The molecular basis of the interactions between luteoviruses and their aphid vectors

Saskia A. Hogenhout

Promotor:	Dr. R.W. Goldbach Hoogleraar in de Virologie
Co-promotor:	Dr. Ir. J.F.J.M. van den Heuvel Senior onderzoeker DLO-Instituut voor Plantenziektenkundig Onderzoek (IPO-DLO)

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Proefschrift

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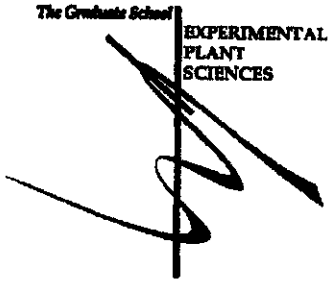

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Stellingen

1) Het gegeven dat hydrofiele aminozuren in het equatoriale domein PLRV-deeltjes binden en niet hydrofobe aminozuren in het apicale domein, is een aanwijzing dat GroEL een beschermende functie heeft in de hemolymfe van bladluizen en niet betrokken is bij eiwitvouwing.
(dit proefschrift)

2) Transgene planten zullen in de toekomst een bijdrage leveren tot de beperking van verspreiding van vector-afhankelijke plantpathogenen.
(dit proefschrift)

3) Het lijkt meer dan toeval dat de aanwezigheid van extracellulair GroEL en het bezit van een type III secretiemechanisme gemeenschappelijk kenmerken zijn van pathogene gram-negatieve bacteriën.
(dit proefschrift)

4) Luteovirussen binden aan GroEL van *Buchnera* spp. om het immuunsysteem van bladluizen te omzeilen.
(dit proefschrift).

5) De conclusie dat het recombinante doorleesprodukt van het *Barley yellow dwarf virus* manteleiwit GroEL van *Buchnera* spp. bindt maar niet GroEL van *Escherichia coli* is slecht verdedigbaar.
(Filichkin *et al.*, 1997).

6) De aanwezigheid van extracellulair GroEL bij het infectieproces van *Legionella pneumophila* is belangrijk om de overleving van deze pathogene bacterie in de gastheer mogelijk te maken.
(Fernandez *et al.*, 1996).

7) Na 250 miljoen jaren van co-evolutie is het onwaarschijnlijk dat bacteriële symbionten van bladluizen onder stress-condities leven.
(Baumann *et al.*, 1996).

8) Het is beter om wetenschappers in teams te groeperen op basis van de doelstellingen van hun onderzoek en niet op basis van de technieken die zij bezigen of de organisatie waarbinnen ze werken.

9) Het voordeel van stellingen op een los blaadje is dat de lezer nooit zonder bladwijzer zit.

10) I haven't failed, I've found 10,000 ways that don't work.
Thomas Edison

Stellingen behorende bij het proefschrift van Saskia A. Hogenhout: The molecular basis of the interactions between luteoviruses and their aphid vectors. Wageningen, 15 juni, 1999.

Aan mijn ouders, Tanja
en Sophien
uit liefde en dankbaarheid

Contents

Chapter 1

General Introduction

RATIONALE

A considerable number of insects and other arthropods transmit pathogenic organisms to man, cattle or plants and are therefore responsible for severe human and economical losses. Arthropod-borne pathogens include protozoa, helminthes, bacteria, arboviruses and circulative plant viruses. Effort has been invested into curative methods for controlling arthropod transmitted diseases such as the applications of pesticides, drugs and medicines. In order to develop vaccines or other medical treatments, the replication cycle of pathogens are investigated and host receptors have been characterized. However, effective vaccines, especially those for the control of protozoan diseases in cattle and humans are, due to epitope variability, far from application (Reeder and Brown, 1996).

