 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.2

2.4.2.2. Protein expression and purification buffers

Bacteria lysis buffer

Buffer B: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjust pH to 8 using NaOH.

Washing buffer

Buffer C: for 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjust pH to 6.3 using HCl.

Elution buffer

Buffer D: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjust pH to 5.9 using HCl.
Buffer E: 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, adjust pH to 4.5 using HCl.

TEV HC-Pro Extraction Buffer

100 mM Tris-HCl (pH 8.0), 20 mM $MgSO_4$, 500 mM NaCl, 0.5 mM EGTA. Then immediately before use add: PVP M.W. 44,000 (BDH Laboratory Supplies, UK) at 0.1 g/100 ml and sodium sulphite at 0.2 g/100 ml.

2.4.2.3. PVY purification

All operations were carried out at 4 °C. The borate method (Govier *et al*., 1977) was used for purification of PVY. PVY-inoculated *N. tabacum* cv. Samsun plants were harvested 3–4 weeks after manual inoculation and leaves were homogenised with three volumes of 0.1 M ammonium acetate (pH 7.0) containing 0.02 M EDTA, M/15 Sodium diethyldithiocarbamate (Na DEICA). The extract was passed through 3 layers of muslin fabric and centrifuged for 10 min at 8,000 rpm. The supernatant was recovered and Triton X-100 was added to 2.5 % (v/v). The solution was stirred for 20 min then centrifuged at 35,000 rpm in an ultra centrifuge using type 50.2 Ti rotor (Beckman) for 1.5 h at 4 °C. The supernatant was discarded and the pellet was covered with 1–2 ml of 0.01 M sodium borate buffer (pH 8.0) and kept overnight at 4 °C to allow sedimented virus particles to resuspend.

Sucrose gradients (10–40 % w/v in sodium borate buffer, pH 8.0) were prepared in SW28.1 tubes (Beckman), and kept overnight at 4 °C. The resuspended virus pellet was centrifuged at 5,000 rpm for 10 min in a bench top centrifuge, and the supernatant centrifuged again at 35,000 rpm in a Beckman ultra centrifuge for 1.5 h. The pellet was resuspended in a small volume of 0.01 M sodium borate (pH 8.0), and 250 µl layered on to each sucrose gradient. Gradients were centrifuged for 2 h at 28,000 rpm in an SW28.1 swinging bucket rotor in the same type of centrifuge. Finally, virus was recovered by collecting 0.5–1 ml fractions manually.

2.4.2.4. Virus quantification

Virus concentration was calculated from measurements of the OD at 260 nm, which was then multipled by the extinction coefficient of this virus (2.9) (CIP Training Manual). Additionally, protein concentration was estimated from the intensity of Coomassie stain following separation through 12.5% NOVEX-PAGE gels by comparison with a standard concentration of BSA.

2.4.2.5. Recombinant protein expression and induction

Protein was produced in bacteria following the QIAGEN expressionest booklet (2001). Briefly, transformed *E. coli* colonies were inoculated into 5 ml of LB or TY medium containing selective antibiotic and incubated at 30 °C for 12–16 h with shaking. The culture was diluted in a larger volume of the same medium and incubated in the same conditions for 2–3 h (OD = 0.4–0.6). Bacteria were then induced with IPTG at 1–2 mM and incubated in the above conditions for 3–16 h (depending on the induced protein). The bacteria were then harvested by centrifugation in a low speed centrifuge at 4,000 rpm, and stored frozen at -20 °C or preceded with purification or inclusion bodies preparation depending where the protein is expressed (e.g. in chapter 3, IB was prepared during expression of HC-Pro in *E. coli,* whereas for V3 expression in chapter 6, the protein was harvested from the supernatant of the culture by extracting the PE from the bacteria.

2.4.2.6. Bradford protein assay

Standard concentrations (0.5, 1, 5, and 10 µg/ml) of BSA protein were prepared, then the Bradford dye (Bio-Rad) was diluted 1:5 in water, and 10 µl of each BSA protein sample was added to 490 µl of the diluted Bradford dye to make the standards. For each tested sample, 10 µl of the protein was mixed with 490 µl of the diluted Bradford reagent and the absorbance was measured estimated by eye.

2.4.2.7. Protein dialysis

Dialysis tubing (Medicell International Ltd) was boiled briefly in water and rinsed in SDW. The dialysis tube was sealed with a plastic clip at one end then protein solution

was added and the tube was sealed similarly at the other end, leaving enough space for the volume increase which may result from buffer exchange. The samples were dialysed in 500–2000 ml buffer (depending on the volume of the protein sample; generally, the volume of buffer was at least 100 times more than the volume of protein sample) with constant stirring. Dialysis was performed with at least two changes of buffer, one of them overnight at 4 °C.

2.4.2.8. Ni-NTA resin protein purification system

Ni-NTA resin **(**QIAGEN) is supplied as a 50% slurry in 30% EtOH. Resin was washed 2 to 3 times with the buffer used to wash protein, then resin (usually 5–10 mg of protein per ml of resin is recommended) was mixed with the protein, and mixture then added to an empty Illustra^{TM} PD-10 desalting column (GE Healthcare) already equilibrated with protein washing buffer. The mixture was agitated gently for 1 h at room temperature, or for longer at 4 °C. Then, the bottom and top covers of the column were removed and the unbound fraction was removed under gravity flow. The column was washed 2 to 3 times with washing buffer; the 3–4 eluates were collected using a specific elution buffer depending on the protein.

2.5. Statistical analysis

The design of aphid transmission experiments was discussed with Biomathematics and Statistics Scotland (BioSS) staff at SCRI to ensure that replication was enough for analyzing results statistically. Statistical analysis was done using the 13th edition of GenStat statistical package (VSN International Ltd, Harpenden).

Chapter 3. Identification of *Myzus persicae* proteins that interact with Potato virus Y helper component

3.1. Introduction

Helper dependent transmission of plant viruses is a subject of great importance, and elucidation of the exact mechanism of virus transmission would have a major impact on development of novel methods for virus control in the future. Potyviruses which belong to the largest known family (*Potyviridae*) of plant viruses are all transmitted by aphids in a non-circulative, non-persistent manner with the help of a multifunctional virus encoded protein known as the helper component (HC-Pro). The requirement for biologically active HC-Pro has been reported for successful aphid transmission of potyviruses (Kassanis and Govier, 1971a,b; Govier and Kassanis, 1974a,b; Pirone and Blanc, 1996). Moreover, electron microscopy studies revealed that virus retention in aphid stylet was always associated with presence of functional HC-Pro (Berger and Pirone, 1986). Recently, different cuticle proteins extracted from *M. persicae* were confirmed to interact with HC-Pro of the potyvirus Zucchini yellow mosaic virus (ZYMV) (Dombrovsky *et al.*, 2007b). In a different virus helper component system, Uzest *et al.* (2007) localized the interaction between Cauliflower mosaic virus (CaMV) and putative aphid receptors on the tip of the stylet of *Acyrthosiphon pisum*, *M. persicae*, and *Brevicoryne brassicae*. They developed a novel *in vitro* assay based on expression of the P2 protein of CaMV as a fusion to the green fluorescent protein (GFP). After an acquisition access period (AAP), aphid stylets were dissected and the GFP-P2 complex was detected on the tip of the stylet by confocal laser scanning microscopy (CLSM). More recently, by employing their assay Uzest *et al.* (2010) confirmed that the GFP-P2 fusion protein was detected in other aphid species which vector CaMV but not in a non-vector species. In addition they proposed the "acrostyle" structure on the tip of the stylet, and confirmed that the GFP-P2 bound to this structure.

During the past few decades scientists have concentrated on investigation of the virus molecular determinants involved in their transmission, and substantial information has been obtained in this respect. However, less effort was directed towards investigating the aphid vector determinants involved in the transmission process. The current

hypothesis indicates that there are at least two virus components which participate in transmission, namely specific motifs in the coat protein (CP) and the HC-Pro. However, the mechanism by which these two proteins contribute to the transmission process is still unclear. Moreover, the possible involvement of other virus-encoded proteins in the transmission process has not been excluded.

Studies on the aphid vectors have included examination of light and electron microscopy sections of aphid mouthparts after virus acquisition and confirmed that virus particles exist in different locations inside the aphid body (Taylor amd Robertson 1974; Berger and Pirone, 1986). Further studies demonstrated that potyvirus was located in the lumen of the aphid's food canal, and showed that virus was transmitted when acquired simultaneously with HC-Pro or sequentially after HC-Pro was acquired, but not if virus was acquired first. However, the exact location and the nature of these receptors have not been identified (Ammar *et al.,* 1994; Wang *et al*., 1996; Blanc *et al*., 1998; van den Heuvel *et al.*, 1999; Harris and Harris, 2001). Several factors have contributed to this gap in knowledge in identification of these putative virus receptors inside aphids. The first factor is the lack of information about aphid proteins in general and in particular aphid cuticle proteins, which are believed to form the core component of these aphid receptors (Dombrovsky *et al.*, 2007b); secondly, the difficulty in handling aphid cuticle proteins; and third is the lack of collaboration among virologists and entomologists to investigate this process.

The cuticle is a unique feature of the insect's body that enables the insect to withstand a wide range of climatic conditions. Insect cuticle is composed of chitin embedded within a protein matrix (Merzendorfer and Zimoch, 2003); chitin also exists in plants and other organisms such as fungi. Thus it has been suggested that the only unique component of the insect cuticle that may interact with virus components is the cuticle proteins. This protein component is now the basis of investigations of the possible interactions between plant viruses and their aphid vectors.

The aim of this Chapter is to identify aphid proteins that may interact with potyvirus HC-Pro. In this work, a portion of an aphid cDNA expression library constructed as described by Ramsey *et al*. (2007) was screened against purified recombinant PVY HC-Pro protein by preparing high-density colony filters using a Genetix Q-Bot robot, and subsequently performing protein overlay assays. In addition, protein of one of the identified clones was expressed and the interaction with HC-Pro belonging to PVY and

other potyviruses was investigated. Moreover, bioinformatics analysis was done on three of the identified clones and their sequences were compared with other proteins in the sequence databases. Obtained results were discussed in the context of aphid transmission by aphids.

3.2. Materials and Methods

General materials and methods are presented in Chapter 2. This section will deal with specific methods used particularly in this Chapter.

3.2.1. Expressed colony screening

A *M. persicae* cDNA expression library containing 7680 clones was screened. These clones represent a cDNA library constructed by Dr. B. Fenton and colleagues (SCRI), one of the sixteen expression libraries described by Ramsey *et al*. (2007).

3.2.1.1. Q-Bot Filter printing

A fresh copy of the library was prepared before individual printing by growing colonies in wells of Genetix 3.5 mm 384-well plates (Genetix, New Milton, Hampshire, UK), and incubation for 16-18 h at 37°C in "freezing" broth (Chapter 2) containing selective antibiotics. Protran nitrocellulose transfer membranes (PerkinElmer ^{TM} Life Science) were cut to fit into Q-Tray vented bioassay plates (22.2 × 22.2 cm; Genetix), and then a piece of Whatman 3mm paper was put on top of the Q-Bot printing cassette. Water was added to the Whatman paper until it became moderately wet. Afterwards the nitrocellulose membrane was placed on to the cassette and air bubbles were removed by rubbing with a glass pipette. The microtitre plate containing the freshly copied library was placed inside the Q-Bot hotel in numbered order, and the chamber was closed. Printing was started by selecting the appropriate programme. The process was completely automated (Fig. 3.1) and included three repeated steps. These were: (1) picking the 384-well plates placed in the Q-Bot hotel; (2) immersing the Q-Bot printing head into the fresh cultures grown in the 384 well plates and printing on the nitrocellulose membranes; (3) passing the printing head through cleaning solutions placed in the Q-Bot trays then drying. The cycle was repeated 40 times to print all plates.

After printing, the membranes were placed on a Q-Tray containing either LB AMP or LB AIX medium (Chapter 2, section 2.4.1.1) for protein induction, and then the plates were incubated for 16 to 18 h at 37°C to allow colonies to grow (Fig. 3.2).

Each large filter was subdivided into 6 small filters, and each one of these small filters contains 384 clusters in a 24x16 array. Each cluster contains 16 spots, representing eight clones in duplicates from each of eight Genetix 384-well plates (See Fig. 3.3 for membrane layout). Each large membrane can therefore accommodate up to 48 Genetix 384-well plates, divided into 6 sub-areas each with clusters of clones from 8 plates. Therefore the whole library of 7680 clones can be screened on one large filter. If the duplicate wells in a mini-cluster were positive, then the clone was selected. Dark purple colour shows a reaction; blue colour shows that the clone did not contain an insert. Each Genetix 384-well plate itself has numbers from 1 to 24 for rows, and letters from A to P for columns. Thus each clone will have a name such as P72F which means that it was selected from plate 7 with a match to row 2 and column F. Each plate is printed in 24 mini-clusters of 16 wells in duplicate.

Figure 3.1. The Q-Bot robot used for the filter printing process (Picture from SCRI Annual Report, 2007).

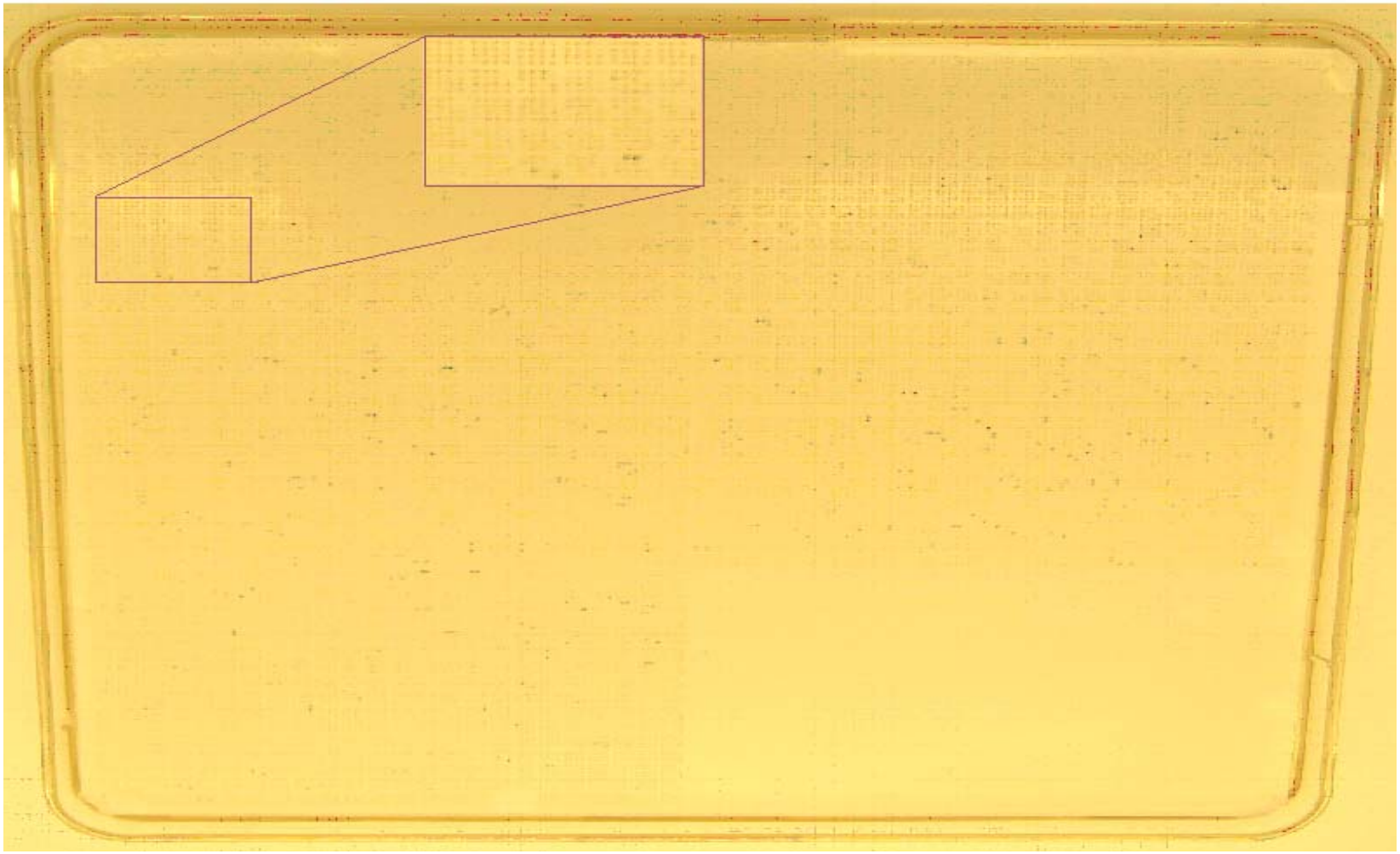
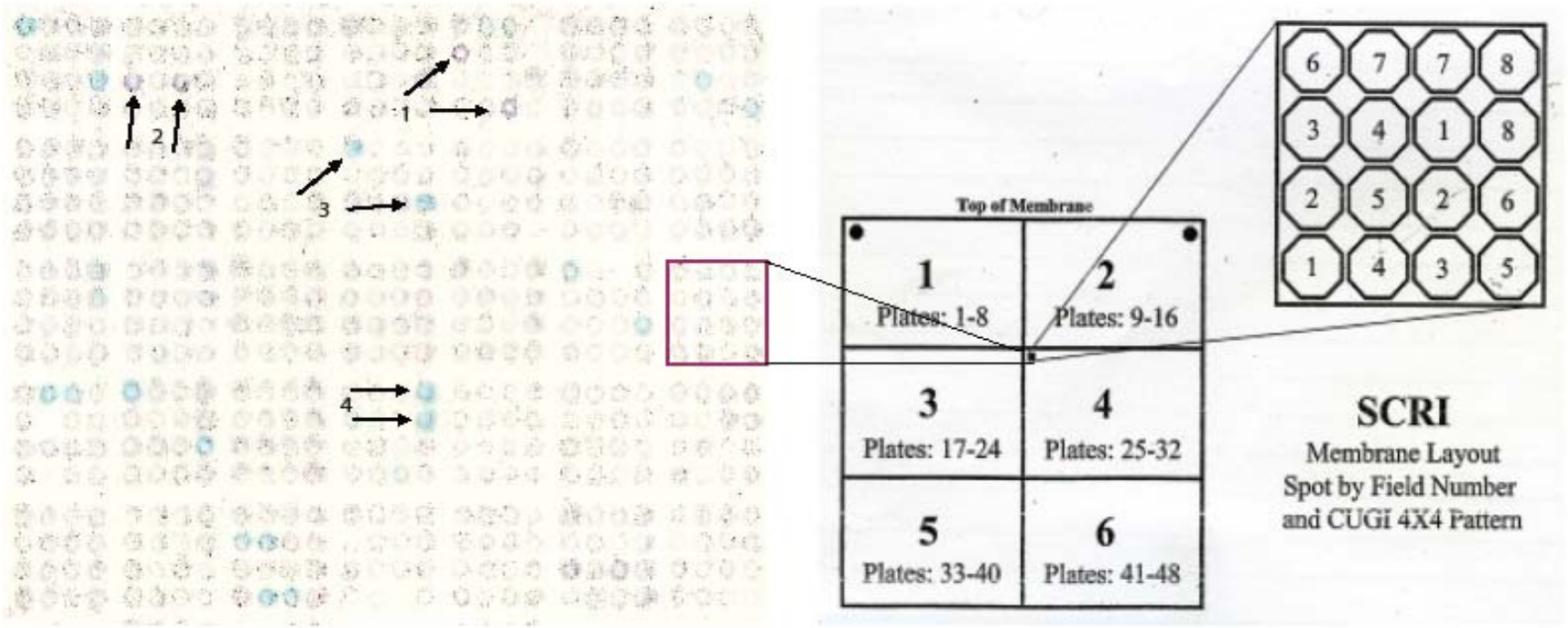


Figure 3.2. Printed bacterial colonies on the filter in the Q-Tray 16-18 h after induction.
The magnified part shows growing bacterial colonies.



SCRI membrane layout and magnified part of the induced filters
1,2: duplicate positive spots
3,4 duplicate spots with no inserts

Figure 3.3. SCRI Q-Bot membrane layout and the basis for selection of positive clones.

The magnified square on the right shows the arrangement of the duplicate clones from plates 1 to 8 in each clust

3.2.1.2. Filter processing and overlay assay conditions

Membranes carrying induced colonies were exposed to an atmosphere of saturated chloroform for 10 min by hanging over a beaker containing chloroform, then placed on Whatman 3mm paper to allow the chloroform to evaporate. The membranes were incubated for 1 h in 150 ml of lysozyme buffer (Chapter 2) with gentle agitation, and any remaining debris was removed by rubbing with a clean gloved finger. Membranes were then transferred to a fresh lysozyme buffer solution, and incubated for a further hour, then washed three times for 10 min each in TBST buffer. Blocking with 1% BSA (Sigma, UK) in TBS was performed for 1 h with gentle agitation. After blocking, membranes were incubated with 150 ml of N-HC-Pro produced in *E .coli* (10 ng/ml) in TBS containing 1% BSA for 16 h at 4^{o}C with gentle agitation, then washed three times with TBST and incubated with commercial α-histidine monoclonal antibody or α-HC-Pro polyclonal antiserum (Table 2.2) in PBSTM for 2-3 h at RT. Filter was then washed as before and incubated with either α-mouse-AP or α-rabbit-AP in PBSTM for 2-3h at RT then filter was washed as before. Colour development was carried out as described for Western blots (Chapter 2).

3.2.2. Cloning aphid proteins into the pQE-30 vector

The pSPORT vector, which harbours the cloned aphid protein sequences (Ramsey *et al*., 2007), does not incorporate any epitope tags to assist investigation of whether the protein is expressed or to estimate the level of expression. To overcome this problem the gene sequences of three identified clones (P72F, P817P, and P820P) were amplified by PCR from the pSPORT vector using primers described in Table 2.3, then inserts were cloned into the pGEM-T Easy vector, and finally sub-cloned into pQE-30 (QIAGEN) to enable protein expression assessment through the 6-histidine tag fused to the N-terminus of the expressed protein. In addition, the sequence of the Myc-tag (Hilpert *et al.*, 2001) was added to the 5′ end of the sequence of the primers in order to create a second fusion protein with c-Myc at the N-terminus, which is recognized by α-Myc commercial monoclonal antibody (Table 2.2).

3.2.3. Aphid cuticle protein expression and purification

Aphid protein clones were expressed from the pSPORT and pQE-30 vectors after induction by IPTG for 1, 4, and 16 h following protein expression under conditions

described in Chapter 2. In addition, native CUP proteins were extracted from the aphid body. Unless otherwise mentioned, all protocols used to express recombinant proteins in *E. coli* were from the QIAexpressionest handbook (QIAGEN, 2001).

3.2.3.1. Whole cell extract

One ml of induced culture was centrifuged at 14,000 rpm for 1–2 min, and the pellet was resuspended in 100 µl of SDS-PAGE sample buffer and boiled for 5 min. The denatured protein was loaded onto an SDS-polyacrylamide gel (15 µl per well) or stored frozen at -20 °C for subsequent analysis.

3.2.3.2. Periplasmic extract (PE)

Bacterial cells were harvested as before, and then resuspended in 30 mM Tris-HCl (pH 8), 20% sucrose, 20 mM EDTA. The mixture was incubated on ice for 30 min then centrifuged at 12,000 rpm for 20 min at 4^{o}C. The supernatant was discarded and the pellet was resuspended in a small volume of ice-cooled 5 mM $MgSO_4$, and shaken for 10 min at 4^{o}C, then centrifuged as before. An equal volume of SDS-PAGE sample buffer was added to the supernatant, then the mixture was boiled and used directly (15 µl per well) for PAGE.

3.2.3.3. Cleared lysate under native conditions

After protein induction and harvesting, bacterial cells were subjected to freezing then thawing cycles in ice. Lysis buffer under native conditions (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8 with NaOH), containing lysozyme (Sigma) to 1.5 mg/ml) was added at 0.5 ml/0.25 g pellet for resuspension. The mixture was incubated on ice for 30-60 min then centrifuged at 12,000 rpm for 10 min. The supernatant was retained. An equal volume of SDS-PAGE sample buffer was added and the mixture was boiled for 5 min then used directly for SDS-PAGE (15 µl per well) and western blot analysis.

3.2.3.4. Cleared lysate under denaturing conditions

Induced cultures were centrifuged, then the resulting pellets were lysed by freeze/thaw treatment and resuspended in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8M urea (pH 8 with NaOH), then kept on ice for 30 min. The mixture was vortexed until the solution became translucent, and was then centrifuged at 10,000 rpm for 20 min, and the supernatant was retained. For SDS-PAGE analysis, equal volumes of sample buffer and the supernatant were mixed and 15 µl of the boiled mixture was loaded per well.

3.2.3.5. Inclusion body (IB) method

Inclusion bodies were prepared from bacterial cultures as follows. Protein production was induced in the bacterial culture by addition of 1 mM IPTG and incubation for 3-4 h at 30 ^{o}C. Cells were harvested by centrifugation at 10,000 rpm for 30 min, resuspended in 4 ml/g of 0.1 M Tris-HCl pH 7.0, 1 mM EDTA, 1.5 mg/ml lysozyme (Sigma), and incubated on ice for 1 h. Then EDTA was added to 20 mM, Triton X-100 to 2%, and NaCl to 0.5 M. The mixture was incubated on ice for 30 min. Viscosity of the mixture was decreased by a further incubation step with protease inhibitor (RNasin ribonuclease inhibitor, Promega) or by passing through a large syringe needle. Inclusion bodies were harvested by centrifugation at 10,000 rpm for 10 min at 4^{o}C, then resuspended and washed three times in 0.1 M Tris-HCl pH 7.0, 20 mM EDTA then resuspended in SDS sample buffer for analysis. Each inclusion body pellet was resuspended in SDS sample buffer (100 µl per 0.1 mg), then the mixture was boiled for 5 min, and 15 µl of the boiled mixture was loaded per well. The mixture was then stored at -20 ^{o}C, and reused 2–5 times after boiling for 5 min every time.

3.2.3.6. Native CUPs extraction

Metopolophium dirhodum, which is a very poor vector of PVY, was selected to test differences in HC-Pro binding to CUPs extracted from different aphid species.CUP proteins from *M. persicae* and *M. dirhodum* were extracted from the whole aphid body according to the method published by Dombrovsky *et al*. (2007b). Briefly, 1 g of aphids were collected and ground in liquid nitrogen and then mixed with 10 ml (1: 10, w/v) extraction buffer (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5), 3 mM PMSF, 10 mM β-mercaptoethanol. The mixture was centrifuged for 5 min at 4,000 rpm, and the pellet

was washed 4–5 times with 40 ml extraction buffer. The pellet was washed in 20 ml of the extraction buffer but containing a high concentration of NaCl (0.6 M). The pellet was washed twice with 10 mM Tris-HCl (pH 7.0) then extracted using 8 M urea in 10 mM Tris-HCl, pH 7.0 (5 v/w). The mixture of aphid pellet with 8 M urea was shaken for 2 h at room temperature (RT), after which the supernatant was collected by centrifugation at 12,000 rpm. Supernatant was mixed 1:1 v/v with SDS sample buffer for Western blot analysis.

3.2.4. HC-Pro protein expression and purification

Different systems were used to express potyvirus HC-Pro in order to test the interaction between expressed virus protein and expressed aphid proteins.

3.2.4.1. HC-Pro expression in *E. coli*

Full length (52 kDa) and N-terminal (full length with deletion of the 14 kDa at the C-terminus) recombinant HC-Pro containing 6-His tag at N-terminus, which were previously cloned in the pQE vector by G. Cowan, SCRI, were used first for aphid library protein screening.

N-terminus HC-Pro expression and protein harvesting

A single colony of bacteria harbouring a plasmid that contained the region coding for the N-terminus of HC-Pro was inoculated into 5 ml LB Amp medium (Chapter 2, section 2.4.1.1) containing 2% glucose and incubated overnight at 30^{o}C. Then the culture was sub-cultured into 200 ml LB Amp containing 1% glucose, and incubated for 3 h at 30^{o}C. LB Amp medium was replaced after centrifugation of the culture for 15 min at 4000 rpm at 4 ^{o}C, then protein was induced by addition of 1 mM IPTG and incubation for 16-18 h at 30 ^{o}C. Inclusion bodies were prepared as shown in section 3.2.3.5. To solubilise the inclusion bodies, inclusion body pellets were resuspended in 6 M urea, 0.1 M Tris-HCl (pH 8.0), 100 mM DL-dithiothreitol (DTT), 1mM EDTA to give a protein concentration of 10 mg/ml using Bio-Rad protein assay reagent with BSA standards diluted in urea buffer. Then 1 ml of solubilised inclusion bodies was diluted with 9 ml of 6 M urea, 0.1 M Tris-HCl (pH 8.0) and purified using Ni-NTA as described in Chapter 2.

Full length HC-Pro expression

Bacteria carrying plasmid harbouring the full length HC-Pro were induced, and the full length HC-Pro was expressed and purified under denaturing conditions following the QIAexpressionest handbook (QIAGEN, 2001). The step was similar to that used for N-HC-Pro except that harvested protein was resuspended in lysis buffer B (Chapter 2,) at 5 ml per gram wet weight and stirred for 60 min at 20–22 ^{o}C. The suspension was centrifuged at 4,000 rpm for 30 min and the supernatant was collected for purification of denatured protein over Ni-NTA resin as described in Chapter 2 except that washing was performed with buffer C, and elution was performed 4 times with buffer D and 4 times with buffer E (Chapter 2).

3.2.4.2. HC-Pro expression in plant

To overcome potential problems associated with poor yields possibly due to improper folding of expressed protein in bacteria, HC-Pro was expressed *in planta* as follows.

3.2.4.2.1. PVY-HC-Pro in PVX system

The PVX vector (Chapman *et al.*, 1992) which harbours the PVY-HC-Pro (Sasaya *et al*., 2000) was first linearized by overnight digestion with *Spe*I restriction enzyme.DNA template was purified using the QIAGEN PB buffer, then infectious RNA transcript was then prepared using the RiboMAX^{TM} large scale RNA production system-T7 (Promega, UK). Briefly, 4 µl (1–2µg) of template was added to 1.5 microcentrifuge tube, then the following were added, 8 µl of T7 5x buffer, 3 µl of each of Atp, Ctp, Utp, 1 µl of Gtp, 4 µl of T7 transcription anzyme, 4 µl of 30mm Cap, and the volume was completed to 40 µl by SDW. Transcription reaction was incubated at 37°C for 2h. *N. benthamiana* plants at the 4–5 leaf stage were mechanically inoculated with the infectious RNA transcript after dusting with carborundum. One week after infection, the leaves which showed severe PVX symptoms were harvested and purified over Ni-NTA resin as detailed in Sasaya *et al*. (2000) with some modification as follows: PVX infected leaves (20 g) were harvested and disrupted using a blender in 50 ml of chilled 0.3 M potassium phosphate (KPB; section 2.4.2.1) containing one protease inhibitor cocktail tablet (Roche, UK). The mixture was then centrifuged at low speed (14,000 rpm) for 30 min in a Sorvall® RC-6 Plus1 centrifuge (F14-6X2 50Y rotor, Thermo

Scientific) then at high speed (35,000 rpm) for 60 min using a 50.2 Ti rotor (Beckman). The supernatant was mixed with Ni-NTA resin and incubated overnight at 4°C; the resin was then washed three times with chilled KPB. Elution was performed using a high concentration of imidazole (400 mM). Eluted fractions were combined and centrifuged at 12,000 rpm for 10 min to remove the plant components which bind non-specifically to the resin by discarding the pellet and keeping the supernatant which contains the soluble HC-Pro in imidazole solution. The preparations were concentrated ten-fold by covering wet dialysis tubing containing the preparation with crushed polyethylene glycol (PEG 4000, BDH laboratory supplies) powder and allowing the volume to decrease to the required level.

3.2.4.2.2. TuMV HC-Pro

To purify TuMV HC-Pro, the method of Kadouri *et al.* (1998) was used with some modifications. This method exploits the ability of native TuMV HC-Pro to bind to Ni-NTA resin. Briefly, *N. benthamiana* plants at the 4–5 leaf stage were mechanically inoculated with TuMV infected sap as indicated in Chapter 2**.** About 10 days after inoculation, leaves showing severe symptoms were collected and disrupted in a blender using an adequate volume of pre-chilled 0.3 M K_2HPO_4, pH 8.8 (designated HCB for HC-Pro buffer). Plant debris was separated by centrifugation at 14,000 rpm for 30 min at 4°C in a Sorvall® RC-6 Plus1 centrifuge (F14-6X2 50Y rotor, Thermo Scientific); HC-Pro was purified from the supernatant by Ni-NTA resin as described before. The mixture was gently agitated overnight at 4°C, and elution performed with 250 mM imidazole in extraction buffer. Eluted fractions were checked by SDS-PAGE, and Western blotting was used to confirm HC-Pro interaction with α-histidine antibody. The protein was stored at -20 °C until needed.

3.2.4.2.3. TEV HC-Pro

His-tagged TEV HC-Pro was expressed in plants, harvested and purified as described by Blanc *et al.* (1999) and according to the protocol modified by Ruiz-Ferrer *et al.* (2005). Briefly, *N. tabacum* cv. Xanthi (N/N) plants at the 4–5 leaf stage were mechanically inoculated with TEV infected leaf samples as previously described. Fully infected leaves showing virus symptoms were harvested 3–4 weeks after inoculation, and disrupted in a blender with 4 volumes of chilled extraction buffer (Chapter 2) after

adding 1 g/100 ml of solid PVP (M.W. 44,000, BDH Laboratory Supplies, UK), and 2 g/100 ml Na_2SO_3 directly to the leaves. The mixture was then passed through a funnel covered with buffer-wet Miracloth (Calbiochem®) and 4 layers of cheesecloth into an ice-cold beaker. Leaf extract was then transferred to 250 ml centrifugation bottles and centrifuged for 2.5 h at 14,000 rpm at 4 ºC using a Sorvall® RC-6 Plus1 centrifuge (F14-6X2 50Y rotor, Thermo Scientific). The supernatant was kept for protein precipitation by adding solid ammonium sulphate to 20% (w/v), and then the mixture was stirred at low speed in a cold room for 1–2 h. The mixture was centrifuged at 14,000 rpm for 30 min at 4 ºC in the same centrifuge. The supernatant was transferred to a chilled beaker, then ammonium sulphate was added to reach 40% (w/v). The mixture was incubated for 1-2 h with gentle stirring and centrifuged at 14,000 rpm for 30 min at 4 ºC. The pellet was then resuspended in cold buffer and kept with gentle stirring for 16 h at 4 ºC. Protein was then centrifuged at 2000 rpm for 10 min to remove plant debris which may block elution from Ni-NTA resin.

Protein was then purified using Ni-NTA resin as described before except that the elution buffer was extraction buffer supplemented with 400 mM EGTA. In order to solubilise protein, it was important that pH was adjusted to 8.0 with NaOH. Eluted protein was checked by Western blotting using antiserum against 6-histidine tag and specific polyclonal antiserum against the TEV HC-Pro provided by J.J. Lopez-Moya (CSIC, Centre for Research in Agricultural Genomics (CRAG), Barcelona, Spain).

3.2.5. Overlay assay

The assay was done as follows: after SDS-PAGE and electroblotting, the membranes were blocked for 1–2 h at room temperature (RT) in PBS containing 5% BSA (Fraction V, Sigma). Then the membranes were incubated with the HC-Pro protein preparations for 16 h at 4^{o}C with gentle agitation. Membranes were washed three times with PBS containing 0.5% Tween 20 (PBST). The membranes were then incubated with either α-6-histidine monoclonal or α-HC-Pro polyclonal antibody (Table 2.2) and kept for 2–3 h at 20–22 ^{o}C, then washing was performed as in the previous step. Conjugates, α-mouse-AP or α-rabbit-AP, were incubated with the membrane for 2 h at 20–22 ^{o}C. Finally Sigma BCIP /NBT substrate was added to the membranes after washing in PBST, and colour development was stopped by rinsing in colour stopping solution or SDW after 20–30 min of incubation at 20 –22 ^{o}C.

3.3. Experimental Results

3.3.1. Clones interacting with the full-length HC-Pro expressed in *E. coli*

The first attempt to screen the aphid expression library for proteins that would bind to HC-Pro was performed with full length HC-Pro produced in *E. coli*. No obvious positive reaction was observed. Some faint positively reacting clones were picked and sequences of the inserts were determined (not shown). Sequence analysis of these clones did not reveal any match to EST sequences that are likely to be candidates for an interaction. The full length HC-Pro readily became insoluble forming large aggregates. Since the attachment between HC-Pro and the aphid's mouthparts was shown to be controlled by the Lys sequence of the conserved region (KITC) in the N-terminus of HC-Pro (Blanc *et al*. 1998), it was decided, to express only the N-terminal part of the HC-Pro (N-HC-Pro), which contains the domains necessary for interaction with aphid stylets, then the library was screened against this protein fragment.

3.3.2. Clones interacting with the N-terminus of HC-Pro expressed in *E. coli*

N-HC-Pro was expressed from the pQE-30 vector. Soluble N-HC-Pro was produced in *E. coli* and the concentration of N-HC-Pro was estimated to be about 1 μg/ml as judged from Coomassie blue staining (Fig. 3.4, panel A, lanes 6-9), and 1:100 dilution was used to incubate the filter. Eight aphid clones were selected from both induced (Fig. 3.5) and non-induced (Fig. 3.6) clones after incubation with N-HC-Pro. DNA was prepared from the candidate clones, and the inserts were sequenced. The nucleotide sequences are presented in appendix 1. Table 3.1 presents the results BLAST searches of the NCBI database (See Section 3.4). This analysis revealed that three of the selected clones (clones P72F, P817P and P94A) gave good matches with aphid cuticular proteins that were previously characterized and reported to interact with HC-Pro of ZYMV (Dombrovsky *et al*., 2003, 2007a,b). These clones, in addition to clones P820P (exoskeleton protein) and P515A (beta-tubulin), might be candidates involved in the transmission process. The remaining clones were ATP citrate lyase (clone P136B), serine/threonine-protein phosphatase (clone P1424E), and a membrane protein (clone P58L). These other interactions may be non-specific binding or related to other

functions of HC-Pro since this protein is multifunctional. Thus, it was decided to concentrate on the CUP clones, which were considered to be the best candidate genes.

The products of four clones which interacted with HC-Pro, including clone P820P initially identified as coding for an exoskeleton protein, were confirmed to be classified as cuticular proteins by searching in the cuticle protein database http://bioinformatics.biol.uoa.gr/cuticleDB/ (Magkrioti *et al*., 2004). Their predicted protein sequences were aligned together. Fig. 3.7 shows that there was little similarity among the selected clones, except for P817P and P94A. This indicates that the selected clones represent at least three different aphid CUPs.

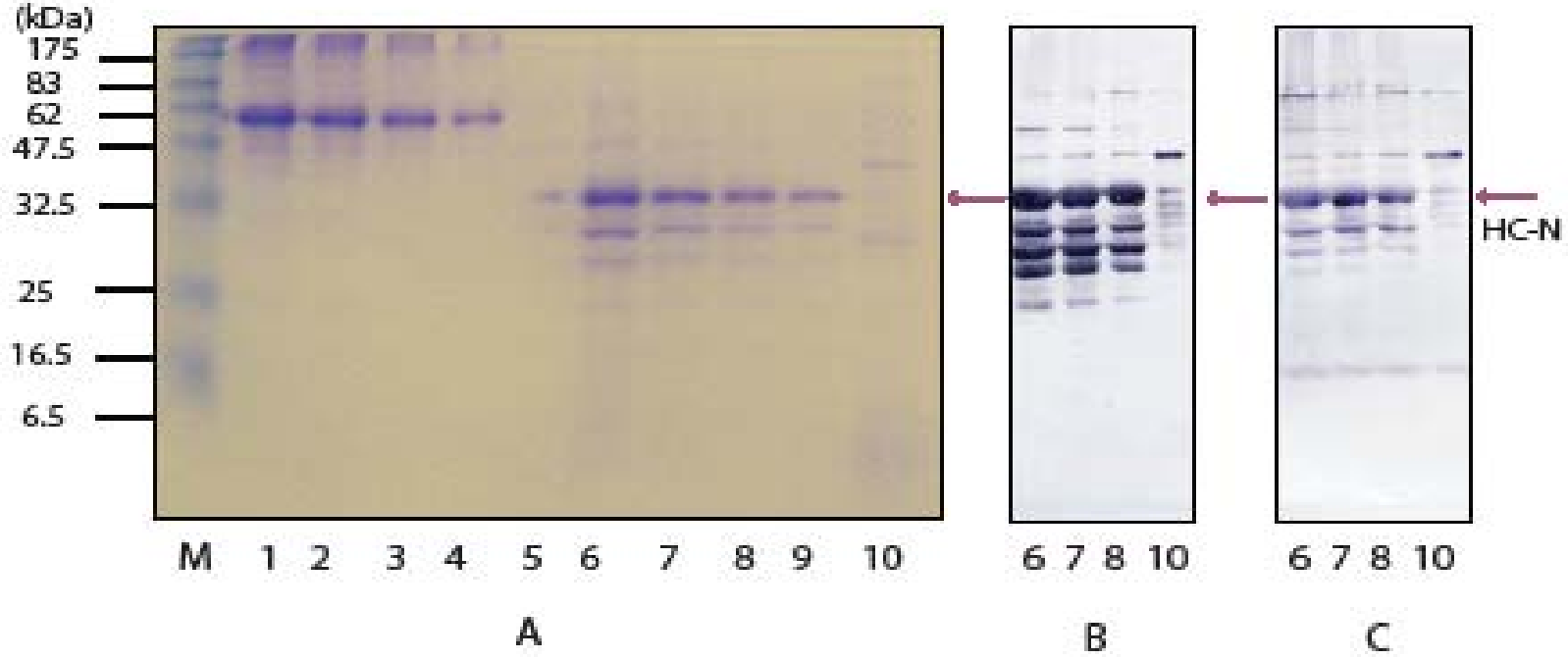


Figure 3.4. SDS-PAGE and Western blot of N-HC-Pro expressed in *E. coli* used to screen the aphid cDNA expression library.

A: Coomassie blue stain of HC-Pro elution fractions from Ni-NTA resin (lanes 6-9) compared with known concentrations of BSA protein (lanes 1-4). B: Western blot confirming the ability of expressed HC-Pro to bind to commercial α-6-histidine antiserum. C: Western blot incubated with specific polyclonal antiserum raised against recombinant HC-Pro. Lanes M: molecular size markers. 1, 2, 3, and 4: 1, 0.5, 0.25, and 0.125 µg/ml of BSA respectively in SDS sample buffer. 6, 7, 8, and 9: elution fractions of N-HC-Pro bound to Ni-NTA resin in SDS sample buffer. 10: non-bound to Ni-NTA resin fraction.

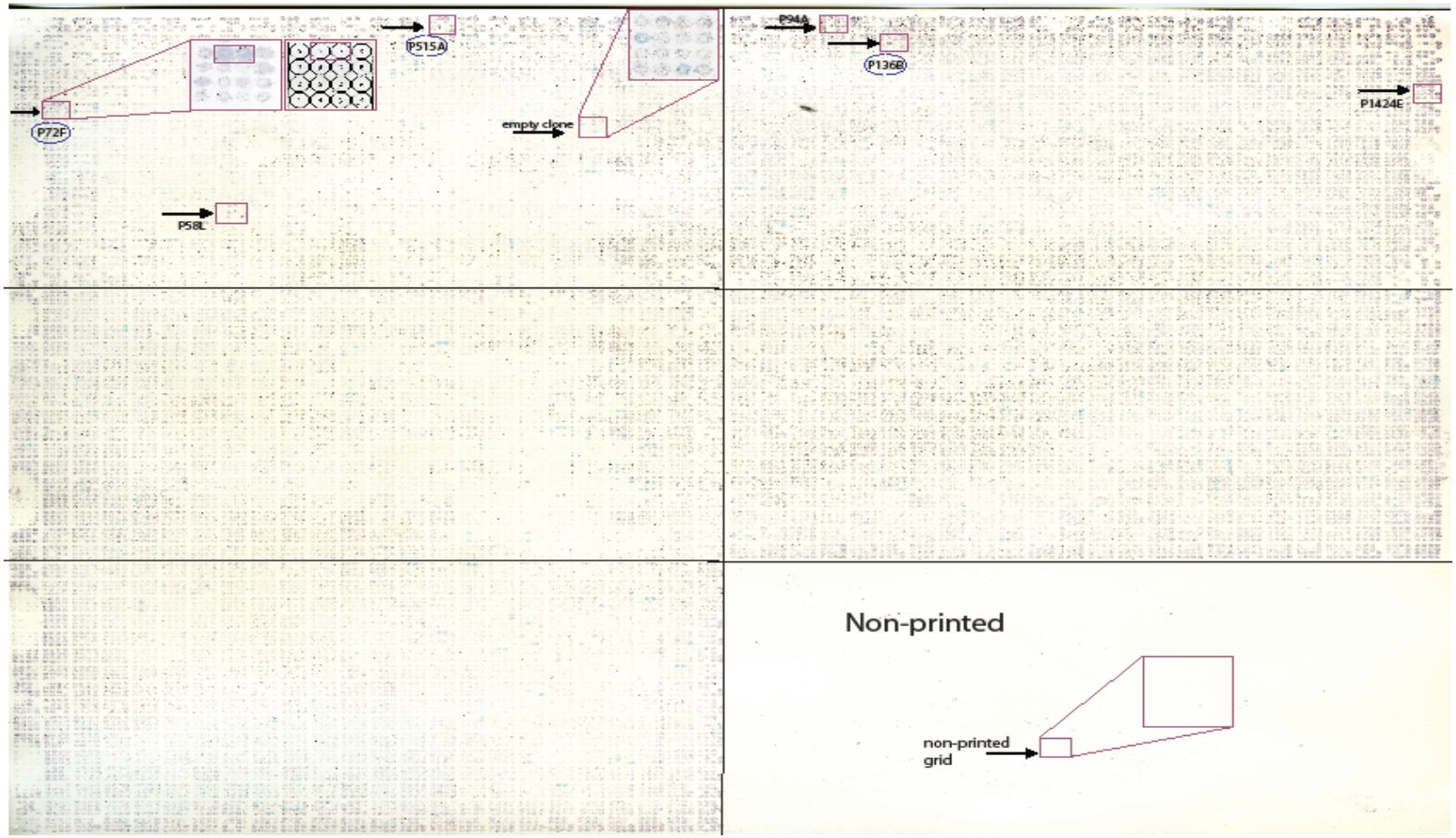


Figure 3.5. Filter showing clones that reacted with N- HC-Pro expressed in *E. coli* after first screening of the induced aphid library.

Clones which were selected for sequencing are indicated by arrows. The clusters containing a clone which was selected for sub-cloning into the pQE-30 vector is magnified (P72F). One magnified cluster contains blue spots representing examples of colonies with no inserts inside. The clones which appeared in the induced and non-induced filters (P515A, P72F, and P136B) are circled. HC-Pro concentration was 10 ng/ml (1:100 dilution of the original preparation in Fig. 3.4).

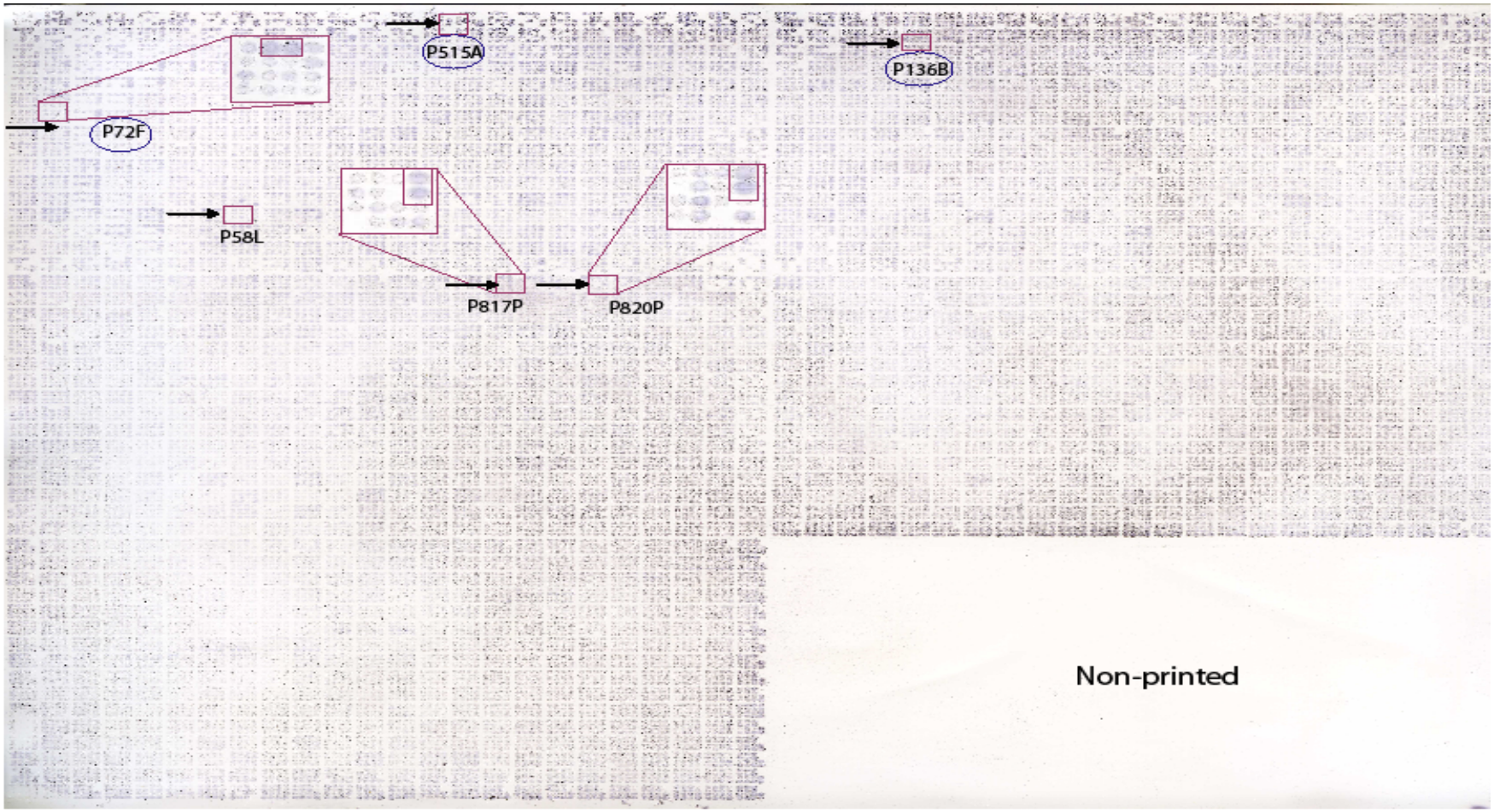


Figure 3.6. Filter showing clones reacting with N-HC-Pro expressed in *E. coli* after first screening of the non-induced aphid library.
Clones which were selected for sequencing are indicated with arrows, and clusters containing clones which were selected for sub-cloning into pQE-30 are magnified (P72F, P817P, and P820P). The clones which appeared in the induced and non-induced filters (P515A, P72F, and P136B) are circled. HC-Pro concentration was 10 ng/ml.

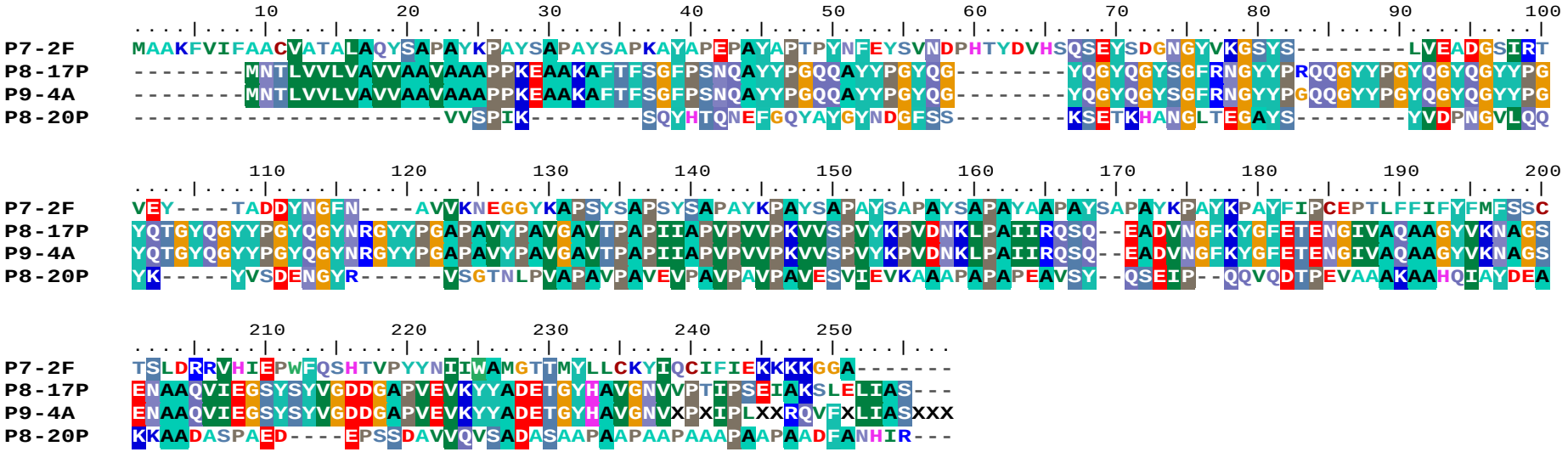


Figure 3.7. Amino acid sequence alignments between selected aphid cuticle proteins that interacted with N-HC-Pro produced in *E. coli*.

Nucleotide sequences of selected clones were translated, and then protein sequences were aligned, and similarities between selected clones were highlighted using the Bioedit programme. Amino acids are colour coded according to their identities and similar chemical properties in conserved sequences.

3.3.3. Expression of cloned cDNA inserts in pQE-30

Three candidate genes were sub-cloned into the pQE-30 vector, so that on expression they would to be fused to the epitop tags 6-His and c-Myc. The first clone selected was P72F, which matches with MPCP1 (Dombrovsky *et al*., 2003) and MPCP3 (Dombrovsky *et al*. 2007a). P817P is the second clone which was selected for sub-cloning; it gave a top match in BLAST search with MPCP5 (Dombrovsky *et al*., 2007a). The last clone which was selected is P820P, which encodes an exoskeleton protein/ RR3 CUP protein (See Section 3.4). Selection was made based on the strength of the interaction with the HC-Pro reflected by the strong colour in the duplicate spots, and based on the likelihood that the putative virus receptors on the aphid stylet are of a cuticle protein nature. Cloning of this kind of protein with much repetitive sequence was difficult and only clone P72F was sub-cloned successfully (Fig. 3.8). Recombinant protein was expressed and purified as indicated in section 3.2.3.1. Gel staining and Western blot analysis showed that the P72F gene product was not toxic to the bacterial cells as protein was induced with IPTG after 1-4 h, but the protein disappeared from the culture after the longer induction time of 16 h (Fig. 3.9). However, the molecular mass of induced proteins separated by SDS-PAGE was greater than expected from the sequence. The detected bands were between 36 and 45 kDa. This main band of 36-45 kDa was observed when induced proteins were incubated with α-His or α-Myc, but the bands were weaker when the α-Myc was used (Fig. 3.9). The deduced molecular mass of the cloned product of the P72F clone is approximately 20 kDa (Table 3.2). Due to the cloning strategy additional sequence of 20 amino acids from the vector, as well as the 6-Histidine and the c-Myc tags given an estimated mass of 23.2 kDa, which is not recognized as one of the main bands by any of the antibodies which were used (Fig. 3.9). This discrepancy between the migration of proteins in SDS gels and their predicted size from amino acid sequence is a characteristic of cuticular proteins, which typically migrate 20-40% more slowly than expected (Cox and Willis, 1987; Andersen *et al*., 1995; Dotson *et al*., 1998; Rebers and Willis, 2001). Therefore, it was concluded that it would be appropriate to induce the bacterial cultures for 3-4 h, and that the size of P72F protein was greater than what was expected. It is possible that the protein does not bind SDS in the expected ratios, and therefore migrates more slowly in the gel.

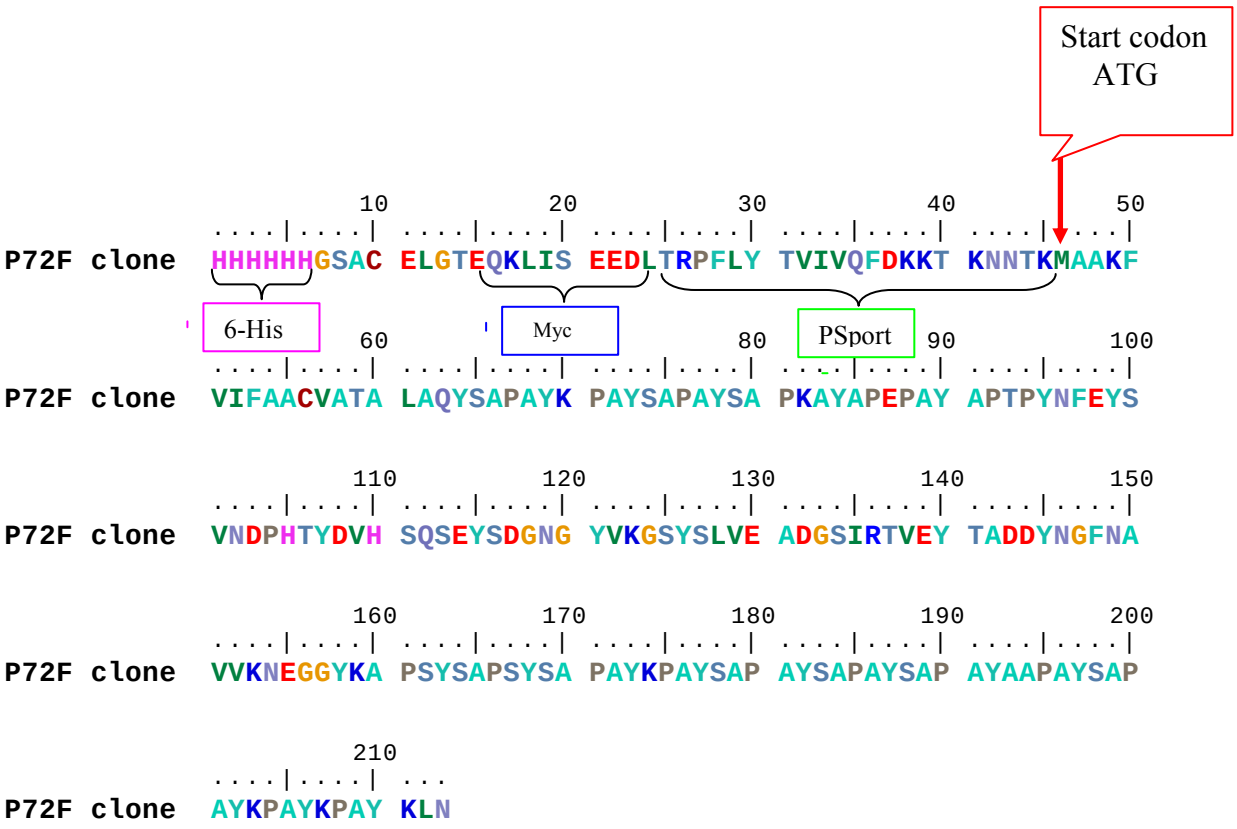


Figure 3.8. The sequence of expressed P72F clone in pQE-30 vector

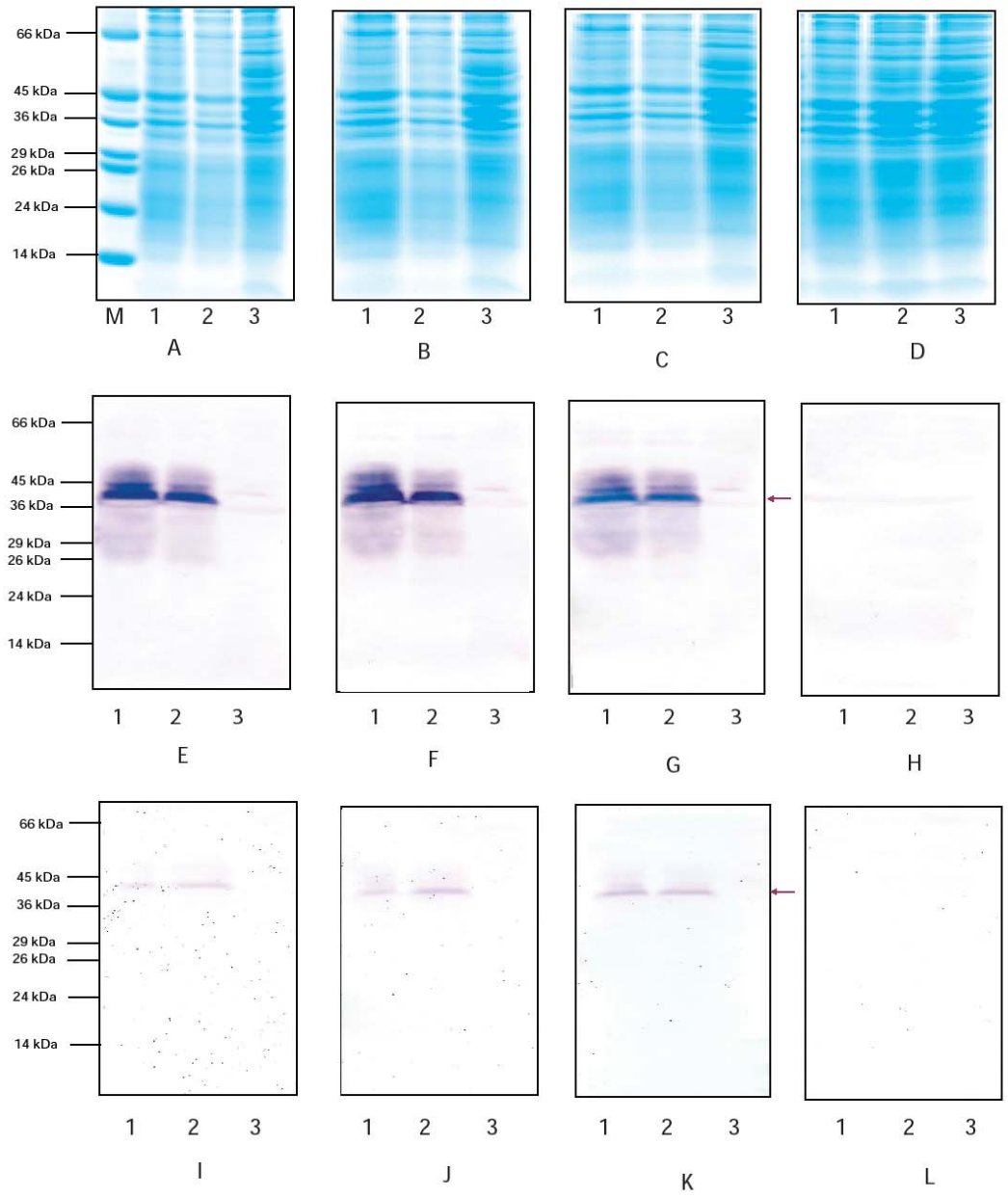


Figure 3.9. Over expression of protein from aphid clone P72F in pQE-30 and interaction in Western blots.

Whole cell extracts prepared from three separate clones of P72F: P72F-1 (A, E, I), P72F-2 (B, F, J) and P72F-3 (C, G, K) after 1 h of induction (1), 4 h (2), and 16 h (3). Extract from control non induced empty pQE-30 is indicated as D, H, L. The first panel (A-D) is Coomassie blue staining of protein, the second panel (E-H) is α-His western blots, and the last panel (I-L) is α-Myc western blots. M: Molecular size markers.

3.3.4. Interaction between aphid proteins and antisera to aphid cuticular proteins

Antisera against two *M. persicae* cuticular proteins expressed in *E. coli* characterized by Dombrovsky *et al*. (2007b), MPCP5 which contains an RR1 consensus (GenBank accession no. DQ108939), and MPCP2 containing an RR2 consensus (GenBank accession no. DQ108935), were provided by A. Dombrovsky (see section 3.4.3 for explanation of RR consensus sequences). For each protein, antisera were prepared against either native (N) or denatured (D) proteins. Western blots showed that both α-MPCP2 and α-MPCP5 reacted with multiple bands in whole cell extract (WCE) of *E. coli* preparations of the P72F clone, The α-MPCP2-D and α-MPCP5-N gave stronger interactions when inclusion body preparations were tested (Fig. 3.10). This may suggest that the secreted proteins of the aphid CUP clones are directed to inclusion bodies inside the *E. coli* cells. However, the main band of molecular weight about 36 kDa was also recognized in the induced proteins from the empty-vector transformed *E. coli*. This suggests that the antisera are interacting non-specifically with *E. coli* proteins of similar size to the identified P72F protein as the interaction was absent when α-His or α-Myc antibodies were used instead (Fig. 3.10).

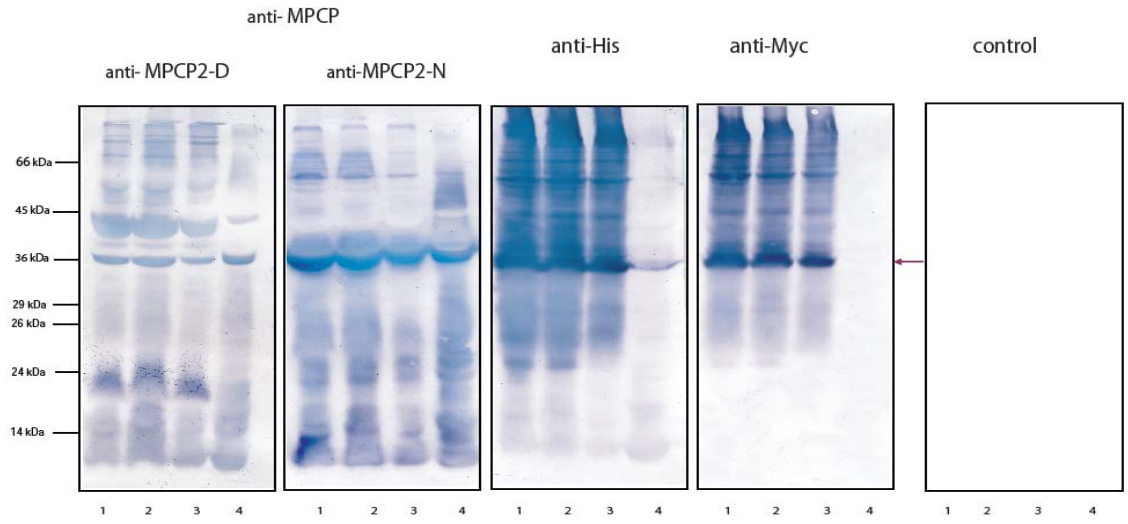


Figure 3.10. Western blots showing the reaction of inclusion bodies from P72F sub-clones with different antisera.

Lanes 1-4 are inclusion bodies prepared of three separate clones of P72F sub-clone and the empty pQE-30 vector, respectively. Control blot was incubated with α-rabbit-AP only. Positions of molecular size markers were shown on the left.

3.3.5. Interaction between aphid protein from clone P72F and PVY-HC-Pro expressed from PVX

Previously, functional full length PVY HC-Pro protein was expressed in *N. benthamiana* using PVX as vector (Sasaya *et al.,* 2000). We used this system to express the HC-Pro protein in the expectation that the HC-Pro expressed in plants might be more soluble than *E. coli* expressed protein and give higher yields. HC-Pro was expressed in tobacco and purified over Ni-NTA resin using a modified protocol as described in section 3.2.4.2.1. The yield was about 0.5 µg/ml (Fig. 3.11). The protein was concentrated about 20 times by decreasing the volume over PEG. Elution of HC-Pro from Ni-NTA resin is normally performed with 200 mM imidazole. However, when the concentration was increased 20 times, the imidazole concentration increased to 4 M. Thus dialysis in TSM buffer containing 200 mM imidazole and 5% sucrose was performed to remove the excess imidazole, which may block the biological function of HC-Pro (S. Blanc, INRA-CIRAD-SupAgro Montpellier, France, personal communication 2009).

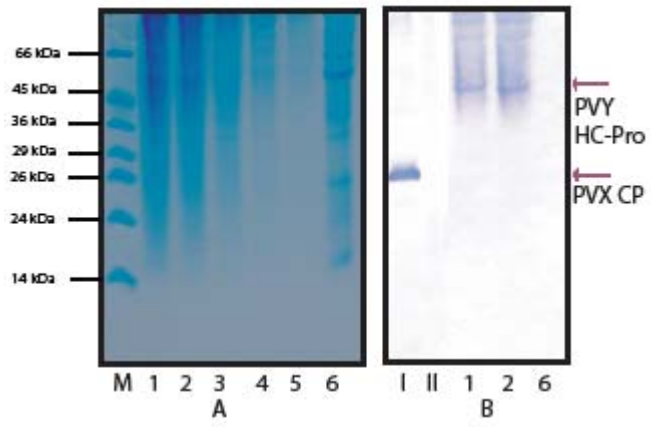


Figure 3.11. Expression of PVY-HC-Pro in the PVX system.

Panel A: Coomassie blue staining of different HC-Pro elution fractions, Panel B: Western blot of the HC-Pro preparation. I, II: leaf extract of PVX infected and healthy tobacco leaf sample incubated with α-PVY CP antiserum. 1, 2, and 6 incubated with α-HC-Pro.M: protein size markers. Lanes 1–5: HC-Pro elution fractions, lane 6: non-bound fraction.

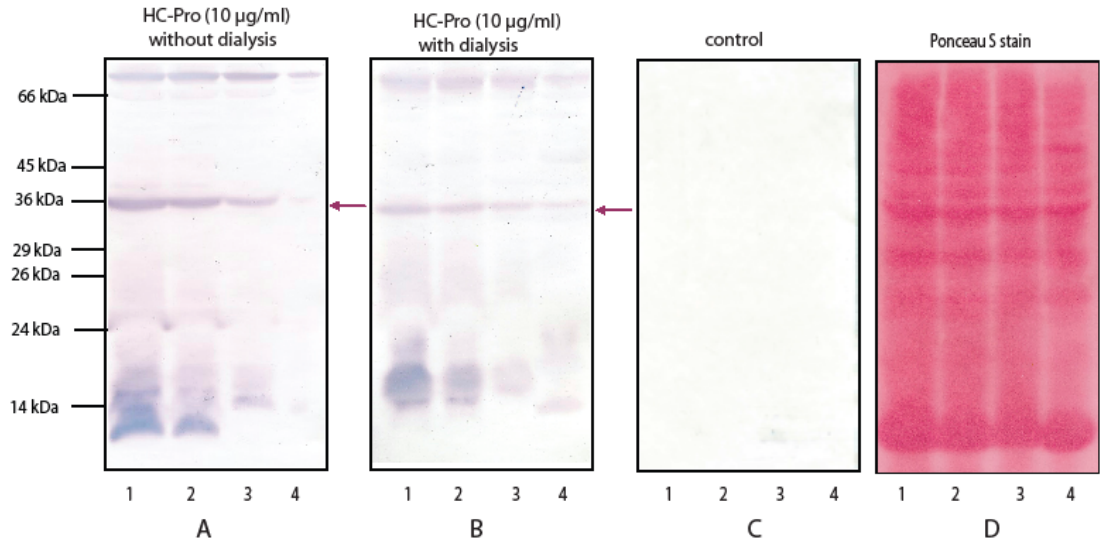


Figure 3.12. Western blots showing interaction of P72F protein with PVY HC-Pro expressed in PVX system.

Blots of protein extracted from inclusion bodies of P72F sub-clones and separated by SDS-PAGE were incubated with HC-Pro preparations: A, HC-Pro (10 µg/ml) purified on NI-NTA resin and PEG concentrated without dialysis; B, as A but with dialysis against 200 mM imidazole and 5% sucrose in TSM buffer; C, negative control incubated with α-HC-Pro + α-rabbit-AP; D, membrane stained with Ponceau S. Numbers 1-4 represent three replicates of P72F sub-clones and the empty pQE-30 vector respectively. Positions of molecular size markers were shown on the left.

Fig 3.12 shows that HC-Pro expressed from PVX in *N. benthamiana* interacted with the inclusion body preparations from the induced P72F clones. Interaction was with a protein of about 36 kDa in molecular mass, which is consistent with the result obtained with the α-MPCP, α-His, and α-Myc antisera (Fig. 3.10). No difference was observed when the concentrated HC-Pro was dialyzed to remove the excess imidazole (Blot B) compared with HC-Pro containing a high concentration of imidazole (Blot A). This finding suggests that imidazole has no harmful effect on HC-Pro biological activity and can be used at higher concentration to keep the protein in solution. In addition, HC-Pro interacted with protein products of smaller mass (14-26 kDa), which may represent degraded CUP protein products. Similar size bands were found when α-MPCP antisera were incubated with P72F protein (Fig. 3.10).

3.3.6. Experiments to study binding of P72F to HC-Pro of other potyviruses

Preliminary experiments were done to test interaction of TuMV HC-Pro and P72F protein, again a low yield of HC-Pro was obtained, approx. 0.5 µg/ml. Therefore, His-tagged HC-Pro belonging to another potyvirus (TEV) was purified from tobacco plants (Blanc *et al*., 1999; Ruiz-Ferrer *et al*., 2005) as presented in section 3.2.4.2.3. The yield of the HC-Pro was much greater (approx. 100 µg/ml), as shown in Fig. 3.13.

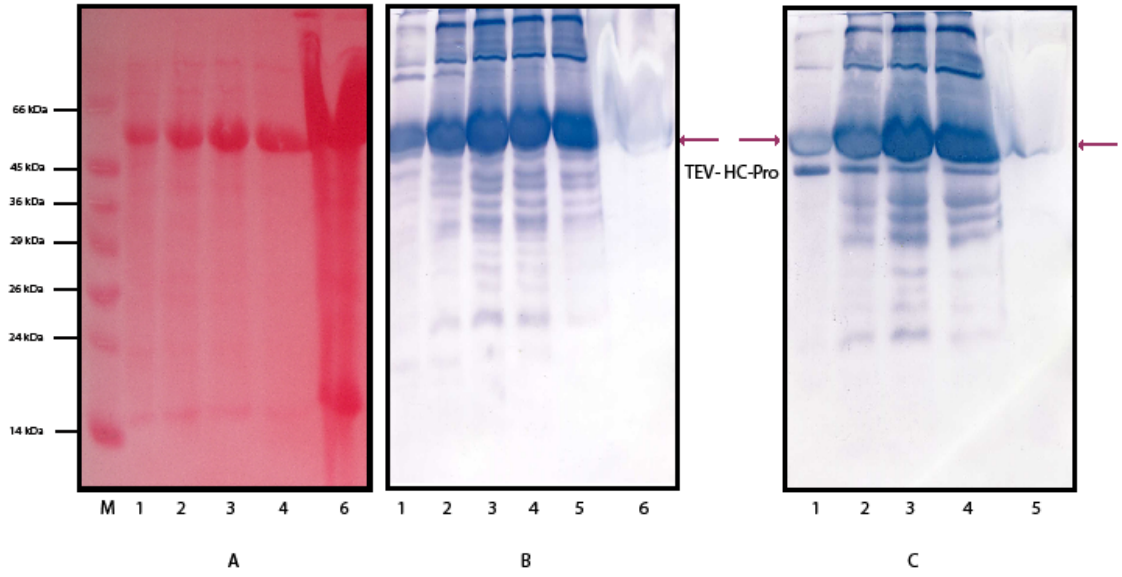


Figure 3.13. Preparation of His-tagged TEV HC-Pro from tobacco by elution from Ni-NTA resin.

Panel A: Ponceau S stained protein, Panel B: blot incubated with α-His, Panel C: blot incubated with α-TEV-HC-Pro. M: marker size markers, 1-5 HC-Pro elution fractions, and 6: non-bound fraction.

It was decided to use His-tagged TEV HC-Pro in all future experiments because of the high HC-Pro yield produced from this engineered clone. TEV HC-Pro was incubated with electroblotted aphid proteins prepared from the inclusion bodies of P72F sub-cloned in pQE-30. Fig. 3.14 suggests that TEV HC-Pro interacts with P72F IB protein preparations at the same position as did PVY HC-Pro derived from the PVX vector. There also appears to be a reaction with smaller mass products which may be degradation products, as seen before in Fig. 3.12 and in the interaction with anti-MPCP antisera (Fig. 3.10).

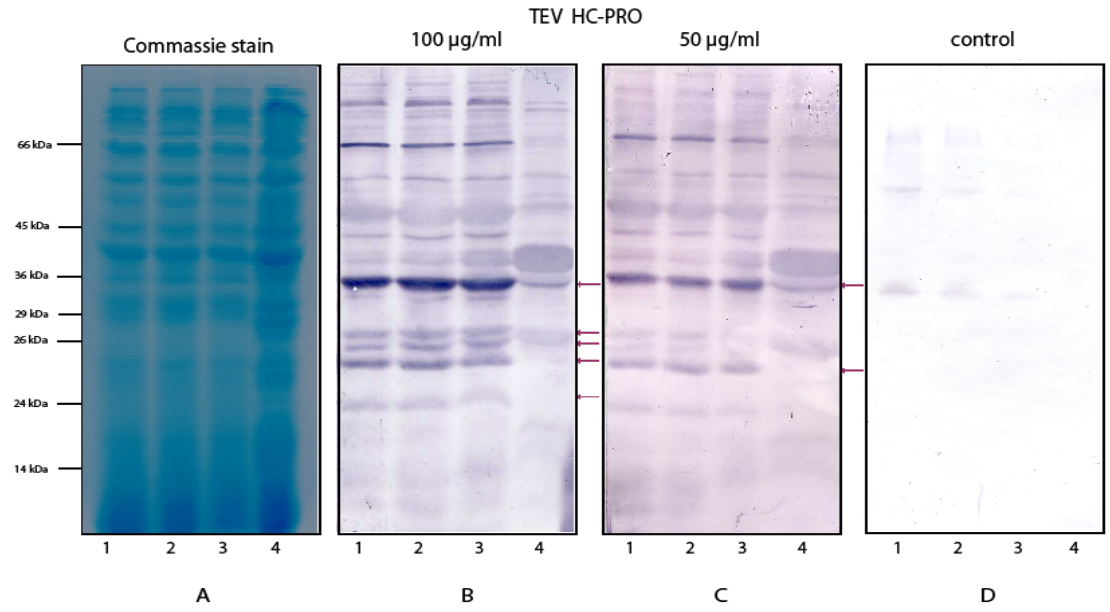


Figure 3.14. Interaction between P72F protein and the His tagged HC-Pro of TEV expressed in tobacco.

Panel A**:** Coomassie blue stain, panel B: blot was incubated with 100 µg/ml of purified TEV HC-Pro, panel C: blot was incubated with 50 µg/ml of purified TEV HC-Pro, panel D: blot was incubated exactly the same as in blots B and C but without HC-Pro.
Lanes 1, 2, and 3 represent inclusion bodies from three replicates of P72F clone, lane 4 represents empty pQE-30 vector.

Since different bands reacted with TEV HC-Pro, a further experiment was done to confirm that P72F protein preparations react at the same position as with PVY HC-Pro. A portion of the gel was stained with Coomassie blue and directly compared with sections of the same gel electroblotted and incubated with α-His, α-Myc or TEV HC-Pro preparation followed by α-TEV HC-Pro antiserum. The results (Fig. 3.15) indicate that the band of mass of 36 kDa does react.

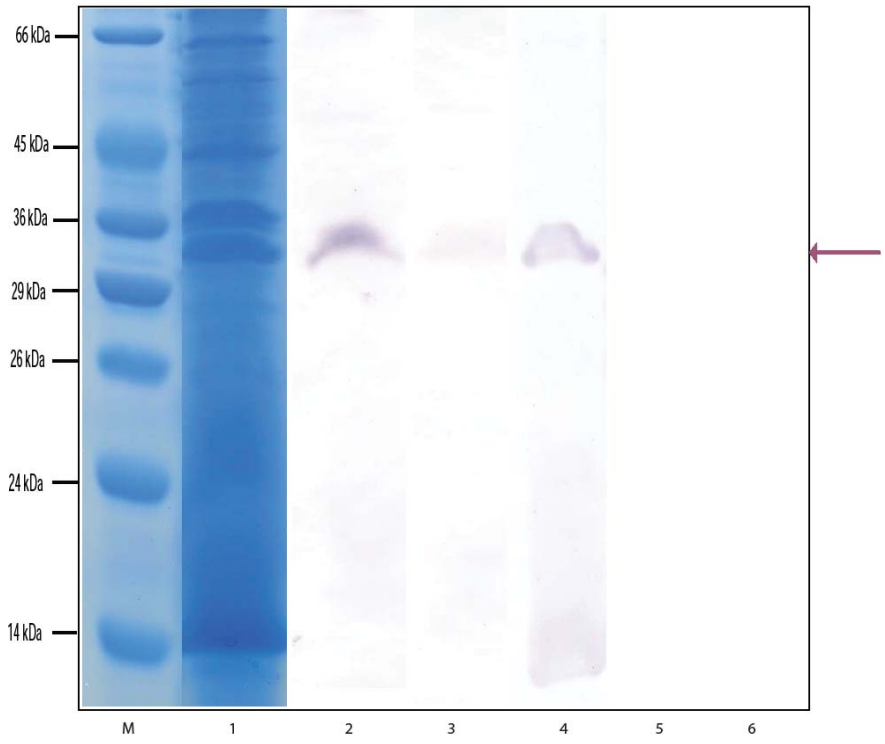


Figure 3.15. Comparison of P72F preparation stained with Coomassie blue or incubated with specific antisera.

M: Molecular size markers. Lane 1: Coomassie blue stain of expressed P72F inclusion body protein, lane 2: protein was incubated with α-His antibody, lane 3: protein was incubated with α-Myc antibody, lane 4: protein was incubated with TEV HC-Pro, lane 5: protein was incubated with α-mouse-AP only, lane 6: protein was incubated with α-TEV HC-Pro and α-rabbit-AP.

3.3.7. HC-Pro binding to native aphid proteins

CUP proteins were extracted from two different aphid species following the method published by Dombrovsky *et al.* (2007b). In addition to *M. persicae*, the poor PVY vector *M. dirhodum* was used.

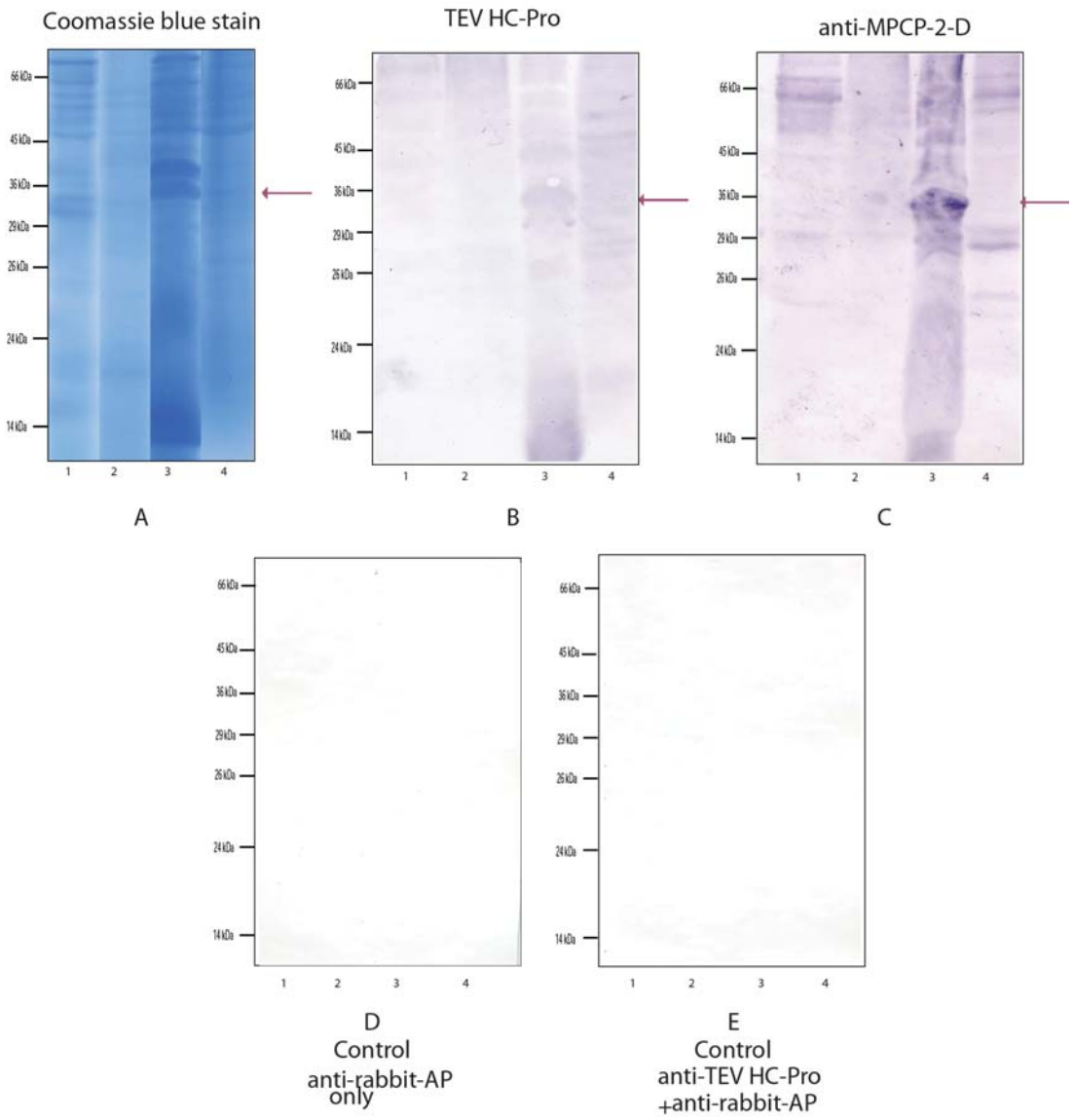


Fig. 3.16. HC-Pro binding to native aphid proteins

Panel A: Coomassie blue stain, panel B: blot was incubated with 2–3 months frozen TEV-HC-Pro (100 µg/ml), panel C: blot was incubated with α-MPCP-2-D antiserum, panel D: blot was incubated with α-rabbit-AP only, panel E: blot was incubated with α-TEV HC-Pro antiserum and α-rabbit-AP. Lane 1: native *M. persicae* CUP preparation, lane 2: native *M. dirhodum* CUP preparation, lane 3: Inclusion bodies of recombinant P72F protein, lane 4: whole *M. persicae* protein extract in SDS-PAGE buffer. Positions of molecular size markers were shown on the left.

Fig. 3.16 shows that α-MPCP-2-D antiserum interacted with different CUP bands extracted from both *M. persicae* and *M. dirhodum* (panel C, lanes 1, 2), and with protein prepared from the whole aphid extract of *M. persicae* (Panel C, lane 4). CUPs extracted from both *M. persicae* and *M. dirhodum* interacted weakly with TEV HC-Pro (panel B,

lanes 1, 2), but whole aphid extract prepared from *M. persicae* (Panel B, lane 4) interacted with HC-Pro at different positions.

3.4. Bioinformatic analysis of identified clones in NCBI

3.4.1. General view of HC-Pro interacting clones

All eight clones whose products were found to interact with N-HC-Pro preparations were sequenced (Table 3.1). The sequence data were subjected to a Basic Local Alignment Search Tool (BLAST) search via the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/). The programme BLASTX was used by selecting the WU-BLAST2 or the NCBI-BLAST2 protein databases. Sequence analysis by BLAST revealed that three of the selected clones (clones P72F, P817P and P94A) gave good matches with *M. persicae* cuticular proteins that have been previously characterized by Dombrovsky *et al*. (2003, 2007a). The remaining clones were exoskeleton protein (clone P820P), beta-tubulin (clone P515A), ATP citrate lyase (clone P136B), membrane protein (clone P58L) and serine/threonine-protein phosphatase (clone P1424E).

From this search it appears that P72F matches MPCP1 (91%) and MPCP3 (52%) RR2 cuticle proteins (Table 3.1). In addition, clones P817P and P94A, which appear to be duplicate clones, matched with MPCP5 (90%), which is an RR1 CUP. P820P clone gave a match with an RR3 CUP characterized from insects other than aphids.

A BLAST search of the EST sequence database gave a large number of EST matches to the P72F clone; interestingly two of the matched clones (ES451638.1, ES450274.1, 96% identity) were sequences from the *M. persicae* cDNA library which was used for the initial screening. This large number of matches may indicate that this gene is highly expressed or represents a multi-copy gene family. Similarly a BLASTN search gave many EST sequences which matched clone P817P, with one of them matching the ES450857.1 sequence (96% identity) from the aphid cDNA library used in this study. The rest were genes from different lineages of *M. persicae* and *A. pisum* aphids. Clone P820P gave matches to many homologues when searched with BLASTN using the non-human and non-mouse ESTs database. The first match (99% identity) is with ES450462.1, which is a sequence from the aphid cDNA library used in this study.

cDNA clone	Nucleotide search		Protein sequence search		
	EST match	**ID**	**EMBL protein match**	**Organism**	**ID**
P72F	*M. persicae* ES451638.1	96%	RR2 cuticle protein/ Q95V16_MYZPE (MPCP1, Dombrovsky *et al.*, 2003)	*Myzus persicae*	91%
	M. persicae ES450274.1	96%	RR2 cuticle protein/Q45V97_MYZPE (MPCP3, Dombrovsky *et al*., 2007a)	*M. persicae*	52%
P817P	*M. persicae* ES450783	97%	RR1 cuticle protein/Q45V94_MYZPE (MPCP5, Dombrovsky *et al*., 2007a)	*M. persicae*	99%
	M. persicae ES450857.1	96%			
P94A	*M. persicae* ES450783	97%	RR1 cuticle protein/ Q45V94_MYZPE (MPCP5, Dombrovsky *et al*., 2007a)	*M. persicae*	90%
P820P	*M. persicae* ES450462.1	99%	Exoskeleton protein (Nousiainen *et al*., 1998)	*Homarus americanus*	66%
			Full cuticular protein 111, RR-3 family (Nousiainen *et al*., 1997)	*Anopheles gambiae*	72%
P515A	*M. persicae* ES224251.1	95%	*A. pisum* tubulin beta-1 EMBL: ADI24738 .1	*A. pisum*	95%
P136B	*A. pisum* FF330144.1	90%	Putative uncharacterized protein (Richards *et al.*, 2008)	*Tribolium castaneum*	78%
			ATP-citrate synthase (Nene *et al*., 2007)	*Aedes aegypti*	75%
			ATP citrate lyase, isoform C (Adams *et al*., 2000)	*Drosophila melanogaster*	75%
P1424E	*A. pisum* FF332657.1	88%	Serine/threonine-protein phosphatase (Carninci *et al*., 2005)	*Mus musculus*	74%
			Serine/threonine-protein phosphatase (Ghedin *et al.*, 2007)	*Brugia malayi*	73%
P58L	*M. persicae* EE571212.1	95%	Conserved *Plasmodium* membrane protein (Gardner *et al.* ,2002)	*Plasmodium falciparum*	28%

Table 3.1. Results of BLAST sequence searches with clones selected after screening aphid library against N-terminal HC-Pro.

ID: identity of the sequence, cDNA clone: clones from the cDNA expression library that interacted with HC-Pro.

3.4.2. Sequence alignment of CUP clones with protein matches

The sequence of each selected clone (P72F, P817P, and P820P) was compared against other protein databases (EMBL, NCBI) and the closest matches were aligned together using the ClustalW multiple alignment programme. The alignments are presented in Figs. 3.17, 3.18, and 3.19.

The closest protein matches with clone P72F obtained from sequence databases (EMBL, NCBI) were aligned together (Fig. 3.17) using the ClustalW multiple alignment programme through the BioEdit program or on the EMBL website. There is a high level of identity among different sequences and the P72F clone. Fig. 3.18 shows that clone P817P has a high level of identity with both aligned proteins. In contrast, clone P820P is less similar to the best matching proteins than are the other two cuticle proteins (P72F, P817P; Fig. 3.19).

In addition, protein sequences of the three selected aphid clones were compared with the other *M. persicae* cuticle proteins characterized previously by Dombrovsky *et al.* (2003, 2007a). The published cuticle proteins designated MPCP, MPCP1, MPCP2, MPCP3, MPCP4, and MPCP5 with accession numbers and some properties are presented in Table 3.2. Sequence analysis of *M. persicae* CUPs (Table 3.2) revealed that they share some motifs at the N-terminus (Fig. 3.17, Fig. 3.18), but they are more likely to be distinct different protein particularly clone P72F.

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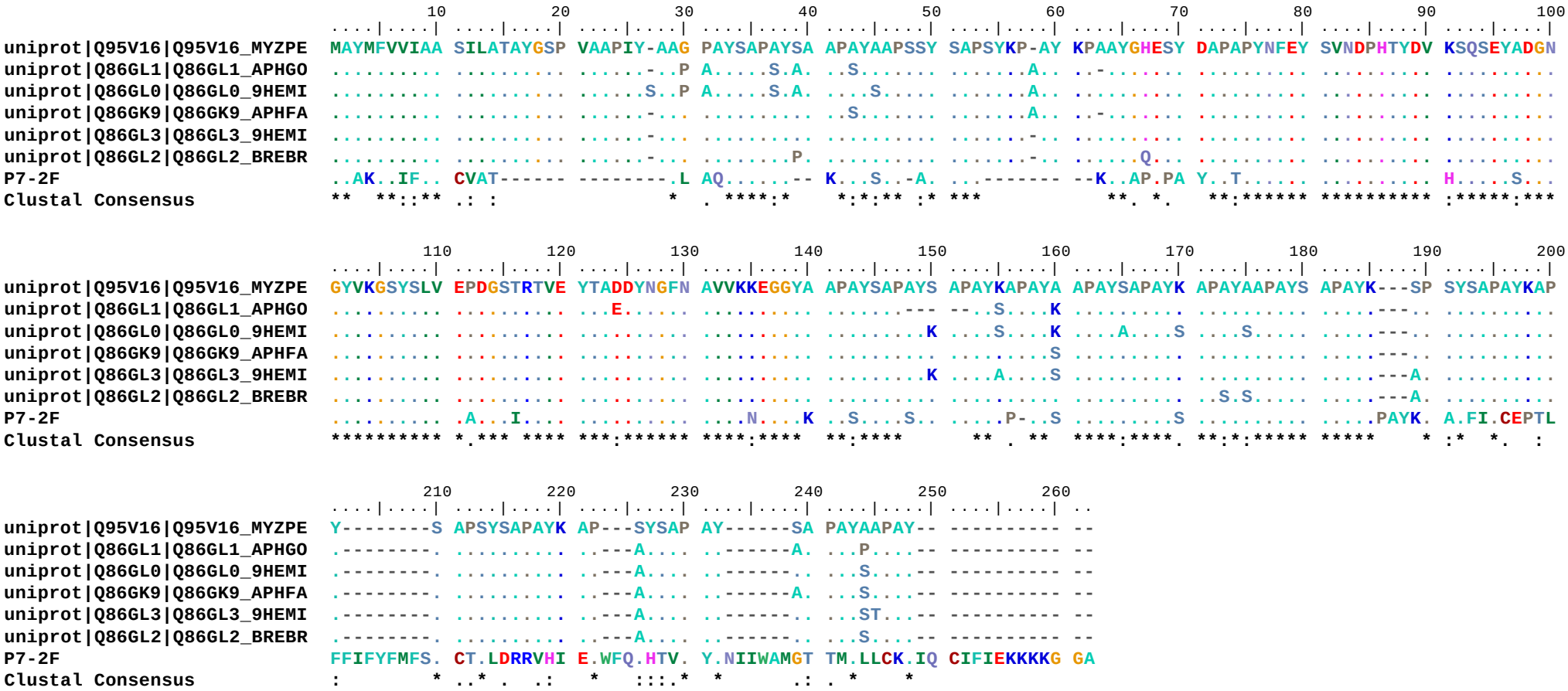


Figure 3.17. Differences and similarities in protein sequences between the P72F clone and the top protein matches in BLAST.

Amino acids are colour coded according to properties. Amino acids identical to those in the reference sequence are represented by dots; gaps are represented by dashes.
* indicates identity, and dots indicate alteration in conserved sequence

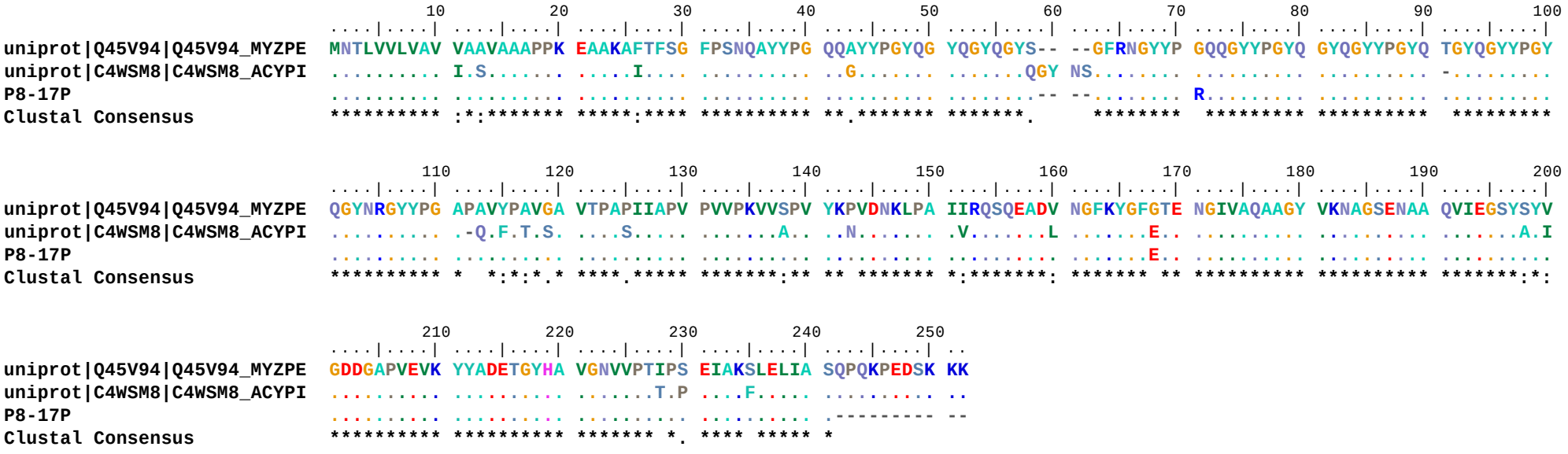


Figure 3.18. Alignments of the protein sequences of the P817P clone with the closest two matches by BLAST.

Amino acids are colour coded according to properties. Amino acids identical to those in the reference sequence are represented by dots; gaps are represented by dashes.
* indicates identity, and dots indicate alteration in conserved sequence.

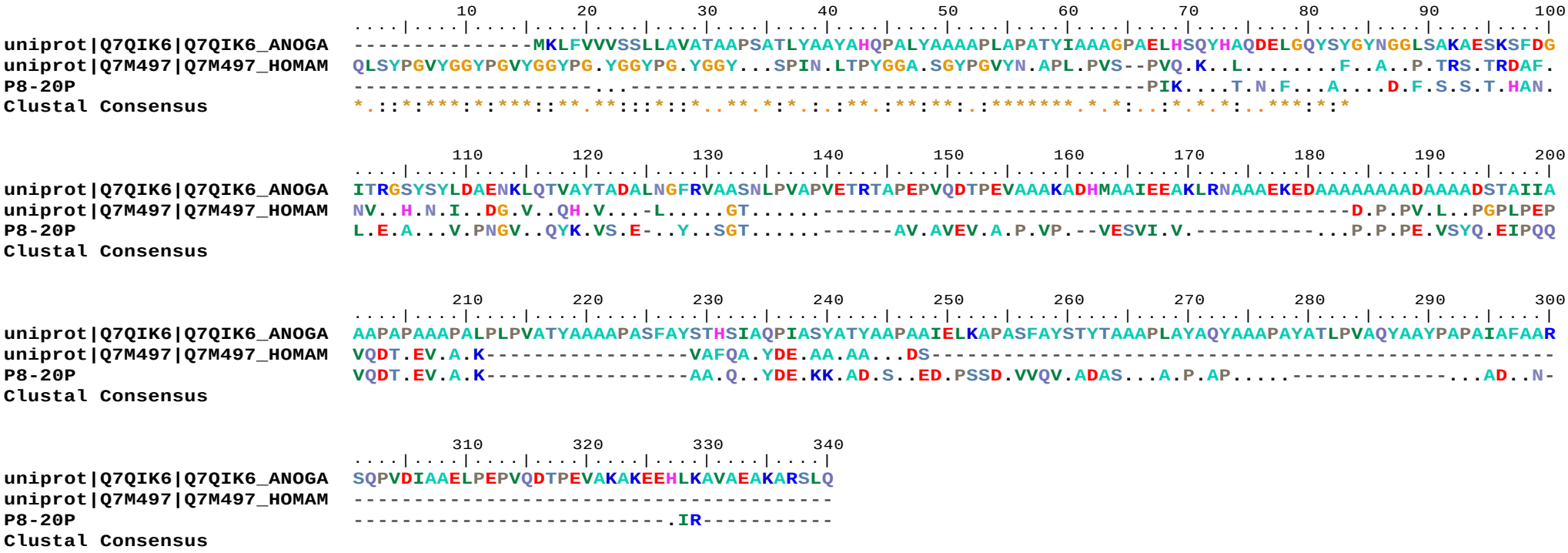


Figure 3.19. Protein sequence alignments of the P820P clone with the two closest matches in BLAST.

Amino acids are colour coded according to properties. Amino acids identical to those in the reference sequence are represented by dots; gaps are represented by dashes.
* indicates identity, and dots indicate alteration in conserved sequence.

Cuticle protein name	EMBL Accession number	UniProt Accession number	R&R type	Deduced amino acids	Estimated mass
MPCP	DQ108937	Q45V96	None	118 aa	13.2 kDa
MPCP1	AF435075	Q95V16	RR2	226 aa	25 kDa
MPCP2	DQ108935	Q44V98	RR2	228 aa	24.3 kDa
MPCP3	DQ108936	Q45V97	RR2	205 aa	22.7 kDa
MPCP4	DQ108938	Q45V95	RR1	135 aa	14.2 kDa
MPCP5	DQ108939	Q45V94	RR1	248 aa	26.49 kDa
P72F	NA	NA	RR2	186 aa*	20.0 kDa*
P817P	NA	NA	RR1	247 aa	26.6kDa
P820P	NA	NA	RR3	193 aa	19.9 kDa

Table 3.2. Information about the selected *M. persicae* cuticle proteins.

*The sequence of the cloned insert into the PQE vector, the actual protein size will be 23.2 kDa because of the added sequences of the added and Myc tags (See Fig. 3.8).

3.4.3. The R & R consensus sequences of selected clones

Conserved motifs in the protein sequence were confirmed to be a characteristic of cuticle proteins of insects and are referred to as R & R (for Rebers-Riddiford) consensus sequences (Rebers and Riddiford, 1988). There are several types of these conserved regions which are named RR1, RR2, and RR3 (Andersen, 1998). The function of these conserved sequences was shown to be binding to chitin (Rebers and Willis, 2001; Togawa *et al*., 2004), which may enhance the physical properties of the aphid cuticle. Dombrovsky *et al.* (2007a) compared the insect RR consensus with the *M. persicae* cuticle proteins that they characterized. They found that MPCP4 and MPCP5 have the RR1 consensus, but the motifs are not identical to those reported generally in insects. Moreover, MPCP5 shows some differences in characteristic motif within the RR1 consensus: a substitution from P to D in the PDG motif, the QP motif is absent, and there is a substitution from N to T in the ADENG motif. P817P shows some similar changes which may indicate differences in the RR1 sequence between *M. persicae* and other insects. MPCP2 and MPCP3 were reported to have a conserved RR2 motif, and the identified P72F was grouped in the same category. However, there are many differences in amino acid sequence between the MPCP proteins and the P817P protein and between both proteins and the published RR2 sequence (See Figures 3.20, 3.21, and 3.22).

In agreement with Dombrovsky *et al*. (2007a), the P72F protein, which was classified as an RR2 protein, contains three domains. The N-terminal region of 58 amino acids contains the YSAP motif, which was reported to be unique for cuticle protein isolated from *M. persicae* (Dombrovsky *et al.*, 2007a). The second domain of the P72F protein is a central domain that consists of 73 amino acids and represents the RR2 consensus. The C-terminus of P72F exhibits PSYSA and AYSAP repeats resembling those reported to exist at the C-terminus of MPCP1, which is a previously characterized RR2 CUP (Dombrovsky *et al.*, 2007a). The P817P clone has an identical sequence to MPCP5 at the N-terminus starting with MNTLVVLVAVVAAVAAAPPKEAAKAFTFSGFPSNQ, a central area with identical sequence to MPCP5, and a C-terminal region containing the RR1 consensus. Clone P820P has the third type of conserved R & R sequence known as RR3. Cuticular

proteins having this type of R & R sequence have never been reported in aphid species. However, it was reported to occur in other insects such as the mosquito.

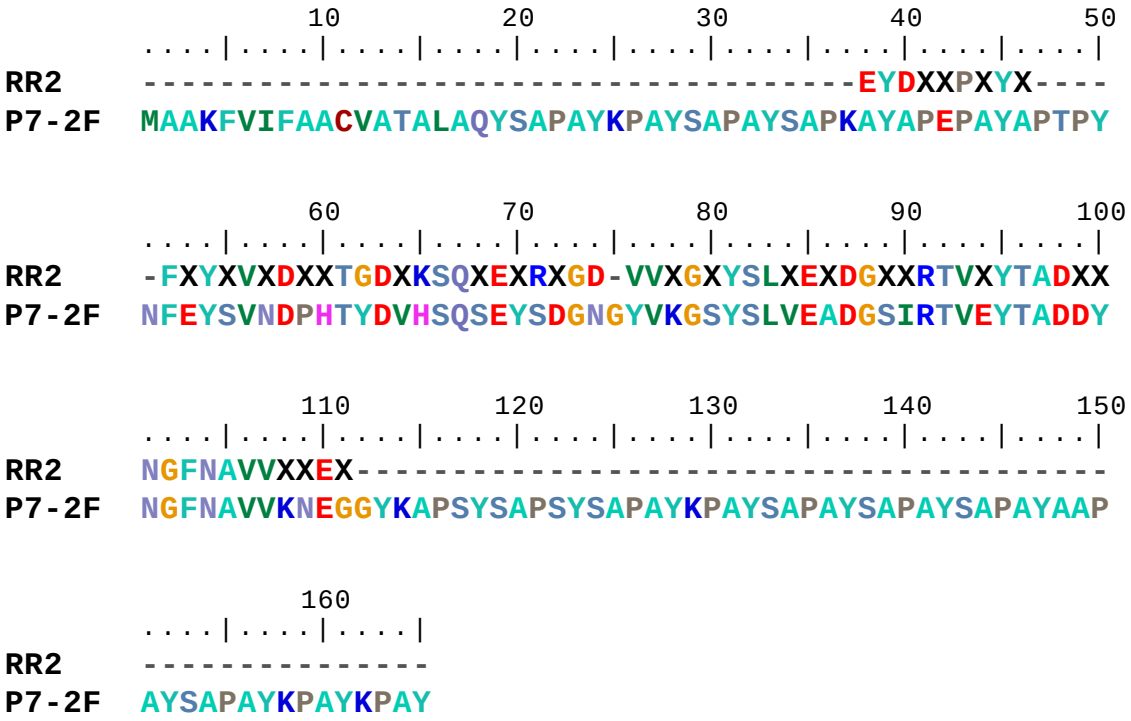


Figure 3.20. RR2 consensus of the P72F Mp protein and the top match of *M. persicae* CUPs

Similarities between the RR2 consensus sequence and the MPCP1 and P72F sequence were found by alignment of the protein sequences with the published RR2 sequence using the ClustalW multiple alignment programme. Amino acids are colour coded according to properties.

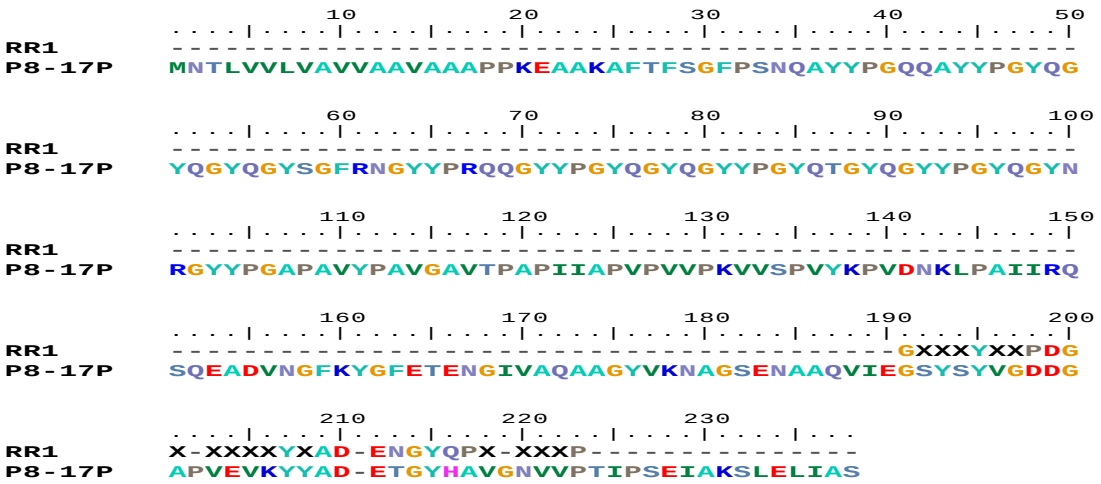


Figure 3.21. RR1 consensus of the P817P Mp protein and the top match of *M. persicae* CUPs

The P817P and published MPCP5 sequences were aligned with the RR1 consensus published for insects using the ClustalW multiple alignment programme. Amino acids are colour coded according to properties.

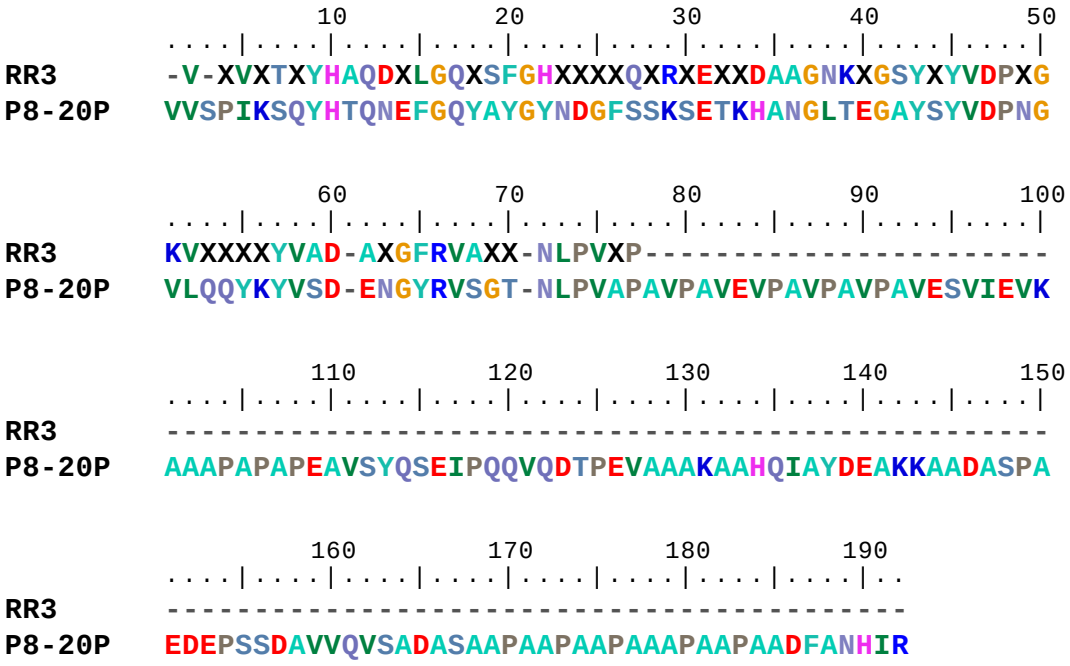


Figure 3.22. RR3 consensus of the P820P aphid protein clone

The P820P sequence and the published RR 3 consensus were aligned using the ClustalW multiple alignment program. Amino acids are colour coded according to properties.

3.5. Bioinformatics analysis of CUP sequences in the aphid genome

The pea aphid (*A. pisum*) genome sequence was published in April 2010 (The International Aphid Genomics Consortium, 2010). A further bioinformatics analysis on the sequence of the three CUP clones which I isolated was done using the aphid genome database: http://www.aphidbase.com/aphidbase/. Screening the pea aphid genome can help identify gene sequences that are related to enquiry sequence and which may be difficult to clone or identify in expression libraries. It is also useful to help identify members of closely related genes (such as gene families).

3.5.1. Clone P72F

Searching the aphid genome database using the BLASTN programme and Scaffolds (nuc.) databank option showed many scaffold matches. The top match was a region of EQ126624.1, (100% identity, E-value = 0) is presented as an example in Fig. 3.24. This genome region contained three CUP sequences repeated one after the other. The sequences of these genes was almost identical (See Fig 3.25 (three of these sequences correspond to the genes on EQ1266241 are: XM_001951994, XM_001951976, and XM_001951964). Using the reference annotation option (transcript), sequences coding for three homologous *A. pisum* genes which are similar to cuticular proteins were identified in different locations in the EQ126624.1 sequence (Fig. 3.23). These represent a multigene family repeated in tandem and each sequence is almost identical to the next. As a result some ESTs are artefactually being mapped across all three genes. BLASTX search results for EQ126624.1 presented in Fig 3.23 show that the predicted gene products from this scaffold sequence are equivalent to *M. persicae* CUP proteins (MPCP1, MPCP3) characterized previously (Dombrovsky *et al.*, 2003, 2007a). The sequence corresponding to one of the putative CUP protein matches (XM_001951994) is illustrated in Fig 3.24, which shows that the gene contains two exons separated by a small intron. This structure is identical in the other two genes as shown in Fig. 3.23.

All of the nucleotide sequence matches found from the Scaffolds search were aligned together with the P72F sequence and presented in Fig. 3.25. It is clear that the identified clone shares an identical 5′ terminal sequence with the first three matches. However, there are differences in the sequence at the 3′ end (Fig. 3.25). Similarly, protein sequences of the first three matches were also compared (Fig. 3.26). It is clear from Fig 3.26 that the P72F clone shares an almost identical protein sequences with the top putative CUP sequence of *A. pisum* in the N-terminal part of the protein when the frame shift is modified which suggest a frameshift error in the database sequence.

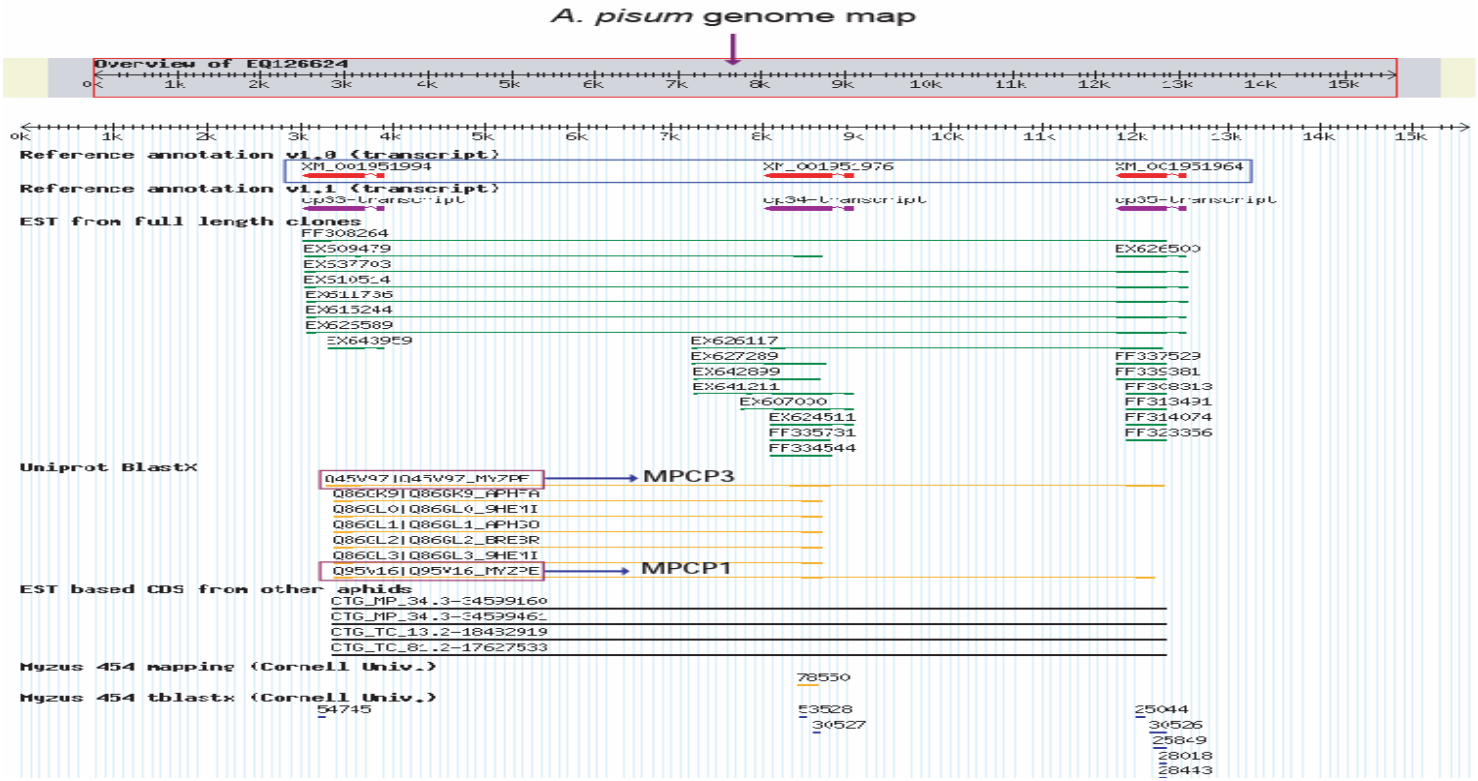


Figure 3.23. EQ126624.1, the top match to P72F using the Scaffolds (nuc.) databank and the BLASTN programme on the aphid genome database website.

Blue rectangle highlights the positions in the *A. pisum* genome of the three matches to P27F. The purple rectangles highlight matches with *M. persicae* proteins. The thick red lines in the reference annotation indicate exons and the thin red lines introns. The direction of transcription is indicated by the taper on the exon.

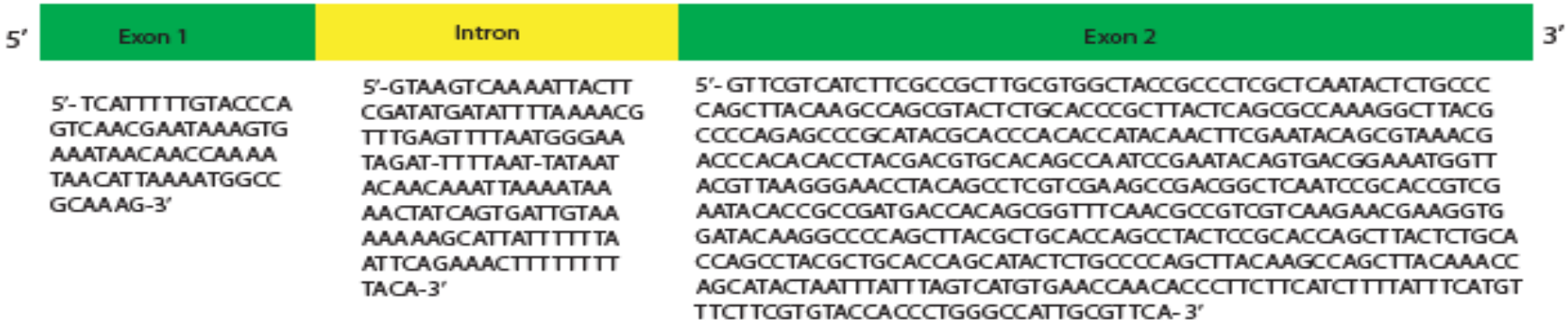


Figure 3.24. Gene map and sequence of the top Scaffolds match (XM_001951994).

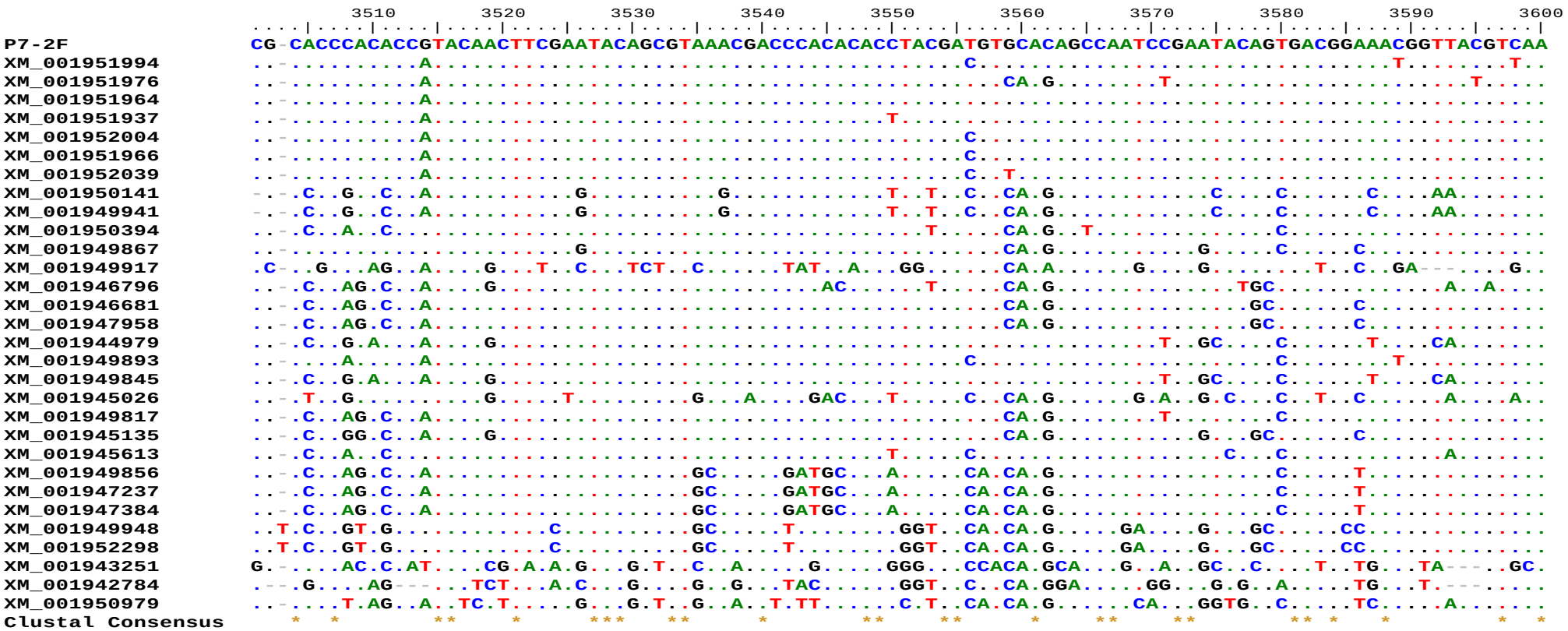


Figure 3.25. Similarity in nucleotide sequence between putative CUP sequences from the *A. pisum* genome and the identified P72F clone from *M. persicae.*

Nucleotide sequence of genes for CUPs in the *A. pisum* genome database found to match with the P72F sequence (30 sequences) were aligned together. Identical nucleotides are represented by dots. Aligned sequence represents the region of high similarity between genes.

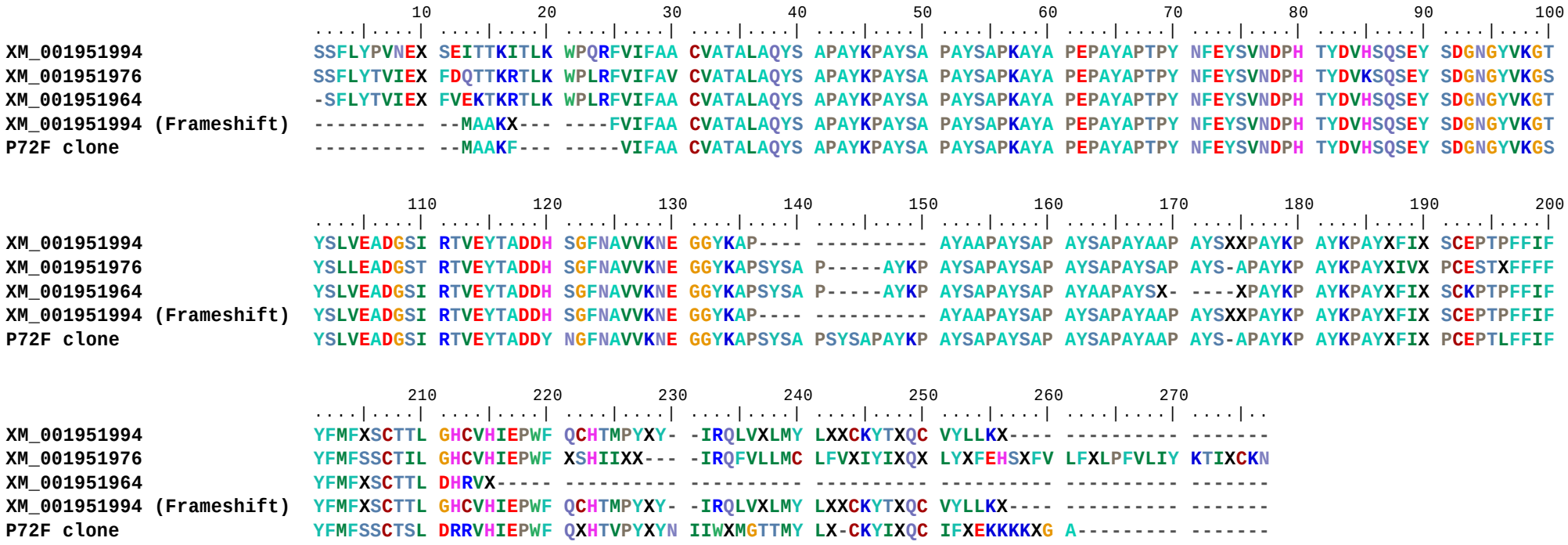


Figure 3.26. Protein alignments of the P72F protein sequence with the top three scaffold matches in BLAST using the ClustalW multiple alignment program and BioEdit.

Amino acids are colour coded according to properties. Dots represent identical amino acids, tildes represent gaps, and asterisks represent stop codons. The alignments include the sequences before the starting methionine.

3. 5. 2. Clone P817P

Searching against the aphid genome database and using the same options as in the P72F search found only a single gene match located on the EQ124291.1 scaffold (90% identity, E-value e-166). This scaffold is presented in Fig 3.28.

Using the reference annotation option, three matches (one is a match the other two it is coincidence) were found to be similar to cuticular proteins in the RR1 family. Only one of these genes (XM_001951031) was a match for P817P.

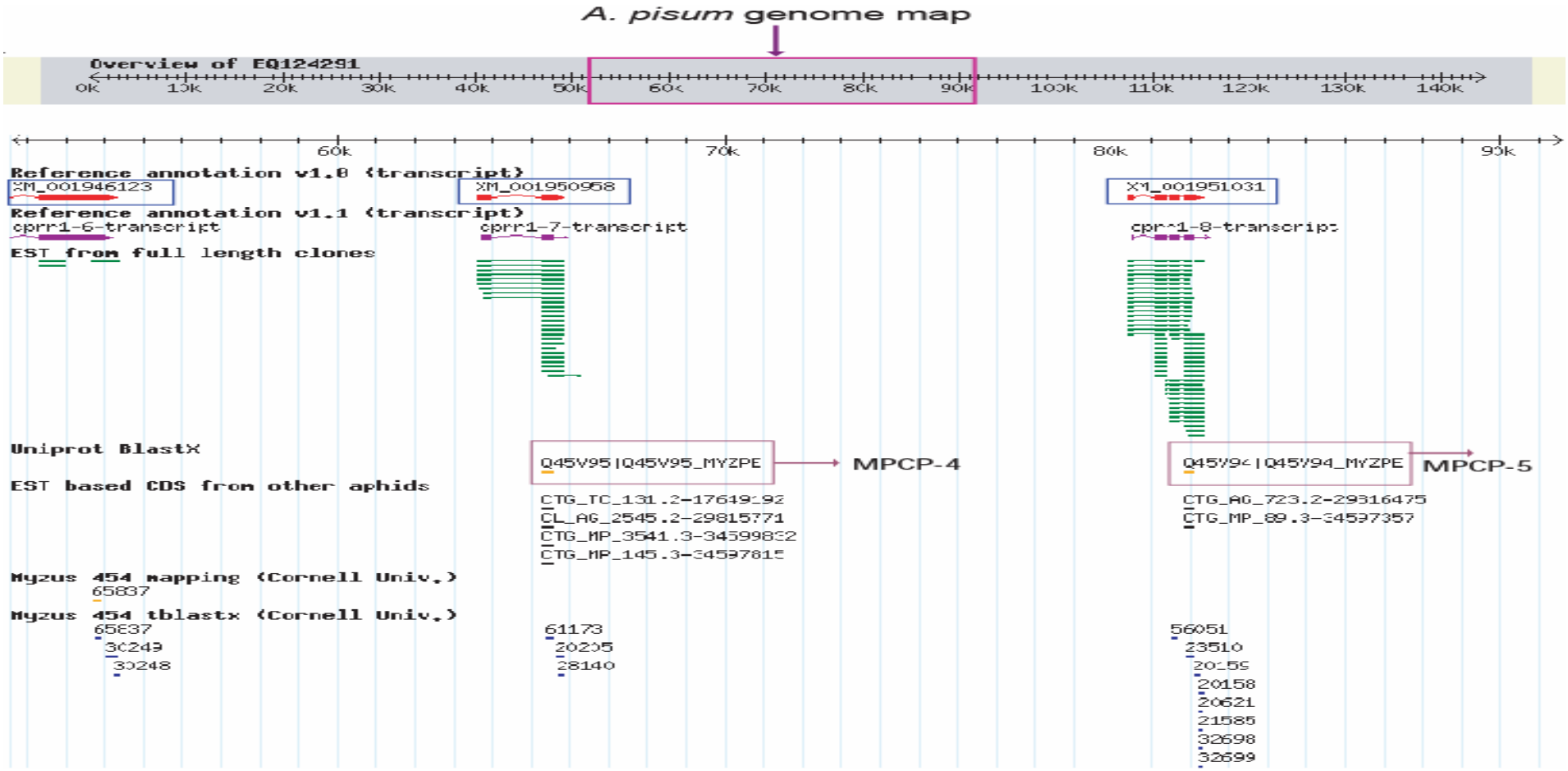


Figure 3.27. EQ124291.1, the only match to P817P using the Scaffolds (nuc.) databank and the BLASTN programme on the aphid genome database website.

Blue rectangle highlights the positions in the *A. pisum* genome of the three matches to P817P. The Purple rectangles highlight matches with *M. persicae* proteins.

3. 5. 3. Clone P820P

Similarly to the result found with the P817P clone, only a match to scaffold sequence EQ124381.1 (94% identity, E-value = 4e-93) was obtained (Fig. 3.28) when using the aphid genome database, and by searching with the BLASTN programme and Scaffolds (nuc.) databank option, which suggests that this gene(XM_001950803) is unique

Also when using aphid genome database, only one protein match was verified for the P820P clone. This match corresponds to the full cuticular protein 111 of the RR-3 family (Nousiainen *et al*., 1998) of *Anopheles gambiae*.

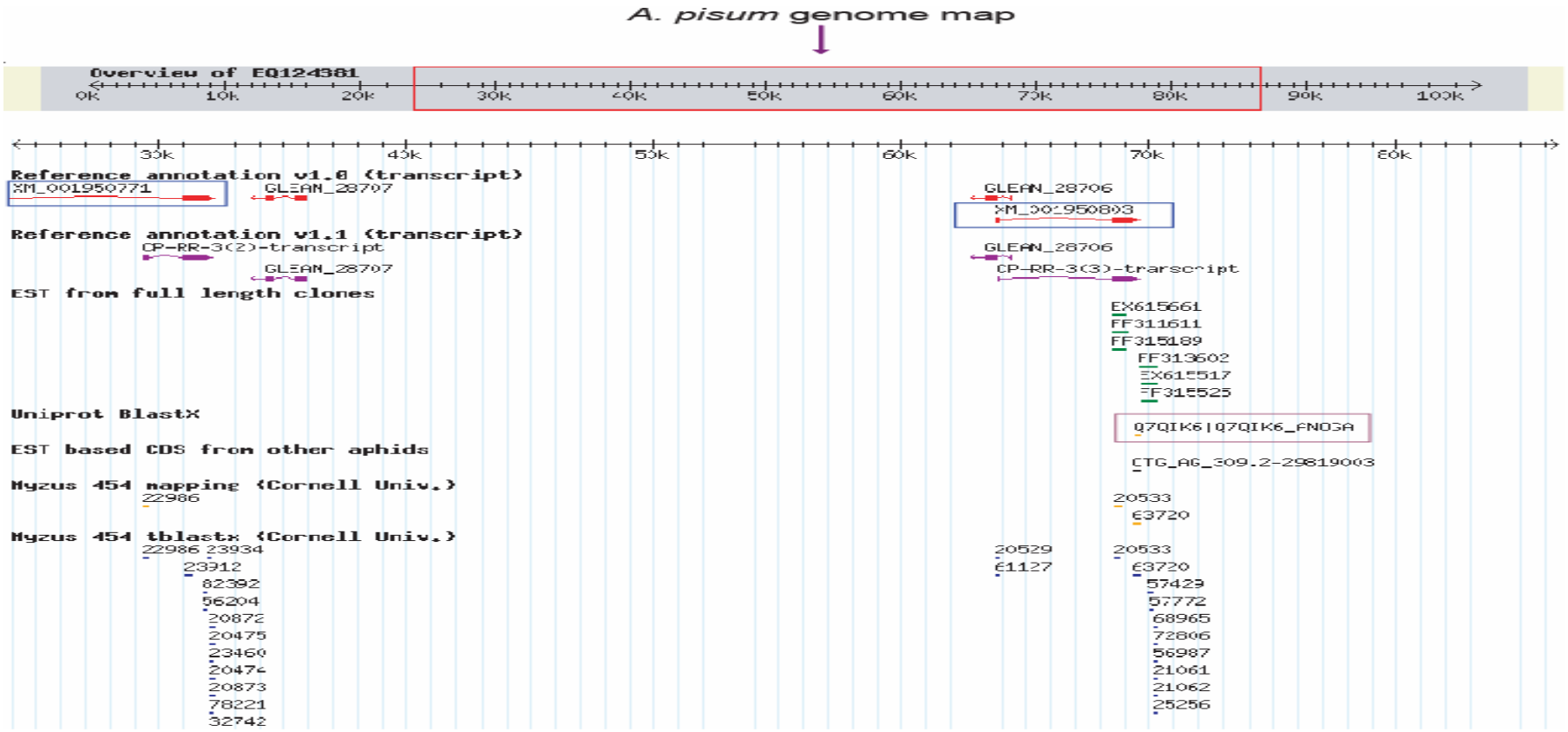


Figure 3.28. EQ124381.1, the only match to P820P) using the Scaffolds (nuc.) databank and the BLASTN programme on the aphid base website.

Blue rectangles highlight the positions in the *A. pisum* genome of the two matches to P820P. The purple rectangle highlights the protein match obtained through aphid genome database search.

3.6. Discussion

In this chapter, the Q-Bot high density filter screening method proved to be efficient in screening large numbers of clones in a short time. Using this system, products of eight EST clones within an aphid cDNA library were identified to interact *in vitro* with the N-terminal part of PVY HC-Pro expressed in bacteria. These identified clones belong to different groups, including aphid CUPs, other *A. pisum* proteins of unknown function, and some enzymes. This apparent diversity in HC-Pro interactions may reflect the multifunctional nature of this viral protein reported previously (reviewed by Maia and Bernardi, 1996). Since aphid CUPs were reported to be the most likely candidates to be the aphid receptors (Dombrovsky *et al.*, 2007b; Uzset *et al.*, 2007), three of these identified clones that represented different RR repeats were selected to sub-clone into the expression vector pQE-30 to express the proteins with epitop tags, in order to investigate their interaction with different antisera and with HC-Pro. The molecular mass of the protein that interacted with specific antisera was greater than the deduced molecular weight. However, CUP proteins when separated on SDS-PAGE have been reported to migrate 20–40% more slowly than expected (Cox and Willis, 1987; Andersen *et al*., 1995; Dotson *et al*., 1998; Rebers and Willis, 2001). This discrepancy between the migration of proteins in SDS gels and their predicted size from amino acid sequence was not reported by Dombrovsky *et al.*, (2007b) when they expressed *M. persicae* protein in *E. coli*.

On the Q-Bot filter, HC-Pro was found to interact with three different CUP proteins; this interaction was confirmed with one of these, P72F. There were differences in the results using different assays and when the clones were expressed in different vectors; in particular, weak and inconsistent results were obtained using the PVY HC-Pro preparations. The discrepancy can be explained on the basis of the sensitivity of the protein assay. On the Q-Bot printed filters, the size of the colony is very small; therefore a very small amount of protein would be enough to interact with the aphid protein. In contrast, using Western blotting, larger amounts of protein are employed and consequently more HC-Pro is required to differentiate between colours that result from interaction and those that result as a background. Therefore, HC-Pro expressed in plants from PVX was tested to try to improve yields of HC-Pro.

It was reported that HC-Pro of one potyvirus may help transmission of other potyviruses (Harrison and Robinson, 1988). This fact was exploited by using the HC-Pro of other potyviruses. TuMV HC-Pro can be readily purified from virus-infected leaf material using Ni-NTA Resin even though it does not contain a hexa-histidine tag (Kadouri *et al*., 1998). Although TuMV HC-Pro was reported to be purified readily by Ni-NTA resin (Kadouri *et al*., 1998), the quality of the preparation purified by these methods was poor in my hands. In addition, the absence of a specific antiserum against this TuMV HC-Pro restricted its usage in our overlay assay system as the expressed aphid protein has a 6-histidine residue, and the purified TuMV HC-Pro has the same tag.

M. persicae can transmit different potyviruses. Moreover, PVY HC-Pro was reported to assist transmission of TEV (Pirone, 1981); thus it was anticipated that HC-Pro derived from TEV may interact with the identified CUP clones. An engineered TEV clone with a 6-histidine tag to assist purification of protein (Blanc *et al.*, 1997) was used. The yield of HC-Pro expressed using this system was high enough to initiate recognizable interaction between the electroblotted P72F protein and purified HC-Pro protein. This result is in contrast to that reported by Dombrovsky *et al*. (2007b), who did not detect any interactions between aphid proteins expressed from a cDNA library and ZYMV HC-Pro. Therefore, they adopted an alternative method to isolate protein from the whole aphid body. By using this approach they confirmed an interaction of CUPs of *M. persicae* with HC-Pro of ZYMV. The isolated proteins were confirmed to interact with antiserum raised to cuticle proteins characterized from a cDNA library. They suggested that the characterized cuticle protein from the cDNA library may not exist in the aphid stylet (the proposed location of the virus receptors), or that cuticle proteins expressed in bacteria differ in binding properties from those extracted from the aphid body. In contrast to the first explanation, Csikos *et al*. (1999) reported that CUPs of insects are not restricted to the organ they are synthesized in as these proteins are mobile inside the insect body. The inconsistency between our result and what was reported by Dombrovsky *et al*. (2007b) can be explained by the fact that although PVY and ZYMV are both potyviruses, HC-Pro belonging to each virus may have different binding properties as the conserved region in the ZYMV HC-Pro is KLSC not KITC.

It has been some time since Bradley and Ganong (1955) first reported that non-persistent viruses are retained at the distal part of the aphid vector stylet, although the methodology by which the result was obtained was criticized because of the physical

damage that may have been caused to the aphid stylet when it was treated with formaldehyde. However, it now seems more likely that this conclusion is valid. This is particularly true after the report by Uzest *et al*. (2007) that confirmed that virus particles of the semi-persistent virus CaMV are retained in the distal part of the *A. pisum* stylet. Semi-persistent viruses were first considered together with viruses that have a non-persistent mode of transmission (Watson and Roberts, 1939). Traditionally it has always been thought that non-persistent viruses are retained inside the aphid stylet (stylet-borne), whereas semi-persistent viruses are retained in the foregut of the aphid's mouthparts (foregut-borne). However, there were more recent reports which indicated that non-persistent viruses were retained in various locations of the food canal (Berger and Pirone, 1986; Wang *et al*., 1996). Recently, Uzest *et al*. (2010) proposed the terminology of the "acrostyle" at the tip of aphid stylet. The acrostyle binds to specific antisera raised against a peptide of 16 amino acids (GSYSLLEADGSTRTVE), termed pepL. This peptide represents the RR2 conserved motif in 20 *A. pisum* CUPs. Clone P72F, which was identified in this study, shares the same RR consensus and contains the sequence GSYSLVEADGSIRTVE, which is almost identical to the pepL sequence; there are only two differences between the two sequences, substitutions from L to V and from T to I at positions 5 and 10, respectively. In addition, clone P817P, which has the RR1 motif, was found to have a sequence (GSYS) which matches pepL at the N-terminus. This resemblance may suggest a more specific binding motif inside the conserved RR2 motif itself. Taken together with those reported by Uzest *et al*. (2007, 2010), my findings strongly support the concept of a receptor-like structure of a cuticle nature which mediates potyvirus transmission by aphid vectors.

The binding of cuticle proteins to chitin is a characteristic of CUPs containing the R & R consensus. The RR1 motif is reported to exist in soft cuticle protein while the RR2 protein occurs in hard cuticle only. The ability of HC-Pro to bind to both kinds of protein may suggest that it binds to a region conserved between the two types. Since the RR regions are different and moreover, cuticular proteins belonging to other arthropods were reported to have similar RR1 and RR2 sequences, the proposed area of interaction is most likely to be in the regions flanking this RR sequence. In the recent work by Uzest *et al*. (2010), the pepL sequence was derived from the RR2 region, which is conserved among different insects, was localized at the tip of the aphid stylets. In addition GFP-fusion protein to the CaMV helper factor was localized in 9 aphid species belonging to 8 genera at the same location indicating that the helper component bind to

this RR motif. Database search and sequence alignment studies of the cuticle proteins identified in this thesis showed strong similarity with other CUPs belonging to the same aphid species or to other species. In addition, the homologous gene products are identified from different locations of the *M. persicae* body and not just from the head. This may suggest that these genes are highly expressed in aphids.

Another interpretation of these results could be that instead of a specific receptor for virus like a cuticle protein in the aphid stylet, there is a non-specific attachment between the cuticle proteins covering the interior lining of the aphid body and virus particles, but only particles which attach to the stylet may participate in the transmission process. One reason for proposing that only particles which attach to the stylet are transmissible is that cuticle protein ligands that might potentially bind to virus in other parts of the insect body may be damaged or inhibited due to the enzymes inside the aphid gut, while on the aphid stylet the exposure to such enzymes is less frequent. This assumption correlates well with the fact that aphid fasting enhances non-persistent virus transmission (Watson and Robert, 1939); fasting may decrease the activity of these enzymes on the virus transmissible complex (virus particle/HC-Pro/ligands). In addition, the finding in Chapter 5 of this thesis about the long retention of non-transmissible virus inside different parts of the insect body and after different times of acquisition adds evidence in support of the existence of CUP ligands elsewhere in the insect body. Thus the virus may bind non-specifically to any cuticle proteins inside the aphid gut. More work is required to identify whether there are specific receptors or not, and my results can be utilized to design experiments to block virus transmission via approaches such as feeding insects on antibodies prepared against these identified proteins or genetically through silencing of the genes encoding such putative receptors if they exist in specific locations and not covering the whole insect body.

The work presented in this chapter has provided evidence that HC-Pro interacts with proteins of the aphid cuticle, which is supportive of other reports which state that the virus receptors of aphids are of cuticle nature (Dombrovsky *et al*., 2007b; Uzest *et al*., 2007; Uzest *et al*., 2010). Additionally, the non-specificity in binding to CUP proteins among aphid species and the abundance of these proteins in the aphid body may indicate either the existence of more than one receptor inside the aphid or the absence of specific receptors as the virus particles can be retained equally on any CUP proteins covering the food canal. However, due to some unknown reasons only those virus

particles which are retained on the tip of the stylet contribute to the transmission process.

Because of the time limit for the PhD project, it was not possible to look at the function of the identified clones in detail. Future experiments could be designed to try to block virus transmission through aphid feeding on antisera prepared against aphid CUPs. In addition, site-directed mutations of TEV-HC-Pro and aphid CUP protein domains would be helpful to reveal amino acid motifs that are important to the interaction. Ultimately, continuing this work could contribute to the development of novel methods of controlling spread of non-persistent plant viruses. Further experiments are required to confirm the specificity of interaction and to investigate the function of these proteins inside the aphid body.

Chapter 4. Effect of host plant species and virus isolate on potato virus Y acquisition and transmission by aphids

4.1. Introduction

The mechanisms regulating non-persistent virus transmission by aphids are not completely understood. Some of the viral determinants involved in transmission have been identified. However, there is a lack of information about the effects of vector and host plant.

The host plant can affect virus multiplication rate and systemic movement. Thus some plants or plant varieties enable high multiplication and translocation while others restrict virus multiplication or virus movement. Aphid transmissibility of PVY was reported to be dependent on virus concentration in the source plant (Bawden and Kassanis, 1946; Bagnall and Bradley, 1958, Bradley, 1962; De Bokx *et al.*, 1978), and there are some reports which show that plants differ in suitability as a virus host (Watson, 1956, Bagnall and Bradley, 1958). Others have suggested that some potato cultivars (Bawden and Kassanis, 1946; Bagnall and Bradley, 1958) or certain tissues within the same plant (Bradley, 1962) are better virus sources than others for aphid transmission. Moreover, there are many other factors which control or determine virus transmissibility, such as the age of the virus source plant, aphid vector species and colony growing conditions, and the aphid life stage (Mackinnon, 1961; Orolob, 1962; Swenson, 1963; Matthews, 1991). In addition to the effect on virus acquisition, the host plant can also affect the efficiency of virus inoculation. Matthews (1991) stated that "the species and even the variety of plant used as a source of virus or as a test plant may affect the efficiency of transmission". There is very limited information about this topic in the literature, and to my knowledge, no detailed study has been done to investigate the effect of the host plant when used as a virus source or as recipient. In addition, the effect of the host plant used to rear aphid colony on their efficiency in vectoring potyvirus was not fully investigated.

Changes to virus populations in the field occur in response to host genotype, vector pressure and environment whereas, in laboratory isolates, the selection pressures are different. It is known that a virus isolate may lose the capacity to be vector transmitted after successive manual passages on host plants (Pirone and Blanc, 1996). This was reported for some potyviruses (Watson, 1956; Atreya *et al*., 1991; Deborre *et al*., 1995)

and other non-persistent viruses such as Cucumber mosaic virus (CMV) (Badami, 1958; Ng *et al.*, 2005). Loss of aphid transmissibility of viruses was associated with mutations in the HC-Pro or the coat protein sequence in the potyviruses, whereas no major change was observed in the coat protein of CMV.

A proof-reading mechanism is lacking in RNA plant viruses (Roossinck, 1997), so that the possibility of changes due to mutation is high (Sanjuán *et al*., 2009). In addition to mutation there is another source of variance in plant virus populations resulting from their ability to recombine RNAs (Revers *et al*., 1996; Aaziz, 1999), and the latter source of variance in virus populations is an important feature among PVY isolates (Glais *et al*., 2002). There are well documented examples of modification in virus populations by passaging on host plants (Yarwood, 1979; García-Arenal *et al*., 2001). For example, Matthews (1949) found that the virulence of Potato virus X (PVX) towards potato decreased when the virus was passaged continuously on tobacco, and its ability to infect potato was lost completely after 19 passages through tobacco. This host adaptation was reported to be reversible in some viruses. For example, CMV was found to infect cowpea non-systemically, but after four passages on this host the virus caused more damage to cucumber than to cowpea. Moreover, the virus returned to its original form after about four passages through cucumber (Yarwood, 1979).

Patterns of virus infectivity can be complex. PVY isolates from potato were shown to be able to infect tobacco but not peppers, and the pepper strains were unable to infect potato (Selassie *et al*., 1985). Similarly, Marte *et al*. (1991) found that PVY isolates in central Italy were better adapted to tobacco, whereas isolates from southern regions infected pepper more readily than tobacco. The term "founder effect" is used to explain the bottleneck in a virus population which may occur as a result of infecting a new plant or when the virus is introduced into a new geographical area. Host plant and vector passages of plant viruses can contribute to the founder effect as well (García-Arenal *et al.*, 2001). Recently, Ohshima *et al*. (2010) reported that adaptation of TuMV (a potyvirus) to new plant hosts was associated with high diversity in nucleotide sequence compared to the isolate propagated on the original host.

M. persicae is well known to show some level of preference or selection towards some plants compared to others (Margaritopoulos *et al*., 2005). For example, potato plants are favoured more than tobacco plants. However, preference for tobacco was observed for

the sub-species *M. persicae nicotianae.* Despite being a poor host for aphids, tobacco is extensively used in the laboratory for many kinds of virus studies including aphid transmission. The effect of the host on the aphid vectoring ability is often mitigated by using large numbers of individual aphids per plant for aphid transmission studies. Host plants can directly affect aphid vectors by affecting their fecundity or reproduction, generation time, and morphology. This kind of effect has been reported (Margaritopoulos *et al*., 2005; Fenton *et al*., 2010) and will not be discussed further here. However, the host plant may also have an indirect effect on the aphid capability to transmit plant viruses. It has been reported that the host plant has an influence on the transmission of the semi-persistent BYV. Gladders and Peters (1986) found that aphids taken from oilseed rape plants were less efficient in BYV transmission than those reared on sugar beet. Host plant effects on aphid vectoring ability have been reported for non-persistent viruses as well even though the aphids do not require a long time to acquire or inoculate virus. Sylvester (1955) found that *M. persicae* reared on peach transmitted lettuce mosaic virus (LMV) less efficiently than did when reared on mustard, sugar beet, or radish. In addition, *A. nasturtii* was reported to be able to transmit PVY in some reports but failed to transmit it in others. The reason for the discrepancy in results is the host plant effect: aphids collected from the primary host were found not to transmit the virus whereas aphids collected from secondary hosts did transmit it (Orlob, 1962).

Although host plant species are known to differ in suitability as virus sources and to influence the vectoring ability of aphid species, the reasons are still unknown. Moreover, the impact of changing the plant species used to rear aphids and as indicator plants is not usually considered. In this Chapter, the effects of using potato or tobacco as virus source are studied in combination with the effects of using different hosts (potato, tobacco, oilseed rape (canola), and *P. floridana*) for rearing the aphid colonies. The implications of these effects on the epidemiology and control of PVY will be discussed.

4.2. Materials and methods

4.2.1. Virus source

Two sub-isolates of PVY^{O} (Ordinary stain) were investigated: the stock SCRI laboratory isolate (PVY-L) and a field isolate (PVY-F) that was obtained from naturally infected potato cv. Rosetta by Adrian Fox, Science and Advice for Scottish Agriculture (SASA), Edinburgh, Scotland, UK. Both isolates were maintained on different host plants. Symptomatic plants (3–4 weeks post inoculation) of potato, tobacco, and *P. floridana* were used as the virus source for aphid transmission. In this chapter, the source plant used to acquire virus is indicated by a superscript letter after the name of the virus. PVY propagated on potato as virus source will be represented as $PVY\text{-}F^{P}$ for the field isolate and $PVY\text{-}L^{P}$ for the laboratory isolate. Similarly PVY propagated on tobacco: $PVY\text{-}F^{T}$ or $PVY\text{-}L^{T}$.

Figure 4.1. An example of symptoms of PVY on a leaf of potato cv. Shula used as virus source for aphid transmission (right) compared with a healthy leaf (left).

4.2.2. Aphid colony maintenance

M. persicae colonies (Fig. 4.2) were maintained on *Solanum tuberosum* cv. Desiree, *N. tabacum* cv. White Burley, *Brassica napus* (Oilseed rape), and *P. floridana* in a glasshouse at 18°C in a 16:8h light: dark cycle with plants replaced on a weekly basis. The aphids were reared on plants in clear perspex cages (40x40x40 cm) as described in Chapter 2. Single aphids were transferred from potato to establish colonies on other plant species. To kill aphids on infested plants, the plant and pot were submerged into a bucket of hot soapy water for 5 min. Plants used for virus cultures were autoclaved before being disposed of.

In this chapter, the aphid colony used for virus transmission is tagged with the first letter of the plant on which it was maintained. Aphids maintained on potato are referred to as *M. persicae*-P, aphids reared on tobacco as *M. persicae*-T, aphids originating from oilseed rape as *M.* persicae-Osr, and aphids from *P. floridana* as *M.* persicae-Phy.

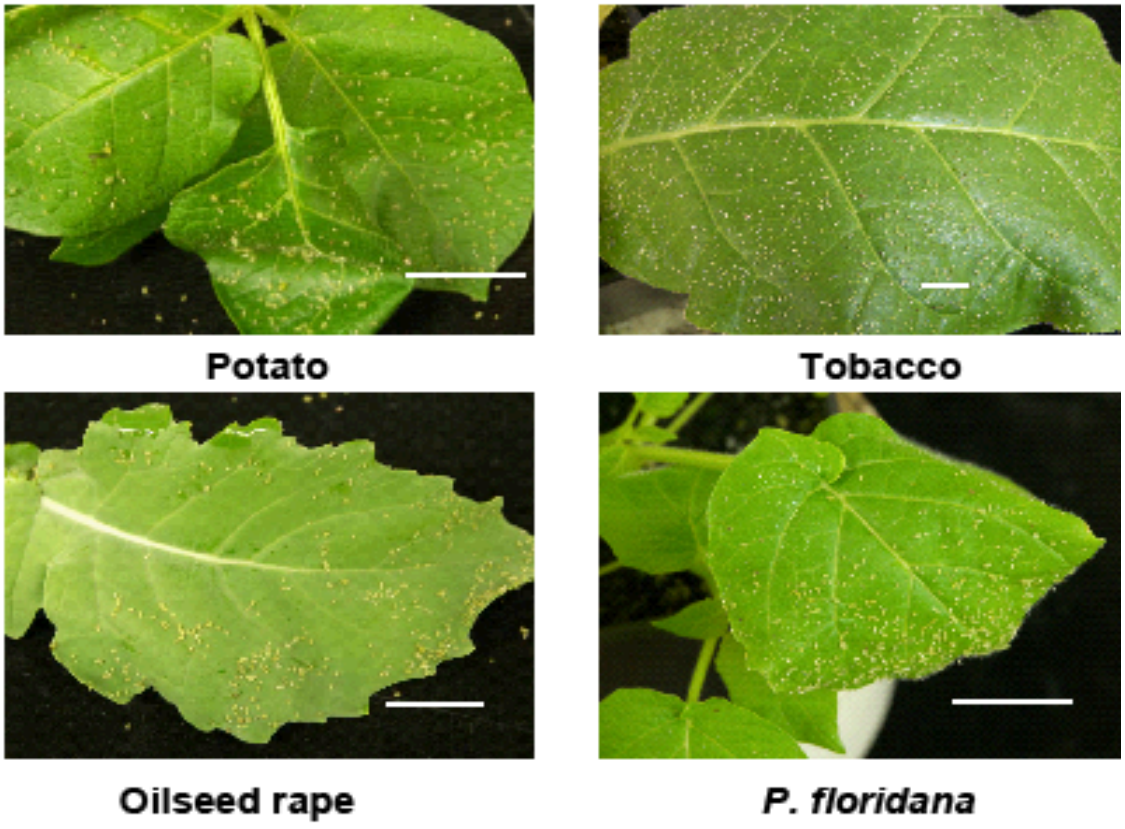


Figure 4.2. *M. persicae* colonies reared on different host plants.
Aphids on tobacco, oilseed rape, and *P. floridana* were originally taken from a potato colony two weeks after initiation of the colony. The white scale bars represent 1 cm.

4.2.3. Production of test plants

All test plants used in this project were produced by the glasshouse staff at SCRI in compost free of Intercept (granular insecticide used routinely for controlling insects at SCRI). Briefly, potato plants, cv. Shula, were produced on request. Eye plugs were removed from healthy tubers after dormancy breaking and transplanted into 12.5–17.5cm pots containing insecticide-free compost. Tobacco plants (*N. tabacum* cv. White Burley) were produced continuously throughout the year. The other host plants, oilseed rape and *P. floridana*, were produced on request by transplantation from a pre-germinated seed pan. All the plants were kept in glasshouse conditions of 18°C in a 16:8h light: dark cycle.

For each aphid transmission experiment young plants (3–4 leaf stage) of similar size were selected. Plants of similar size or slightly bigger were used for transmission by mechanical inoculation.

4.2.4. Aphid transmission

Detailed information on aphid selection and transmission is presented in Chapter 2. In general, for each transmission experiment aphids were taken from a single plant, 5 aphids per plant and 10 replicate plants were used and each experiment was repeated three times. Aphids were allowed for 5 min acquisition access period (AAP) from detached leaf taken from a single infected plant (3–4 weeks post inoculation or newly emerging sprouts from infected plant tubers). Aphids were allowed an overnight (16–18 h) inoculation access period (IAP), and then were killed by Pymetrozine (Pyridine) spray and fumigation overnight with nicotine shreds. Then plants were transferred to the glasshouse for symptom development (2–3 weeks) and ELISA was used to confirm infection.

4.2.5 Virus testing

ELISA was used for virus testing following the methods presented in Chapter 2.

4.3. Results

4.3.1. Effect of the virus source on virus transmission by aphids

Two plant species (potato and tobacco) were tested for their suitability as a PVY source for aphid transmission by aphids reared on different host plants (Fig. 4.3).

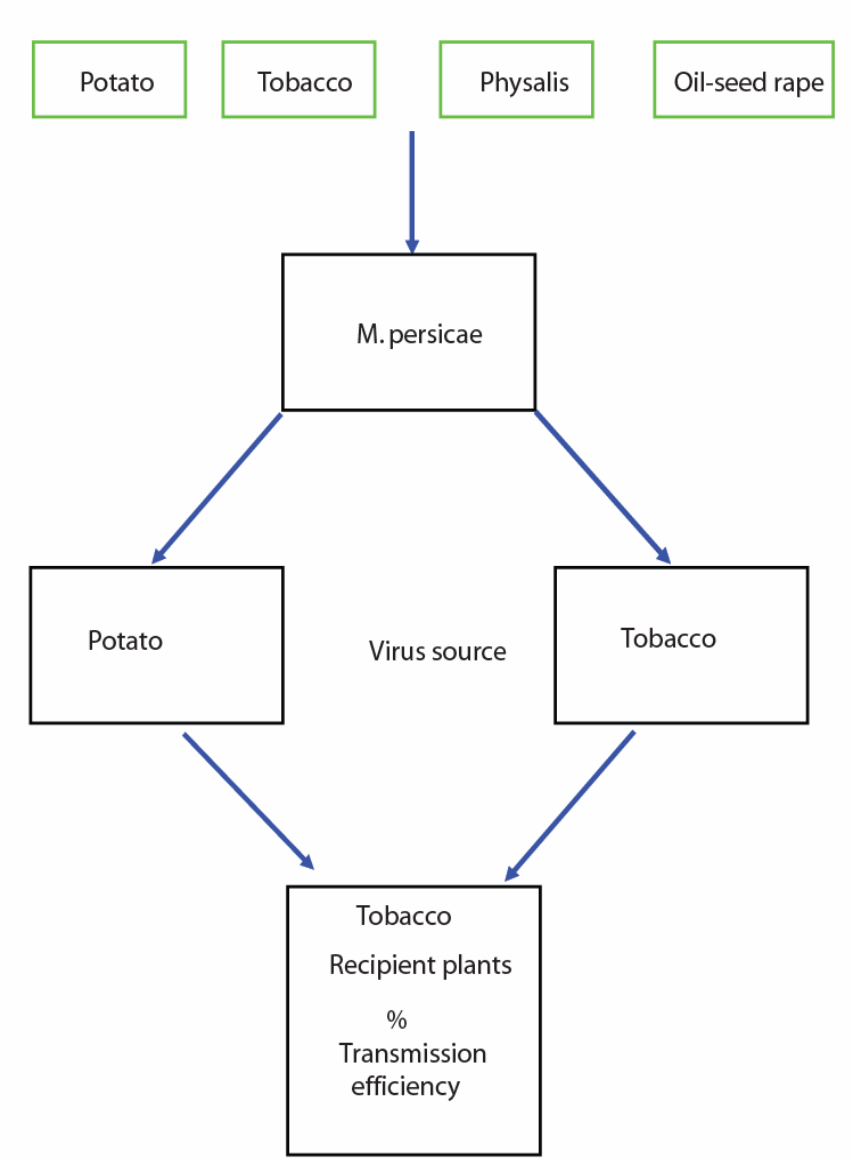
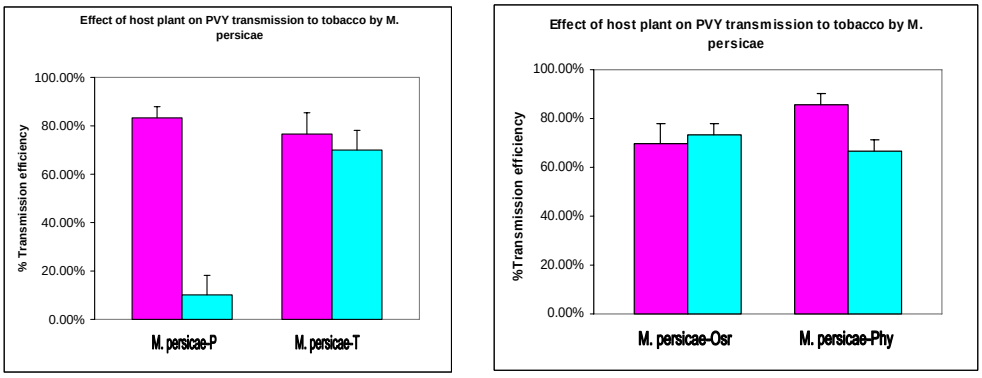


Figure 4.3. Schematic diagram of the experiments conducted to investigate the effect of the host plant used for rearing aphid colonies on virus vectoring ability.

4.3.1.1. Aphids reared on potato

In preliminary experiments, acquisition of the PVY-F isolate from potato was greater than from tobacco. This finding was confirmed by performing three replications of the same experiment (Fig. 4.4, A, left). This result is different from the reports in the literature which concluded that tobacco is a better virus source for PVY than potato (Watson, 1956; Bagnall *et al*., 1958). However, the aphids which were used in my experiments were reared on potato. Rearing aphid clones on tobacco considerably affected the outcome (Fig.4.4, A, right; see section 4.3.1.2). Therefore it was decided to investigate the link between aphid transmission capability and the host plant they originated from, which has been previously reported to be related for some plant viruses (Sylvester, 1955; Gladders and Peters, 1986).



A **B**

Figure 4.4. Effects of the plant species used for rearing aphids and the species used as a virus source on PVY-F transmission to tobacco by *M. persicae.*

Aphids were reared on potato (*M. persicae*-P), tobacco (*M. persicae*-T), oilseed rape *(M. persicae*-Osr), or *P. floridana* (*M. persicae*-Phy). ■: Potato as virus source, ■: tobacco as virus source. Each experiment was repeated three times and for each sub-experiment (A, B) 40 tobacco plants were used. Error bares represent values of standard deviation.

The transmission efficiency of PVY to tobacco was only 10 % when aphids were reared on potato and allowed to acquire PVY from tobacco. In contrast, much higher transmission rates (83.3%) were obtained when aphids were reared on potato and they were allowed to acquire virus from potato (Fig. 4.4 A).

4.3.1.2. Aphids reared on tobacco

There may be several reasons for the low aphid transmissibility of the PVY-F isolate from potato when the virus is propagated in tobacco compared with propagation in potato. The first considered possibility was that aphid probing behaviour is different between the two hosts: aphids may avoid or delay probing tobacco, so decreasing the probability of virus acquisition. However, aphids were monitored during the experiments, and only aphids which were observed to be probing at the end of the AAP were transferred to the test plant. Moreover, acquisition times of 1 to 20 min from different parts of infected tobacco leaves and after different times post infection (1, 2, 3, and 4 weeks) were tested. None of these variations changed the transmission efficiency (not shown).

A colony of aphids was established on tobacco and used to perform similar transmission experiments. When aphids were reared on tobacco, the situation changed and the transmission rate to tobacco was approx. 70% when aphids were allowed to acquire virus from tobacco infected with PVY-F, similar to that of *M. persicae*-P. Moreover, the transmission rate was also high when *M. persicae*-T acquired virus from potato. Thus, *M. persicae*-T acquired PVY-F readily from tobacco and potato but *M. persicae*-P only acquired it readily from potato (Fig. 4.4A).

4.3.1.3. Aphids reared on oilseed rape or physalis

Comparable results to those found with *M. persicae*-T were obtained when either a non-virus host plant (oilseed rape) or the indicator host *P. floridana* was used to rear the aphid colony. Transmission efficiency was about 70% when aphids reared on oilseed rape (*M. persicae*-Osr) were used for PVY-F transmission from either virus source (Fig. 4.4B). Similarly *M. persicae*-Phy transmitted PVY efficiently from potato or tobacco, the efficiency was slightly greater (80%) from potato than from tobacco (65%).

4.3.1.4. Statistical analysis

Genstat was used to analyse the significance of the host plant effect using the logistic regression analysis. Comparison of the host plant effect between potato and tobacco showed that both the host plant used to rear the aphid colony and the plant used as a virus source had affected the transmission significantly ($P < 0.001$), and the interaction was highly significant ($p < 0.001$). The same was obtained when the comparison was made between *M. persicae*-P or *M. persicae*-Phy, and the interaction was highly significant ($P < 0.001$). Comparison between potato and *P. floridana* showed that the effect of the virus source is highly significant ($P < 0.001$), but the host plant used to rear the aphid colony was less significant ($P = 0.064$), and the interaction between them was significant ($P = 0.004$).

4.3.2. Effect of the recipient plant on virus transmission by aphids

In addition to the effect of plant species on virus acquisition, the effect on transmission of the plant species used as a recipient was investigated (Fig. 4.5).

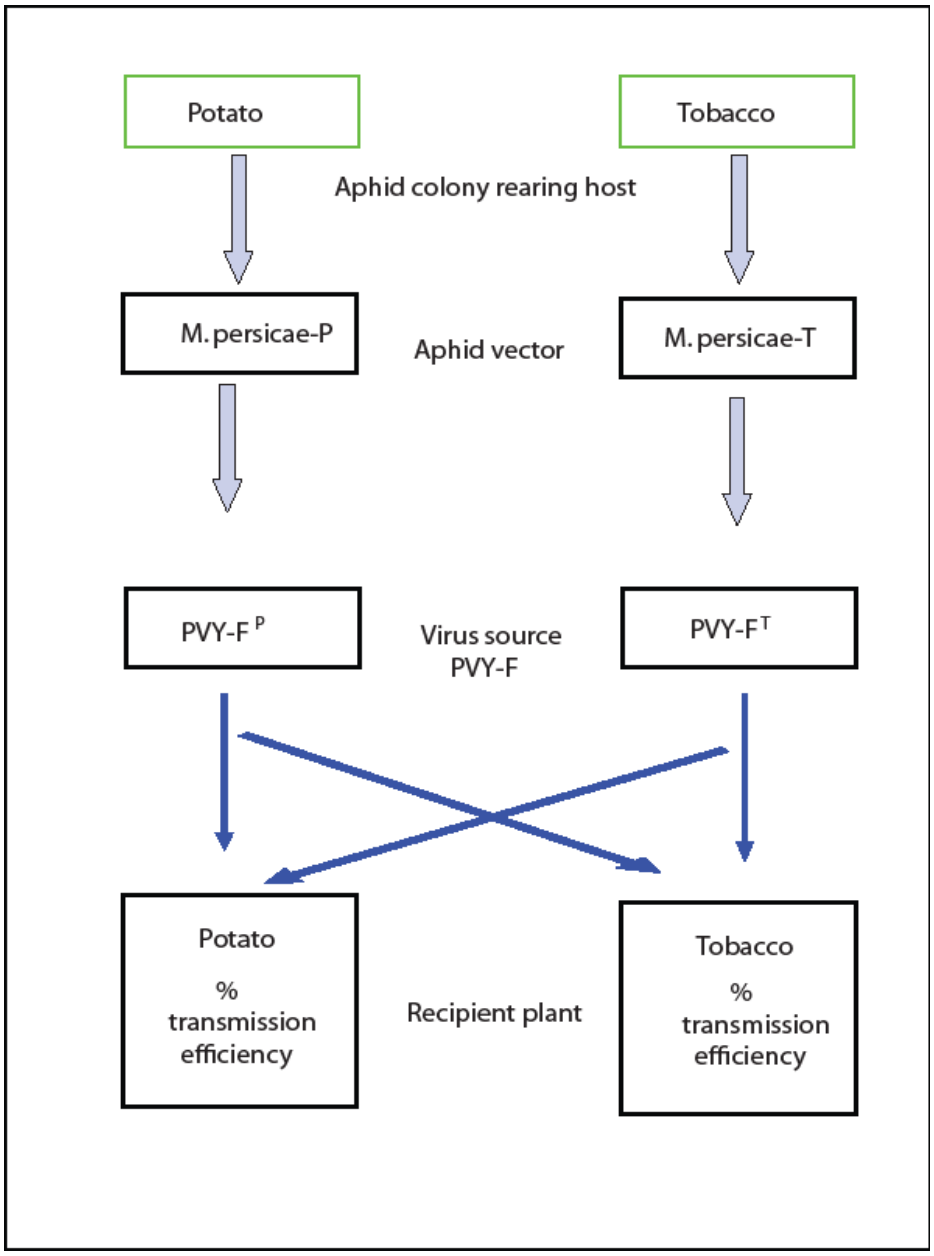


Figure 4.5. Schematic diagram of the experiments conducted to investigate the effect of recipient host plant on aphid transmission ability.

In this experiment, aphids were reared on either potato or tobacco then aphids were allowed to acquire virus from a source plant similar to the one they were reared on (*M. persicae*-P from potato, *M. persicae*-T from tobacco).Then each group of aphids were

allowed to inoculate virus to either potato or tobacco. The transmission rate was measured based on visual symptoms and ELISA 3–4 weeks post transmission.

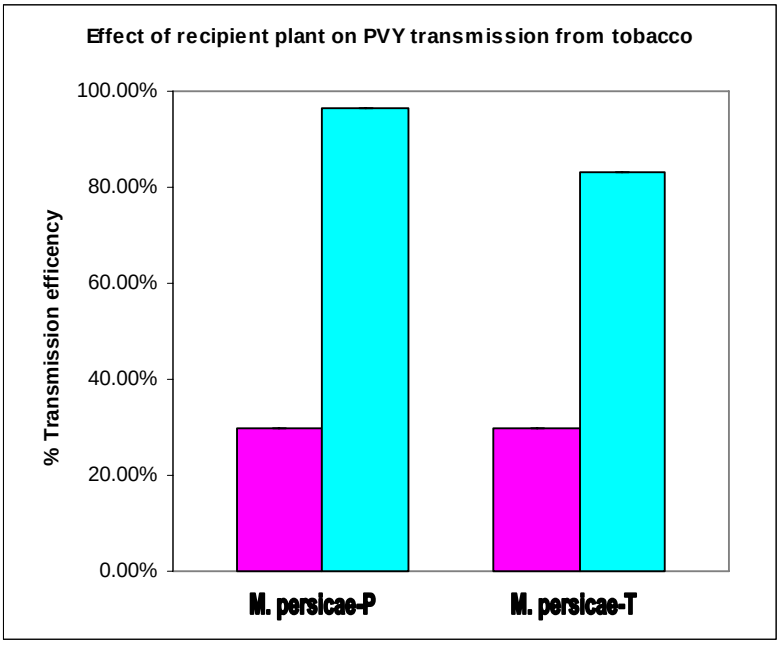


Figure 4.6. Effect of the recipient host plant on PVY-F transmission by *M. persicae.*

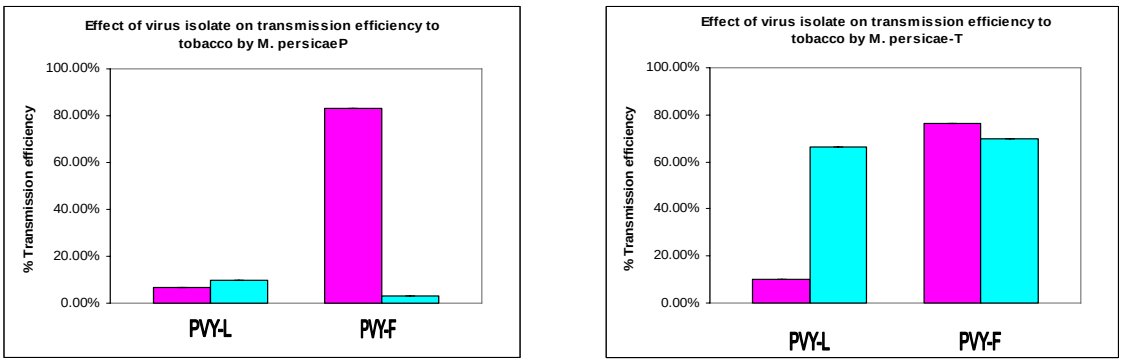
Aphids were reared on potato (*M. persicae*-P*)* or tobacco (*M. persicae*-T) and virus was acquired from potato and tobacco. ■: Potato recipient plants, ■: tobacco recipient plants. The experiment was repeated three times and for each sub-experiment 40 tobacco plants were used.

Fig. 4.6 shows that the transmission rate was higher to tobacco than to potato irrespective of host plant used to rear aphids or for virus acquisition. For both aphid sources, about 80–90% of tobacco plants were infected compared with only 30% of potato plants. Regression analysis revealed that the recipient plant effect is highly significant ($P < 0.001$). However, the host plant effect was not significant ($P = 0.393$).

4.3.3. Difference in aphid transmissibility between PVY-F and PVY-L isolates

During investigation of the host plant effect on PVY transmission by aphids, a difference in aphid transmissibility between the laboratory (PVY-L) and the field (PVY-F) isolates was noted. Initially, it was found that PVY-L was transmitted poorly from potato or tobacco (less than 10%), whereas PVY-F was transmitted efficiently from

potato (approx. 80%) and poorly from tobacco (Fig. 4.7 A). However, aphids used in this experiment were reared on potato. Rearing aphids on tobacco (4.7 B) increased transmissibility of both isolates from tobacco (about 70%), but transmissibility of PVY-L from potato remained low (10%).



A **B**

Figure 4.7. Differences in aphid transmissibility between the PVY isolates.

PVY-L: laboratory isolate, PVY-F: field isolate. Virus source: ■: potato, ■: tobacco. The experiment was repeated three times and for each sub-experiment 40 tobacco plants were used (20 for each virus isolate).A: aphids were reared on potato; B aphids were reared on tobacco.

Statistical analysis for the data in graph A revealed that there is a significant difference between the PVY-F and the PVY-L isolates ($P < 0.001$), and the difference was also significant between potato and tobacco when used as a virus source ($P = 0.008$). The interaction between virus isolate and the host plant used as a source was also significant ($P < 0.001$). For data in graph B, the difference was highly significant ($P < 0.001$), and the interaction was also highly significant ($P < 0.001$).

4.3.3.1. Virus multiplication and concentration in source plants

The reason for the difference in aphid transmissibility between PVY-F and PVY-L was investigated. Virus concentrations in tobacco and potato after infection were determined. Moreover, sequences of virus determinants known to be involved in transmission were obtained and compared with the published sequence of PVY^{O}.

Efficiency of transmission by the vector aphid is partially related to virus concentration in the source plant (Bradley, 1962; De Bokx *et al*., 1978). The finding that PVY-L is not acquired from potato with the same efficiency as from tobacco prompted me to investigate the virus concentration in the two hosts. Both PVY-F and PVY-L were mechanically inoculated to 10 tobacco plants and 10 potato plants, 3–4 weeks after inoculation leaf samples of both plants were tested by ELISA. The experiment was repeated twice and results are presented in Table 4.1.

Test plant	Isolate	Exp. 1	Exp. 2
Tobacco	PVY-L	0.888 (±0.140)	1.067 (±0.049)
	PVY-F	1.107 (±0.117)	1.142 (±0.080)
Potato	PVY-L	0.338 (±0.194)	0.350 (±0.168)
	PVY-F	1.254 (±0.344)	1.231 (±0.261)

Table 4.1. Mean A_{405} values obtained in ELISA of extracts of tobacco and potato leaves infected with different isolates of PVY.

For each experiment 10 plants of each host were inoculated with PVY-L and 10 plants with PVY-F. Samples were tested in duplicate wells and the mean A_{405} values (±SD) are presented. The mean A_{405} value of the healthy controls was 0.07 for both hosts.

No substantial differences were observed in absorbance values obtained in tests on tobacco plants infected with either virus isolate. However, on potato, PVY-F gave values three to four times greater than PVY-L (Table 4.1).

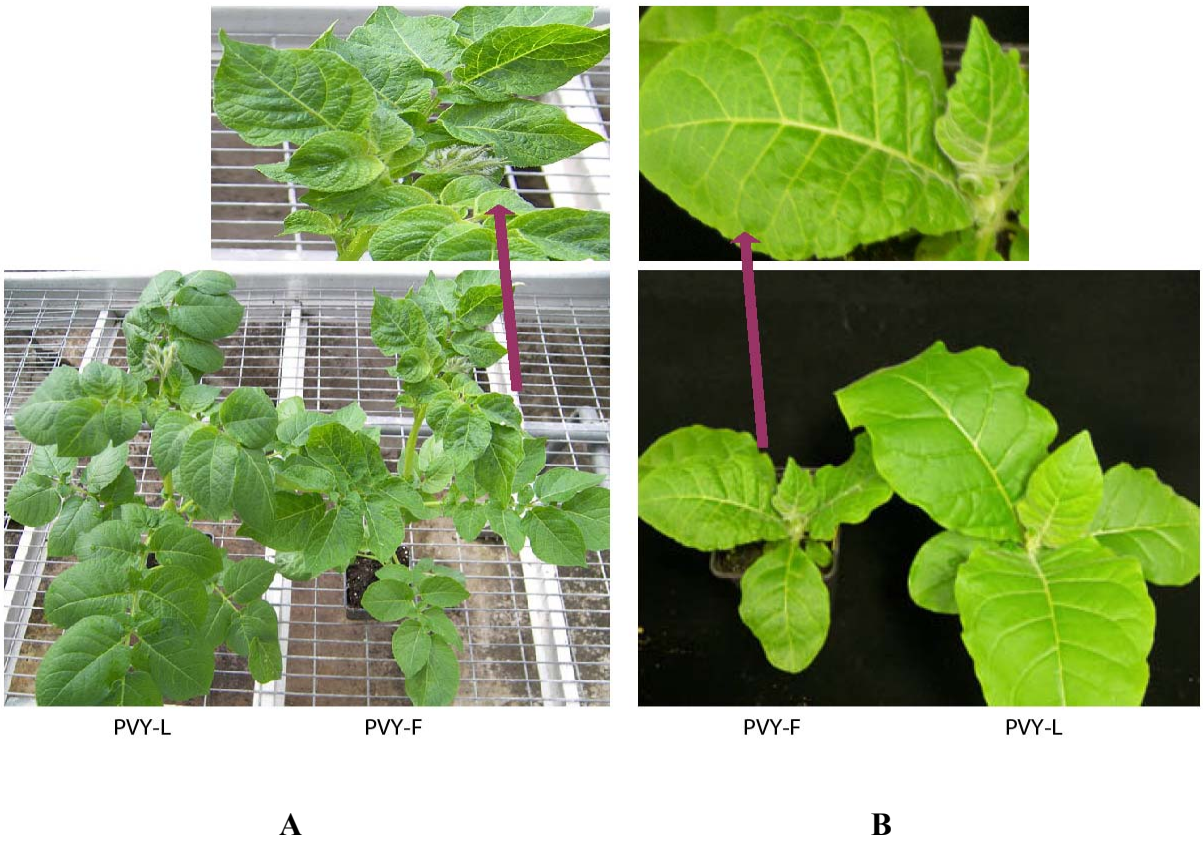


Figure 4.8. PVY symptoms three weeks after mechanical inoculation of PVY-L and PVY-F on potato (A) and tobacco (B).

PVY-F induced severe mosaic symptoms on potato 2-3 weeks after inoculation. On tobacco, PVY-F developed severe mosaic; mottling and stunting.PVY-L induced mild mosaic symptoms on tobacco, and remained symptomless on potato.

Comparing the data in Table 4.1 and the symptoms shown in Fig. 4.8 shows that whereas tobacco was a good host for propagation of both virus isolates, potato was a poor host for the PVY-L isolate. PVY-F also induced more severe symptoms on tobacco than did PVY-L. However, severity of infection decreased after several mechanical passages through tobacco.

The mild symptoms of PVY-L on potato probably reflect an adaptation to tobacco after continuous passage through this host for many years. Moreover, PVY-L did not reach a high concentration in potato and could not be maintained by mechanical inoculations on potato (infectivity was lost after 4–5 mechanical inoculation passages through potato). In contrast, PVY-F multiplied normally on both hosts. However, the symptoms became less severe on tobacco after 8–10 passages by mechanical inoculation. This decrease in

symptom severity after several mechanical passages to tobacco may suggest the start of adaptation.

To investigate after how many passages PVY-F would adapt to multiply on tobacco; it was mechanically inoculated to tobacco every three to four weeks. After two years (24-30 passages), the isolate from tobacco was inoculated to potato and aphid transmissibility was investigated. The results (Fig. 4.9) show that PVY-F retained its aphid transmissibility and ability to multiply in potato after 24–30 passages through tobacco. This suggests that the adaptation observed in PVY-L may have occurred after many years of continuous passages through tobacco. PVY-L is a stock laboratory isolate that has been maintained for more than 20 years on tobacco by mechanical inoculation.

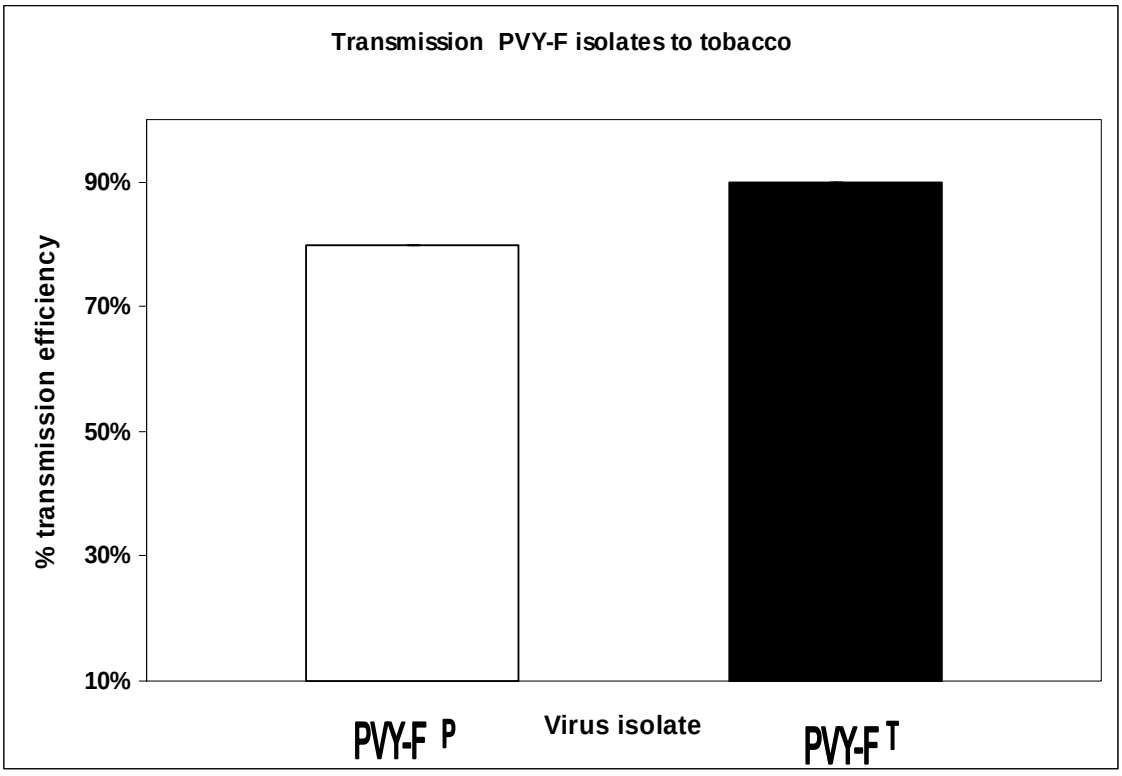


Figure 4.9. Transmission efficiency of PVY-F isolate after sequential manual propagation through tobacco or potato.

PVY-F-P: PVY field isolate propagated continuously on potato. PVY-F-T: PVY field isolate propagated continuously 24-30 times on tobacco then propagated on potato. After two years of mechanical passages of the PVY-F isolate through tobacco, this isolate was mechanically inoculated to potato cv. Shula which was used as a virus source 3-4 weeks after inoculation. Aphids used in transmission were reared on potato. The experiment was repeated twice and in each experiment 10 tobacco plants were used for each virus isolate, then % transmission efficiency was estimated. The difference in transmissibility between PVY-F-P and PVY-F-T was not significant ($P = 0.38$).

4.3.3.2. Sequencing virus components involved in aphid transmission

Two conserved motifs in the HC-Pro and in the potyvirus coat protein, termed the KITC region and the DAG motifs, respectively, have been reported to be critical for aphid transmission (See Chapter 1). The regions surrounding KITC/DAG motifs in PVY-F and PVY-L isolates were sequenced and compared with the published sequence of the PVY^{O} (Fig. 4.10). It is clear from Fig. 4.10 that KITC is retained in both isolates. In addition, Fig 4.11 shows that the DAG region is intact in both isolates, and there are no differences in amino acid sequences directly after or before this region. However, the PVY-L sequence contains five substitutions in the amino acid sequence after the DAG region (labeled 1 to 5 in Fig. 4.11). The sequence of this region of PVY-F is identical to the published PVY^{O} sequence.

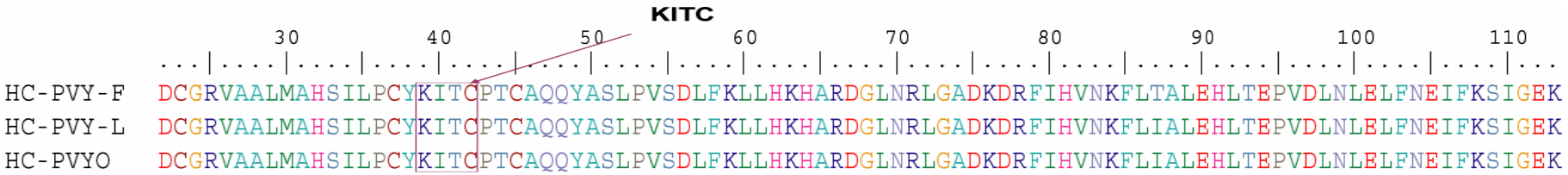


Figure 4.10. Amino acid sequence of the region flanking the KITC sequence of the HC-Pro of the PVY-L and PVY-F isolates, and alignment of both isolates with the published sequence of the SCRI ordinary strain of PVY.

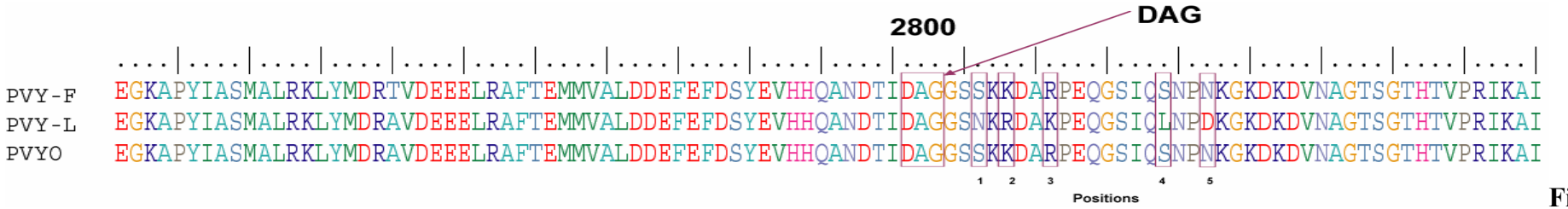


Figure 4.11. Amino acid sequence of the region flanking the DAG sequence of the coat protein of the PVY-L and PVY-F isolates, and alignment of both sequences with the published sequence of the SCRI ordinary strain of PVY.

Figure 4.12. Alignment of the amino acid sequence of the region flanking the DAG sequence of the coat protein of the PVY-L and PVY-F isolates with different published sequences of PVY isolates.

(Accession numbers, from EMBL unless otherwise indicated: PVY-SASA-61, AJ585198; PVY-Tu 660, AY166866; PVY-SASA-207, AJ584851; PVY-SASA-110, AJ585195; PVY-EG, EF455803; PVY^{NTN}-Tobacco, FJ204165; PVY-LYE8402 (tomato isolate-Spain), AJ439545).

Sequences of the DAG regions of different PVY isolates were retrieved from the database and aligned (Fig. 4.12). None of the substitutions found in PVY-F or PVY-L in the region following the DAG motif are present in any of the other isolates, except for the third substitution after the DAG region (R to K). This is present in the Scottish isolates PVY-SASA-110, PVY-SASA-61, and PVY^{N}-SCRI, in the PVY^{NTN} isolate from pepper (PVY-Tu 660), in a recently characterized isolate from egg plant, PVY-Eg (Sadeghi *et al*., 2008), and in a PVY isolate from tomato in Spain (PVY-LYE8402), which has high similarity to PVY-Eg. However, there are substitutions in other PVY isolates from different host plants that are different from the ones found in PVY-L. Interestingly, many of these substitutions are in the same positions as substitutions in PVY-L, which suggests that these positions in the PVY coat protein may be particularly prone to mutation associated with host plant selection. The egg plant PVY (PVY-Eg) isolate does not show any changes at positions 1 and 2, position 3 is mutated to K as in PVY-L, and positions 4 and 6 have substitutions S to P and N to S, respectively, compared with S to R and N to D in PVY-L.

Another substitution at position 1 is present in other PVY isolates (PVY-SASA-61 and PVY^{N}-SCRI), S to T instead of S to N in PVY-L. Similarly, these two isolates have a substitution in the fourth position as in PVY-Eg. The substitutions at positions 2 and 5 (K to R and N to D, respectively) seem to be unique to PVY-L as no corresponding substitutions have been reported in any other PVY isolate published to date, except for the recently published PVY sequence from eggplant (PVY-Eg), where N is substituted with S not with D (Sadeghi *et al*., 2008).

Surprisingly, the PVY isolate characterized from tobacco , PVY^{NTN}-Tobacco (Hu *et al*., 2009) exhibits similar substitutions next to the DAG region to the eggplant PVY-Eg isolate and has no similar substitutions to PVY-L, which seems adapted to tobacco, except the R to K substitution at position 3, which is found in all PVY isolates that are different from PVY^{O}-SCRI.

4.4. Discussion

In this study, the differences among several host plants in virus acquisition and inoculation by *M. persicae* were investigated, and were linked to the host used to maintain the aphid vector colony. Additionally, the difference in transmissibility between two PVY^O isolates was investigated biologically and partially at the molecular level. The results indicate clearly that the host plant used to maintain aphid cultures influences the aphid ability to acquire PVY from certain host plants, and furthermore, there is an influence of the recipient plant on the transmission rate. The results suggest that adaptation to multiply on tobacco is the reason for variation in aphid transmissibility of the field and laboratory isolates of PVY.

4.4.1. Effect of the host plant on virus source/aphid vectoring ability

Potato is a favoured natural host for *M. persicae*. However, aphids reared on potato have a poor ability to transmit the virus from tobacco. The fact that PVY-F reaches a similar concentration in both potato and tobacco hosts removes one important factor to be considered when interpreting this result. Therefore, it is likely that poor ability of aphids reared on potato to transmit virus from tobacco is due to the tobacco host itself. However, when aphids were reared on tobacco, the transmission efficiency from tobacco was restored, which suggests that the poor transmission from tobacco is not solely related to the properties of tobacco as a virus host. When aphids were reared on tobacco, which was initially a poor host for this lineage of aphid, and *P. floridana*, which is less favoured by aphids than potato (Mackinnon, 1960, 1963), virus was acquired equally from both tobacco and potato virus sources. Keeping aphids reared on potato for 2-3 days on tobacco was enough to restore the vectoring ability of aphids in virus transmission from tobacco, showing that *M. persicae* can adapt rapidly to different hosts.

Recent investigations into the aphid transmission of plant viruses have focused on feeding and probing behaviour by electronic monitoring using the EPG technique. Important information has been gained by employing this technique to elucidate aphid

probing behaviour during both acquisition and inoculation (Collar *et al,* 1997; Powell, 2005, Pelletier *et al.*, 2008). It would be of interest to investigate whether the probing behaviour of the poor transmitters or non-transmitters changes if they are forced to switch to different host plants.

Host plant effects on aphid vectoring ability are normally hidden because it is a common practice to maintain aphids on a host plant immune to the virus under study (Matthews, 1991). My results agree with this general rule, as virus transmission from both virus sources (potato and tobacco) was comparable when the aphid culture was maintained on oilseed rape, which is immune to infection by PVY. However, this change between hosts may change aphid feeding behaviour or create variation in the biochemistry of the virus particles and their attachment sites on aphid stylets in a way which may increase or inhibit attachment depending on the host plants under study (E. Jacquot, INRA, France, personal communication 2010). It may be better to use the same type of plant for both virus and aphid maintenance to rule out any possible impact of the host plant on the transmission efficiency of aphids. However, care should be taken to ensure that plants used for aphid rearing are not infected with the virus under study or with any other plant virus which may compete with the studied virus.

It has been argued that rearing aphids on unrelated plants will increase their ability to transmit non-persistent viruses because the shock of changing host may induce changes in probing behaviour (Matthews, 1991). This could be the reason for high transmission efficiency of *M. persicae* in vectoring PVY compared with other PVY vectors (Table 1.3) since *M.persicae* has a wide range of natural hosts. Host plant effects have been reported for *M. euphorbiae* which did not transmit PVY when potato cv. Russet Burbank was used as the source and test plant. However, this aphid species transmitted the virus when *N. tabacum* cv. Samsun was used as the source and recipient plant (Singh and Boiteau, 1984). In addition, Perez *et al*. (1995) found that *D. noxia* which was reared on wheat did not transmit PVY (pepper isolate) from tobacco to either tobacco or pepper, whereas it transmitted the virus to both test plants when the source was pepper. In contrast, *D. noxia* reared on pepper was able to transmit the virus irrespective of the combination of the source and test plants. Furthermore, Van Hoof (1980) reported that tobacco is not a suitable host for *R. padi* to acquire PVY^N. Effects of the host plant from which aphids originate are likely to be very important in field conditions, and this is particularly critical for some aphid species which has wide range

of plant hosts. *M. persicae*, for example, colonizes plants belonging to more than 40 plant families (Blackman and Devonshire, 1978), and so changes in the plant host are likely to cause changes in the aphid's ability to vector different plant viruses.

4.4.2. Recipient plant effects

The effect of the recipient plant on virus inoculation is very important when assessing the REF values for aphid vectors. REFs were first introduced by Van Harten (1983) and are currently being applied widely to assess the ability of different aphid species to transmit particular viruses and for predicting virus pressure in the field. The use of REF is very common in epidemiological studies of PVY in the field. It is used together with information on virus vector species caught in traps in order to estimate the timing of chemical applications or haulm destruction. The results in this Chapter demonstrate that although tobacco was a poor virus source for aphids maintained on potato, tobacco was infected more readily by aphids than potato whether potato or tobacco were used to rear aphids or as a virus source . The bait plant system which is used to assess virus levels in the field routinely employs tobacco to check whether live captured aphids carry virus (Harrington and Gibson, 1989). Also, experiments to estimate the vectoring ability of laboratory raised aphids after feeding on potato as virus source employ further transfer to tobacco (De Bokx and Piron, 1990; Sigvald, 1984; Van Hoof, 1980). Recently, a new system has been introduced to determine REFs for aphid vectors of PVY. This system is based on using *P. floridana* as a recipient plant after allowing aphids reared on laboratory host plants to acquire virus from potato (Verbeek *et al.*, 2010). In view of my findings about the effect of the recipient plant, a bait plant system that involves using indicator plants (tobacco or physalis) may not be accurate in assessing the aphid's vectoring ability as assessment is based upon acquisition from the natural host and transmission to indicator hosts, whereas in the field both acquisition and inoculation normally occur on the natural host. Thus, in order to be more accurate, such systems should use potato as both the virus source and the test plant to avoid overestimation or underestimation of the virus risk in the field.

In addition to affecting the assigned REF values for aphid vectors, and possible errors in estimation of the potential virus risk in the field, the recipient plant effect may be utilized. This would be possible in countries where tobacco can be grown, as this plant

could be used as a trap plant on field edges to deplete aphid charge of the virus. However, the trap plants should be disposed of regularly, for example weekly, to prevent aphids from acquiring virus from them. Moreover, the effect of tobacco in increasing vectoring ability of aphids when they are maintained on tobacco and acquired from tobacco may have an important epidemiological impact on virus transmission in the field. This is true in places like in the Mediterranean, where tobacco fields are located next to potato crops, so that the possibility of virus transmission from tobacco to potato crops is high.

4.4.3. Searching the literature for host effects on aphid vectoring ability

As explained earlier in this chapter, any host plant effect is normally hidden because large numbers of aphids are normally used to perform aphid transmission experiments, and because the common practice is to maintain aphids on an unrelated host plant that is immune to the virus under study. Information was gathered from the literature about host plants used to rear aphids (*M. persicae*) or to be used as virus source and test plants. Data are presented in Table 4.3 and are in agreement with the general rule reported by Matthews (1991) that virologists used to rear aphids on a plant which is immune for PVY and to acquire the virus from tobacco.

A survey of the literature revealed a wide range of efficiencies of PVY transmission by *M. persicae*. Transmission efficiency ranged between 26% and 75% depending on the experimental conditions. However, all experiments used the standard method of rearing aphids on virus-immune plants, particularly brassica species. Table 4.3 presents a list of published experiments performed on PVY to date. It is evident from the table that no study has ever investigated the difference between potato and tobacco as virus sources after maintaining aphids on potato or tobacco. The majority of the studies used brassica species to rear the aphids, and tobacco as the virus source and test plants, and transmissibility was within the range (40–80%) depending on the number of individual aphids used per plant, the aphid clone used, the virus strain, the virus source and recipient plants, the AAP, and many other minor variables in experimental conditions.

The host effect on aphid vectoring ability of plant viruses may be specific to *M. persicae.* Orlob (1962) reared *M. euphorbiae* on potato, and then aphids were allowed to acquire PVY from tobacco cv. White Burley. Aphids reared under these conditions transmitted virus efficiently to tobacco, which is different from the findings with *M. persicae* in the present study. In agreement with Bradley and Rideout (1953), Orlob (1962) found that *A. solani* transmitted PVY inefficiently. However, this low efficiency may be attributed to the host plant effect on aphid vectoring ability as both authors reared aphids on potato and acquired from tobacco.

REF*	Aphid host	Virus Source	Virus Recipient	AAP/min	Aphids/ plant	T. E%
1	Radish, Turnip	Tobacco	Tobacco	2.5	1	34 –55%
2	Potato	Tobacco	Tobacco	2	1	55%
3	Turnip	Tobacco	Tobacco	1	N/A	50 %
4	Oilseed rape	Tobacco	Tobacco	1	5	67.5%
5	Radish, Pepper	Potato	Tobacco	3–10	3	36–41%
6	Chinese cabbage	Potato	Potato	2	5	40%
7	N/A	Potato	Tobacco	3–10	5	50%
8	Trapped aphids	No AAP	Pepper	No AAP		6.3%
9	Trapped aphids	Potato PVY^N	Potato	0.5	1	71%
10	N/A	Tobacco	Tobacco	2	1	65%
11	Trapped aphids	No AAP	Tobacco	No AAP		4.6%
12	Potato	Potato	Potato	0.75–1	1	51%
13	Oilseed Rape	Potato	Potato	N/A		26%
14	Chinese cabbage	Tobacco	Tobacco	5	1	37%
15	Trapped aphids	No AAP	Tobacco	No AAP	N/A	8.4%
16	Trapped aphids	Potato	Potato	0.2	1	51%
17	Potato	Tobacco	Tobacco	N/A	N/A	NA
18	Potato	Potato	Potato	0.15–1	1	7.2%
19	Chinese cabbage	Tobacco	Tobacco	3–10	1	33–37%
20	Pepper	Tobacco	Tobacco	5	5	50%
		Tobacco	Pepper			66. %
		Pepper	Tobacco			66.6%
		Pepper	Pepper			75%
21	Chinese cabbage	Potato	*P. floridana*	2.5	1	34–38%

Table 4.3. Differences in *M. persicae* transmission efficiency of PVY reported by different authors following transmission experiments under different conditions.

*REF: Reference. 1: Watson and Roberts (1939), 2: Bradley and Rideout (1953), 3: Bradley (1959), 4: Bradley (1962), 5: De Bokx. (1977), 6: Kostiw (1979), 7: Van Hoof (1980), 8: Raccah *et al*. (1985), 9: Piron (1986), 10: Katis *et al*. (1986), 11: Harrington *et al*. (1986), 12: Singh and Boiteau (1984), 13: Sigvald (1984), 14: Gibson *et al.* (1988), 15: Harrington and Gibson (1989), 16: de Bokx and Piron (1990), 17: Boiteau *et al*. (1998), 18: Halbert *et al*. (2003), 19: Kanavaki *et al*. (2006), 20: Perez *et al*. (1995), 21: Verbeek *et al*. (2010). T.E: % transmission efficiency. AAP: Acquisition access period, N/A: Not applicable.

4.4.4. The reason: behavioural or molecular?

Although host adaptation does influence transmission, other factors may be involved**.** All current information conclude that aphid transmission of non-circulative viruses involves biological interactions between different components of the virus and the vector, and more recently there is evidence that a plant component is involved in the transmission of Cauliflower mosaic virus (CaMV) (Martinière *et al*., 2009). This biological interaction requires certain conditions, and these conditions should be comparable between the virus and the aphid receptors in order for the transmission to happen.

Whether probing activity controlled by host plant preference is the only reason for host plant effects on aphid vectoring ability needs to be confirmed. In this transmission experiments, the effect of host plant preference was minimized by using aphids that were observed to be probing at the end of the AAP. However, in order to rule out any host preference effect completely, acquisition should be performed with single aphids and by monitoring the stylet during the whole time of the AAP. Alternatively, the EPG technique should be utilized to monitor aphid stylet probing behaviour for aphids that originated on different hosts.

It was reported that sinigrin produced by *Brassicaceae* species can inhibit virus transmission by aphid species that do not colonize these plants, but stimulated uptake by aphids that feed normally on plants containing sinigrin (Nault and Styer, 1972). Nicotine or other tobacco substances may have similar effects; as tobacco is not a favoured host for *M. persicae* virus uptake from tobacco will be inhibited, but when aphids are reared on tobacco, acclimation to this host occurs rapidly, so that virus is acquired readily. If this hypothesis is true, such substances may be useful in controlling plant viruses if they are identified and the active component is isolated.

4.4.5. PVY-L Adaptation

Adaptation of plant viruses after serial passages through host plants is a well documented phenomenon in the literature (reviewed by: Yarwood, 1979; Garcia-Arenal *et al*., 2001). Similarly, the greater ability of the PVY-L isolate to multiply in tobacco

may be explained in the same way. Sequencing results confirmed at the molecular level the identity in the KITC and the DAG regions of the HC-Pro and coat proteins belonging to both isolates and with published sequences.

The low concentration of PVY-L in potato may reflect the adaptation of this isolate to multiply on tobacco after many years of passages in this host. However, such adaptation may have happened after many cycles of mechanical passage through tobacco as no such effect of the host plant on virus accumulation was observed after two years (Fig. 4.9). In addition, this adaptation seems to be irreversible as there was no increase in virus accumulation in potato after successive inoculation of PVY-L to potato. Moreover, PVY-L was not maintained after 4-5 passages into potato. This result suggests that there may be other changes in the PVY genome away from the sequenced regions, possibly in the Nib gene responsible for virus multiplication as reported to happen in PPV (Wallis *et al*., 2007). In the future, it will be interesting to investigate whether any of these substitutions are responsible for limiting virus multiplication or cell to cell movement in potato, as they apparently had no impact on the aphid transmissibility of PVY-L when acquired from tobacco provided that aphids were reared on tobacco or other hosts other than potato.

Chapter 5. Retention of Potato virus Y inside Vector Aphids: Implications for the Mechanism of Transmission

5.1. Introduction

Some determinants of the molecular mechanisms which mediate the transmission of non-persistent plant viruses are still unclear. Early studies concluded that viruses originally classified as being transmitted in the non-persistent manner were lost shortly after the vector aphid fed on a healthy plant for different periods of time (Watson and Roberts, 1939; Bradley, 1959). The concept of short retention of non-persistent viruses inside their vectors has remained unchallenged since then (Pirone and Harris, 1977; Pirone and Blanc, 1996; Ng and Falk, 2006). At that early date there were no molecular biology techniques to enable researchers to investigate clearly what happens to the virus after it was acquired by the vector. Serological methods were also unavailable at that early stage and their application was limited to some important viruses after that. Thus in most experiments, visual symptom induction in test plants was the only method used for virus detection. The question which arises is- Is it really true that non-persistent viruses are lost rapidly from their aphid vectors after they have fed on a healthy plant?

Electron microscopy studies showed that when aphids acquire transmissible combinations of HC-Pro and potyvirus particles, virions are retained within the maxillary stylets (Berger and Pirone, 1986) and potyvirus helper component (HC-Pro) mediated adherence to the cuticular lining of the food canal (Ammar *et al*., 1994; Wang *et al*., 1996). The electrical penetration graph (EPG) technique (Tjallingii, 1978), monitors insect probing behaviour on the basis of the associated changes in electrical signals (potential drop) during different phases of probing and feeding. EPG was widely applied to study the mechanism of non-persistent virus transmission by aphids. The uptake of virions occurs when the maxillary stylet tip punctures the plasma membrane of an epidermal cell (Lopez-Abella *et al*., 1988; Powell, 1991), and further studies confirmed that specific subphases in the potential drop are associated with the acquisition and inoculation of Cucumber mosaic virus (CMV) and PVY

(Martin *et al.*, 1997). Moreover, the volume of sap ingested by aphids was found to correlate with the length of the intercellular stylet puncture, which may increase the probability of virus acquisition (Powell *et al.*, 1995; Collar *et al.*, 1997). Collar and Fereres (1998) reported that subphase II-3 of the potential drop related to virus acquisition is variable and is longer during the first intercellular stylet puncture. On the other hand, subphase II-1 is short and unique during different interacellular stylet punctures. Powell (2005) determined that virus inoculation is associated with the first intracellular activity (subphase II-1 of the potential drop) following maxillary puncture of an epidermal cell which is associated with active injection of saliva directly into the cytoplasm.

Regardless of the mechanism of transmission, there is general agreement among virologists about the retention time of the virus inside its vector. The majority of the literature about aphid-borne viruses concludes that non-persistent viruses are lost shortly after the aphid has fed on a healthy plant. There are very few reports about the likelihood of longer retention time of non-persistent viruses. Examples include, Van Hoof (1980) who reported that PVY^N was retained for 4 and 8 hours in *M. persicae* and *P. humuli* respectively. Winged forms of *A. nasturtii* remained viruliferous up to 17 hours after acquisition (Kostiw, 1975). In addition, Heinze (1959) found that *M. persicae* immobilized at -1°C for 6 days after PVY acquisition were still able to transmit virus once restored to room temperature (Zeyen and Berger, 1990). Similarly, Bradley (1959) found that viruliferous aphids that probed glass lost acquired PVY less frequently than those allowed to probe on plant tissue.

In this chapter, PVY^O was detected inside an aphid vector up to two weeks after acquisition and successive feeding on healthy plants. However, the virus was not transmissible. Aphid vectors were dissected to investigate where the virus had accumulated, and the quantity of virus inside the aphid was estimated and compared with other reports about the virus charge required for an aphid vector to initiate an infection. This new observation is discussed with respect to virus transmission mechanisms and new insights about virus transmission were proposed.

5.2. Materials and methods

5.2.1. Aphid transmission

Information on aphid handling and virus transmission is presented in Chapter 2. All aphids used in these experiments were apterous *M. persicae* at 3–4 instar stage; aphid colonies were reared on potato in clear perspex cages (35x40x45 cm) as mentioned in Chapter 2.

Unless otherwise stated, all aphids used were starved for 2–3 h in groups of 10 in 1.5 ml microcentrifuge plastic tubes.

The PVY source was a detached leaf taken from 3–4 week infected potato plants showing fully developed virus symptoms and infection was confirmed by ELISA.

5.2.2. Aphid dissection

In this experiment, aphids acquired virus from an infected potato leaf during a 5 min acquisition access period (AAP). Aphids were then transferred to fresh healthy plants on a daily basis without having access to a virus source then tested for virus by N-RT-PCR after dissection.

For dissection, aphids were anaesthetized using CO_2 then mini-forceps were used to release the stylet from the bundle under suitable magnification (Zeiss microscope). Each aphid was dissected to separate the head from the body or stylet from the rest of the body on a clean filter paper. To decrease the potential of contamination between different aphids, forceps were rinsed successively in four dilutions of hypochlorite disinfectant solution (2.5 % Chloros) after each dissection, with a final rinse in SDW.

5.2.3. PVY preparations

PVY particles were purified and quantified as described in Chapter 2, and then the virus preparation (1.5 µg /µl) was diluted using Sorensen's phosphate buffer, pH 7.5–8.0 (Chapter 2). Five- and ten-fold dilutions were prepared and virus presence in the diluted samples was investigated using biological assay, ELISA, RT-PCR, and N-RT-PCR.

5.2.4. Virus detection

ELISA of test plants, or RT-PCR, and N-RT-PCR of aphids and the primers used are as described in the Chapter 2. For N-RT-PCR, the primers used were Singh S0, Singh AS480 for the first round and Malloch F, Malloch R for the second round. For RT-PCR, primers Singh S0 and Singh AS480 were used (Table 2.3). RNA extracted from either infected leaf plants or viruliferous aphids were used as a positive control template for RT-PCR or N-PRT-PCR. For negative control, RNA extracted from either healthy plant leaves or from non-viruliferous aphids was used. Plants were either tested by ELISA or inspected by visual symptoms to test virus infection.

5.2.5. Successive feeding experiments

In these experiments aphids that had already transmitted PVY were starved 2-3 h, and allowed to access the virus source a second time, then transferred to a healthy plant to assess their vectoring ability after performing the first transmission.

5.3. Results

5.3.1. Virus retention inside the whole aphid

Aphids were starved in batches of 10 in 1.5 ml microcentrifuge tubes then allowed to acquire PVY from infected potato leaves with a 5 min AAP. The aphids were then transferred to a healthy tobacco plant and then transferred successively to a fresh plant on a daily basis. Ten aphids which were observed to be feeding were collected after 1, 2, 7, and 14 transfers (days) of feeding on successive healthy plants. The collected aphids were tested by nested RT-PCR for PVY (Fig. 5.1), and two experiments were done (replication R1+R2). The results show that virus was detectable in all 10 aphids after 7 successive transfers to healthy plants (7days). After 14 transfers (14 days), which is the longest period tested, virus was detectable in 9/10(R1) and 10/10 (R2) aphids.

Using ELISA, only the first tobacco plant (H1) tested virus positive, and all the other plants (H2-H14) were found to be virus-free.

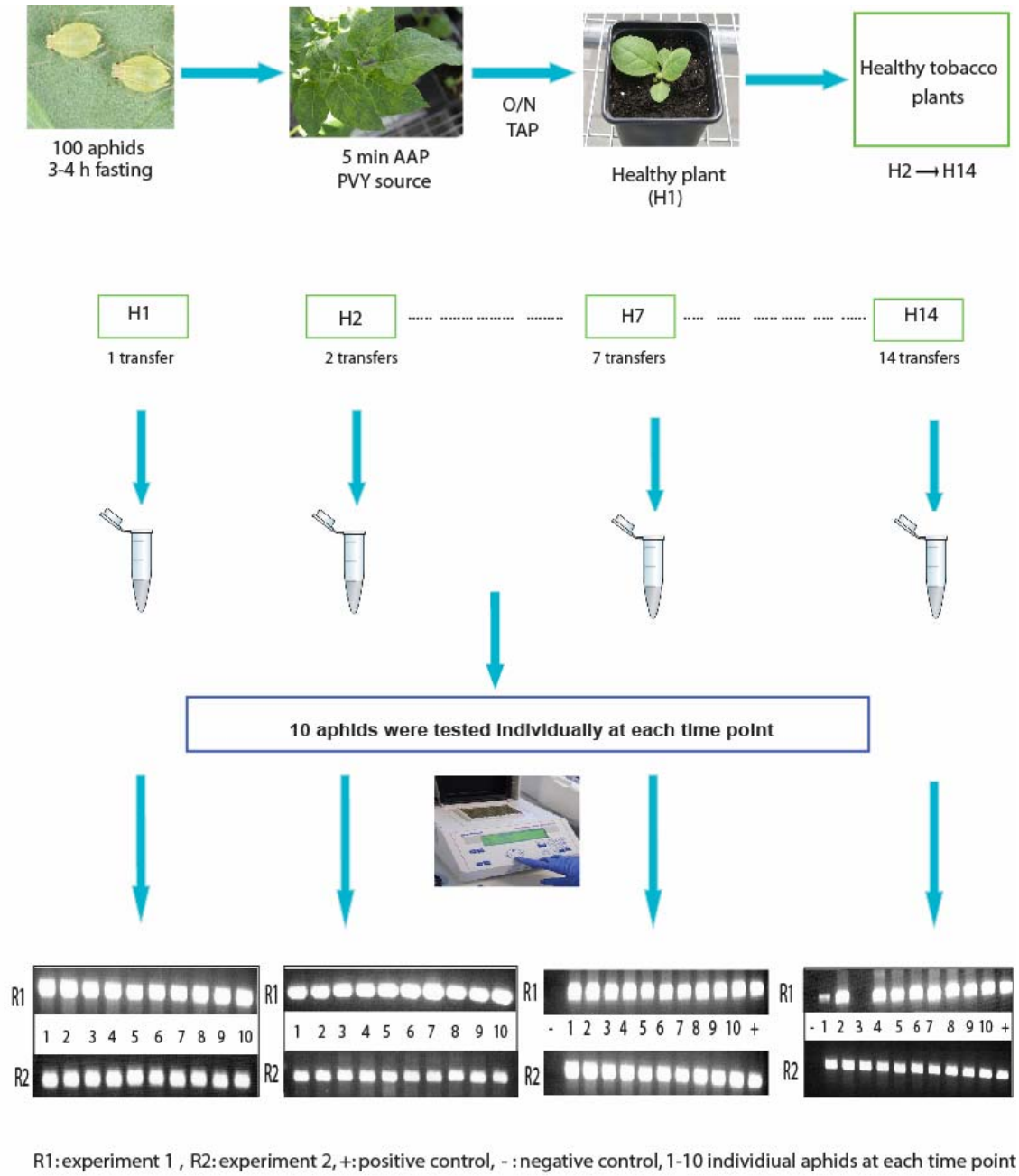


Figure 5.1. Experimental design and N-RT-PCR results of PVY detection in aphids 1, 2, 7, and 14 days post acquisition of PVY.

5.3.2. Retention in different parts of the aphid body

N-RT-PCR was used to investigate virus retention in different parts of the insect body. Aphids were fasted in batches of 10 for 2–3 hours, and then allowed a 5 min AAP. Aphids which were observed to probe were transferred to healthy tobacco plants and kept caged overnight. Aphids were transferred on a daily basis to fresh healthy tobacco plants, and simultaneously aphids which had performed virus transmission were dissected and either the body separated from the head (B), head and the body without stylet (H+B), stylets separated from the rest of the body(S), or stylets attached to the head and separated from the rest of the body(S+H) were tested. These tests were done once after 1, 4, and 7 successive transfers of viruliferous aphids to healthy plants, and 10 individual aphids were dissected for each time point in each experiment.

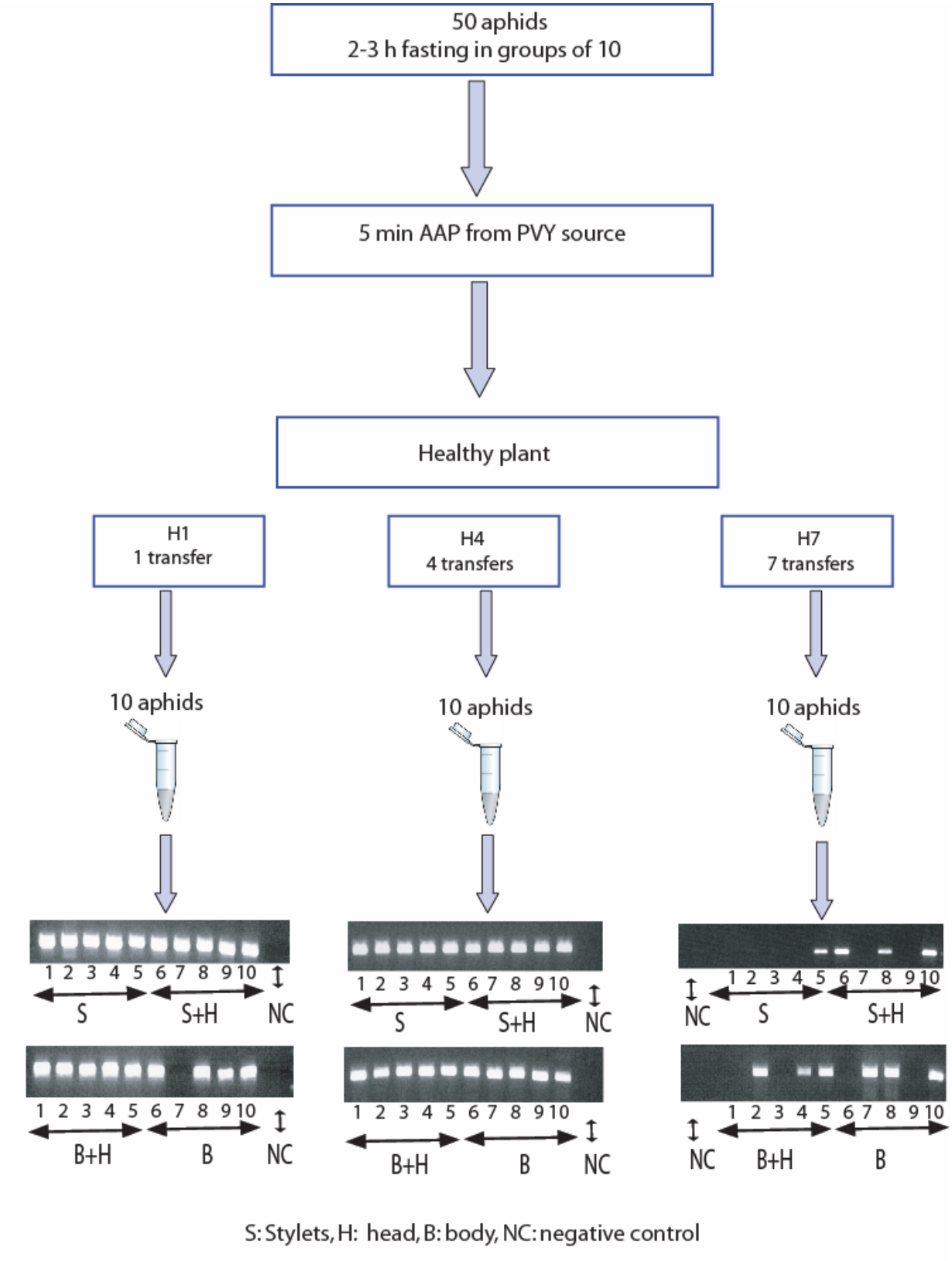


Figure 5.2. Retention of PVY in different parts of the aphid body 1, 4, and 7 days after acquiring the virus and following daily transfer to healthy tobacco plants.

The results show that virus is retained in all parts of the insect body (stylet, head, and body), but was detected in fewer aphids after 7 transfers (Fig. 5.2). This finding is contrary to the currently accepted view of non-persistent viruses being only stylet-borne. However, this result may suggest that more than one type of receptor exists in the aphid's body, but only the one located at the tip of the stylets carries transmissible virus particles as will be discussed further in the next sections.

5.3.3. Retention of a non-transmissible virus isolate inside aphids

A similar experiment was done using the non-aphid transmissible strain of PVY, PVY^C. Twenty aphids were fasted in batches of 10. Aphids were allowed to have 5 min AAP on PVY^C infected potato leaf and then kept on healthy plants overnight, and 10 individual aphids were taken to be tested by N-RT- PCR for virus retention.

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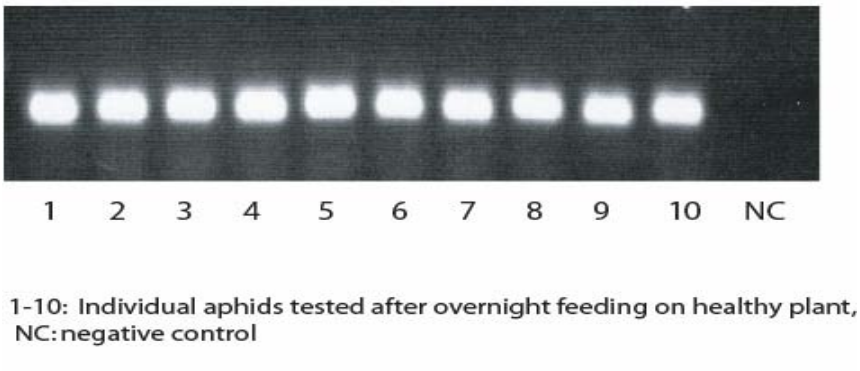

Figure 5.3. Detection of non-transmissible PVY^C in aphids after acquiring the virus and following feeding on healthy tobacco plants for 1day.

The result presented in Fig. 5.3 showed that aphids were also able to retain PVY^C, which may suggest that the detectable form of the virus is not infectious.

5.3.4. Finding the detection limit of the nested RT-PCR technique

Previous experiments showed that aphids were not able to transmit virus detected by N-RT-PCR inside their bodies. Possibly because the technique is highly sensitive it can detect virus quantity below the limit of infection. It was decided therefore to find the detection limit of the N-RT-PCR technique and compare that with available information in the literature about the virus quantities required for an aphid vector to transmit a potyvirus. Obtained data will be helpful to reveal if the detectable virus was not transmissible.

A 10-fold dilution series of PVY preparation (1.5 µg /µl) was tested by N-RT-PCR and RT-PCR and ELISA, and ten- fold dilutions were tested by inoculation to healthy tobacco plants (Table 5.1).

The results from two experiments revealed that the detection limit of the mechanical inoculation method is 0.015 µg /µl compared with 0.00015 µg /µl using ELISA. This means that ELISA is approx. 100 times more sensitive than mechanical inoculation. The sensitivity of detection was increased by a further factor of 10^4 times by using RT-PCR alone. The lowest detection limit was 0.015 ag/µl by using nested RT-PCR (Figs. 5.4 and 5.5)

10-fold dilutions of 1.5 µg /µl prep.		ELISA Values (Titration)	ELISA Values (Plant)±	RT-PCR	N-RT-PCR
1	Exp.1	0.88*	0.70*	-*	-*
	Exp.2	0.91*	0.72*	-*	-*
2	Exp.1	1.05	0.76	+	+
	Exp.2	1.08	0.78	+	+
3	Exp.1	1.12	0.92	+	+
	Exp.2	1.15	0.92	+	+
4	Exp.1	0.74	0.11	+	+
	Exp.2	0.73	0.10	+	+
5	Exp.1	0.24	0.07	+	+
	Exp.2	0.23	0.07	+	+
6	Exp.1	0.10	0.06	+	+
	Exp.2	0.11	0.06	+	+
7	Exp.1	0.07	0.06	+	+
	Exp.2	0.08	0.06	+	+
8	Exp.1	0.06	0.06	+	+
	Exp.2	0.07	0.06	+	+
9	Exp.1	0.06	0.06	+	+
	Exp.2	0.06	0.06	+	+
10	Exp.1	0.06	0.06	+	+
	Exp.2	0.06	0.06	+	+
11	Exp.1	0.06	0.06	-	+
	Exp.2	0.06	0.06	-	+
12	Exp.1	0.06	0.06	-	+
	Exp.2	0.06	0.06	-	+

Table 5.1. Summary table showing detection limit of PVY using different assays.

*: Detection of the virus was inhibited or decreased by PCR or ELISA respectively at higher virus concentration, +: virus positive, -: virus negative.

±Plants were mechanically inoculated with PVY virus particles at 1.5–1.5 x10^{-11} µg /µl in 10-fold dilution series that were tested by ELISA.
Results for dilutions 13-15 were not presented as they were all negatives.

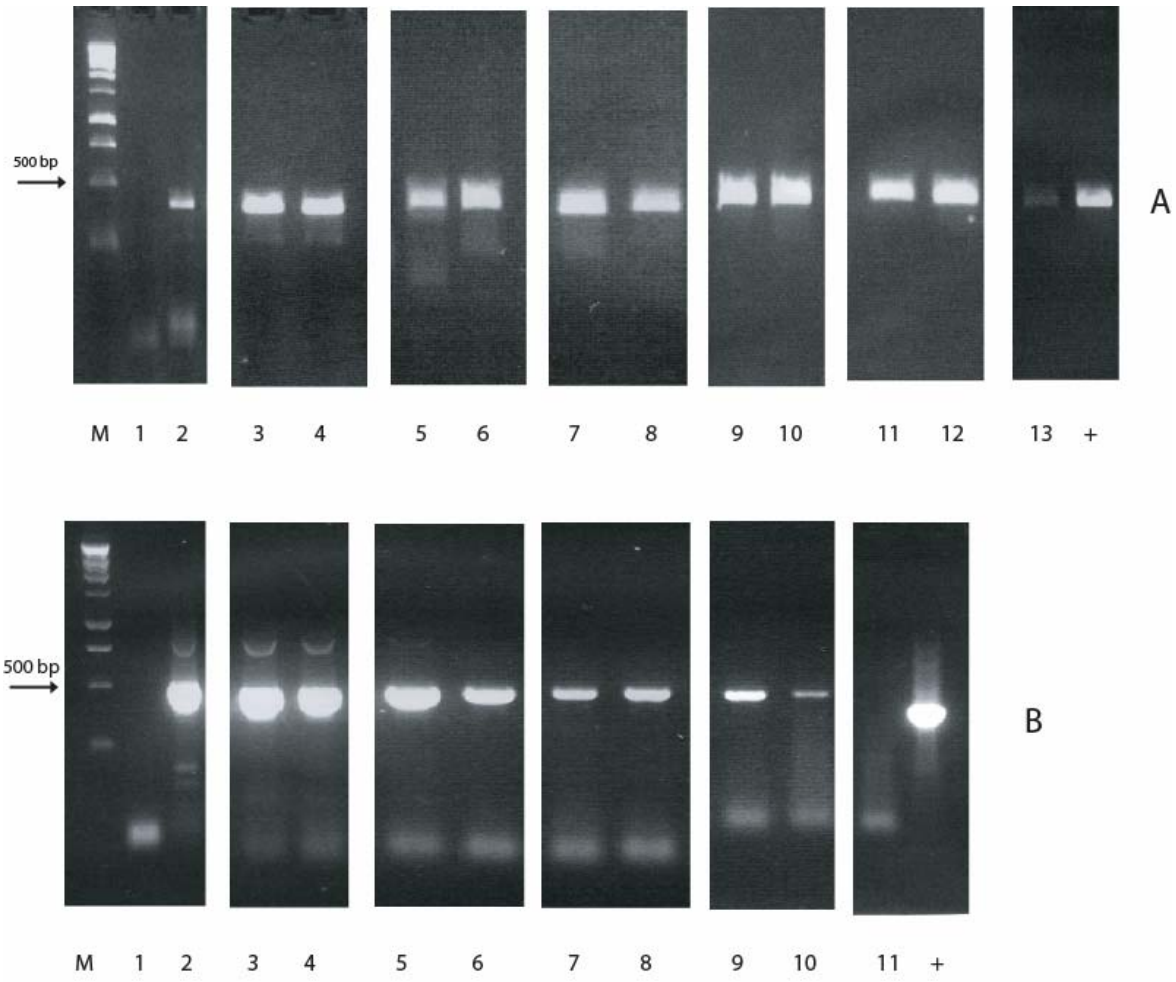

Figure 5.4. Limit of detection of PVY by two RT-PCR techniques.

Panel A: titration of PVY prep by N-RT-PCR, panel B: titration of PVY prep using RT-PCR, M: DNA marker, 1-13: 10-fold dilution series, + positive control.

* Results for samples 14-15 were not presented as they were all negative.

It is clear from Fig. 5.4 that the N-RT-PCR method (panel A) is about 1000 times more sensitive than the standard RT-PCR method (panel B). However, while RT-PCR showed a decreasing quantity of PCR product with virus law concentration in the tested sample, the N-RT-PCR method gave bands of similar quantity in each sample. With both techniques PCR reaction was inhibited when the sample contained a high virus concentration, as can be seen from the results of the first dilution.

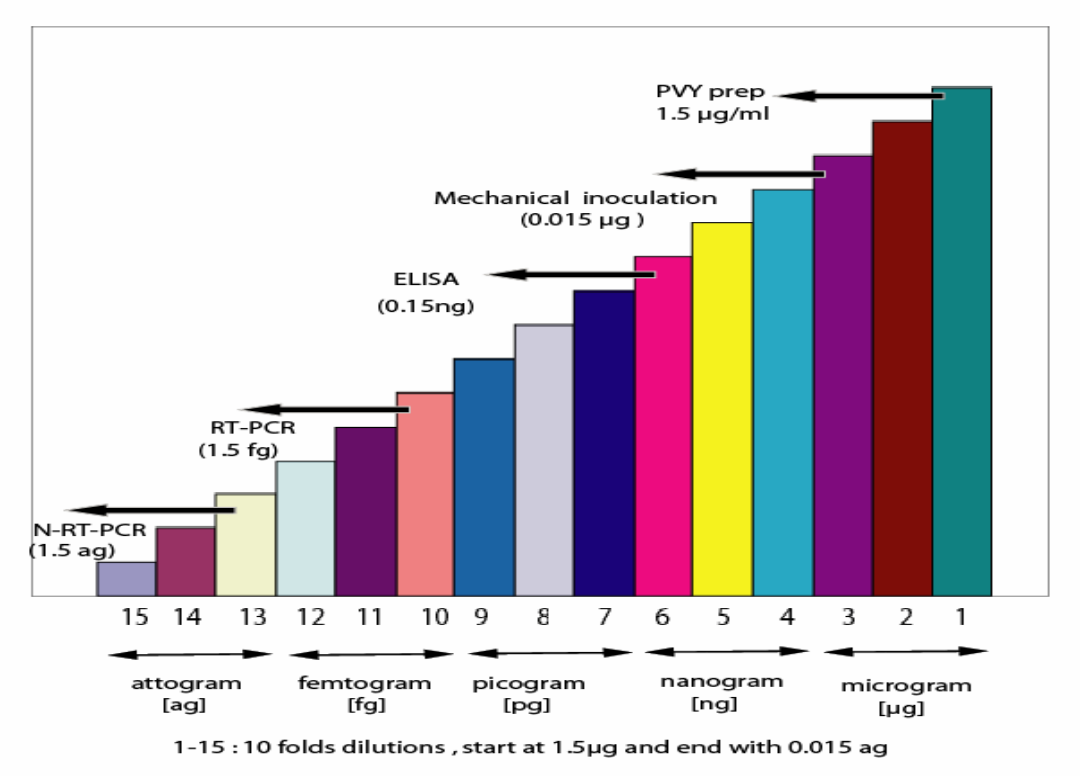

Figure 5.5. Summary diagram showing the sensitivity of different detection methods in measuring the quantities of purified PVY particles.

5.3.5. Investigation of the ability of aphids to acquire and transmit PVY more than once.

Interference between different plant viruses with respect to their vector transmission ability was reported by Katis *et al.* (1986). In contrast, interference between particles of the same virus affecting vector efficiency has not been reported. In fact, Watson (1936) observed a small decrease in efficiency after successive transmission of virus by aphids. The interpretation was that either (1) the aphids (*M. persicae*) lost their appetite to feed on tobacco, which is not a favourable host, or (2) aphids were mechanically damaged after successive transmission, and (3) the aging of aphids after successive transmissions may affect their ability to transmit virus. Watson's experiments on aphid transmission were carried out on hyoscyamus virus 3 which considered sharing the same transmission properties as PVY. However, Hyoscyamus virus 3 was not found in the ICTV taxonomic index as a member of the *Potyviridae* or other virus families which may suggest that this virus has been lost or renamed.

Experiments were done to examine the effect on transmission efficiency by aphids that had already transmitted PVY; the experimental plan is illustrated in Fig.5.6. Aphids were reared on healthy potato plants then fasted 2–3 h and allowed 5 min AAP to acquire virus from infected leaf for 5 min. A prolonged access period was tested by allowing aphids which had been reared on healthy potato plants to have access to a virus infected source for overnight (16–18h) AAP and then they were transferred to healthy tobacco plants for 16–18 h IAP.Then aphids were individually numbered and fasted 2–3 h and allowed an AAP of 5 min to infected virus source and then transferred to healthy tobacco plants. In addition, the transmission behaviour of aphids were tested after continuous access (1–2 weeks) to virus source, then aphids were fasted 2–3 h and were given 5 min AAP to virus source and transferred to healthy tobacco plants, then fasted for 2–3 h and allowed to acquire virus for a second time then transferred to healthy tobacco plants.

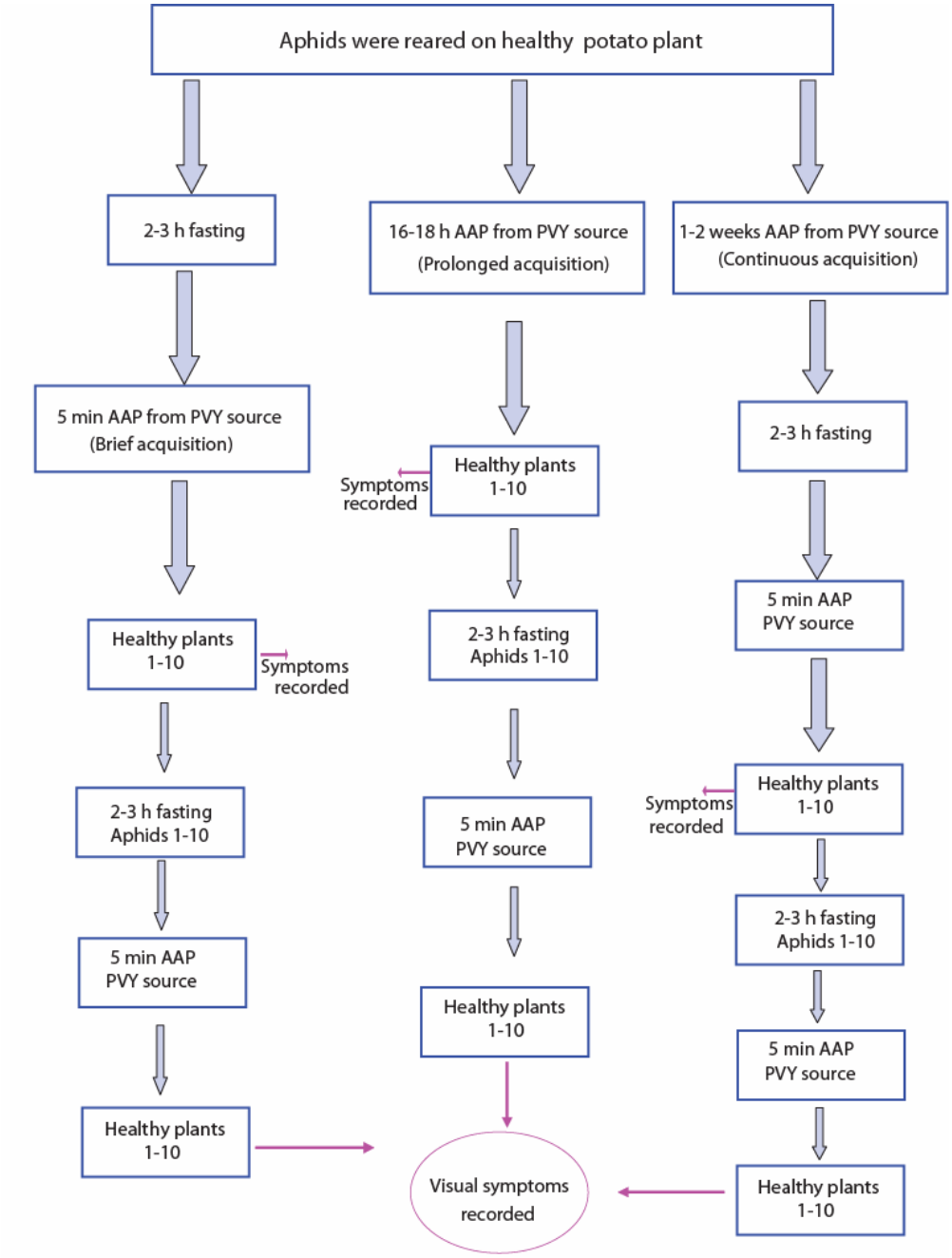

Figure 5.6. An outline of the successive transmission experiments using single aphid.

5.3.5.1. Transmission efficiency after brief acquisition period (5min)

In this study, experiments to test the effect of successive transmission on aphid vectoring ability were performed initially by using 5 aphids per test plant. The transmission rate achieved ranged between 70–90%. There was a slight decrease in

aphid transmission ability at the second transmission to between 60 and 80% (Table 5.2), but the difference was not significant.

Experiment	First transmission%	Second transmission%
Exp. 1	70*	60
Exp. 2	80	70
Exp. 3	90	80
Average	80 %	70 %

Table 5.2. Transmission of PVY by aphids in successive transmissions experiments using 5 aphids/plant.

*Percent plants infected with PVY
Five aphids per plant were used to perform transmission. Aphids were fasted for 2–3 h and allowed to acquire virus for 5 min AAP, then allowed an overnight TAP, then the same aphids were fasted for 2–3 h, allowed to acquire virus again for 5min AAP, and 16-18h TAP. The difference was not significant according to Chi-square test.

The results in table 5.2 show that aphids transmitted virus the second time as efficiently as in the first transmission. However, this result is based on using 5 aphids per plant, which may mask any effect due to difference among individual aphids (Gibson *et al.*, 1988). For this reason, single aphids were used in the following experiments. The results in table 5.3) indicate that aphids which infected a plant in the first transmission were less likely to do so in the second transmission and vice versa. This result does not support Watson's observations about the capacity of a single aphid to acquire the virus twice or three times in successive acquisition and transmission tests. However, this may be explained by the fact that she used a different virus.

Experiment	**First Transmission**	**Second transmission**
Exp. 1	4, 8	6, 2
Exp. 2	1, 6, 10	3, 8
Exp. 3	1, 6, 7, 8	1, 5, 3
Average transmission efficiency	30 %	23.3 %

Table 5.3. Variation in ability of aphids to transmit PVY in successive virus transmissions using 1aphid/plant and brief AAP.

Aphids were individually numbered 1-10 and the number of the aphid that transmitted the virus in the first or the second transmission is shown.

5.3.5.2. Transmission efficiency after prolonged (16–18h) or continuous (1–2 weeks) acquisition periods

Further investigations into the effect of virus acquisition access time on successive acquisition and transmission of the same virus species were conducted. First, the aphid's capacity to acquire the virus after being kept overnight (16–18h) on a virus source was investigated. In these experiments, the aphids were fasted for 2–3 h, kept 16-18h on detached virus-infected leaves, then transferred to healthy plants overnight and fasted for 2–3 h to perform a second acquisition for 5 min before being transferred to healthy tobacco test plants.

The results (Table 5.4) show that few of the aphids which were kept 16–18 h on PVY infected source and succeeded in transmitting the virus and only a few did so when they were given a second AAP of 5 min. However, the results are in agreement with the previous experiment that all aphids which transmitted virus in the first transmission after 5 min AAP failed to transmit after a second 5min AAP. Moreover the average transmission rate dropped from 23–30% (Table 5.3) to about 6.5% or 16.5% when the acquisition period was prolonged to overnight and followed by the first and second transmission opportunity, respectively (Table 5.4).

Experiment	First Transmission	Second transmission
Exp. 1	1	6, 8
Exp. 2	-	3, 5
Exp. 3	2	10
Average transmission efficiency %	6.6 %	16.6%

Table 5.4. Variation in ability of aphids to transmit PVY in successive transmission opportunities after a prolonged AAP for 16–18h.

Aphids were individually numbered 1−10 and the number of the aphid that transmitted the virus in the first or the second transmission is shown.

Similar results were obtained when the aphids were reared continuously on potato leaves infected with PVY. In agreement with the previous experiments, the majority of aphids which were able to transmit after this continuous access period failed to transmit the second time (Table 5.5).

Experiments	First transmission	Second transmission
Exp. 1	1, 5	6, 8
Exp. 2	2, 9	3, 5
Exp. 3	1, 2, 8	2
Average transmission efficiency %	23.3 %	16.6 %

Table 5.5. Variation in the ability of aphids to transmit PVY in successive transmission opportunities after a continuous AAP.

Aphids were individually numbered 1-10 and the number of the aphid that transmitted the virus in the first or the second transmission is shown.

Transmission rate decreased considerably after this continuous association between the virus and the aphids.

5.3.5.3. Transmission effect after acquisition of other potyviruses

PVA is another potyvirus which has similar morphological and molecular properties to PVY. In the following experiments aphids which had performed PVY transmission were given 5 min access to PVA and compared with aphids allowed to acquire PVA directly. Five aphids/plant were used. The aphids were treated the same as in PVY experiments in terms of fasting and acquisition. The experiment was repeated and 10 plants were used in each experiment.

Experiment	% Virus Transmission			
	PVY then PVA	PVY over-night then PVA	PVY only	PVA only
Exp. 1	30%	40%	80%	70%
Exp. 2	40%	50%	70%	50%
average	35%	45%	75%	60%

Table 5.6. Percent plants that became virus infected by aphids that had previously been exposed to potyvirus.

Ten plants were used for each experiment, and transmission was carried out using 5 aphids per plant. PVA transmission was performed by allowing fasted aphids 5 min AAP, and 16–18h IAP. ELISA was used to detect PVY and PVA.

The results summarized in Table 5.6 show that the group of aphids which transmitted PVY transmitted PVA less efficiently (35%) from potato to tobacco than did the aphids which were allowed access to PVA only (60%).

5.4. Discussion

The mechanism of non-persistent transmission of plant viruses by their aphid vectors has been studied extensively by plant virologists. However, these studies have focused on one component of the transmission process (the virus) with less investigation of the other components (vector, plant) in the virus–plant–vector complex. In order to understand the role of the aphid vector in the molecular mechanisms of transmission, better information about all components should be available. In Chapter 3, the possible identification of some aphid receptor proteins involved in virus particle recognition and acquisition was discussed, and the effect of the host plant on the aphid transmission process was investigated in Chapter 4. Early studies reported that potyviruses are lost from their aphid vector within a relatively short time depending on the conditions. For example, Hashiba and Misawa (1969b) used electron microscopy and found that virus-like particles of Bean yellow mosaic virus (BYMV) were visible at the tip of the aphid stylet, and that virus particles were released from the aphids after they were observed to probe when confined in a glass vial for a period of time. The transmission efficiency of those aphids which were observed to probe the glass decreased by half compared with aphids that did not probe. Hashiba and Misawa (1969b) suggested that the most credible hypothesis for inoculation of non-persistent viruses is that virions are released from the stylet tips by salivation. This is possible because, although the salivary canal remains distinctly separate from the food canal for almost the entire length of the aphid stylet bundle, the two canals converge 2–4 μm from the tips (Forbes, 1969, 1977). At the point of convergence, the maxillary stylets form an enclosed common duct (Kimmins, 1986) where mixing of food and salivary canal contents may occur. Ingested virions adhering to the cuticular lining of the common duct may therefore be flushed out during saliva secretion into the plant, providing an 'ingestion–salivation' hypothesis for transmission (Martin *et al*., 1997). According to the ingestion–salivation theory, the virus should be flushed out of the retention sites in the common duct shortly after viruliferous aphids feed on a healthy plant.

In this Chapter, a sensitive nested RT-PCR technique was used to confirm that PVY is retained in the aphid after feeding on healthy plants. Virus RNA was detected within the aphid body (S, S+H, B+H, B) even after 14 successive transfers to healthy plants, but only the first plant became infected. In addition, the aphid non-transmissible strain of PVY (PVY^C) was detected inside aphids after feeding for 1 day on healthy plants. It was found from successive virus transmissions, that an aphid vector became less able to acquire virus for a second time, and this result was the same whether aphids were taken from a healthy plant or allowed to access virus source for 16–18 h or 1–2 weeks before being starved and given 5 min AAP to infected virus source. This finding may indicate that, successful virus acquisition and transmission may lead to blocking of the virus receptors inside aphid vector and thereby decreasing by that the chance for successive virus transmission.

A specific number of virus particles must be introduced by a vector into a host plant in order to initiate infection by a particular virus. If the number of virus particles falls below this threshold an infection will not occur. It was assumed that the virus content inside an aphid after feeding on a healthy plant becomes too low to be infectious. However, due to the extreme sensitivity of the N-RT-PCR technique being used, it was found that such aphids tested positive for viral RNA. Potyviruses were reported to be aphid transmissible when the acquired virus was in the femtogram range, which is equal to a few thousand virus particles (Pirone and Thornbury, 1988). My results suggest that the N-RT-PCR technique can detect virus quantities in the attogram range, which are only a few virus particles and 1000 times less than the threshold reported by Pirone and Thornbury (1988) which means that the virus detected by the N-RT-PCR inside the aphid is less than the infection limit. However, more recently it was reported that approx. 1–3 virus particles (attogram range) were enough for an aphid to initiate an infection with PVY (Moury *et al.*, 2007), and a comparable result was reported by Moreno *et al.* (2009). The threshold reported by Moury *et al.* (2007) is similar to that detected by the N-RT-PCR technique which was used in this study. On balance, these reports suggest that the aphids are carrying sufficient virus to initiate an infection, but the virus was not transmissible. A possible reason could be because it was no longer present on the tip of the stylets and inaccessible to being flushed out during salivation.

5.4.1. Conformational/bridging effect of HC-Pro

The DAG motif located near the N-terminus of the potyvirus coat protein was reported to be involved in the interaction with HC-Pro to initiate aphid transmission of potyviruses (Harrison and Robinson 1988; Lopez-Moya *et al.*, 1999). Shukla *et al*. (1988) found that trypsin digestion of potyvirus particles remove the N-terminus but the treated virus particles are still infectious by mechanical inoculation. Therefore, it is possible that aphid saliva, which is speculated to contain trypsin activity, may cause breakage of the attachment between virus particles and putative receptors on the aphid mouthparts by removing of the the DAG motif and releasing virus particles with the flushed out saliva.

This hypothesis is compatible with both the bridge hypothesis and the conformational change hypothesis. Moreover, it may explain the decrease in transmission efficiency of the virus by aphids between 1 and 10 min or after an overnight acquisition access period. Releasing virus particles from their attachment sites by cleaving the DAG motif with aphid saliva can possibly lead to leaving some part of the transmission components (HC-Pro according to bridge hypothesis and N-terminus of CP with the DAG motif according to the conformational change hypothesis) attached to the receptor site and blocking by that attachment of other virus particles.

As indicated above, this proposal is in agreement with the bridge hypothesis. However, it is more in favour of the direct binding hypothesis which states that interaction between the viral HC-Pro and the DAG motif on the coat protein initiates a conformational change in the coat protein. As a result, direct binding between virus particles and the aphid's receptors on the stylet occurs. This hypothesis is supported by the work of Salomon and Bernardi (1995), who were able to block aphid transmission of Maize dwarf mosaic virus (MDMV) after feeding aphids on the N-terminal part of the virus CP expressed in bacteria, which suggests that this peptide can compete with the virus particles and block the receptor sites on the aphid's stylet. In addition this hypothesis is supported by long retention of virus inside aphid vector which may be a result of releasing the infectious virus particles by trypsin and keeping the DAG with the N-terminus part of the CP attached to the aphid receptors.

5.4.2. Blocking of aphid's receptors

The putative interaction between aphid proteins and virus particles, like any other biological protein-protein interaction, should be controlled by the kinetics of the biochemical reaction. It can be speculated that the interaction between virus particles and aphid receptors which is mediated by the helper component protein of the virus is very weak during the first while of attachment, thus acquired virus can be inoculated by flushed out saliva.

Virus + receptor $\rightleftharpoons$ virus-receptor complex (two-way reversible binding)

However, the interaction starts to become stronger over time until it becomes irreversible after a certain time when the aphid's ability to inoculate the virus is lost. This possibly happens after 1–2 h after aphids acquire PVY under normal acquisition conditions. The interaction stops and the virus bind very tightly (irreversibly) to the aphid receptors. The aphids therefore will no longer be able to infect healthy plants, but they can be detected by N-RT-PCR. Moreover, these defective virus particles attached to aphid mouthparts may block virus receptor sites decreasing by that the chance for aphids to acquire virus again after they transmitted and given an access to virus source again. In support of this hypothesis, Katis *et al*. (1986) found that PVY^O transmission by aphids decreased when the aphids were allowed to access a PVY^N infected plant first. Moreover, it has also been reported that aphids which transmit the first time do not necessarily transmit a second time when they were given another AAP and transferred to healthy plants, and those that did not transmit virus the first time are more likely to transmit PVY in the second transmission (Gibson *et al.*, 1988).

Data presented in tables 5.2 to 5.6 about the effect of successive transmission of virus on aphids vectoring ability are preliminary. Validation of such results would require conducting successive transmission experiments using a large number of replications to confirm the findings that once an aphid has acquired the virus, its ability to transmit again is decreased or inhibited.

5.4.3. Primary and secondary aphid's receptors

In my opinion, the existence of two types of aphid receptors can be proposed, and these can be termed primary and secondary receptors. Primary receptors are probably located at the tip of the stylet, and the interaction between these receptors and virus is proposed to be very weak and short. In addition, this interaction may be sensitive to plant sap materials and aphid inhibitory enzymes. This kind of receptor is speculated to be important for virus transmission, and the interaction is probably controlled by the virus HC-Pro and is suspected to be dominant in both directions (reversible) all the time.

Virus + receptor $\rightleftharpoons$ virus-receptor complex (two-way reversible binding)

On the other hand, the secondary receptors are not important in virus transmission because interaction is suspected to be irreversible all the time due to the direct attachment of virus particles non-specifically to any cuticle protein inside the aphid digestive system which may disable the virus particles by preventing them to release.
This hypothesis is in agreement with the observations about the retention of the virus inside the body, but detecting the virus in dissected stylets disagrees with the proposed nature of a weak interaction between the primary receptors on the stylet and the virus particles via the HC-Pro bridge. However, detection of the virus in the stylet can be explained by the possible contamination from the secondary receptors when the stylet is removed from the stylet bundle. Detecting virus inside the body (without the head) suggests that the proposed secondary receptors might exist anywhere in the cuticle proteins embedded inside the insect body. In addition, detection of HC-Pro defective PVY (PVY^C) long time after acquisition may suggest that PVY^C is possibly retained on the secondary receptors and not on the tip of the aphid stylet.

In conclusion, the newly-observed long retention of virus inside aphids can be related to one of these factors: (1) the virus becomes inactive due to the binding to specific/non-specific targets so that it can not be released by aphid saliva; (2) the virus binds directly to aphid receptors according to the conformational change hypothesis, or (3) only RNA targets were detected inside aphid which is not infectious by itself.

Chapter 6. Application of Recombinant Antibody Technology for Plant Virus Detection

6.1. Introduction

Serological tests are used in virology laboratories worldwide. Nucleic acid-based techniques such as RT-PCR are not as highly applied as ELISA for screening large numbers of samples because of the technical and financial difficulties associated with RNA extraction and the expense of the reagents. Moreover, for rapid field detection, antibody-based kits such as the pocket test developed by FERA for simultaneous detection of five of the most important potato viruses are very practical (Hims and Hill, 2001). However, despite the advantages of antibody-based assays in plant virus diagnosis, there are some problems with antibodies produced by conventional methods. For example, it is known that the N-terminal amino acids of potyvirus coat proteins are surface located, and contain most of the antigenic epitopes (Shukla *et al*., 1988). Degradation of the N-terminus of viral coat proteins of the potyvirus group after purification is common, and this problem is common also in Beet necrotic yellow vein virus (BNYVV) during storage of sugar beet (Uhde *et al*., 2000). Immunization against these degraded antigens may lead to production of less specific but more effective antibodies particularly when monoclonal antibodies are raised for specific epitopes in the coat protein. In addition to that, some viruses are very difficult to purify because of their low concentration, such as Potato leafroll virus (PLRV) (Barker, 1994), or instability, such as Potato mop-top virus (PMTV) (Cerovská *et al.* 2003). Some virus particles, such as BNYVV, aggregate with plant cell materials (Griep *et al*., 1999), and immunization of animals with impure virus preparations can lead to production of antibodies to plant components giving non-specific or false positive results (Harper *et al*., 1997). Another disadvantage of producing antibodies by traditional methods is that some viruses are weakly immunogenic. For example, it has been difficult to produce antibodies by conventional methods to viruses such as Blackcurrant reversion association virus (BRAV) (Susi *et al*., 1998) and Cucumber mosaic virus (CMV) (Ziegler *et al*., 1995). In addition, it is difficult to standardize or devise uniform tests between different laboratories or testing stations when different preparations of antisera are used, since antisera produced by different animals vary. In addition, some hybridoma lines may die or lose their ability to secrete antibodies after periods of

storage in liquid nitrogen. Recombinant antibodies can be used to rescue these lines and provide a continuous supply of a particular antibody. Furthermore, statutory licensing is required in some countries for immunizing animals, and experience is also required when working with animals to ensure their welfare. And in some countries, particularly the UK, there is a desire to find alternatives to using animals for research.

With advances in molecular biotechnology and molecular immunology, it has become easy to isolate the gene sequences coding for antibodies and insert these genes into heterologous expression systems using suitable vectors. After that, it is possible to obtain the antibodies as fusion proteins. Therefore recombinant antibody technology has emerged in the field of plant viral diagnosis. Phage display is the most common technique to select antibody molecules with the required specificity (Hoogenboom *et al*., 1998). The principle of this method relies on displaying a portion of the antibody molecule on the minor coat protein P3 of filamentous phage. The antibody gene is large and so usually a portion that contains only the binding sites of the whole antibody is displayed on the phage surface. The most common format of antibody fragments for phage display is the scFv (Fig. 6.l), which contains the variable regions of the heavy and light chains of immunoglobulin (McCafferty *et al.* 1990; Marks *et al*., 1991). In addition, the stability of a phage library produced from displaying scFv is better than that of libraries produced from other fragments such as Fab (Hoogenboom *et al*., 1998). Furthermore, it is usual to express functional scFv in bacteria rather than the whole antibody, which needs post translational modifications such as glycosylation for proper folding and expression (Daly *et al*. 2001).

Antibody libraries can be constructed for general use by using DNA from non-immunised human donors to construct naïve libraries (Vaughan *et al*., 1996) or from synthetic genes to construct synthetic libraries (Nissim *et al.*, 1994). After production of the phage library, phage ELISA can be used to screen the library for any antigen of interest by conducting several rounds of panning and enriching (Dyson *et al*. 1995). Once a desirable clone has been selected, it can be sub-cloned into a suitable cloning vector to produce fusion proteins with different characteristics. For example, Toth *et al*. (1999) produced α PLRV scFv fusion proteins with different physical properties by sub-cloning into different vectors. For example, fusing the α-PLRV scFv to the CL domain of the light chain of human immunoglobulin improved the stability of the scFv molecule, which tends to denature when used alone to coat ELISA plates. Another

detecting molecule was produced by Toth *et al*. (1999) by fusing scFv to the alkaline phosphatase enzyme and using the resulting product directly in ELISA. Moreover, both scFv-CL and scFv-AP fusions tend to dimerize spontaneously, thereby improving their functional affinity (Ziegler and Torrance, 2002) (Fig. 6.2).

Many systems have been used to express these recombinant molecules. Bacterial expression systems such as *E. coli* are commonly used as they are cheap and easy to maintain and manipulate (Harper *et al.*, 1997; Daly *et al*., 2001). The possession of post-transcriptional modification capacity in eukaryotic systems makes them better than prokaryotic systems, which lack this important machinery (Daly *et al*., 2001). Introducing a translocational signal in *E. coli* which directs the produced protein to the periplasm can lead to a more functional scFv as the secreted antibodies follow a similar route when produced naturally in the endoplasmic reticulum of the lymphocyte (de Haard *et al*. 1998). In addition, many expression systems have been used such as yeast, insect cells (Reavy *et al*., 2000) and plants (Firek *et al*., 1993; Ziegler *et al*., 2000), but such systems are less commonly used than *E. coli*.

Phage display antibody libraries can be considered as a potentially unlimited source of testing reagents with similar efficiency to polyclonal and monoclonal antibodies. In addition, they can be produced cheaply and quickly with simple techniques and without immunization of animals, and they can be stored indefinitely as DNA (Toth *et al*., 1999; Daly *et al*., 2001). Low affinity is the major limitation of recombinant antibody technology for routine virus detection. However, this problem can be overcome by construction of diverse libraries. In addition, the affinity of such diagnostic proteins can be improved before incorporation into virus detection kits by cloning into vectors which enable expression of bivalent or multivalent molecules.

The affinity of biotin towards avidin or streptavidin is extremely high; the dissociation constant (K_d) is ~10^{-15} M/L, one of the strongest known non-covalent bonds, and this high affinity is currently exploited by labelling proteins for detection and purification (Bayer and Wilchek, 1990, Wilchek and Bayer, 1990). Chemical biotinylation has been used to facilitate purification and detection of proteins by targeting biotin to the free amino groups on the lysine residues of the protein. However, because of the problems associated with random biotin labelling, a more targeted approach is sometimes required to maintain biological activity of the biotinylated products. *In vivo*

biotinylation is a mimic of what happens to biotinylated proteins in nature: the biotin acceptor domain of the biotin carboxyl-carrier protein (BCCP) acts as a natural substrate for the biotin ligase enzyme in bacteria (Schatz, 1993). This fact has been exploited by fusion of this substrate sequence to the protein of interest and subsequent biotinylation of the expressed protein by the *E. coli* endogenous biotin ligase (Cronan, 1990). Since it was introduced, the *in vivo* process has been used to biotinylate many proteins for different purposes, such as affinity purification of antibodies (Cronan, 1990; Lesley and Groskreutz, 1997). It has been used in molecular diagnostics as well to improve the functional affinity of some antibody fragments such as the Fab fragment (Weiss *et al*., 1994; Sibler *et al*., 1999) and the scFv fragment (Cloutier *et al*., 2000; Santala and Lamminmaki, 2004).

Previous work at SCRI has revealed the feasibility of recombinant antibody technology for producing reagents for routine testing, but before these reagents are introduced commercially, the stability and affinity of such products must be improved (Torrance, 1999). The aim of the experiments reported in this chapter was to improve the detection efficiency of α-PLRV scFv by coupling it to biotin, in order to devise a detection system based on the biotin/streptavidin binding (Fig. 6.3), and by optimizing ELISA conditions to create a fully recombinant ELISA test for routine PLRV detection, with high sensitivity and low background.

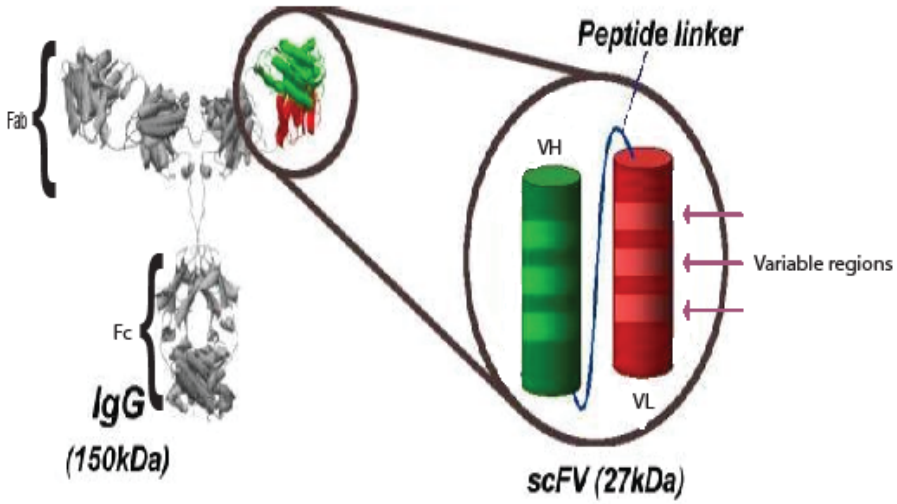

Figure 6.1. Schematic diagram showing the scFv fragment in relation to the whole IgG molecule.

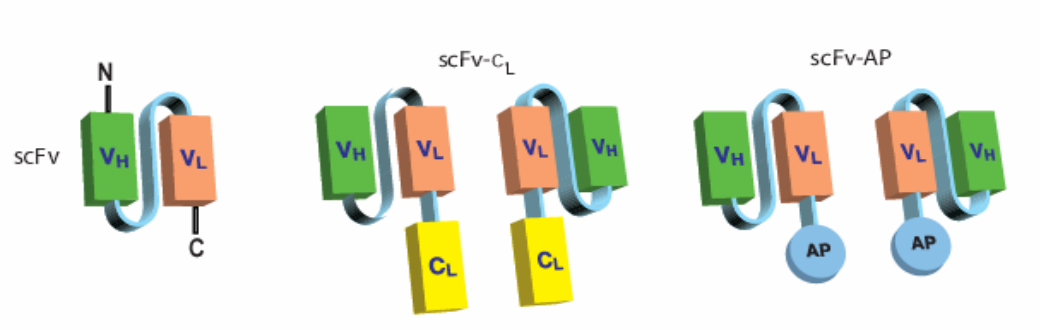

Figure 6.2. Schematic representation of the monomeric and dimeric forms of ScFv.

Adapted from Ziegler and Torrance (2002).

V_H: variable region of the heavy chain, V_L: variable region of the light chain.
AP: Alkaline phosphatase, C_L: constant region of the light chain.

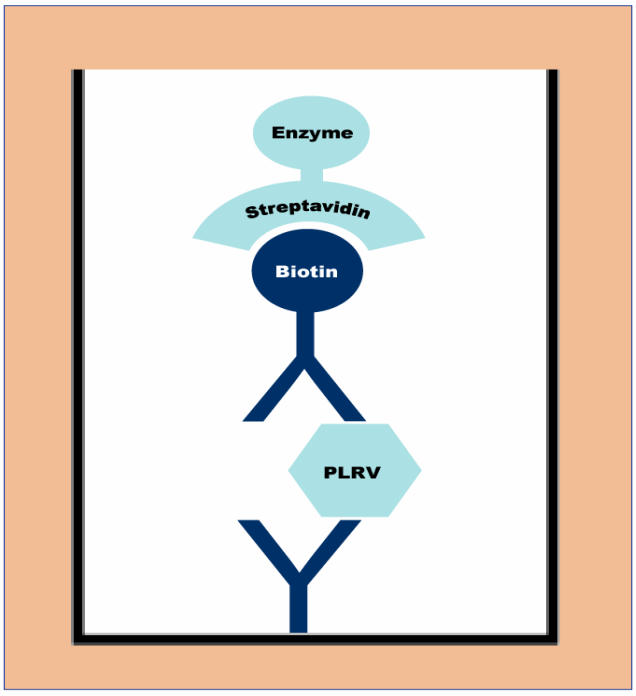

Figure 6.3. Schematic diagram of the proposed recombinant biotinylated ELISA format.

scFv-CL exists in dimeric form, so it is shown as a bivalent y shape.

6.2. Materials and methods

6.2.1. V3HCL (V3) protein expression and purification from *E. coli*

The pCL vector containing the PLRV specific scFv insert was obtained in-house and its construction was previously reported (Toth *et al,* 1999). Competent *E. coli* cells (XL10 Gold, Stratagene) were transformed with pCL-V3 which already been constructed (Toth *et al*., 1999) as explained in Chapter 2. Transformed colonies were grown in 5 ml LB medium containing 100 µg/ml kanamycin and 1% glucose overnight at 30°C with shaking. 10 ml of each culture was diluted into 100 ml 2x TY medium containing 100 µg/ml kanamycin and 0.1% glucose and grown with shaking at 30°C for 2 h. The culture was centrifuged in a bench top centrifuge for 20 min at 4,000 rpm, and the cells were resuspended in 250 ml fresh 2x TY medium containing 100 µg/ml kanamycin. Expression of scFv was induced by the addition of IPTG to 1 mM, with the culture grown overnight (16-18h) with shaking at 30°C.
Cells were harvested from induced cultures by centrifugation (Eppendorf 5810R centrifuge) at 4,000 rpm for 30 min at 4°C. The supernatant was retained on ice, and the harvested bacterial cells were resuspended in ice-cold PBS containing 1 mM /L EDTA at 1ml/per 1g of dry weight of the bacterial pellets. Then 2 ml/ g of 1: 20 dilution of PBS-EDTA in water was added to the mixture and the mixture was incubated in ice for 30 min then centrifuged for 10 min at 14,000 rpm at 4°C to produce the periplasmic extract (PE). Supernatant and PE were combined and protein precipitated by addition of solid ammonium sulphate (30 g/100 ml) with stirring. Precipitated protein was collected by centrifugation at 4,000 rpm for 30 min at 4°C then resuspended in 1–2 ml of PBS and dialyzed against PBS with several changes.

Ni-NTA resin (Qiagen) was used for purification following the manufacturer's instructions (see Chapter 2). The protein was incubated with resin for 60 min at 4°C. The resin was washed twice in 10 mM imidazole in PBS and gently shaken at 4°C for 5 min, then resuspended in 500 mM imidazole in PBS and shaken at 4°C for 10 min to elute protein. The eluate was added to a PD-10 desalting column containing Sephadex™ G-25 (GE Healthcare) previously equilibrated in 20 ml PBS. Then 20 fractions each containing 10 drops, were eluted from the desalting column with PBS.

The A_{280} of each fraction was measured and protein concentration estimated according to the protein extinction coefficient (1 absorbance unit = 0.7 mg/ml). Fractions with protein concentrations greater than 1 mg/ml were combined and total protein concentration was calculated.

6.2.2. Chemical biotinylation of V3

Chemical biotinylation was performed by using the EZ-Link NHS-Biotin preparation (Pierce) following the manufacturer's instructions. Briefly 1.5 mg of V3 was dialyzed with several buffer changes (one overnight and 2 for 2 h each) against 1 L of Sorensen's phosphate buffer at 4°C. Then biotin was dissolved in DMF at 1 mg/ml, and added to protein samples at different biotin: V3 molar ratios (10:1, 20:1, and 40:1). The solution was incubated in ice for 2 h, and finally dialyzed against PBS as before.

6.2.3. *In vivo* biotinylation of V3

6.2.3.1. Constructing the V3-CL insert

PCR was used to amplify the insert coding for the variable heavy and light chains and the C_L domain using pCL plasmid, which contains this insert, as template. Primers (V3FWDSfi, V3REVNot) which contain *Sfi*I and *Not*I sites were designed (Table 2.4). Taq DNA polymerase (Roche) was used following these conditions: initial denaturation at 94°C for 2 min; 24 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C; and final extension at 72°C for 1 min.

After successful amplification, the V3-C_L insert was cloned into the pGEM-T Easy vector (Promega Corporation) and sequenced. Qiapreps (QIAGEN) were prepared from white colonies, and after restriction enzyme digestion three of these clones were chosen for sequencing. One clone was selected to prepare a midiprep which was used to release the V3 insert by restriction enzyme digestion for cloning into the biotinylation vector.

6.2.3.2. Biotinylation vector

The pAK300Bio plasmid, which is a modified version of the pAK300 vector (Krebber *et al.* 1997*)*, was modified as described below to enable *in vivo* biotinylation of expressed protein by adding a biotin acceptor domain before the His-tag coding sequence.

6.2.3.3. Introducing a *Not*I site into pAK300Bio plasmid

In order to be able to clone the V3-C_L *Sfi*I/*Not*I insert, it was necessary to introduce a *Not*I site into the pAK300Bio vector instead of its second *Sfi*I site. The QuikChange II Site-Directed Mutagenesis kit (Stratagene) was used to change the *Sfi*I site into a *Not*I site using the primers BIOFWD and BIOREV (Table 2.4), by following the manufacturer's instructions and the method presented in Chapter 2. The PCR conditions were: initial denaturation at 95°C for 1 min; 17 cycles of denaturation for 50 s at 94°C, annealing for 50 s at 60°C, and elongation for 6 min at 68°C; and final extension at 68°C for 7 min.

6.2.3.4. Mutation of NotI site in the insert

The V3 insert sequence contained a *Not*I restriction site which interfered with cloning. This site was removed by mutagenesis using the primers V3NOTMutFwd and V3NOTMutRev (Table 2.4), and by following the site directed mutagenesis method presented in Chapter 2. PCR conditions were similar to those mentioned above except that the elongation cycles at 68°C lasted for 5 min only.

6.2.3.5. Cloning V3 into biotinylation vector

The V3-C_L insert and pAK300-Bio-Not plasmid were ligated overnight at room temperature using Promega T4 DNA ligase enzyme following the method presented in Chapter 2. The ligation products were electroporated into the *E. coli* AVB100 strain (Avidity), which has the *birA* gene incorporated into the chromosome under the control of L-arabinose inducer. A clone which was confirmed to have the right size insert was used for expression a scFv-CL

6.2.3.6. V3B protein expression and purification

V3B was induced and purified according to the method published by Warren *et al*. (2005) with some modifications. Prior to induction, the bacteria were grown in 2x TY containing 1 g/l glucose, 30 mg/L chloramphenicol, 50 mM/l K_2HPO_4 pH 7.2), and 5 mM/l $MgSO_4$ for 3-4 h until the OD reached A600/cm = 0.6-0.8 Then protein production was induced by addition of both IPTG to 1 mM and L-arabinose to 1.5 µM/l

in the presence of 50 μM/l D-biotin (Sigma). Recombinant protein was harvested 16-18 h after induction at 30°C by centrifugation of cultures for 20 min at 4000 rpm at 4°C. Supernatant (S1) was collected, and the pellet was resuspended (2:1 V/W) in ice-cold 200 mM/l Tris-HCl (pH 8) containing 0.5 mM/l EDTA and 500 mM/l sucrose, then 1:5 dilution of the previous buffer was added, and the mixture was incubated in ice for 30 min then centrifuged for 10 min at 14,000 rpm at 4°C. Supernatant (S2) was collected, and combined with S1. Protein was precipitated by addition of solid ammonium sulphate (30 g/100 ml) with stirring and collected by centrifugation at 3000 rpm for 30 min at 4°C. Finally protein was resuspended in a small volume of PBS and dialyzed against PBS for 2 h, then buffer was changed into 1 L fresh PBS, and dialysis was continued overnight at 4°C. Afterwards, the absorbance of the protein was measured and protein concentration was determined, then biotinylated protein was ready to be used.

A simpler method was also used which excluded the ammonium sulphate precipitation step. The harvested protein was resuspended (2:1 V/W) in ice-cold 200 mM/l Tris-HCl (pH 8) containing 0.5 mM/l EDTA and 500 mM/l sucrose, then the mixture was diluted in 1: 5 dilution of the ice-cold 200 mM/l Tris-HCl, and then the mixture was incubated for 30-60 minutes at 4°C. Supernatant was collected afterwards by centrifugation at 14,000 rpm for 10 min at 4°C. Protein was dialysed as above, and then was used directly for detection.

6.2.3.7. ELISA and Western blot procedures

General ELISA conditions are presented in Chapter 2. Control (standard) ELISA with immune reagents used 1:1000 dilution of SCRI α PLRV polyclonal antibody for coating wells and 1:1000 diluted α PLRV SCR1 monoclonal antibodies with 1:1000 diluted α mouse AP for detection; recombinant ELISA used biotinylated V3-CL and streptavidin-AP for detection (Table 2.2). Incubation of the conjugates was performed together with the detecting reagent as the sequential incubation of the recombinant reagents was poor. Unless otherwise mentioned, leaf extract was used at 1:10 dilution in extraction buffer, and absorbance values (A_{405}) were routinely measured after 1h of incubation with substrate but in some experiments readings were recorded after 1, 2, and after overnight. Absorbance values were measured for duplicate samples and mean values are presented; in addition to ensure reproducibility, each experiment was repeated three

times and representative results were presented in each table. Absorbance values of extracts from infected plant leaves were compared with absorbance values of non-infected leaf extract controls. Samples were considered positive if the value was more than two times greater than the value obtained from the negative control.

Western blot was performed as indicated in Chapter 2; protein electroblotted on membranes was incubated with α-histidine antibody (α-His) then with anti-mouse-AP (α-mouse-AP) conjugate or streptavidin-AP (S-AP) at working diltions stated in Chapter 2.

6. 3. Results

6.3.1. Chemical biotinylation

Recombinant ELISA		Biotin:V3 Molar Ratio					
		10:1		20:1		40:1	
V3/V3B*	Incubation Time	I	NI	I	NI	I	NI
1 µg/ml	1 h	0.11	0.08	0.14	0.15	0.11	0.14
	2 h	0.17	0.09	0.23	0.15	0.15	0.20
	12 h	0.43	0.25	0.54	0.51	0.35	0.47
5 µg/ml	1 h	0.20	0.08	0.1	0.08	0.12	0.11
	2 h	0.35	0.09	0.15	0.11	0.19	0.16
	12 h	1.00	0.85	0.38	0.25	0.47	0.39
10 µg/ml	1 h	0.42	0.08	0.08	0.06	0.13	0.09
	2 h	0.74	0.09	0.09	0.07	0.20	0.16
	12 h	2.01	0.34	0.20	0.20	0.55	0.38

Control (P/M)			
	Incubation Time	I	NI
	1 h	0.56	0.08
	2 h	0.86	0.10
	12 h	2.34	0.20

Table 6.1. Absorbance values (A_{405}) obtained from ELISA plate readings when *in vitro* biotinylated V3 preparations were used for detection of PLRV in infected *P. floridana* plant leaf extracts.

∗ V3/V3B: V3 coating and V3B detecting (See Fig. 6.3). I: infected *P. floridana* leaf extract, NI: non-infected *P. floridana* leaf extract. 10:1, 20:1, and 40:1 biotin: protein molar ratio.

P/M: polyclonal antibody for coating and monoclonal antibody for detection.
Different ratios of biotin to V3 in the biotinylation reaction were tested as indicated. S-AP was used at 1:4000 dilution.

V3 protein was biotinylated *in vitro* using different molar ratios (Table 6.1) of biotin to V3 protein and the resulting products were tested in ELISA for PLRV detection at a range of concentrations. Results were compared with those obtained in a standard ELISA test with polyclonal antibody for coating and monoclonal antibody for detection. Table 6.1 shows that only the ratio with the lowest biotin concentration (10:1 biotin: V3 molar ratio) gave good discrimination between positive and negative samples. The reason could be that at higher concentrations of biotin, over-biotinylation might occur, and as a result the scFv binding site is inactivated by modification of any accessible lysine residues, especially if they are located in the active site of the protein (Chames *et al*. 2002). The preliminary results (Table 6.1) showed that the best working concentrations of both V3 and V3B were 10 µg/ml, which gave high sensitivity and low background when a 10:1 biotin:V3 ratio had been used in the biotinylation reaction.

6.3.1.1. Optimizing ELISA conditions

6.3.1.1.1. Reagent concentrations

The effect of using different dilutions of V3 and S-AP was investigated as well as blocking and other conditions. Previous work at SCRI showed that the optimum concentration of V3 for coating was 10 µg/ml, and this was confirmed here by coating with 5, 10, and 20 µg/ml (not shown) of V3, and detecting with 5 or 10 µg/ml of V3B. ELISA results from a typical experiment are presented in Table 6.2. They show that 10 µg/ml V3B gave lower non-specific background readings than 5 µg/ml. In addition, different concentrations above and below 10 µg /ml were tested. However, the concentration (10 µg/ml of both V3 and V3B) gave better discrimination between infected and non-infected samples, and was used subsequently for coating with V3 and detecting with V3B.

Incubation time	V3/V3B 10/10 µg/ml		V3/V3B 5/10 µg/ml		V3/V3B 10/5 µg/ml		V3/V3B 5/5 µg/ml		P/M	
	I	NI	I	NI	I	NI	I	NI	I	NI
1 h	0.38	0.08	0.24	0.08	0.32	0.1	0.19	0.09	0.44	0.06
2 h	0.6	0.12	0.37	0.1	0.51	0.16	0.3	0.12	0.71	0.06
12 h	1.88	0.33	1.18	0.27	1.61	0.44	0.96	0.3	2	0.08

Table 6.2. A_{405} values obtained from ELISA plate readings when different concentrations of V3 and V3B were used for coating and detection of PLRV in plant leaf extracts.

S-AP was used at 1:4000 dilution.
I: infected *P. floridana* leaf extract at 1:10 dilution in extraction buffer, NI: non-infected *P. floridana* leaf extract at 1:10 dilution.
P/M: polyclonal antibody for coating and monoclonal antibody for detection.

	S-AP Dilution								P/M	
	1:1000		1:2000		1:4000		1:8000			
Incubation Time	I	NI	I	NI	I	NI	I	NI	I	NI
1 h	0.23	0.22	0.24	0.18	0.29	0.09	0.27	0.08	0.35	0.06
2 h	0.41	0.37	0.4	0.28	0.52	0.09	0.46	0.09	0.64	0.06
12 h	1.14	1.17	1.22	0.85	1.45	0.32	1.42	0.7	1.77	0.06

Table 6.3. A_{405} values obtained when different concentrations of streptavidin-AP were used in the recombinant ELISA assay with V3 for coating and V3B for detecting.

P/M: polyclonal antibody for coating and monoclonal antibody for detection. I: PLRV-infected *P. floridana* leaf extract; NI: *P. floridana* non-infected leaf extract.

S-AP was tested at dilutions from 1:1000 to 1:8000. The lowest background readings were obtained with a 1:4000 dilution of S-AP, and so this was considered to be the best dilution for the conjugate (Table 6.3). The values from the samples showed some difference between recombinant (0.29) and immune reagent (0.35) antibodies 1 h after incubation with the substrate. In addition, higher background was obtained when using

the recombinant antibodies (0.08 compared with 0.06 for the immune reagents). It was concluded that a combination of 10 µg/ml of V3 for coating and 10 µg/ml of V3B for detecting, with a 1:4000 dilution of the S-AP, gave the best results in terms of difference between specific and non-specific reactions.

6.3.1.1.2. Effect of blocking and leaf extract concentration

ELISA format	Leaf extract dilution	V3/V3B 10/10µg/ml			Control (P/M)		
		I	NI1	NI2	I	NI1	NI2
+ blocking	1:10	0.32	0.08	0.08	0.59	0.06	0.06
	1:20	0.22	0.09	0.08	0.34	0.05	0.06
	1:40	0.15	0.09	0.09	0.23	0.05	0.06
- blocking	1:10	0.25	0.09	0.08	0.57	0.06	0.06
	1:20	0.2	0.09	0.08	0.3	0.06	0.06
	1:40	0.15	0.09	0.07	0.22	0.06	0.06

Table 6.4. A_{405} values obtained from ELISA plates when a blocking step was included in the recombinant ELISA assay.

V3/V3B: V3 (10 µg/ml) coating and V3B (10 µg/ml) detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection.
I: infected *P. floridana* leaf extract, NI1: non-infected *P. floridana* leaf extract, NI2: non-infected potato leaf extract. S-AP was used at 1:4000 dilution.

The effect of blocking the nonspecific binding sites with 3% non-fat dried milk (Marvel) on the performance of the assay with *in vitro* biotinylated V3 was investigated. It can be concluded from Table 6.4 that including a blocking step had little impact on increasing efficacy of biotinylated protein in virus detection, and the background in the non-infected leaf samples remained the same. Moreover, increasing the dilution of plant extracts from 1:10 to 1:40 decreased the absorbance about two times for infected material but did not reduce the background reading.

6.3.1.1.3. Antibody buffer composition

For immune reagents antibody, it is recommended to perform incubation steps in PBST buffer. In addition, it is recommended to add PVP to the extraction buffer to prevent interferance by components of plant extract. In the following experiments (Table 6.5) the effect of different buffers for incubation of the recombinant antibodies was investigated.

ELISA Format	Incubation Time	Detection buffer conditions								
		PBSTM			PBST			PBSM		
		I	NI1	NI2	I	NI1	NI2	I	NI1	NI2
V3/V3B	1 h	0.25	0.08	0.09	0.25	0.13	0.13	0.09	0.06	0.07
	2 h	0.42	0.09	0.1	0.42	0.21	0.2	0.12	0.07	0.08
	12 h	2.62	0.52	0.53	2.63	1.34	1.34	0.61	0.25	0.33
P/M	1 h	0.85	0.07	0.07	0.98	0.07	0.07	1.23	0.06	0.06
	2 h	1.48	0.07	0.07	1.71	0.08	0.08	2.11	0.07	0.06
	12 h	5.54	0.18	0.16	4.84	0.28	0.28	4.43	0.15	0.11

Table 6.5. A_{405} values obtained in ELISA to test the effect of blocking agents in the buffer on virus detection.

PBSTM: 1x PBS + 0.05% Tween 20 + 0.1% non fat powdered milk. PBST: PBS + 0.05% Tween 20.

PBSM: PBS + 0.1% non-fat powdered milk. I: infected *P. floridana* leaf extract, NI1: non-infected *P. floridana* leaf extract, NI2: non-infected potato leaf extract. 10 µg/ml of V3 was used for coating and 10 µg/ml of V3B for detecting, and S-AP was used at 1:4000 dilution.

The effect of addition of Tween 20 and non-fat powdered milk to the detection antibody (V3B) buffer was tested. Table 6.5 indicates that although both PBSTM and PBST buffers gave the same absorbance for infected samples, PBSTM performed better as the background was much lower. In the assay with V3 and V3B, absorbances for infected samples were much lower with PBSM than with PBST and PBSTM.

The next experiment (Table 6.6) was designed to investigate the effect of adding PVP to the extraction buffer on the performance of the recombinant test when an incubation step with 3% Marvel was included or not.

Leaf extract dilution		No blocking				Blocking			
		+PVP		- PVP		+PVP		- PVP	
		V3/V3B	P/M	V3/V3B	P/M	V3/V3B	P/M	V3/V3B	P/M
I	1:10	0.12	0.85	0.29	0.98	0.15	0.91	0.32	0.94
	1:20	0.1	0.59	0.23	0.7	0.17	0.53	0.19	0.6
	1:40	0.08	0.35	0.15	0.42	0.12	0.32	0.14	0.36
NI1	1:10	0.06	0.06	0.06	0.05	0.06	0.07	0.06	0.05
	1:20	0.06	0.06	0.06	0.05	0.06	0.06	0.05	0.06
	1:40	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.06
NI2	1:10	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
	1:20	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
	1:40	0.06	0.06	0.06	0.05	0.06	0.06	0.06	0.06

Table 6.6. A_{405} values obtained in recombinant ELISA to test the effect of adding PVP to extraction buffer on the efficiency of recombinant ELISA.

V3/V3B: V3 coating and V3B detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection.
I: infected *P. floridana* leaf extract, NI1: non-infected *P. floridana* leaf extract, NI2: non-infected potato leaf extract. 10 µg/ml of V3 was used for coating and 10 µg/ml of V3B for detecting, with 1:4000 S-AP.

Table 6.6 shows that adding PVP to the infected leaf extract reduced the absorbance values of infected samples to approximately half their values without PVP when the recombinant assay was used. On the other hand, little difference was observed in the immune reagent control (P/M) whether PVP was added or not. Moreover, the blocking had no effect in decreasing the background reaction.

6.3.1.2. Comparison of the performance of *in vitro* biotinylation with immune reagents

Table 6.7 shows that fully recombinant antibodies gave less absorbance values compared to immune reagents when readings were made after 1 h incubation, and the background non-specific reaction was higher and became stronger after a prolonged incubation period.

Leaf extract dilution	Incubation time	V3/V3B			P/M		
		I	NI1	NI2	I	NI1	NI2
1:10	1 h	0.45	0.08	0.09	0.68	0.06	0.06
	2 h	0.83	0.12	0.13	1.22	0.06	0.06
	12 h	3.08	0.4	0.44	2.2	0.09	0.09
1:20	1 h	0.3	0.09	0.09	0.38	0.06	0.06
	2 h	0.53	0.12	0.13	0.75	0.06	0.06
	12 h	2.08	0.39	0.41	1.45	0.09	0.09
1:40	1 h	0.2	0.09	0.09	0.15	0.06	0.06
	2 h	0.35	0.13	0.13	0.25	0.06	0.06
	12 h	1.4	0.41	0.44	0.93	0.09	0.09

Table 6.7. A_{405} values obtained from recombinant ELISA in comparison with immune reagent ELISA for detection of PLRV in different concentrations of leaf extract.

V3/V3B: V3 coating and V3B detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection. I: infected *P. floridana* leaf extract, NI1: non-infected *P. floridana* leaf extract, NI2: non-infected potato leaf extract.
10 µg /ml of V3 was used for coating and 10 µg/ml of V3B for detecting, with 1:4000 S-AP.

For example, the background in the recombinant assay was relatively low (0.08-0.09) after 1h of incubation with the substrate; it became higher with prolonged incubation, and this was similar with potato and physalis extracts at all dilution of leaf extracts.

6.3.1.3. Variation between chemically biotinylated batches

Three separate batches of chemically biotinylated V3 were compared. Table 6.8 shows that although ELISA readings were similar for infected samples, the performance varied between different prepared batches.

Incubation time	Batch 1		Batch 2		Batch 3	
	I	NI	I	NI	I	NI
1 h	0.34	0.14	0.27	0.08	0.3	0.06
2 h	0.63	0.23	0.45	0.1	0.55	0.06
12 h	1.79	0.61	1.41	0.25	1.51	0.07

Table 6.8. A_{405} values obtained from recombinant ELISA showing variation in detection performance between different chemically biotinylated V3 batches.

I: PLRV-infected *P. floridana* leaf extract; NI: non-infected *P. floridana* leaf extract.
10 µg/ml of V3 was used for coating and 10 µg/ml of V3B for detecting, with 1:4000 S-AP.

This is because of the background non-specific reaction readings. In batch 1, the background reading after 1 h was high (0.14) compared to 0.08 in batch 2 and 0.06 in batch 3. After overnight incubation, the background was 0.61, 0.25, and 0.07 respectively for the three batches. This means that batch 3 was the best as the background remained at a low level and comparable with the control's background reading (0.08). Batch variation can be attributed to either differences in proportion of functional V3 protein relative to inactive *E. coli* protein, or the number of biotinylated lysine residues in the scFv fragment, and possible interference with the scFv active sites after biotinylation as discussed in Section 6.4.

6.3.2. *In vivo* Biotinylation

6.3.2.1. Construction of the expression vector

The expression vector pAK300 was fused with the sequence coding for the biotin acceptor domain (BAD) to enable *in vivo* biotinylation of expressed protein. In addition, a *Not*I site was introduced successfully (Fig. 6.4) into the pAK300 vector using site-directed mutagenesis. The modified version of this vector is named pAK300-Bio-Not.

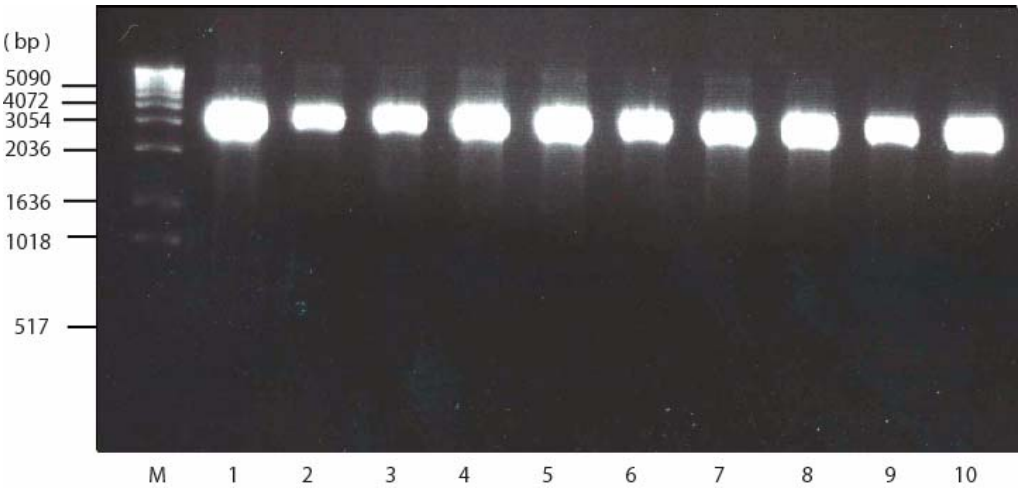

Figure 6.4. Agarose gel showing linearized pAK300-Bio-Not vector after restriction enzyme digestion with *Not* I.

M: DNA molecular size markers, Lanes 1-10: *Not*I digests of randomly chosen clones after a *Not*I site was introduced inside the vector.

The V3 gene sequence contains an internal *Not*I site. This site was destroyed by site-directed mutagenesis in order to be able to perform complete digestion of the vector harbouring the insert (Fig. 6.5), as partial digestion resulted in low quality insert. The mutated V3 insert was ligated into the pAK300-Bio-Not vector to create the pAK300-Bio-Not-V3 expression plasmid (Fig. 6.6). Successful ligation of the insert was confirmed by restriction enzyme digestion. The expected size insert (1.2 Kb) was released by digestion of the ligated vector (Fig. 6.7).

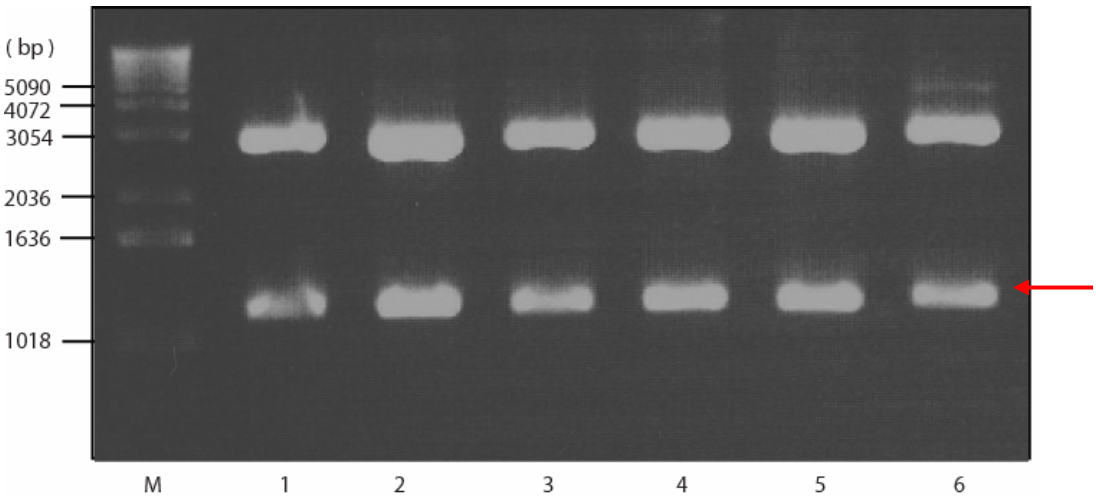

Figure 6.5. Agarose gel showing successful mutation of the *Not* I site in the scFv-CL insert.

M: DNA molecular size markers, Lanes 1-6: *Not*I digests of randomly chosen clones after mutation of the *Not*I site inside the insert. Upper band: vector. Lower band: insert arrowed.

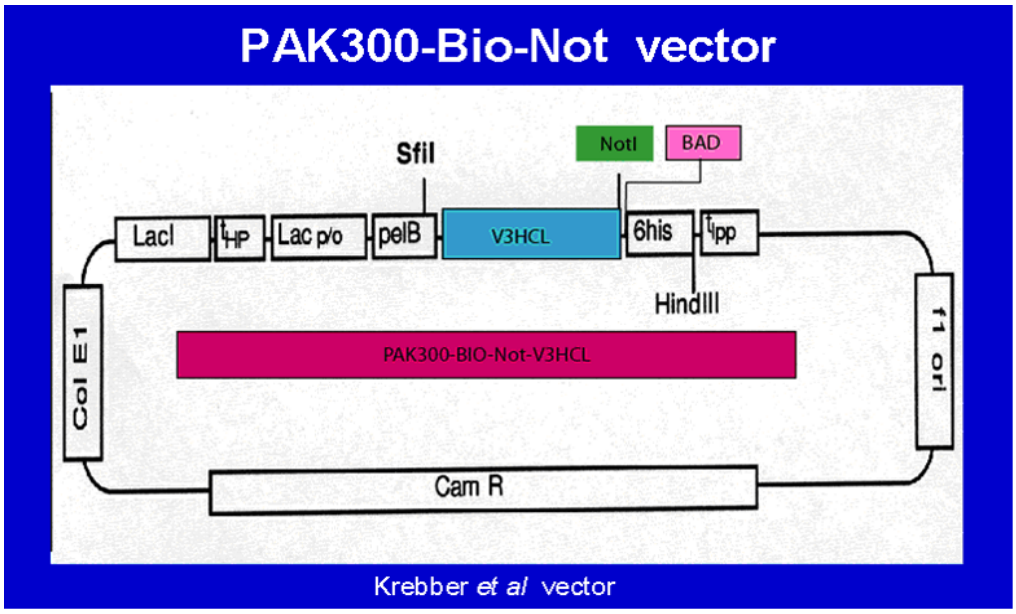

Figure 6.6. Schematic representation of the pAK300-Bio-Not expression vector map, and the ligation of the insert.

The key elements of the pAK300-Bio-Not vector are,the pelB signal peptide, chloramphenicol antibiotic resistance marker (Cam R), the Lac I promoter, the Coi E1 origin of replication, Not I and SfiI cloning sites, the 6-Histidine tag. In addition, the BAD was fused, and the the *Not*I site was introduced to enable V3 cloning.

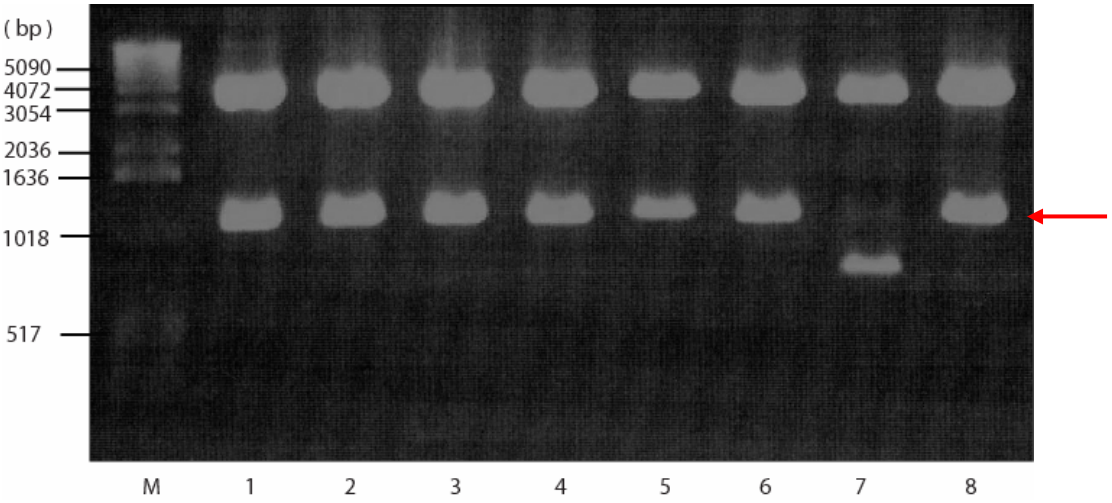

Figure 6.7. Successful ligation of the scFv-CL (V3) insert into the pAK300-Bio-Not vector.

M: DNA molecular size markers, Lanes 1-8: *Not*I / *Sfi*I digest of randomly chosen clones after transformation. Upper band: vector. Lower band: insert arrowed.

6.3.2.2. Optimizing V3B expression conditions

Induction of V3B production was tested under several different conditions, for example, addition of K_2HPO_4 and $MgSO_4$ for enrichment expression cultures to increase bacterial cell growth, induction with different concentrations of IPTG and L-arabinose or adding only one of them (data not shown). Both inducers are required for V3B production as the IPTG induces expression of V3 whereas arabinose induces the BirA enzyme. The optimal conditions for V3B production were in accordance with the results published by Warren *et al.* (2005) who used the system to biotinylate a scFv to be used as a capturing reagent for minimizing the interference from heterophilic antibodies in immunometric assays. Induction was for 16 h after addition of IPTG and arabinose at 1 mMl/l and 1.5 µM/l respectively to the culture, which is supplemented with 50 µM/L D-biotin, 50 mM/l potassium phosphate (pH 7.2), and 5 mMl/l $MgSO_4$.

Fig. 6.8 shows that biotinylated protein was successfully expressed, and the protein was detected using α-histidine antibody and streptavidin-AP or anti-mouse-AP conjugates. However, the detection was better using the streptavidin-AP conjugate. The protein was

detected after 1h induction and reached the maximum concentration after 16 h post induction.

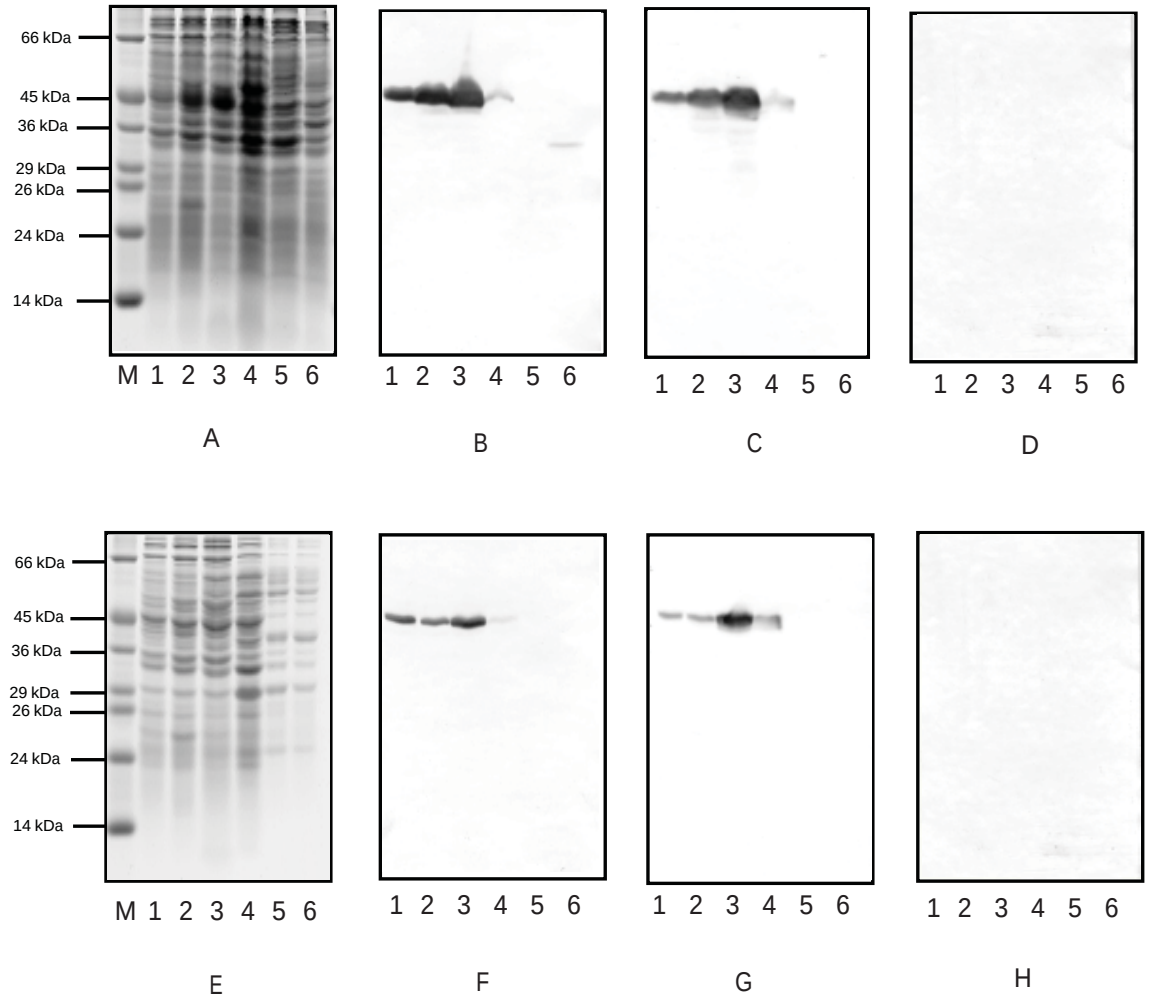

Figure 6.8. SDS-PAGE and western blot analysis of V3B expression in *E. coli*.

Upper panels (A-D): whole cell extracts. Lower panels (E-H): periplasmic extracts. Coomassie blue stained SDS-PAGE (A, E); western blots with α-histidine and α-mouse-AP conjugate (B, F) and streptavidin-AP (C, G); control blots minus α-histidine (D) or streptavidin-AP (H). Lane M: protein size markers (molecular mass of markers indicated on the left). Lanes 1, 2, 3, 4: samples harvested 1, 3, and 16 h after induction or after 16 h without induction respectively; Lane 5: Non-transformed AVB100 bacterial strain grown for 16 h without induction; Lane 6: AVB100 strain harbouring the pAK300-Bio plasmid after 16 h without induction.

6.3.2.3. Performance of the *in vivo* biotinylated V3

Protein was prepared in different batches. The maximum yield (6 mg/l) was obtained by increasing the concentration of EDTA in the buffer used to extract the periplasmic extract from 0.5 mM/l to 1 mM/l and applying a second periplasm extraction. In addition, the protein elution step from Ni-NTA resin was repeated and the products combined.

ELISA format	V3/V3B 10/5 µg/ml		V3/V3B 10/10 µg/m		V3/V3B 10/20 µg/ml		P/M	
	I	NI	I	NI	I	NI	I	NI
1h	1.58	0.07	1.79	0.07	1.98	0.07	1.12	0.08

Table 6.9. A_{405} values obtained from recombinant ELISA when diferent concentration of V3/V3B were used.

V3/V3B: V3 coating and V3B detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection.
I: PLRV-infected potato; NI: non-infected potato leaf extract.
S-AP 1:4000

Table 6.9 shows that V3/V3B worked as well as the P/M antibody combination for PLRV detection on potato using a 1:4000 dilution of the S-AP conjugate. V3B performance in virus detection was comparable using 5, 10 or 20 µg/ml of this protein.

6.3.2.4. Optimization of ELISA conditions with *in vivo* biotinylated V3

In order to find the best conditions for the recombinant ELISA test, all coating and detecting reagents and the conjugate were titrated with PLRV-infected *P. floridana* and potato leaf extracts.

6.3.2.4.1. Using infected *P. floridana* leaf extract

Optimization was carried out to decide the best concentration of the *in vivo* prepared V3B and the S-AP conjugate only. The best concentration of the V3 for coating was 10 µg/ml; this was similar to the results found during optimization of the *in vitro* biotinylated reagent and some as found previously by Toth *et al*. (1999). Frozen PLRV-infected physalis leaf tissue was initially used to optimize the recombinant ELISA assay conditions.

ELISA format		V3/V3B			P/V3B		
Streptavidin-AP		1:1000	1:4000	1:8000	1:1000	1:4000	1:8000
V3B 10 µg/ml	I	0.44	0.38	0.07	0.96	0.68	0.09
	NI	0.15	0.08	0.07	0.08	0.07	0.06
V3B 20 µg/ml	I	0.75	0.47	0.09	1.29	0.83	0.12
	NI	0.1	0.07	0.07	0.07	0.07	0.06
P/M	I	0.85					
	NI	0.07					

Table 6.10. A_{405} values obtained from recombinant ELISA with different concentrations of V3B and S-AP.

V3/V3B: V3 coating and V3B detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection, P/V3B: polyclonal antibody for coating and V3B for detecting.
I: PLRV-infected *P. floridana* leaf extract; NI: non-infected *P. floridana* leaf extract.
10 µg/ml of V3 for coating and 10 µg/ml of V3B for detecting, and 1:4000 S-AP.

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Table 6.10 shows that the detection ability of the fully recombinant assay was less than the immune reagent assay, but the problem is with the combination of V3 for coating and V3B for detecting. For example, when V3B was applied for detecting but polyclonal antibody was used for coating (partially recombinant assay) and with 1:4000 of streptavidin, the reading was 0.68 compared with 0.85 for the P/M control, but this value decreased to 0.38 when V3 was used for coating and V3B for detecting (fully recombinant assay). Moreover, the background problem which appears when the S-AP concentration is increased seems to be related to the coating reagent not the biotinylated reagent. This was concluded because with a 1:1000 dilution of the S-AP, the reading for the half recombinant test (P/V3) was 0.96 and the background reading was 0.08, whereas in the fully recombinant test (V3/V3B) with the same dilution of S-AP, the reading was 0.44 but the background became 0.15.

Although using either 10 or 20 µg/ml V3B for detection combined with 10 µg/ml V3 for coating gave comparable absorbance readings, there was a slight increase in the absorbance reading when the V3B concentration was increased to 20 µg/ml. The data presented in Table 6.10 show that increasing the V3B concentration from 10 to 20 µg/ml slightly increased the reading from 0.38 to 0.47 when 10 µg/ml of V3 was used

for coating and 1:4000 S-AP as a conjugate. In general, the best combination is 10 µg/ml of V3B for detecting and 1:4000 diluted streptavidin-AP

V3/V3B		V3B concentration (µg/ml)									P/M
		2.5	5	10	2.5	5	10	2.5	5	10	
		1:1000 S-AP			1:4000 S-AP			1:8000 S-AP			
1:10 sap	I	0.44	0.61	0.91	0.43	0.66	0.56	0.13	0.2	0.17	0.88
	NI	0.51	0.45	0.42	0.12	0.09	0.07	0.07	0.08	0.07	0.07
1:40 sap	I	0.39	0.42	0.46	0.19	0.23	0.2	0.08	0.1	0.09	0.41
	NI	0.55	0.5	0.45	0.11	0.09	0.08	0.07	0.07	0.06	0.07
1:160 sap	I	0.43	0.39	0.38	0.12	0.13	0.1	0.07	0.07	0.07	0.18
	NI	0.58	0.54	0.45	0.11	0.1	0.07	0.07	0.07	0.06	0.07
1:640 sap	I	0.51	0.5	0.39	0.11	0.1	0.09	0.06	0.07	0.08	0.1
	NI	0.64	0.55	0.51	0.14	0.11	0.08	0.09	0.07	0.08	0.08

Table 6.11. A_{405} values obtained from fully recombinant ELISA during optimization of assay conditions and finding the detection end point with *P. floridana* leaf extract.

V3/V3B: V3 coating and V3B detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection.
I: PLRV-infected leaf extract (sap); NI: non-infected leaf extract (sap).
10 µg/ml of V3 was used as the coating reagent

A further experiment was carried out to investigate the effect of using lower concentrations of V3B and to determine the dilution endpoint of the fully recombinant assay. The experiment was repeated three times. Table 6.11 shows the results of a representative experiment, which are consistent with the results in Table 6.10: again, the combination of 10 µg/ml of V3B for detecting and 1:4000 S-AP was the best result in term of discrimination of infected samples and low background reaction. Table 6.11 also shows that the detection limits of the recombinant ELISA was 1:40 compared with 1:160 for the assay based on immune reagents on frozen infected *P. floridana* leaf extract.

P/V3B		V3B concentration (µg/ml)									P/M
		2.5	5	10	2.5	5	10	2.5	5	10	
		1:1000 S-AP			1:4000 S-AP			1:8000 S-AP			
1:10 sap	I	0.36	0.89	1.54	0.78	1.1	1.22	0.21	0.42	0.29	0.77
	NI	0.1	0.08	0.08	0.07	0.07	0.07	0.07	0.07	0.06	0.07
1:40 sap	I	0.15	0.22	0.39	0.19	0.3	0.29	0.12	0.13	0.11	0.34
	NI	0.08	0.08	0.08	0.07	0.07	0.07	0.1	0.06	0.06	0.07
1:160 sap	I	0.09	0.09	0.11	0.08	0.09	0.09	0.08	0.08	0.07	0.17
	NI	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.06	0.07
1:640 sap	I	0.08	0.08	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.1
	NI	0.07	0.07	0.08	0.07	0.07	0.07	0.12	0.07	0.07	0.07

Table 6.12. A_{405} values obtained from partially recombinant ELISA during optimization of assay conditions and finding the detection end point with *P. floridana* leaf extract.

V3/V3B: V3 coating and V3B detecting, P/M: polyclonal antibody for coating and monoclonal antibody for detection. 10 µg/ml of V3 for coating and 10 µg/ml of V3-B for detecting, and 1:4000 of the S-AP. I: PLRV-infected leaf extract; NI: non-infected leaf extract.

To investigate further the effect of coating antibody on the assay, the experiment in Table 6.11 was repeated except that coating was with polyclonal antibody instead of V3. Table 6.12 shows that the absorbance values of recombinant reagent were higher than the control immune reagent. The absorbance values (1.22 compared to 0.77) when 1:4000 S-AP was used, and these values increased to 1.54 compared to 0.77 when the S-AP dilution was 1:1000. In contrast to what was found in the fully recombinant assay, the background reaction remained the same whether S-AP was used at 1:1000 or 1:4000 dilution. It was concluded that, the high background reaction with V3B is related to the weak ability of the coating reagent (V3) rather than in the detection ability of the V3B.

In addition, the results in Table 6.12 provides futher confirmation of the findings shown in previous tables: it indicates that increasing the concentration of detecting reagent from 5 to 10 µg/ml had little impact on increasing absorbance values in infected samples and decreasing the background in the healthy samples.

Test format		V3/V3B				P/V3B				P/M
V3B µg/ml		10	20	10	20	10	20	10	20	
S-AP		1:4000		1:8000		1:4000		1:8000		
1:10 sap	I	0.59	0.51	0.19	0.32	1.29	1.17	0.39	0.58	0.81
	NI	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.08
1:40 sap	I	0.22	0.19	0.11	0.15	0.36	0.39	0.2	0.17	0.38
	NI	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
1:160 sap	I	0.12	0.1	0.08	0.09	0.13	0.12	0.1	0.08	0.13
	NI	0.08	0.07	0.07	0.07	0.07	0.07	0.07	0.06	0.07

Table 6.13. A_{405} values obtained from fully/partially recombinant ELISA during optimization of assay conditions and finding the detection end point with *P. floridana* leaf extract.

V3/V3B: V3 for coating and V3B for detection, P/V3B: polyclonal antibody for coating and V3B for detection, P/M: polyclonal antibody for coating and monoclonal antibody for detection.
I: PLRV-infected leaf extract (sap); NI: non-infected leaf extract (sap).
10 µg/ml of V3 was used for coating.

The results shown in Table 6.13 confirm that with a 1:4000 dilution of S-AP, there is little difference in absorbance reading whether 10 or 20 µg/ml of V3B is used for detection, and 10 or 20 µg/ml of V3 is used as coating reagent. Thus it was decided to use the lower rate (10 µg/ml) for both coating and detecting with V3 and V3B respectively.

6.3.2.4.2. Using infected potato leaf extract

All previous optimization experiments were performed with extracts from infected leaves of *P. floridana*, which is an indicator plant used to study PLRV in the laboratory. Thus it was important to test the performance of the recombinant reagents with leaf extracts from infected potato, which is the major natural host for PLRV in the field.

ELISA format		V3/V3B		P/V3B		P/M
Leaf extract		1:2000 S-AP	1:4000 S-AP	1:2000 S-AP	1:4000 S-AP	
1:5	I	1.17	1.3	1.86	2.15	2.05
	NI	0.12	0.08	0.07	0.08	0.07
1:10	I	0.73	0.78	1.13	1.33	1.2
	NI	0.13	0.08	0.07	0.07	0.07
1:20	I	0.52	0.55	0.74	0.86	0.68
	NI	0.13	0.08	0.07	0.07	0.07

Table 6.14. A_{405} values obtained from fully/partially recombinant ELISA during optimization of assay conditions for potato leaf extract.

V3/V3B: V3 for coating and V3B for detection, P/V3B: polyclonal antibody for coating and V3B for detection, P/M: polyclonal antibody for coating and monoclonal antibody for detection. I: infected potato leaf extract frozen at -20 °C in ELISA extraction buffer, NI: non-infected potato leaf extract.
10 µg/ml of V3 was used for coating and 10 µg/ml of V3B for detecting.

The preliminary results on frozen potato leaf extract, presented in Table 6.14 are in agreement with what was found with frozen physalis leaf extract that using V3 for coating gave higher values for non-infected background compared with the polyclonal antibody and stronger A_{405} values were obtained from infected samples.

The best combination of reagents seems to be in agreement with the combination chosen after titration on physalis: coating with 10 µg/ml of V3 and detection with the same concentration of V3B. The data in Table 6.14 confirm that although the fully recombinant (V3/V3B) assay gave rather lower absorbance reading as the immune reagent assay, the partially recombinant assay (P/V3B) performed better than the immune reagent assay. In Table 6.14 the reading for the fully recombinant when 1:4000 dilution of S-AP was used was 0.78 compared with 1.2 for the control, but this value became 1.33 when V3 was replaced by polyclonal antibody for coating, suggesting that a superior recombinant assay could be designed if the coating condition was improved.

It was decided to use fresh potato leaf extract to investigate the effect of freezing leaf samples on the recombinant assay sensitivity. The data in Table 6.15 show that while A_{405} values for infected samples in the fully recombinant assay were lower compared with the partially recombinant assay, but the values were similar to those obtained with immune reagents and the end point was 1:160 for all tests.

ELISA Format		V3/V3B				P/V3B				P/M
Leaf extract		1:2000 S-AP	1:4000 S-AP	1:6000 S-AP	1:8000 S-AP	1:2000 S-AP	1:4000 S-AP	1:6000 S-AP	1:8000 S-AP	
1:5	I	1.35	1	0.3	0.09	2.54	1.77	0.61	0.3	0.82
	NI	0.09	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.07
1:10	I	0.81	0.59	0.18	0.07	1.61	1.08	0.33	0.16	0.49
	NI	0.09	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.07
1:20	I	0.49	0.34	0.12	0.07	0.94	0.64	0.2	0.11	0.31
	NI	0.09	0.07	0.06	0.06	0.07	0.07	0.07	0.06	0.07
1:40	I	0.32	0.23	0.1	0.07	0.52	0.33	0.12	0.08	0.22
	NI	0.09	0.06	0.06	0.06	0.07	0.07	0.07	0.06	0.07
1:160	I	0.21	0.14	0.08	0.06	0.32	0.2	0.09	0.07	0.15
	NI	0.1	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.08
1:320	I	0.16	0.09	0.07	0.06	0.12	0.1	0.07	0.06	0.11
	NI	0.11	0.07	0.06	0.06	0.06	0.06	0.06	0.06	0.08

Table 6.15. A_{405} values obtained from fully/partially recombinant ELISA during optimization of assay conditions and finding the detection end point for potato leaf extract.

V3/V3B: V3 for coating and V3B for detection, P/V3B: polyclonal antibody for coating and V3B for detection, P/M: polyclonal antibody for coating and monoclonal antibody for detection. I: infected potato leaf extract, NI: non-infected potato leaf extract.
10 µg/ml V3 was used for coating and 10 µg/ml V3B for detection.

The data shown in Table 6.15 confirm clearly that the detection limits are comparable between the recombinant assay and the standard immunoassay when fresh leaf extract was prepared. For example, with 1:160 dilution of potato leaf infected sap and S-AP diluted 1:4000; the reading was 0.14 for with the fully recombinant assay compared with 0.15 for the control immune reagent assay

From the combined results of the optimization experiments, it was concluded to use 10 µg/ml V3 for coating, 10 µg/ml V3-B for detection, and 1:4000 dilution of streptavidin-AP and to use freshly prepared leaf extract.

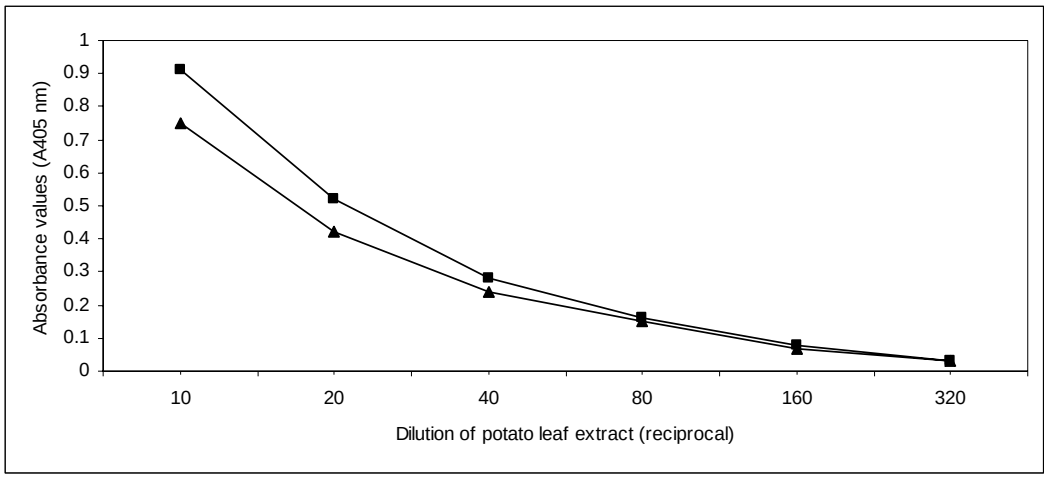

Figure 6.9. Comparison of the detection of PLRV by the recombinant ELISA (V3/V3B) with an assay based on immunoglobulins.

(■), V3 coating and V3 detecting reagents; (▲), polyclonal anti-PLRV coating and MAb SCR1 detecting reagents; for each data point the absorbance value (A_{405}) of the non-infected samples was subtracted from the infected sample values (mean non-infected value 0.07). Absorbance values were recorded 1 h after incubation at RT. Freshly prepared potato leaf extract was used.

Data in Fig. 6.9 are means from three experiments (a typical one of them was presented in Table 6.15) about the sensitivity of PLRV detection between the recombinant and the immune reagent ELISA.

6.3.2.5. Sensitivity and batch variation between *in vivo* biotinylated V3 preparations

In an attempt to track batch-to-batch variation, and to compare detection limits between fully recombinant and immune reagent assays, two preparations of V3B were used to test different dilutions of PLRV-infected potato leaf extract and the results were compared with the immune reagent assay. Both V3B batches and the immune reagent assay were able to detect virus in dilutions up to 1:160 of infected potato leaf extract.

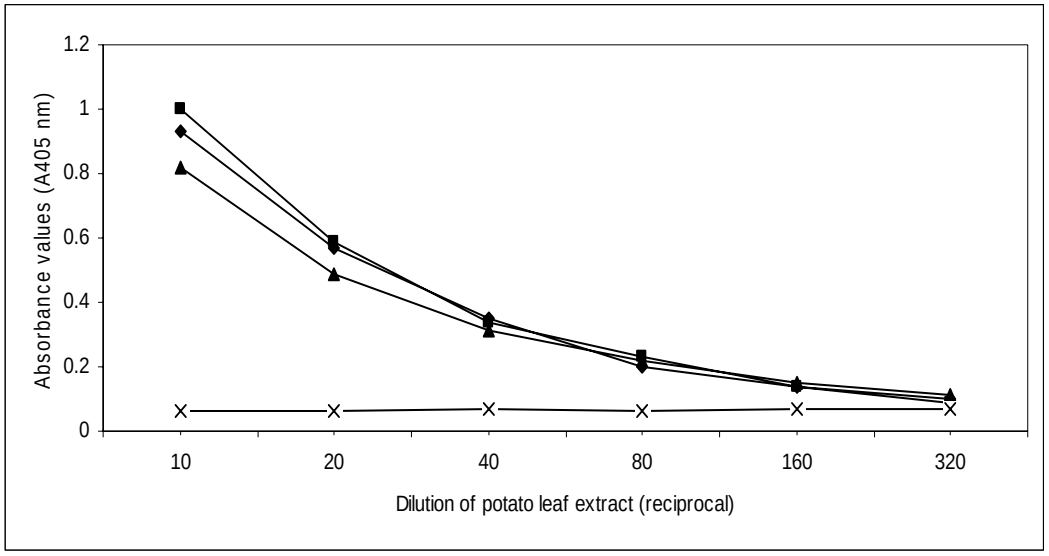

Figure 6.10. Comparison of detection of PLRV in potato leaf extract using two independent preparations of *in vivo* biotinylated V3B.

Two preparations of V3B (■, ♦) were used in the V3/V3B assay; P/M (▲), polyclonal anti-PLRV coating and MAb SCR1 detecting reagents; non-infected samples (X) tested in both assays. 10 µg/ml V3 used for coating, 10 µg/ml V3B for detecting, with 1:4000 dilution of S-AP. Absorbance values were recorded 1 h after incubation at RT. Freshly prepared potato leaf extract was used.

Fig. 6.10 shows that with 10 µg/ml of V3 for coating, 10 µg/ml of V3B for detecting, and 1:4000 dilution of the S-AP, there was no difference between the two biotinylated V3 batches, and both were as sensitive as the immune reagents.

6.3.2.6. Stability and Storage

To check the stability and to optimize storage conditions of biotinylated products, it was decided to store samples under different conditions and test them periodically. The storage conditions were: 4°C, -20°C, -20°C in 50% (v/v) glycerol, and 37°C. Samples were tested after 1 day, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 4 months, 8 months, and 1 year of storage. Only results of storing after 1 day, 1, 2, 6, and 12 months are presented in Fig 6.11.

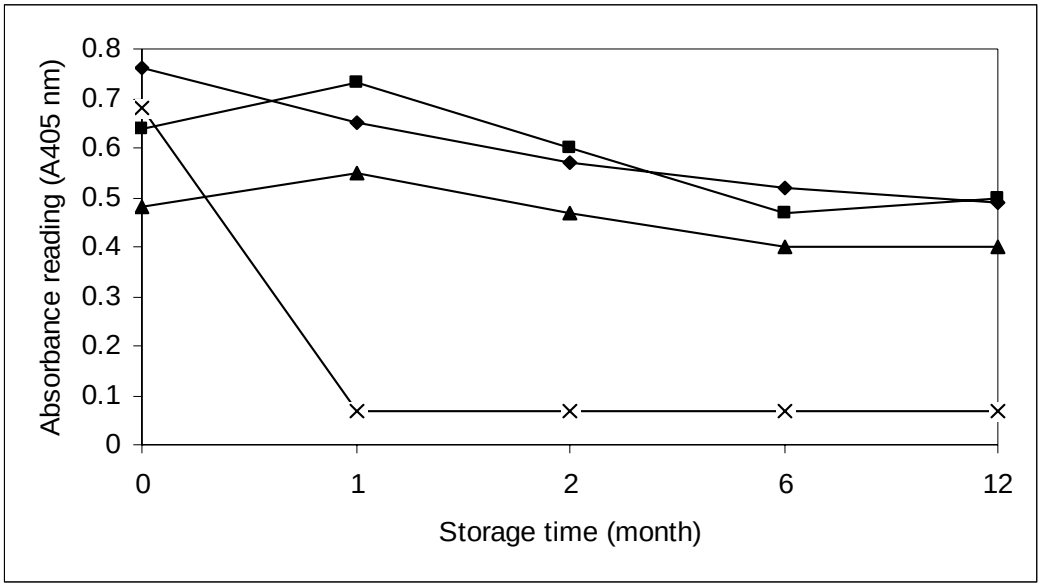

Figure 6.11. Absorbance values (A_{405}) obtained in ELISA with *in vivo* biotinylated protein preparations after storage under different conditions.

10 µg/ml V3 used for coating, 10 µg/ml V3B for detecting, with 1:4000 dilution of S-AP. Reagents were stored at 4°C (♦), -20°C (■), -20°C in 50% (v/v) glycerol (▲), or 37°C (X). For each data point the absorbance value (A_{405}) for the non-infected samples was subtracted from value for the infected samples (mean non-infected value 0.07). Absorbance values were recorded 1 h after incubation at RT. Frozen *P. floridana* leaf extract was used.

V3B did not retain its functionality after 1 day of storage at 37°C. However, V3B was stable for up to 1year in all other combinations.

6.3.2.7. Recombinant versus immune reagents

After finding the best conditions, I compared the performance of fully recombinant and immune reagent ELISA formats to detect PLRV in naturally infected potatoes. Field-infected potato leaf samples obtained from SASA were used to compare the efficiency of the recombinant test and our immune reagent antibodies at SCRI.

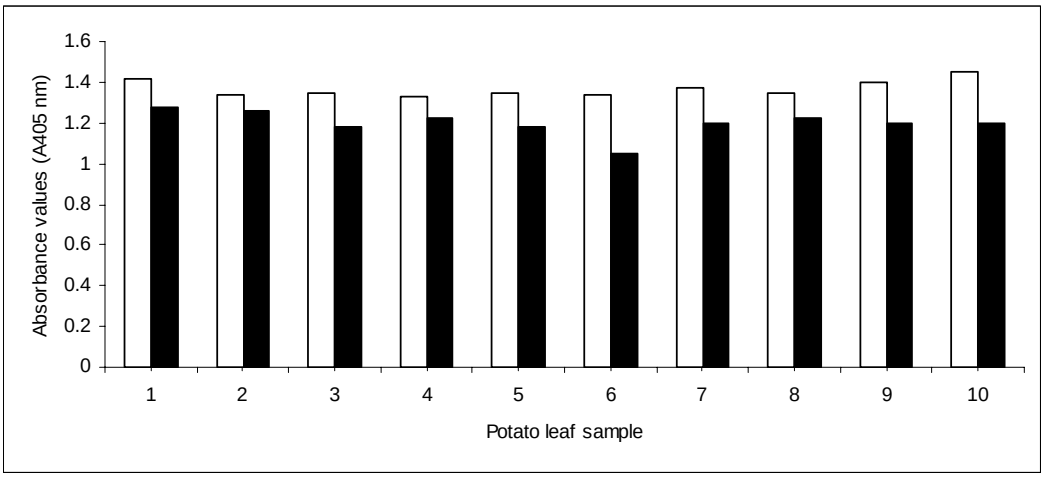

Figure 6.12. Comparison of the detection of PLRV in potato samples by recombinant and immune assays.

Ten fresh potato leaf samples were diluted 1:10 in extraction buffer and tested in both ELISA formats (□, V3/V3B; ■, P/M). The mean value of the healthy samples (0.07) was subtracted from the infected samples.

It is clear from Fig. 6.12 that the recombinant assay gave similar absorbance reading to that obtained from the immune reagent assay.

6.3.2.8. Naturally infected leaf samples

In order to compare performance of the recombinant test with ELISA procedures in other labs, 54 field-infected potato leaf samples which had been previously tested by SASA were tested using the recombinant assay.

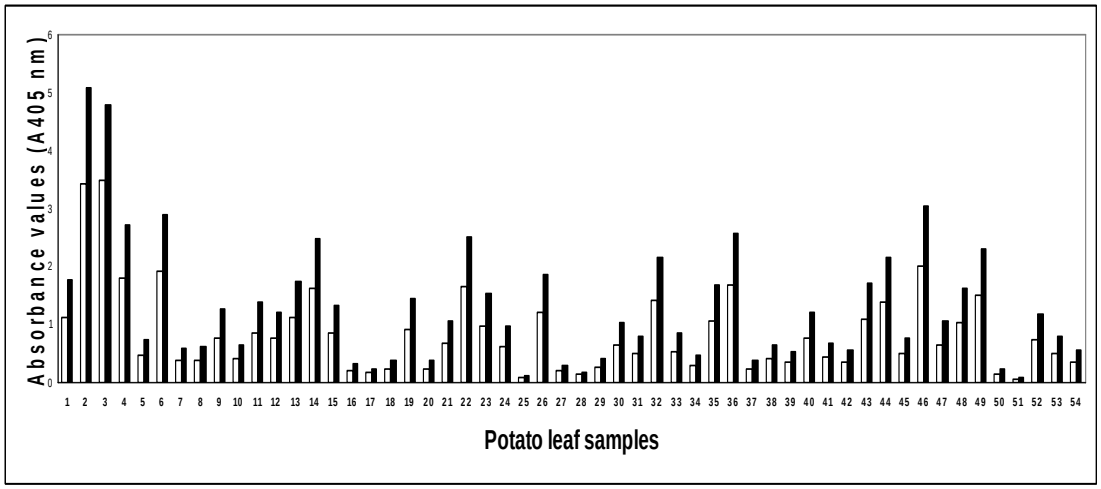

Figure 6.13. Comparison of the detection of PLRV in naturally-infected potato leaf samples by recombinant and immune assays.

Absorbance values obtained in the V3/V3B ELISA (□) or the SCRI immune reagents test (■). Mean non-infected control values were 0.07 in both tests. Samples 25 and 51 were considered to be negative (less than twice mean control value)**.** Frozen potato leaf extract were used

Fig. 6.13 shows that there was about 100% agreement between recombinant and immune assays done at SCRI. There are 96% agreement in discrimination of infected samples between SCRI tests and those done at SASA. Two samples scored positives in SCRI tests were negatives in SASA tests. The difference could be because the leaves were frozen at -20°C before being tested and possibly PLRV was detected more efficiently using the recombinant test in the fresh samples (Fig. 6.12). An RNA based test (such as RT-PCR) could be used to verify the results.

6.4. Discussion

ScFv-based immunoassays for plant viruses have been reported (Harper *et al*., 1997; Terrada *et al*., 2000). However, despite the potential advantages in terms of assay standardization and limitless supply, these assays have not been widely applied to date (reviewed by Ziegler and Torrance, 2002). For PLRV one of the reasons is that the scFv-AP fusion proteins are not stable over time. In order to produce a robust, fully recombinant test, an alternative method of labelling scFv was investigated by exploiting the well known streptavidin-biotin system (Bayer and Wilchek, 1990). Preparations of V3 were modified successfully by chemical coupling to biotin but the results were not reproducible between batches. Chemical biotinylation is a process that targets lysine residues of proteins and it is difficult to control precisely which residues are modified. Moreover, for antibodies, biotinylation of lysine residues within the domains conferring binding specificity (the complementarity determining regions; CDRs) can lead to loss of function due to disruption of the antigen binding site (Bulow and Linbladh, 1998). The scFv V3 contains 7 lysine residues (Fig. 6.14) and one of them is located within the CDR2 of the light chain; differential biotin labelling of these various lysine residues, including that present in the CDR2, may explain the observed variation in performance of different batches of chemically biotinylated V3.

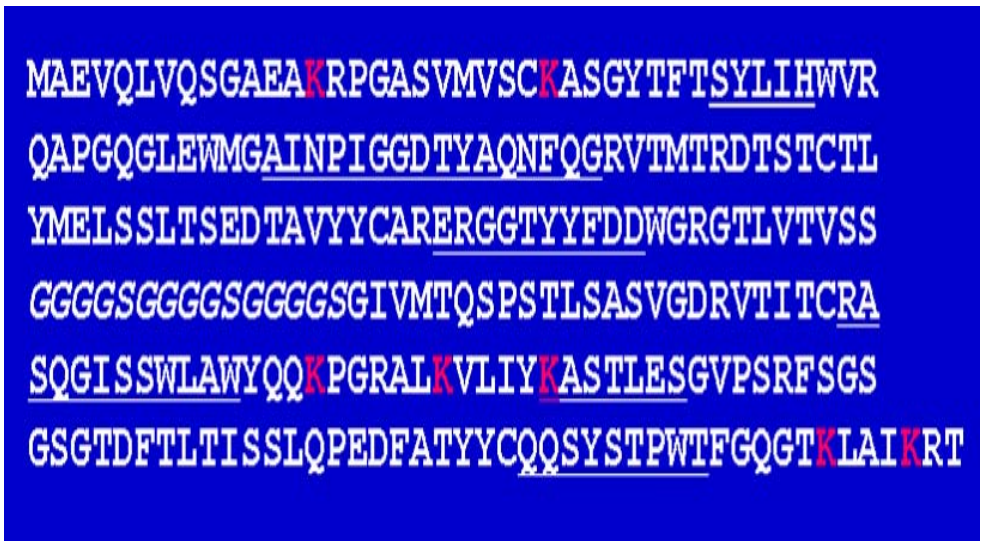

Figure 6.14. Amino acid sequence of the anti-PLRV scFv-CL (V3HCL).
Taken from A. Ziegler, SCRI.
The complementarity determining regions (CDRs) are underlined, and the letters in italics represent the hinge region between the light and heavy chains of the scFv. Lysine residues are highlighted in red.

It can be concluded that chemically biotinylated scFv can give results comparable to immune reagents, but a limitation of chemical biotinylation is the lack of reproducibility. A more reproducible method is needed to biotinylate this protein in order to devise a robust, fully recombinant ELISA assay to detect PLRV. *In vivo* biotinylation can be achieved by adding a short biotin acceptor sequence to the C-terminus of a protein sequence (Schatz, 1993). On production of the protein in an *E. coli* strain that carries the *birA* gene for the enzyme biotin ligase, the protein becomes labelled with biotin. In this system the protein is modified specifically at the C-terminus, thereby decreasing the likelihood of loss of function. This work demonstrated that *in vivo* biotinylation can be effectively applied to V3 and that the process is reproducible between different experiments. The *E. coli* expressed V3B preparations were used together with preparations of V3 and streptavidin-AP to devise a fully recombinant assay to detect PLRV. The recombinant ELISA gave results that were comparable to those obtained by an assay based on immunoglobulins for the detection of PLRV in *P. floridana* and naturally-infected potato. A partially recombinant ELISA format in which polyclonal antibody was used for coating and V3B was used for detection was also tested. In this assay it was shown that the coating ability of V3 was weak compared with the detecting performance of the V3B. The reason for that could be because the polyclonal antibody is less inhibited by components of plant extract compared with the recombinant V3. The weak coating ability would both increase the non-specific and reduce the specific reactions. Thus the performance of the recombinant assay could be improved if the coating capacity of V3 was improved by coating with mixtures of scFv or increasing the affinity of the molecule.

V3 was shown previously to be stable for 6 months at 4°C (Toth *et al*., 1999) and here the V3B preparations were found to be stable for more than one year. Although greater amounts of recombinant proteins were used in the biotin assay compared with monoclonal antibodies, the recombinant proteins were expressed in reasonable yields in *E. coli* shake-flask cultures quickly and cheaply. This system could be readily applied to produce other recombinant antibodies for virus detection.

The results demonstrate that scFv reagents derived from synthetic phage display platforms can provide effective alternatives to assays incorporating immune reagents. In addition, this work showed that recombinant ELISA based on the scFv can be as effective as immune reagent ELISA for routine testing of PLRV if biotinylation of the

scFv is performed *in vivo*. In addition, this recombinant test can be applied for other plant viruses, with a potential to replace immune reagent immunoassays.

Chapter 7: Effect of Agrochemicals on PVY Transmission by Aphids

Pages 207-221

This Chapter was taken out due to the confidential agreement with the insecticide company.

Chapter 8. General Discussion and Future Recommended Work

8.1. General discussion

Aphid-borne viruses cause economically important diseases on potato crops through yield reduction and the direct damage which some viruses may induce in potato tubers. In addition, viruses are largely responsible for the degeneration of vegetatively propagated seed potatoes. PVY in particular is now the main problem affecting potato production in many countries around the world. In the UK, for example, it is estimated that PVY alone causes 45% of the total loss caused by all potato diseases (Valkonen, 2007), and this is estimated to cost £34 million annually (Anonymous, 1999). In addition, considerable amounts of insecticides are being employed in controlling the vector aphids with limited effect due to the short time required by aphids to acquire and transmit the virus between infected and healthy plants. Breeding for resistance is the most durable way to control virus transmission in potato crops. However, most commercial potato cultivars still lack resistance genes because breeding in potato is a lengthy process and most breeding programmes have considered viruses as a relatively low priority, including breeding programmes in the UK.

The overall aim of this PhD project was specifically to support improved virus control in potato crops by increasing the general understanding of the transmission mechanism, developing a more sensitive and robust serological assay for virus diagnosis, and investigating the effect of agrochemicals on the aphid vectoring ability of PVY.

The mechanism by which potyviruses are transmitted has not been fully elucidated, and this is a focus of research groups in many laboratories across the world. The transmission is known to be mediated by the well known virus encoded protein known as HC-Pro; the most widely accepted hypothesis postulates that HC-Pro regulates the transmission process by forming bridge-like structures between the virus particles on the one hand and the putative receptors in the aphid mouth-parts on the other hand. However, no adequate information is available yet about the exact nature

and the location of these putative aphid virus receptors. A detailed understanding of the mechanism of virus transmission could greatly enhance the control of this important pathogen. By using the knowledge to interfere with the acquisition or inoculation process by the aphid vector, it would be possible to decrease or block the process. Interfering with the transmission process can be performed genetically for research purpose through silencing the genes responsible for encoding virus receptor proteins inside the aphid vector if there are specific receptors. Alternatively, transmission can be blocked through competition to interact with or disable the function of virus receptors. However, before that the putative receptors need to be characterized and fully studied. To date, there is limited information about the composition or location of such proteins inside aphid vectors, but evidence has accumulated that the putative virus receptors inside the aphid are of cuticle nature (Dombrovsky *et al*., 2007b; Uzest *et al*., 2007, 2010).

In this project several putative receptors for PVY were identified in the aphid *M. persicae* by screening an aphid cDNA library, and their possible binding to HC-Pro of other potyviruses was demonstrated. The high level of homology of these identified proteins with other CUPs belonging to other aphids (Chapter 3) may suggest non-specific binding. Non-specificity of binding between HC-Pro and aphid CUPs is supported by the long retention time of PVY inside its aphid vector (Chapter 5). In addition, detection of virus inside aphids after a long time of feeding on healthy plants is a novel finding and could be useful in understanding the transmission mechanisms of potyviruses. However, further investigations are required to enable precise localization of the virus retention sites inside the aphid body, and to fully characterize the range of CUPs that HC-Pro can bind to.

Bioinformatic work on the aphid cuticle protein identified in Chapter 3 suggests that clone P72F, shares a high level of similarity with other aphid CUP proteins, which supports the evidence from the genomic sequence that there is a multi-gene family or highly expressed genes. The CUP protein represents about 1% of the total sequenced clones in the screened library. Thus, HC-Pro can theoretically interact with any CUPs that cover the interior part of the aphid digestive system if the nonspecific binding theory is correct. However, only those virus particles which stick on the stylet are transmissible. One possible explanation for that is the inhibitory effect of aphid enzymes on the infectivity of the virus particle, which may be more abundant in the other interior parts of the aphid feeding system than on the tip of the stylet. The fact that transmission efficiency of non-persistent and semi-persistent viruses is enhanced by fasting supports this hypothesis. Powell (1993) found that while starvation increased transmission efficiency of non-persistent viruses, the occurrence of electrically-recorded membrane punctures during acquisition access did not change, which can be explained by the assumption that fasting does not interfere with the probing behaviour of the aphid but may provide enzyme-free active virus attachment sites which become available for the virus. It is possible to test the inhibitory effect of aphid enzymes on PVY activity *in vitro*. This can be conducted by collecting aphid saliva and incubating it with purified virus particles under different conditions then allowing aphids to acquire saliva-incubated virus particles through membranes in the presence of biologically active HC-Pro.

The localization of the GFP-P2 fusion of CaMV on the tip of the stylet only (Uzest *et al*., 2007) is evidence that only viruses that stick on the distal part of the stylet are transmissible, despite earlier work which reported that semi-persistent viruses are foregut-borne while non-persistent ones are stylet borne. Berger and Pirone (1986) showed that aphids acquired on average similar amounts of purified virus particles whether the HC-Pro was present or not. However, when active HC-Pro was present, virions were detected in the stylet, the foregut, and to some extent the gut, whereas virions were detected only in the gut regions of aphids which were allowed to have access to defective HC-Pro. The explanation may be that virions retained in the gut bind non-specifically and do not participate in the transmission process, and only those that are retained in the stylets and the foregut may contribute to the transmission process.

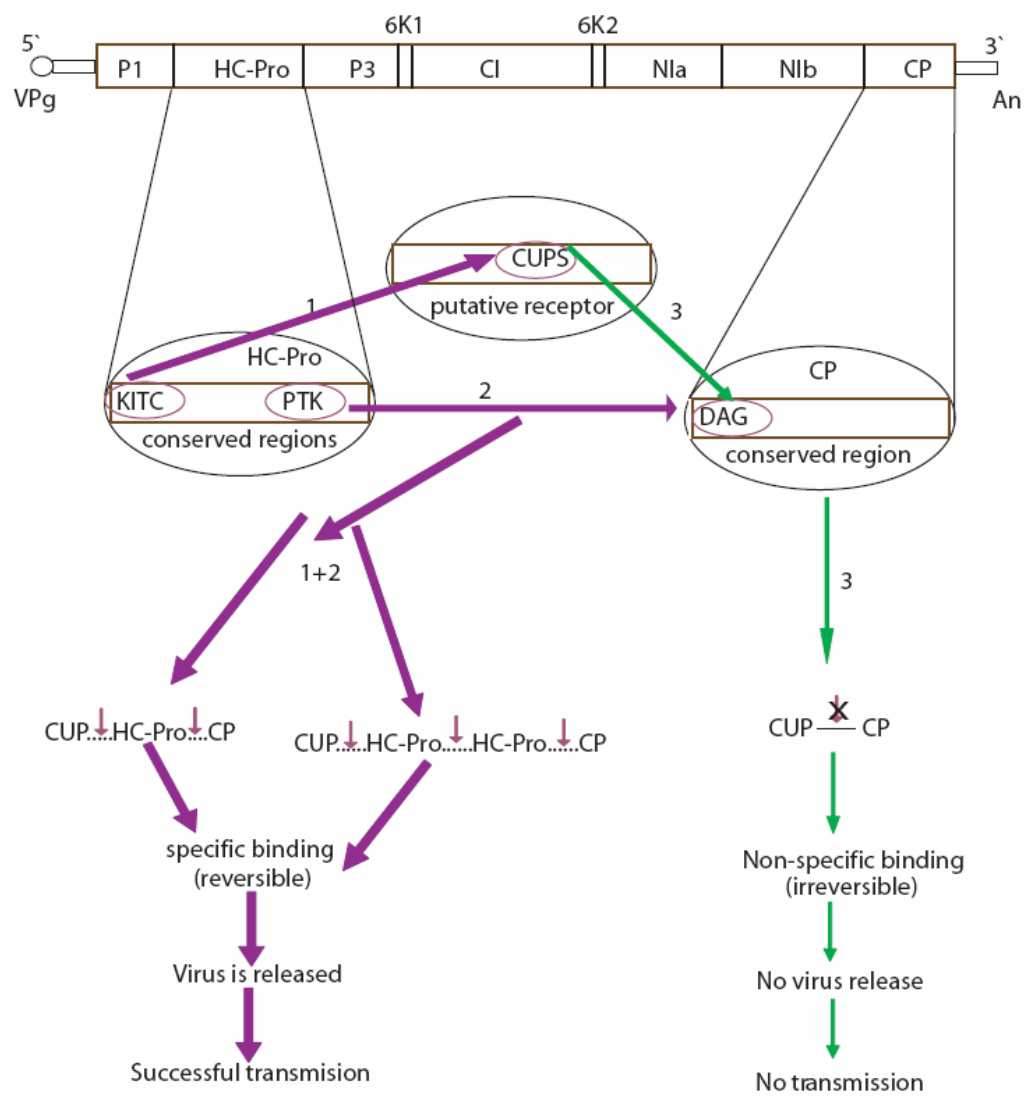

Figure 8.1. Schematic putative representation of the mechanisms of potyvirus transmission by aphids.

CUP: aphid cuticle protein, putative virus receptors on the aphid mouthparts, KITC, PTK: two conserved motifs in the HC-Pro, DAG: a conserved motif in the coat protein. Purple arrows: interaction which can lead to successful transmission, Green arrows: the interaction may occur but does not contribute to the transmission process.1 and 2: interaction leads to successful virus transmission, 3: interaction leads to unsuccessful transmission. Small arrows refere to possible sites of release of attached virus particles in the virus/HC-Pro/ CUPs complex.

Fig. 8.1 is based on Fig. 1.2 and outlines the proposed mechanism for the interaction between potyvirus and aphid putative receptors. In this proposed diagram successful virus transmission by the aphid can occur only if the KITC region of HC-Pro interacts with a specific motif on the aphid CUPs, and the PTK region interacts with the DAG region on the virus coat protein. Binding of the virus particles directly to CUPs can lead to non-specific interactions which can be responsible for the observed long retention of

virus inside the aphid. However, this hypothesis is based on assumption although it is partially supported by findings obtained from this project about the abundance and similarity between identified CUPs and other CUPs, and the retention of the PVY in different part of the aphid vectors after long time of acquisition and feeding on healthy plant. If more time had been available, investigations would have been carried out to localize the identified CUPs inside aphid stylets using antisera prepared against them. Moreover, site-directed mutagenesis could be employed to identify specific motifs inside the CUPs which may be responsible for the interaction with HC-Pro and mediating virus transmission.

Virus transmission by aphids is a complicated process. There are three participants involved in this process: the virus, the aphid vector, and the host plant. All components may influence each other (Fig. 8.2). Thus it is important to study how the three components do affect each other in order to understand the transmission process. In this project valuable biological observations relating to the transmission process were made, and highlighted some critical areas where the plant may affect the outcome of the transmission process. The key finding is that the host plant used to maintain an aphid vector colony has an impact on the aphids vectoring ability from different host plants particularly rearing aphids on potato significantly decreased transmission efficiency from tobacco, but rearing aphids on tobacco restored aphid transmissibility from tobacco. In addition, recipient host plant influenced the transmission efficiency of the aphid vector, thus aphids transmitted PVY to more tobacco plants than potato. The recipient plant effect is usually overlooked by plant virologists as they normally focus only on the source plant used for acquisition and use a high number of aphids to perform virus transmission, which masks the recipient plant's effect on efficiency of virus transmission by aphids. This point is critical for estimating the REF values of different virus vectors, and for assessing virus infection pressure in the field, which may lead to either over or underestimation of the virus risk if the aphid culture or the recipient plant was not selected properly. It was an important finding that rearing aphids on potato decreased their ability to acquire virus from tobacco. However, the opposite was not true: aphids which were reared on tobacco acquired virus readily from either potato or tobacco. This result may suggest that aphids which reared on potato adapted to this host so that their appetite to feed on tobacco and acquire the virus from it is decreased significantly. However, this cannot exclude the involvement of other unknown molecular factors which remains an opened question.

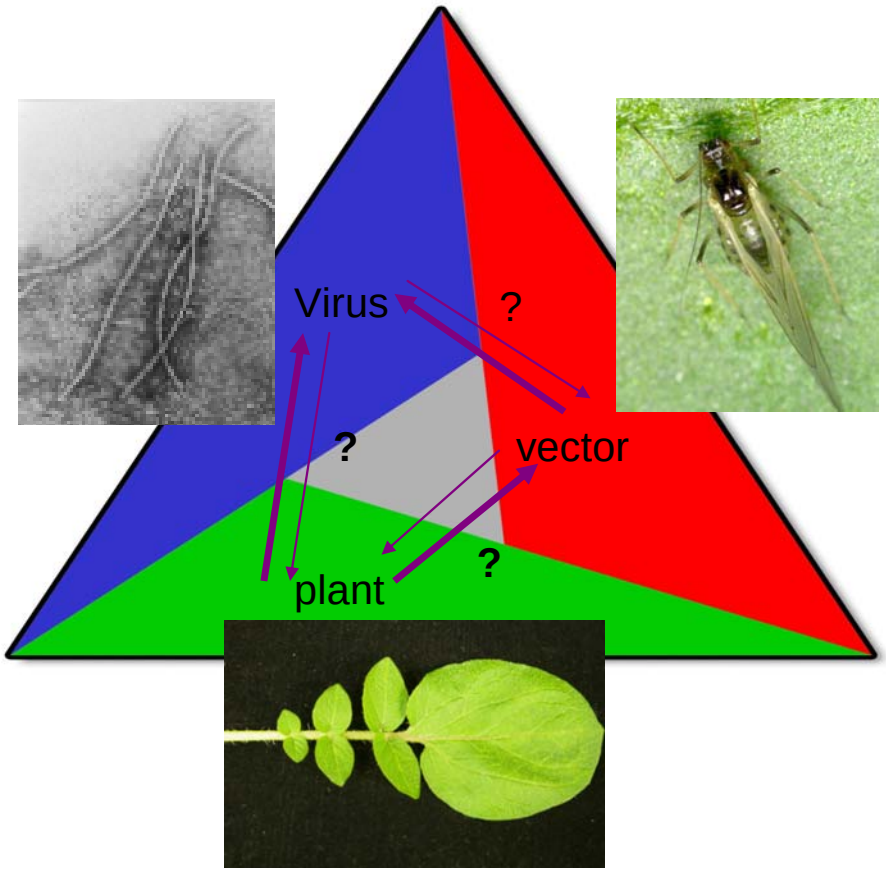

Figure 8.2. Schematic diagram shows the three components of the transmission process.

Arrows represent the inter-relationships between the virus, the vector, and the host plant Thicker arrows were the relationships studied in this project.

As mentioned earlier the purpose of this project was to improve control of potato viruses. The first Chapters (3, 4, and 5) focused on controlling non-persistent plant viruses indirectly by elucidation of how transmission happens. On the other hand, in Chapters 6 and 7 the focus was to tackle the virus transmission directly. In this respect, developing cheaper and more robust diagnostic assays based on recombinant antibody fragments was investigated in Chapter 6. The work demonstrated that anti-scFv fragment selected from a naïve phage library against PLRV was as effective as conventional immune reagent antibodies provided that the scFv was biotinylated *in vivo*. The reagents produced in this way have high yields, which compensates for the fact that the working concentration is 5–10 times higher than for the monoclonal

antibodies, and they can be stored for a long time under different conditions. This method should theoretically apply to any plant virus, and provide an unlimited source of diagnostic reagents cheaply and rapidly if access to antibody libraries becomes routinely available. This step is very important for controlling plant viruses as to control viruses efficiently; detection methods should be available cheaply and continuously.

In conclusion, this project has contributed to the pool of knowledge related to aphid transmission mechanisms of plant viruses, and control. In addition, new windows of research were opened, and further ideas and issues were raised and discussed in the context of aphid transmission mechanisms, detection, and control of plant viruses on potato crops.

8.2. Future recommended work

These studies have raised new ideas and hypotheses that could be explained in the future.

8.2.1. Investigations of virus receptors in aphids

8.2.1.1. Specificity of interaction between HC-Pro and CUPs

This work started to investigate the specificity of interaction between aphid CUPs and HC-Pro by studying the interaction between native proteins extracted from two aphid species with different virus vectoring ability. However, the experiment can be expanded to include non-vector aphid species as a negative control and repeated enough to validate the findings. Moreover, the cDNA library used for screening contains sequences coding for about 20 CUPs. It would be useful to express these proteins and incubate them with HC-Pro to find out whether they interact with HC-Pro or not.

The above experiments should be useful in revealing the range of CUPs to which HC-Pro can bind. To investigate the specificity of the HC-Pro interaction, it would be helpful to study the interaction between the PVY coat protein and the identified CUPs. This can be achieved by incubating the CUPs with whole virus particles, but it is better to use CP preparation from virus particles. Alternatively, the gene for coat protein can be cloned into a suitable expression vector and the protein can be produced in *E. coli.*

In Chapter 5, PVY was detected inside the aphid vector at attogram levels using N-RT-PCR after transmission but the detected virus was not transmissible. It is important to check the content of aphid honeydew for virus particles to support the findings in Chapter 5, and to eliminate the possibility that virus particles were detected in the aphid gut not on virus receptors.

It is possible also to feed aphids through membranes on purified PVY preparations in the attogram range in the presence of active HC-Pro to find out if aphids can transmit virus at this low virus concentration. If aphids are able to transmit virus at the attogram level this will indicate that either the infectivity of virus particles was disabled or virus particles are firmly attached to aphid CUPs which prevents them from initiating infection after hours of feeding on healthy plants. In addition, investigation of virus retention inside non-vector aphid species and poor vectors is required to determine

whether virus can be retained as frequently as in the efficient vector, and this will help to inform whether virus particles can bind to any cuticle protein structure inside the insect body. Moreover, electron microscopy can be utilized to localize the virus retention sites inside aphids by preparation of immunogold-labelled (to anti-coat protein antibody) sections of aphids which were allowed to acquire the virus and kept overnight on healthy plants. Examination of sections under electron microscopy will reveal whether or not there are any virus particles after the aphid has fed on the healthy plant.

Another way to confirm the specific interaction between HC-Pro and the aphid cuticular proteins would be to mutate the KITC domain in the HC-Pro to render it non-functional. If deficient HC-Pro is expressed and purified as normal, protein overlay with aphid proteins should reveal no interaction. Similarly, to determine the motif in the aphid protein responsible for binding HC-Pro, mutation into suspected motifs (YSAP) which are abundant at the N and C-terminus of aphid CUPs could be used. Then interaction with functional HC-Pro should be absent. In addition, yeast two hybrid system could be a useful *in vivo* technique to investigate interaction between HC-Pro and indentified CUPs.

8.2.1.2. Localization and blocking of virus receptors

Nucleic acids can be extracted from different parts of the aphid body (head or body without head) then PCR can be used to perform differential gene expression studies to partially localize the CUPs that interact with HC-Pro. Furthermore, it would be useful to employ the system developed by Uzest *et al*. (2007) in order to investigate the interaction between aphid stylets and PVY HC-Pro. A fusion of the green fluorescent protein (GFP) to the protein sequence of the PVY HC-Pro can be constructed. The construct then can be agroinfiltrated into *N. benthamiana* plants. Then functionality of the GFP-HC-Pro fusion will be revealed by feeding aphids on the agroinfiltrated leaves then on PVY purified particles through parafilm membranes. The GFP-HC-Pro binding site in the aphid stylets can be investigated under microscopy.

Blocking of aphid receptors can be tested by preparing antisera against aphid proteins from clones P72F/P817P, which are the best candidates. Then blocking of aphid transmission of PVY can be tested by feeding aphids on the antibody then testing transmission efficiency compared to the controls. Another thought would be to fuse the antibody with a fluorescent tag and investigate the position of interaction by looking at the aphid stylet under the confocal microscope. Alternatively an immunogold conjugate

can be prepared in order to label the place of interaction on the aphid stylet by using the electron microscope.

8.2.2. Host plant effect on virus transmission

Investigation of the host plant effect on poor aphid vectors (e.g. *R. padi*, *M. dirhodum*) was of great interest to this project. However, due to the time limit this effect was investigated only in the efficient vector *M. persicae*. Further work is recommended to determine whether analogous host differences are found with other aphid species or other field isolates of PVY.

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10. APPENDIXES

10.1 APPENDIX 1

Sequencing and bioinformatics data

10.1.1. Nucleotide Sequence of the identified aphid protein clones from cDNA library (raw sequence data).

>P7 2F

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AGTAAGCTGGCGCGGAGTAAGCTGGCTTGTAGGCTGGTGCGGAGTATGATG
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GAAACCGTTGTAGTCGTCAGCGGTGTATTCGACGGTGCGGATGGAGCCGTC
GGCTTCGACGAGGCTGTAGGATCCCTTGACGTAACCGTTTCCGTCACTGTAT
TCGGATTGGCTGTGCACATCGTAGGTGTGTGGGTCGTTTACGCTGTATTCGA
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CNANTAAGCGGGCGCANANTACGCTGGCTTGTAAGCTGGGGCGGANTATTG
GGCGAGGGCGGTANCNCNCAGCGGCGAAATGACNACTTANCGCCATTTAGN
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AACTG

>P817P
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TTCGGTTTCGAATCCGTATTTGAATCCGTTCACGTCAGCTTCTTGGGATTGTC
TGATGATAGCTGGCAATTTGTTGTCTACTGGTTTGTACACTGGAGAAACAAC
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NCNTGGAA

>P8 20P
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CCGAGTATCTGGTACCAACTTGCCAGTGGCTCCAGCCGTCCCCGCAGTAGA
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GCGCCAGCCGCACCAGCCGCACCAGCCGCCGCACCCGCTGCACCAGCCGCC
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>P9 4A
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>P5 15A
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GCTCNGTAGANACGTCCAGNCTGGGNCCATGCTGCNTCAGNTTTGGCACAA
CATTGTGGGAAGTCGGGACGNTAGGCCTGATGTGCTCCCANAGTAAAGACG
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GGACNGAN

>P13 6B
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>P14 24E
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> P6 13B
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AAAAAAAAAAA

10.1.2. Protein sequences of CUP identified clones

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VVKNEGGYKAPSYSAPSYSAPAYKPAYSAPAYSAPAYSAPAYAAPAYSAPAYK
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>P8-17P
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VNGFKYGFETENGIVAQAAGYVKNAGSENAAQVIEGSYSYVGDDGAPVEVKY
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>P9-4A
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GYYPGAPAVYPAVGAVTPAPIIAPVPVVPKVVSPVYKPVDNKLPAIIRQSQEAD
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>P8-20P
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VSADASAAPAAPAAPAAAPAAPAADFANHIR

10.2. APPENDIX 2

Production and Characterization of a Polyclonal Antiserum for PVY Non-Structural Fusion Protein*

* Unless otherwise stated, all information about antisera production was derived from Harlow and lane, (1988), and Leenaars *et al*., 1999.

10.2.1. Introduction

Polyclonal antisera are produced by the immunization of suitable animals such as mice, rats, rabbits and goats with an antigen in order to obtain high titer, high affinity antisera for use in experimentation or in diagnostic tests. The production of antibodies in animals must be carried out in strict accordance with the legislation of the country concerned.

There are many specialized companies which offer polyclonal antibody production (examples, Eurogentec, Open Biosystems, and Precision Antibody), the customer only having to provide purified antigen sufficient for the immunizations involved and for initial analysis of the antiserum. In addition, ready to use antibodies for the most important plant viruses are available to purchase from some companies such as Agdia, Agden, and Bioreba.

10.2.1.1. Selecting animal

The selection of the animal is typically made depending on the amount of antiserum needed, usually; rabbits are the most common choice for laboratories. However, many other animals may be used including, mice, rats, hamsters, horses, goats, and guinea pigs. Chickens are sometimes used, with the advantage that the chicken antibodies are extracted from the egg yolk. However, chicken IgY antibodies are more difficult to purify than mammalian antibodies. For commercial purposes horses, sheep, goats and pigs are used.

As different animals even off the same species react differentially to certain antigen, it is advisable to use more than one animal for immunization with the same antigen (Harlow and lane, 1988)

10.2.1.2. Antigen production

Microgram to milligram quantities of protein in adjuvant are necessary to elicit high titer antibodies. The amounts of antigen used differs depending on the animal species e.g. it is recommended to inject 50–1000 µg of the antigen when rabbit is used. The purity of the antigen is the most important factor that influences the quality of the antiserum that is produced. Small proteins (<3000-5000 Daltons) and non-protein antigens generally need to be conjugated or crosslinked to a larger carrier moleculein order to increase their immunogenicity (Harlow and Lane, 1988). Plant virus antigens may often be purified from infected plants, but when this is not possible then cloning of the protein of interest gene into a bacterial expression system is a useful alternative

10.2.1.3. Selecting Adjuvant

Producing high affinity antisera for diagnostic use requires the use of adjuvants to help improve and enhance the immune response. The role of the adjuvant is to protect the antigen from degradation and to prolonge the immunogenic response. In addition, adjuvants are used for non-specific stimulation of the immune system. There are many types of adjuvants including oil emulsions, mineral salts, microbial-like products, saponins, synthetic products and adjuvant formulations containing mixtures of products (Leenaars *et al*., 1999). The adjuvants that are used for routine polyclonal production include Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), Quil A, Titermax and RIBI. Freund's adjuvants are mineral oil based solutions with the FCA containing, in addition, inactivated and dried mycobacteria. This is recommended for poorly immunogenic weak antigens, however, its use is tightly controlled because intradermal injections may cause skin ulceration and necrosis and it can induce anaphylactic shock.

10.2.1.4. Immunisation

Before beginning an immunisation schedule reference to the relevant local rules must be made in order to ensure that the correct procedures are being followed. This is to ensure the antigen does not contain any toxic or harmful contaminants. The site of injection is

determined by the volume of injected antigen, the type of adjuvant, and how quickly the immunogen should be released. For immunizing rabbits, antigen is normally given at multiple subcutaneous sites, typically, 500 μg of antigen is used. Following this initial immunisation, the animal's primary immune response will produce only IgM antibodies with a lower affinity. It is the subsequent booster immunisations, given typically 2-3 weeks later that lead to a class switch to the more desirable higher affinity IgG antibodies. Booster immunisations may be given until the antibody titre has reached a usable level for the purposes for which it was designed.

10.2.1.5. Bleeding and antiserum collection

As indicated before handling animals for the purpose of antibody production is subjected to legal regulations, therefore blood sampling to monitor the immune response is controlled to ensure minimum stress for the animal. No more than 15 % total blood volume may be taken over any 28 day period. When an antiserum with the desired titre has been confirmed then exsanguination is performed under general anaesthesia. Blood samples are allowed to clot at 4 °C then centrifuged to separate the serum from the clot. Then antiserum can be stored at -20 for many years.

In this appendix an antigen of bacterially-expressed PVY cylindrical inclusion protein (CI) was used to prepare a polyclonal antiserum.

10.2.2. Material and methods

10. 2.2.1. Preparation of CI insert

Total RNA was extracted from PVY^{O} infected tobacco plants using the RNeasy Mini kit as described previously. cDNA was synthesized from the total RNA by Transcriptor reverse transcriptase as described previously, then used as a template in PCR to amplify CI gene using primers PVYOCIFWD and PVYOCIREV (Table 2.3) that include *Sac*I and *Hind*III restriction sites used for cloning. The PCR was done using Taq DNA polymerase (Roche), and the following conditions:
94 °C for 2 min; 29 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; then 72 °C for 3 min. The PCR product was electrophorised through 1% agarose gel and stained with Ethidium bromide, as described previously, and a band corresponding to the CI

insert excised and purified from the gel using the Zymo-spin I column kit. The purified insert was ligated into pGEM-T easy vector (Promega) and used to transform XL10 Gold competent cells (Invitrogen) as described previously. Plasmid preparations from transformed clones were digested with SacI and *Hind*III and the insert gel purified as as described previously.

10.2.2.2. pQE30 vector cloning of CI insert

*Sac*I/*Hind*III digested pQE30 and pGEM-T derived CI inserts were ligated using T4 DNA ligase and used to transform XL10 Gold competent cells (Invitrogen) as described previously.

10.2.2.3. Protein expression and purification

CI expression and purification analysis was performed using both native and denaturing conditions as described previously.

10.2.2.4. CI protein dialysis and concentration

CI protein (5 ml) was dialyzed for four hours with two changes of 10 mM Tris-HCl (pH 7.0) at 4°C. Then concentration was calculated according to the protein coefficient: 1 A280 = 1.61 mg/ml.

To concentrate the protein the volume was decreased by placing the prep (in pre-wetted dialysis tubing) in crushed Polyethylene glycol (PEG). CI concentration became 0.43 mg/ml which is close to the recommended concentration for immunization (0.5 mg/ml). Then samples of concentrated protein were analyzed with SDS-PAGE and western blot (Fig 10.2.1, Fig 10.2.2).

10.2.2.5. ELISA & Western blot analysis

For antiserum titration, plate trapped ELISA (PTE) was used. In this ELISA format, the antigen was used to coat the plate at different concentrations which then was used to trap the antibody which was similarly used at different dilution to detect immobilized antigen on ELISA plate. All washing and incubation steps were performed according to the standard ELISA methods presented in Chapter 2.

Wester blot was carried as explained in Chapter 2.

10.2.2.6. Preparatiojn of α -CI polyclonal antiserum

The anti-CI serum was prepared in a New Zealand White rabbit. Approximately, 500 ng of purified protein was emulsified in Freund's Incomplete Adjuvant and injected subcutaneously at four sites on the back on days 0 and 14. Antiserum was taken 2 weeks after the second injection. Seven weeks after the first immunization an exsanguination was performed.

10.2.2.7. Preparation of IgG fraction of CI antiserum

A 1:5 dilution of crude CI antiserum was prepared in water and an equal volume of saturated ammonium sulphate (pH 7.0) slowly added with gentle stirring. Gentle stirring was continued for 1 h, before pelleting the precipitated IgG by centrifugation for 10 min at 10,000 rpm. The pellet was resuspended and then dialysed overnight in PBS. The solution was clarified by centrifugation as before and the supernatant IgG fraction retained and stored at 4°C with the inclusion of 0.05 % Sodium azide. The IgG concentration was determined spectrophotometrically using the extinction coefficient of 1.4.

10.2.3. Results

10.2.3.1. Bacterial expression of CI

The expected size of the CI protein is about 70 kDa. Our product is about 25-30 kDa. This means that the CI protein has degraded see figure (10.2.1). To prevent degradation expressing of the protein we repeated for clone 3 under denaturing condition in the presence of Protease inhibitor (Roche). However, this step was not useful to prevent degradation, thus it was decided to raise the antiserum against the Histidine-tagged terminus of the CI.

Western blot was used also to check the reaction of series of dilution antiserum (1:100, 1:500, 1:2500, and 1:12500) towards CI antigen. Purified CI protein has been separated by SDS-PAGE and transferred to membrane as mentioned previously. Bands of smaller molecular weight than the expected CI protein were detected by the first antiserum. This result (not presented) confirms that the raised antiserum is against the His-tag part only of the CI protein due to the proteolysis of the CI protein during production. There was

some background reaction towards *E.coli* protein, but this is not a problem, as the prepared antiserum will be used to detect CI in plant or to localize CI in aphid sections.

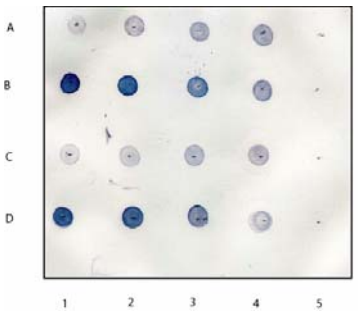

Fig 7.1a detected CI Protein by α-His antibodies in dot blot assay.
A: clone 3 eluted with buffer D.
B: clone 3 eluted with buffer E.
C: clone 8 eluted with buffer D.
D: clone 8 eluted with buffer E.
1-4: number of elutions.
5: Negative control.

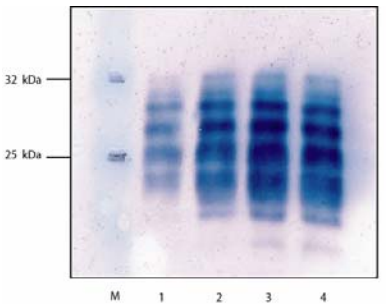

Fig. 7.1b the size of detected CI protein by **α**-His antibodies in Western blot.

M: protein marker

1: clone 3 first elution buffer E
2:clone 3 second elution buffer E.
3:clone 3 third elution buffer E.
4: clone 3 fourth elution buffer E.

Fig 10.2.1. Overexpression of the CI Protein in bacteria and estimation of the protein fragment size by binding to α-His antibodies

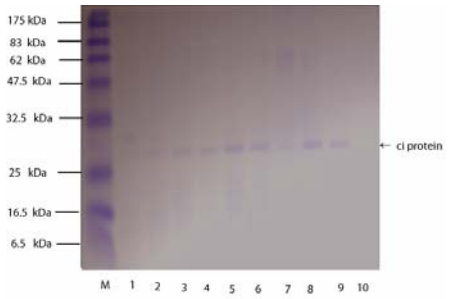

Fig. 7.2a PAGE staining of CI protein before dialysis.

M: protein marker
1-4: clone 3 eluted after adding Protease inhibitor to purification buffers.
5-7: clone 3 eluted before adding Protease inhibitor.
8-12: clone 8 without adding Protease inhibitor.

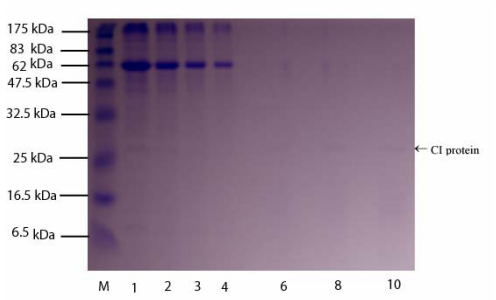

Fig. 7.2b PAGE staining of CI Protein after dialysis and concentration.

M: protein marker
1-4: BSA: 1, 0.5, 0.25, 0.125 µg respectively.
6: CI protein after dialysis.
8: CI first concentration.
10: CI second concentration.

Fig 10.2.2. Coomassie blue staining of overexpressed CI Protein in bacteria before and after dialysis

10.2.3.2. Antiserum production and characterization

10.2.3.2.1. Characterization by ELISA

First bleed

Blood samples collected after two weeks of immunization were used to detect the immunogenic response to injected antigen.

Antigen µg/ml	CI antiserum					Pre-immune blood				
	1:100	1:400	1:1600	1:6400	1:25600	1:100	1:400	1:1600	1:6400	1:25600
2	**0.58**	**0.22**	**0.10**	0.07	0.06	0.12	0.08	0.06	0.06	0.06
1	**0.35**	**0.15**	0.08	0.07	0.06	0.11	0.08	0.06	0.06	0.06
0.5	**0.23**	**0.12**	0.08	0.07	0.06	0.11	0.08	0.06	0.06	0.06
0.25	**0.15**	0.09	0.07	0.06	0.06	0.11	0.08	0.06	0.06	0.06
0.125	**0.12**	0.08	0.07	0.06	0.06	0.11	0.08	0.06	0.06	0.06
0.0625	**0.12**	0.08	0.07	0.06	0.06	0.11	0.08	0.06	0.06	0.06

Table. 10.2.1 Titration of the first bleed of CI antiserum

Absorbance values were recorded after 1 hour of incubation with the substrate

Second bleed

Similarly, after two weeks from the first bleeding another blood sample was collected and immunogenic reaction was checked.

Antigen µg/ml	CI antiserum					Pre-immune blood				
	1:100	1:400	1:1600	1:6400	1:25600	1:100	1:400	1:1600	1:6400	1:25600
2	**0.99**	**0.48**	**0.20**	0.10	0.07	0.12	0.08	0.07	0.06	0.06
1	**0.62**	**0.36**	0.18	0.09	0.07	0.12	0.08	0.07	0.06	0.06
0.5	**0.42**	**0.24**	0.13	0.08	0.07	0.12	0.08	0.07	0.06	0.06
0.25	**0.27**	0.17	0.12	0.07	0.06	0.11	0.08	0.07	0.06	0.06
0.125	**0.17**	0.10	0.09	0.06	0.06	0.11	0.08	0.07	0.06	0.06
0.0625	**0.13**	0.09	0.07	0.07	0.06	0.11	0.08	0.07	0.06	0.06

Table. 10.2.2 Titration of the second bleed of CI antiserum

Third bleed

One week after the second bleed, it was decided to collect the final bleed as the immunogenic reaction was enough to use the produced antiserum for CI testing.

Antigen µg/ml	CI antiserum					Pre-immune blood				
	1:100	1:400	1:1600	1:6400	1:25600	1:100	1:400	1:1600	1:6400	1:25600
2	**0.67**	**0.37**	**0.21**	0.12	0.09	0.17	0.10	0.07	0.09	0.09
1	**0.47**	**0.28**	0.15	0.09	0.07	0.15	0.09	0.07	0.09	0.09
0.5	**0.32**	**0.18**	0.11	0.08	0.06	0.14	0.09	0.07	0.09	0.09
0.25	**0.26**	0.14	0.10	0.07	0.06	0.13	0.09	0.07	0.09	0.09
0.125	**0.16**	0.11	0.08	0.07	0.06	0.13	0.08	0.07	0.09	0.09
0.0625	**0.13**	0.09	0.07	0.06	0.06	0.12	0.08	0.07	0.09	0.09

Table. 10.2.3 Titration of the first bleed of CI antiserum

It cam be concluded from the ELISA results that the second bleed contained the better quality antisera while the quality of the antiserum in the third bleed was decreased. It has been decided to use bleed 2 at 1:400 dilutions as a working concentration to detect CI.

10.2.3.2.2. Characterization by Western blot

Recombinant CI protein, and other virus recombinant proteins were SDS-PAGE electrophoresed, then proteins were transferred to Hybond-ECL^{TM} nitrocellulose membrane. Membranes were then overlaid with 1:400 or 1:1000 dilution of the crude antiserum. Non-recombinant *E. coli* protein was produced following the same conditions of CI production except adding the inducer, and this protein was used as a control.

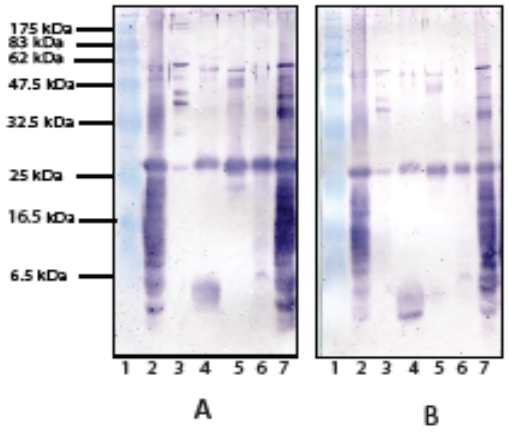

Fig. 10.2.3. Characterization of the third bleeding of CI antiserum

A blot was incubated with 1:400 dilution of the crude CI antiserum
B: blot was incubated with 1:1000 dilution of the crude CI antiserum

1- Prestained protein marker.
2- PVY-CI preparation produced in E.coli used to inject the rabbit
3- PVA recombinant CI produced in E.coli
4- PVY recombinant HC-Pro produced in E.coli
5- PVY recombinant VPG produced in E.coli
6- Non-recombinant *E. coli*
7- PVY- recombinant CI new preparation

Protein of two weeks infected N. Tabacum cv. Samsun leaves were extracted by adding 1 ml of PAGE sample buffer (Laemmli buffer) to 1 g on tissue and the mixture homogenized by using a mortar and a pestle. Then mixture was boiled for 5 min and centrifuged for 5 min at 5,000 rpm. Then the supernatant was used directly in PAGE gel. Protein was transferred to nitrocellulose membrane and overlaid with different dilutions of the crude antiserum.Similary; a protein sample was extracted from a healty tobacco leaf and used as a control.

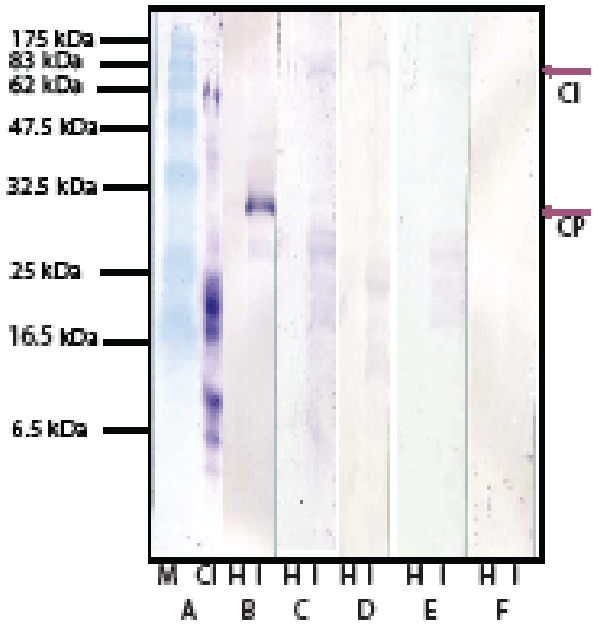

Fig. 10.2.4. Characterization of the third bleeding of CI antiserum using PVY infected plant

A control blot of recombinant CI overlaid in 1:400 of the CI antiserum
B: control blot of healthy and infested plant overlaid with 1:2000 of α-CP antiserum
C, D, and E represent titration of the CI antiserum at 1:400, 1:800, and 1:1600.
F: negative control overlaid in 1:400 of the pre-immune blood
M: Prestained protein marker. CI: recombinant protein produced in E.coli,
H: Healthy tobacco leaf sample, I: PVY infected tobacco leaf sample

It can be concluded that the produced antiserum can recognize natural CI as well as recombinant when used at dilutions between 1:400 to 1: 1600 but better result was obtained at 1:400. Moreover, CI was detected in the plants as a multiband rather than one single band which may indicate that degradation of this protein occur in plant as well as when expressed in bacteria.

It was impossible to detect CI in infected plant sap using ELISA coated with either Polyclonal or Mab which may either indicate that the CI protein compromise a low portion of the virus particle can not be detected by ELISA or it is existed free in the plant sap unattached to the virus particles, thus it is washed during the washing steps in ELISA

10.2.4. Discussion

Production of high affinity antisera for diagnostic purpose or for investigation of protein interaction mechanisms is very important to control plant viruses. The cylindrical inclusion protein (CI) is one of the non-structural proteins produced by potyviruses. There are many reported function of this protein in the potvirus life cycle including formation of pinwheel structures and potyvirus movements. CI is reported to self interact, and the domains responsible for this interaction have been identified at the N-terminus (Lopez *et al*., 2001). In addition, potyvirus CI is reported to interact with the CP, and with the HC-Pro protein (Lin *et al*., 2009).

HC-Pro is a multifunctional potyvirus protein, and one of the most important function is mediating the aphid transmission of potyviruses, and the mechanisms by which HC-Pro is working is the main focus of my study in this thesis. There is no comprehensive study which indicates clearly that CI interfering with the aphid transmission of potyviruses. However, the interaction of the HC-Pro with this protein, and the multifunctional nature of the potyvirus proteins in general, and HC-Pro in particular were the reason to investigate if CI has any role in the transmission mechanism. Thus it was important to prepare an antiserum which can recognize this protein in order to detect any binding between this protein and the aphid proteins or the others domain that participates in the transmission process (HC-Pro, CP). CI exists in low concentration in the infected plant, so it is difficult to purify this protein to obtain enough amounts for antiserum production. Thus, it was decided to express a fusion of the 6-Histidin tag at the N-terminus of this protein of this viral gene, and express the protein in bacteria, then purification over Ni-NTA resin. In this study, the method produced sufficient amount of the antigen. However, the protein was degraded, and all the attempts to stop degradation by adding protease inhibitor enzymes were unsuccessful. Thus it was decided to proceed with the antiserum preparation against the N-terminus part of the protein which is believed to be responsible for protein-protein interaction.

Characterization of the produced antiserum by ELISA and western blot revealed that the produced antibody can be used detecting CI at dilution between 1:400 to 1:1000. However, the interaction was stronger when the antiserum was used at 1:400 .So it was recommended that to use 1:400 routinely as working concentration for measuring CI interaction with the other proteins. Due to the time restriction, I was not able to use the produced antiserum to investigate if CI protein can interact with aphid CUPs or with HC-Pro.

10.3. APPENDIX 3

Scientific contributions

10.3.1. Research paper from chapter 6

Al-Mrabeh, A., Ziegler, A., Cowan, G. and Torrance, L. (2009) A fully recombinant ELISA using in vivo biotinylated antibody fragments for the detection of potato leaf roll virus. J. Virol. Methods 159, n°2, pp. 200-205.

10.3.2. Review paper adapted from the literature review chapter

A Literature Review of Insecticide and Mineral Oil Use in Preventing the Spread of Non-persistent Viruses in Potato Crops

Report Authors: Ahmad Al-Mrabeh[1, 2], Eric Anderson[3], Lesley Torrance[1], Andy Evans[4] and Brian Fenton[1]

Date report submitted: February 2010

[1]Scottish Crop Research Institute (SCRI), [2]Newcastle University, [3]Scottish Agronomy Ltd, [4]Scottish Agricultural College

10.3.3. Oral presentation - The BIT Life Sciences' 2nd Annual International Congress of Antibody 2010, which was held on Mar. 21-23, 2010 Beijing, China

Title: A fully recombinant ELISA based on in vivo biotinylated antibody fragments is effective for routine virus detection.

Authors: Lesley Torrance, Ahmad Al Mrabeh, Graham Cowan and Angelika Ziegler

Abstract

Recombinant antibody fragments (ScFv) derived from naïve phage display libraries have the potential to replace antibodies derived from animal immunizations in routine assays for pathogen detection and diagnosis. There are many advantages of this approach including the limitless supply of reagents compared with polyclonal sera, economy of production and storage compared with monoclonal antibodies and assay standardization to ensure quality and reproducibility of tests within and between testing stations. Moreover, they allow the replacement of animals for antibody production which is of ethical importance in some countries. A perceived disadvantage is that these reagents are of relatively low affinity and stability compared to antibodies obtained from the serum of hyperimmune animals and so the limit of detection of such assays is not adequate for some purposes. Here we report an enzyme-linked immunosorbent assay (ELISA) based on scFv that have been genetically modified to create fusion proteins with the constant domain of the immunoglobulin light chain and a biotin tag. The biotin tag was introduced by chemical coupling in vitro or by an in vivo method and the assay incorporated a streptavidin-alkaline phosphatase conjugate. We found that the in vivo introduction of biotin was superior to chemical coupling and the limit of detection of this 'fully recombinant' assay was similar to an assay based on immunoglobulins for virus detection in potato. The scFv-biotin preparations were stable and retained specific activity for more than one year when stored at 4˚C or -20˚C. The results demonstrate that scFv reagents derived from synthetic phage display platforms can provide effective alternatives to assays incorporating immune reagents.

10.3.4. Oral presentation- The 14th triennial meeting of the Virology Section of the European Association for Potato Research (EAPR), Hamar, Norway 4–9th July 2010
Session: Virus Transmission

Title: Studies on molecular and biological aspects influencing aphid transmission and control of potato virus Y

Ahmad Al-Mrabeh[1, 2], Angelika Ziegler[1], Brian Fenton[1], Graham Cowan[1], and Lesley Torrance[1]

[1]Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, Scotland, UK, DD2 5AD
[2]Institute for Research on Environment and Sustainability (IRES), School of Biology, University of Newcastle, Newcastle upon Tyne, UK, NE1 7RU

Potyviruses are a group of non-persistently transmitted viruses which require the virus encoded protein helper component-proteinase (HC-Pro) in order to be transmitted. The molecular mechanism of non-persistent transmission of plant viruses by aphid vectors is not fully understood. A better understanding of the potyvirus transmission mechanism requires more knowledge about the three components involved in the transmission process (virus/host/vector). In this work, we have identified aphid cuticle proteins (CUPs) that may be potential virus receptor proteins and the impact of the host plant on the vectoring ability of the aphid was investigated. Three CUPs that interacted *in vitro* with PVY HC-Pro were identified by screening a *Myzus persicae* cDNA library (Ramsey et al., 2007). Identified CUP protein genes were cloned and the recombinant proteins were purified and the interaction was confirmed to occur with HC-Pro of another potyvirus. The choice of host plant can influence virus transmission by aphids because virus concentration may vary and aphid vectors have different feeding preferences. We investigated the influence of different plants (potato, tobacco, oilseed

rape and *Physalis floridana*) as virus sources and for aphid colony propagation. Our results suggest that the host plant used for maintaining aphids influenced their capacity for virus acquisition and there was also an influence of recipient plant host on virus transmission. The results will be discussed in the context of understanding the transmission process and methods of controlling non-persistent viruses. In addition, the data highlight the importance of the choice of plant species used in assessment of aphid vectoring ability.

Key words

PVY, HC-Pro, potyvirus, virus receptors, host effect.

References

Ramsey *et al*. 2007. BMC Genomics 8, 423.

10.3.5. Poster presentation- Crop Protection in Northern Britain Conference (CPNB) in Dundee west park conference centre from 26 - 27 February 2008.

Site specific biotinylation is a powerful technique to improve performance of a recombinant antibody

Ahmad Al-Mrabeh[1, 2], Angelika Ziegler[1], Graham Cowan[1], Angharad Gatehouse[2], Ethan Hack[2], and Lesley Torrance[1].

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[2]Institute for Research on Environment and Sustainability, School of Biology, University of Newcastle, Newcastle upon Tyne, UK, NE2 7RU.

V3HCL is a fusion protein comprising an α- PLRV ScFv and the C_L domain of the immunoglobulin light chain. Both chemical and *in vivo* biotinylation were performed on the fusion protein with the aim of devising an ELISA assay for PLRV detection based only on recombinant antibody fragments. For chemical coupling, standard methods were used to modify the V3HCL proteins with EZ-Link NHS-Biotin. The PAK300Bio vector, containing a biotin acceptor domain, was modified to enable cloning of the scFv-C_L sequence and biotinylation of the protein *in vivo*. The chemically biotinylated product gave comparable result to immune reagents but different preparations varied in

the level of background non-specific reactions. On the other hand, no variation was observed when different batches were biotinylated *in vivo*, and their performance was as sensitive as the immune reagents. Furthermore, biotinylated protein remains functional for at least two months under different condition of storage. It was concluded that *in vivo* biotinylation gives the best results in terms of reproducibility and stability compared to the *in vitro* process.

Abbreviations: PLRV*:* Potato leafroll virus*,* scFv: single chain antibody fragment, ELISA: enzyme-linked immunosorbent assay

10.3.6. Plant Virus workshop, 162nd Society for General Microbiology (SGM) Meeting - Edinburgh International Conference Centre - 31 March - 3 April 2008.
The same poster as above.

Site specific biotinylation is a powerful technique to improve performance of scFv

Ahmad Al-Mrabeh[1,2], Angelika Ziegler[1], Graham Cowan[1], Angharad Gatehouse[2], Ethan Hack[2], and Lesley Torrance[1]

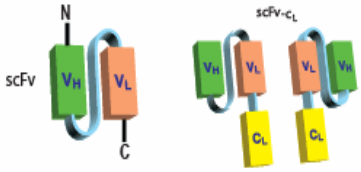
[1]Scottish Crop Research Institute, Invergowrie, Dundee, Scotland, UK, DD2 5AD
[2]Institute for Research on Environment and Sustainability, School of Biology, University of Newcastle, Newcastle upon Tyne, UK, NE2 7RU.

Introduction

Recombinant antibodies (scFv) derived from "naive" libraries of antibody genes by phage display technology can be used as an alternative to conventionally produced antibodies, as they are cheaper and can be produced without immunization of animals . However, to be a viable alternative, they must perform at least as well as the immune reagents.

Hypothesis

Recombinant antibody technology can provide us with cheap, easy to produce, and efficient reagents for plant virus detection that are comparable with currently used antibodies.



Project's aim

To devise a fully recombinant assay for PLRV detection by biotinylating anti-PLRV V3HCL (a scFv which we selected from a phage display library), and using a detection system based on biotin/streptavidin binding.

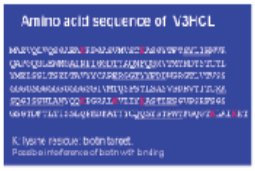
Abbreviations: PLRV: Potato leaf roll virus, scFv: single chain antibody fragment, V3HCL: fusion of anti-PLRV scFv to the C_L domain of light chain of immunoglobulin.

Materials and methods:

1- Chemical biotinylation:

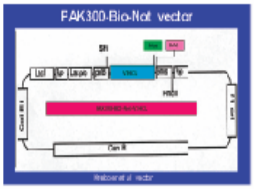
Biotinylation has been performed by using EZ-Link NHS-Biotin (Pierce).

The letters in italic represent the hinge region between the heavy and light chains of our scFv. Complementarity determining regions (CDRs) were underlined.



2- *In vivo* biotinylation:

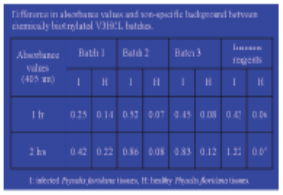
The sequence of V3HCL was fused to a biotin acceptor domain. The construct was expressed in *E.coli* AVB100 strain.
The yield was 5-6 mg/L.



Results and discussion

Chemical biotinylation:

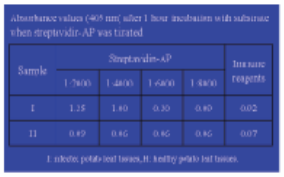
Chemically biotinylated products gave comparable results with the immune reagents. However, their performance was not consistent between batches. We think this is due to uncontrolled reaction with the NHS-Biotin,and possible interference with the V3HCL binding sites.



In vivo biotinylation:

1- Optimisation:

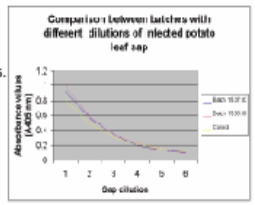
Experiments were performed to find the best conditions for the fully recombinant assay.This table shows for example titration of conjugate. The combination chosen was: 10 µg/ml of scFv-CL for coating, 10 µg/ml of scFv-CL-Biotin for detecting, and 1:4000 of the streptavidin-AP as a conjugate.



Results and discussion

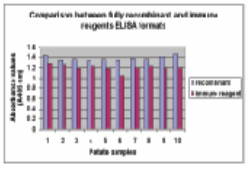
2-Sensitivity and batch variations:

Detection limits were similar in both assays. Moreover, there was no variation between different biotinylated batches.



3- Recombinant versus immune reagents:

Recombinant reagents performed as well as the immune reagents on different infected potato leaf samples.

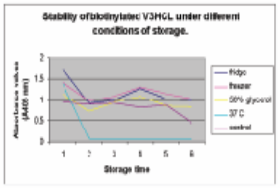


4- Comparison with SASA on naturally infected potato leaf samples:

There was 90 % agreement between both formats. The differences could be due to the samples being frozen at -20°C before being tested in the recombinant test. An RNA based test (such as RT-PCR) could be used to verify the result.

5- Storage:

V3HCL-B remains functional for at least two months.



Summary:

Chemically biotinylated V3HCL can give results comparable to immune reagents, but are limite by lack of reproducibility. However, *in vivo* biotinylation gave a more reproducible and stal product than the *in vitro* process. The sensitivit of recombinant antibodies is comparable with their conventional counterparts. In conclusion, they are applicable for plant viruses testing.

10.3.7. Poster presentation- Potatoes Viruses and their vectors Conference, association of applied biologists (AAP), at SASA, Edinburgh, UK on 16 September 2009.

Effect of the host plant on PVY^{O} acquisition and transmission by *Myzus persicae*

Ahmad Al-Mrabeh[1,2], Brian Fenton[1], Graham Cowan[1], and Lesley Torrance[1]

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INTRODUCTION

Indicator plants are used for virus propagation and virus transmission studies by plant virologists worldwide. These model plants are usually not the natural virus host. However, the virus disease is usually more easily studied using indicator plants that can be grown readily in glasshouses.

Changes to virus populations in the field occur in response to host genotype, vector pressure and environment. Whereas in laboratory isolates the selection pressures are different and it is known that a virus isolate may lose the capacity to be vector transmitted after successive manual passage on host plants.

Selective mutation and adaptation of RNA viruses by continuous passage through their host plants has been reported. For example, PVY isolates from potato were shown to be able to infect tobacco but not peppers, and the pepper strains were unable to infect potato (Gebre Selassie et al., 1985). Similarly Marte et al. (1991) found that PVY isolates in central Italy are better adapted to tobacco, whereas isolates from southern regions infected pepper more readily than tobacco.

Aphids are well known to show some level of preference towards some plants compared to others. Potato plants for example, are more favoured by *M. persicae* compared to tobacco plants. On the other hand, tobacco is extensively used in the lab for different kind of virus studies including aphid transmission, the effect of the host is often mitigated by using large numbers of individual aphids per plant for aphid transmission studies.

The **aim** of this study was to investigate whether the acquisition or transmission of field and lab isolates of PVY differ and the effect of plant host species.

CONCLUSIONS AND FUTURE WORK

1. PVY-F and PVY-L achieved similar concentrations in tobacco leaves as judged by ELISA absorbance values. However, PVY-F accumulated to a greater extent on potato compared with PVY-L. This result suggests that PVY-L has adapted to propagation on tobacco.
2. PVY-F was more readily aphid transmissible from potato.
3. Aphids acquired PVY-F more readily from infected potato plants compared to tobacco although the virus reached similar concentration in both hosts. No such obvious effect was observed with the poorly transmissible PVY-L.
4. There was an influence of recipient host for virus transmission; more tobacco plants became infected than potato with both virus isolates. It is possible that tobacco is a more sensitive host and can be infected with less PVY than potato. This is supported by experiments using one aphid per plant, where 30-40% of tobacco plants and 0% of potato plants were infected.

Future work:

- Obtain sequences of PVY-F and PVY-L coat protein and helper component genes (implicated in aphid transmission) that may help explain differences in transmission efficiency.
- Determine whether the host differences are found in other field isolates of PVY
- Determine whether these differences are found with other aphid species.
- Investigate the behaviour of the PVY-F isolate which has been maintained by manual inoculation on tobacco plants for one year to find out if this isolate has accumulated mutations that may affect aphid transmissibility or host adaptation.

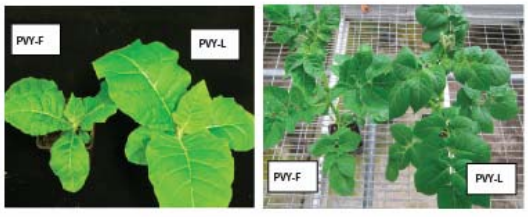
RESULTS

Virus concentration in source plants:
no difference was observed in absorbance values obtained in tests on tobacco plants infected with either virus isolate. However,on potato, the PVY-F isolate gave values 3-4 times greater than PVY-L.

Mean A405nm values obtained in ELISA of extracts of tobacco and potato leaves infected with different isolates of PVY

Test plant	Isolate	Exp.1	Exp.2
tobacco	PVY-L	0.888 (0.140)	1.067 (0.049)
	PVY-F	1.107 (0.117)	1.142 (0.08)
potato	PVY-L	**0.338 (0.194)**	**0.35 (0.168)**
	PVY-F	1.254 (0.344)	1.231 (0.261)

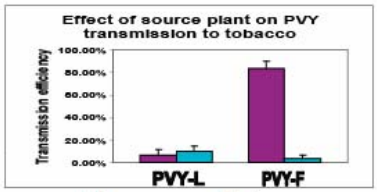
For each experiment 10 plants of each host were inoculated with PVY-L and 10 plants with PVY-F.Samples were tested in duplicate wells and the mean A405nm value and SD are presented. The mean A405nm value of the healthy controls were 0.07 for both hosts



PVY-F gave strong symptoms on tobacco and potato; PVY-L induced mild symptoms on both host species

Effect of source plant on aphid transmission of PVY-F and PVY-L

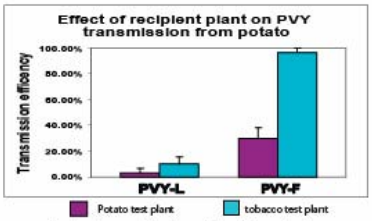
PVY-L was poorly transmissible by aphids only approx. 10% of plants became infected and the source plant (tobacco or potato) had no obvious effect on transmission efficiency. PVY-F was efficiently transmitted by aphids when potato was used as a source of virus with >80% of plants infected.



Experiment was repeated three times and for each sub-experiment 40 tobacco plants were used (20 for each virus isolate)

Effect of recipient host species on aphid transmission of PVY-F and PVY-L

PVY-L transmission was relatively poor but more tobacco plants (10%) were infected compared to potato (5%). With PVY-F the effect of host was more apparent and 90-100% tobacco plants were infected compared to 30% potato plants.



Experiment was repeated three times and for each sub-experiment 40 plants were used (20 for each host plant)

MATERIALS AND METHODS

PVY isolates: Two isolates of PVYO (ordinary strain) were investigated; the stock SCRI laboratory isolate (PVY-L) and a field isolate (PVY-F) that was obtained from naturally infected potato cv Rosetta by Adrian Fox, Scottish Agricultural Science Agency (SASA) Edinburgh. PVY-F was maintained in potato plants grown from infected tubers and PVY-L isolate was maintained on tobacco and propagated by mechanical inoculation. Both isolates were mechanically inoculated into potato and tobacco plants to be used as a virus source.

Plants: Small plants of N. tabacum cv White Burley at the two-three leaf stage were used for aphid transmission studies. Infection by PVY induces symptoms of mild to severe mosaic after 1-2 weeks of infection. Potato plants cv Shula were produced by removing eye plugs from infected tubers after dormancy break and transplanted into 5-7 inch pots. Small plants at the three-four leaf stage were used for transmission studies.

Aphid transmissions: young wingless aphids (3-4 instar) of *M. persicae* (genotype E) were fasted for 2-3 hours at room temperature. Fasted aphids were allowed to acquire virus from detached leaves of potato or tobacco for 5 min acquisition access period (AAP) in groups of 5 individuals. Aphids were then removed and placed directly on test plants. After overnight transmission access period (TAA), the plants were transferred to fume chamber and aphids were killed with Plenum and fumigated with smoke from nicotine shreds. The plants were then left for 3 weeks in a glasshouse and tested for PVY by ELISA using anti-PVY-SCRI polyclonal antibody for coating and monoclonal antibody SCR39 for detecting.

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2- MARTE M., BELLEZA G. and POLVERARI A., 1991. Infective behaviour and aphid transmissibility of Italian isolates of potato virus Y in tobacco and peppers. Ann. appl. Biol., 118: 309-317.

10.3.8. Poster presentation: International Advances in Plant Virology to be held at Arnhem, the Netherlands 5-7 September 2010

Investigations into molecular interactions between Potato virus Y and *Myzus persicae*

Ahmad Al-Mrabeh[1, 2], Brian Fenton[1], Angelika Ziegler[1] Graham Cowan[1], and Lesley Torrance[1]

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The molecular mechanisms of potyvirus transmission are not fully understood, and there are a number of reports which speculate the involvement of virus-like receptors in the transmission of potyviruses by aphids. However, limited information is available about their nature or their location inside the aphid vector. In this study, three aphid cuticle proteins (CUPs) which interacted with the potyvirus PVY helper component (HC-Pro) were identified. Sequence analysis of the identified clones revealed that the selected clones belong to three different families of insect CUPs. The clone which has RR-2 consensus was sub-cloned into an expression vector and expressed protein was confirmed to interact with HC-Pro of TEV potyvirus. In another study, a nested RT-PCR technique developed to detect PVY inside aphids was used to investigate virus retention. PVY was detected in aphids that were allowed access to infected plants and were then transferred several times to healthy plants, but the detected virus was not transmitted. Aphid dissection revealed that virus was retained in the aphid stylet as well as in the aphid body. Similar results were obtained with a non aphid-transmissible virus isolate.

These findings will be discussed in the context of aphid transmission mechanisms, and virus control.

Key words

PVY, HC-Pro, potyvirus, aphid virus receptors.

Investigations Into Molecular Interactions Between Potato Virus Y and *Myzus persicae*

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INTRODUCTION

One way to control the spread of aphid-borne viruses is to control their aphid vector, but this is not always effective as many aphid-borne potato viruses, including the most important ones, are transmitted non-persistently, being acquired within a very short time before agrochemicals can act. Thus in order to devise alternative approaches to control this class of viruses a better understanding of the interaction between the virus, the host plant and the aphid vector is required. In this respect a study was undertaken to identify aphid proteins that may be involved in the virus-vector interaction and some aphid cuticle proteins were identified to interact with potato virus Y helper component (HC-Pro) through screening of an aphid cDNA expression library, and their potential role in virus transmission was discussed. Additionally, virus RNA was detected by a nested RT-PCR assay within aphids that had transmitted PVY but could no longer transmit after sequential transfer to healthy plants. This result challenges the concept of short retention of non-persistent viruses inside their aphid vectors.

CONCLUSIONS

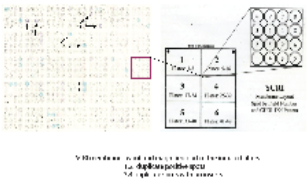
1-Library screening revealed that HC-Pro interacted with eight proteins including aphid cuticle proteins, beta-tubulin, some enzymes, and other genes of unknown function.
2-One of the cuticle proteins, clone P72F, shares a high level of similarity with other aphid CUP proteins and bioinformatic analysis suggests that these proteins may form a multi-gene family. The amino acid sequence shows that P72F clone is different from other CUP proteins characterized before from *M. persicae*.
3. Overlay blot analysis showed that P72F interacted with HC-Pro preparations from PVY and TEV.
4. PVY was detected within the head and body of aphids that had transmitted PVY to healthy tobacco but were no longer able to transmit virus although aphids were thought to be carrying sufficient virus to initiate an infection.

5-Taken together, the observations suggest:
I. That HC-Pro can bind to several different aphid cuticle proteins.
II. This may point to the existence of more than one type of virus receptor inside aphid vector and that the virus detected was defective for transmission or was not available for transmission (possibly it could not be released because it was not accessible to aphid saliva thought to be important in virus release from stylets).

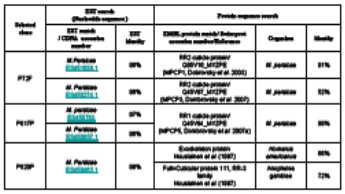
SUGGESTED FUTURE WORK

Specificity of interaction between HC-Pro and CUPs
To find the range of CUPs to which HC-Pro can bind, experiments should be expanded to other aphid vector and non-vector species.
Localization of virus receptors
The system developed by Uzest et al. (2007) can be employed to localize the interaction between aphid stylets and PVY HC-Pro. A fusion of the green fluorescent protein (GFP) to the protein sequence of the PVY HC-Pro can be constructed. The construct then can be agro-infiltrated into *N. benthamiana* leaves, than functionality of the GFP-HC-Pro fusion may be revealed by feeding aphids on the agroinfiltrated leaves than on PVY purified particles through paraffin membranes. If GFP-HC-Pro is functional for transmission, the GFP-HC-Pro binding site in the aphid stylets can be investigated by fluorescent microscopy.
Aphid feeding studies through membranes
This technique can be used to investigate whether aphids can transmit purified PVY preparations in the attogram range in the presence of active HC-Pro. This will indicate whether the infectivity of virus particles was disabled or virus particles are firmly attached to aphid CUPs. Additionally, blocking of aphid receptors can be tested by preparing antiserum against P72F protein, then blocking of aphid transmission of PVY can be tested by feeding aphids on the antibody then testing transmission efficiency compared to the controls. Another thought would be to fuse the antibody with a fluorescent tag and investigate the position of interaction by looking at the aphid stylet under the confocal microscope.

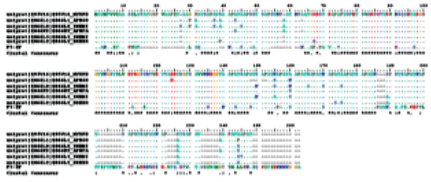
cDNA LIBRARY SCREENING



Each cluster contains 16 spots, representing eight clones in duplicates. Each large membrane can accommodate up to 48 Genetix 384-well plates, divided into 6 sub-areas each with clusters of clones from 8 plates. Therefore the whole library of 7680 clones can be screened on one large filter. Dark purple colour shows a reaction; blue colour shows that the clone did not contain an insert

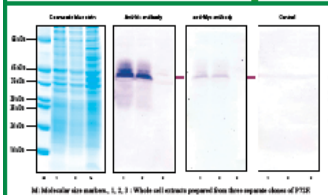


Using this system, eight clones were identified to interact with N-HC-Pro, and inserts were sequenced. The above table presents BLAST result searches of the NCBI database . P72F and P817P gave high matches with *M. persicae* cuticular proteins that were previously characterized and reported to interact with HC-Pro of ZYMV (Dombrovsky et al., 2007). P820P gave match with exoskeleton protein belong to other insects

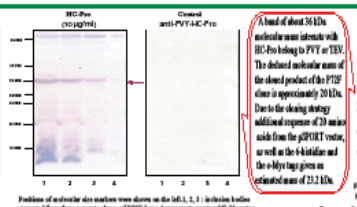


The sequence data of clone P72F were subjected to BLAST search via the NCBI website, BLASTX programme was used. The closest protein matches with clone P72F obtained from sequence database were aligned together using the ClustalW multiple alignment programme through the BioEdit program or on the EMBL website. The result indicates that there is a high level of identity among different sequences and the P72F clone on the N-terminus. However, clone P72F is different sequence at the C-terminus.

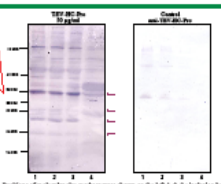
CONFIRMING INTERACTION WITH HC-PRO OF PVY AND TEV



P72F clone gene was sub-cloned into the pQE-30 vector, so that on expression it could be fused to the epitope tags 6-His and c-Myc. Recombinant protein was expressed and whole cell extracts prepared. However, the molecular mass of induced proteins separated by SDS-PAGE was greater than expected from the sequence. The detected bands were between 36 and 45 kDa. This discrepancy between the migration of proteins in SDS gels and their predicted size from amino acid sequence is a characteristic of cuticular proteins, which typically migrate 20-40% more slowly than expected (Cox and Willis, 1987).

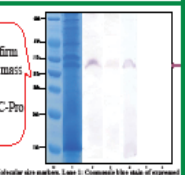


HC-Pro expressed from PVX in *N. benthamiana* (Sasaya et al., 2000) interacted with the inclusion body preparations from the induced P72F clones. Interaction was with a protein of about 36 kDa in molecular mass, which is inconsistent with the result obtained with the α-His, and c-Myc antisera. In addition, HC-Pro interacted with protein products of smaller mass (14-26 kDa), which may represent degraded protein products.



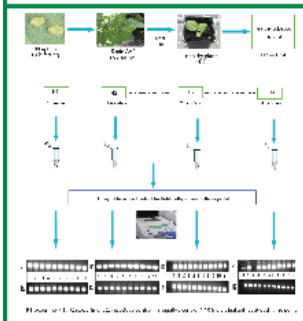
It was decided to use His-tagged TEV HC-Pro(Blanc et al., 1999) because of the high HC-Pro yield produced from this engineered clone. TEV HC-Pro was incubated with electroblotted aphid proteins prepared from the inclusion bodies of P72F sub-cloned in pQE-30. the result suggests that TEV HC-Pro interacts with P72F IB protein preparations at the same position as did PVY HC-Pro derived from the PVX vector. There also appears to be a reaction with smaller mass products which may be degradation products, as seen before in the interaction with PVY HC-Pro

The results confirm that the band of mass of 36 kDa does interact with HC-Pro



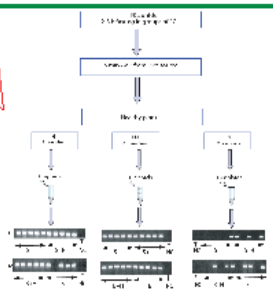
Since different bands reacted with TEV HC-Pro, a further experiment was done to confirm that P72F protein preparations react at the same position as with PVY HC-Pro. A portion of the gel was stained with Coomassie blue and directly compared with sections of the same gel electroblotted and incubated with α-His, α-Myc or TEV HC-Pro preparation followed by α-TEV HC-Pro antiserum.

RETENTION OF PVY INSIDE APHIDS



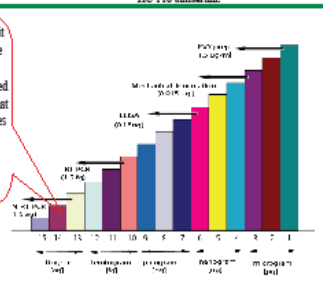
The results show that virus was detectable in all 10 aphids after 7 successive transfers to healthy plants (7days). After 14 transfers (14 days), which is the longest period tested, virus was detectable in 9/10(R1) and 10/10 (R2) aphids. Using ELISA, only the first tobacco plant (H1) tested virus positive, and all the other plants (H2-H14) were found to be virus-free.

PVY was detected within the head and body of aphids that had transmitted PVY to healthy tobacco but were no longer able to transmit virus. Possibly it could not be released because it was not accessible to aphid saliva which is important in virus release from stylets.



The results show that virus is retained in all parts of the insect body (stylet, head, and body), but was detected in fewer aphids after 7 transfers. This finding is contrary to the currently accepted view of non-persistent viruses being only stylet-borne. However, this result may suggest that more than one type of receptor exists in the aphid's body, but only the one located at the tip of the stylets carries transmissible virus particles.

N-RT-PCR detection limit was in the attogram range which is in agreement with the threshold reported by Moury et al. (2007) that approx. 1–3 virus particles (attogram range) were enough for an aphid to initiate aninfection with PVY



A 10-fold dilution series of PVY preparation (1.5 µg /µl) was tested by N-RT-PCR, RT-PCR, and ELISA. And ten- fold dilutions were tested by inoculation to healthy tobacco plants. The results from two experiments revealed that the detection limit of the mechanical inoculation method is 0.015 µg /µl compared with 0.00015 µg /µl using ELISA. This means that ELISA is approx. 100 times more sensitive than mechanical inoculation. The sensitivity of detection was increased by a further factor of 10^6 times by using RT-PCR alone. The lowest detection limit was 0.015 ag/µl by using nested RT-PCR.

MATERIALS AND METHODS/ LIBRARY SCREENING

A portion of the cDNA expression library described by Ramsey et al. (2007), 7680 clones constructed by Dr. B. Fenton, and colleagues (SCRI), was screened using the Q-bot for colony printing in a completely automated process. Antisera used in this work were: Anti-6-histidine (Sigma), anti-Myc (SCRI), anti-PVY-HC-Pro (SCRI), and anti-TEV-HC-Pro (J.J. Lopez-Moya, Spain).

MATERIALS AND METHODS/ VIRUS RETENTION

For N-RT-PCR, the primers used were Singh 50, Singh AS480 (Singh et al., 1998) for the first round and Malloch F, Malloch R (Malloch, unpublished, SCRI) for the second round. For RT-PCR, Singh et al (1998) primers were used.

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