The application of preventive methods such as reduction of the insect population size and/or changing the vectorial capacity of the insect population is a more durable approach to control disease development (Wellde *et al.*, 1989; Davies, 1982). So far, reduction of insect population size relies largely on the application of pesticides but those affect beneficial insect species as well and, therefore, are harmful for the environment. Furthermore, resistance against pesticides develops quickly. Releasing predatory insects for controlling insect vector populations, or changing the habitat such that the breeding possibilities of the vector insects are reduced are good alternatives. The latter was successful for the eradication of malaria in The Netherlands (van Seventer, 1969; 1970). Studies on the critical factors influencing the developmental or circulative processes of pathogens in vectors are expected to provide strategies to influence the vectorial capacity of insect vector populations. Surprisingly, very little research has been performed on the characterization of molecular components related to vector-pathogen interaction.

In this thesis the molecular relationships of luteoviruses and aphids have been investigated. Luteoviruses cause major problems on plant crops and knowledge obtained from the molecular interaction of luteoviruses and aphids may provide new strategies to specifically obstruct the vectorial capacity of aphids. The interaction of *Potato leafroll virus* (PLRV) and its aphid vector, *Myzus persicae*, was used as a model system in most of the experiments. PLRV is considered to be a serious virus disease of potato; worldwide crop losses caused by PLRV is estimated at 10% (Kojima and Lapierre, 1988). If infection occurs, the upper leaves roll, turn pale green and are stiffer than normal. Moreover, PLRV particles are transported to potato tubers. To be able to control spread of PLRV, seed potatoes should be virus-free when

planted. Consequently, a low incidence of PLRV in seed crops already leads to exclusion from certification schedules. Immunity to the virus is not present in current potato varieties and as a consequence, measures to restrict the dispersal of virus in the field relies largely on frequent insecticide applications against the aphid vector during the growing season.

Myzus persicae, *Myzus euphorbiae*, and *Myzus nicotianae* are capable of transmitting PLRV (Harrison, 1984; Halbert *et al.*, 1995). The green peach aphid, *M. persicae*, is the most efficient and major vector of PLRV. It occurs worldwide, is polyphagous, and is resistant to many pesticides. In addition, *M. persicae* is an efficient vector of other economically important luteoviruses e.g. *Beet western yellows virus* (BWYV) and many other viruses including potyviruses, cucumoviruses, tymoviruses and alfamoviruses.

Previous studies have lead to the unraveling of the genetic and molecular properties of the luteovirus genome and the characteristics of luteovirus circulation in aphids before transmission to plants. In the following paragraphs the current knowledge of luteoviruses and their transmission by aphids is described from which the scope of the thesis has been deduced.

LUTEOVIRUSES

Luteoviruses were first recognized as a separate plant virus group in 1976 (Fenner, 1976) and later, in 1995, as a genus by the International Committee on Taxonomy of Viruses (Randles and Rathjen, 1995). Luteoviruses were originally divided into two subgroups (Table 1). Recently, the luteovirus classification changed by creating a family to replace the previous genus and by distributing the luteoviruses among the new genera (Table 2) (D'Arcy and Mayo, 1997). Some of the virus species previously belonging to Subgroup II are classified as unassigned within the family *Luteoviridae*. Moreover, the genus *Enamovirus* is now part of the family *Luteoviridae*. The old nomenclature for luteoviruses will be used in chapters 2, 3, 6 and 7 as these were published before 1999, and the recent nomenclature in all other chapters of this thesis.

Table 1: Species in the genus *Luteovirus* (Randles and Rathjen, 1995)

Subgroup I:	
barley yellow dwarf virus – MAV	
barley yellow dwarf virus – PAV	
barley yellow dwarf virus – SGV	
Subgroup II:	
barley yellow dwarf virus – RGV	indonesian soybean dwarf virus
barley yellow dwarf virus – RPV	potato leafroll virus
bean leafroll virus	solanum yellows virus
beet western yellows virus	soybean dwarf virus
carrot red leaf virus	tobacco necrotic dwarf virus
groundnut rosette assistor virus	tomato yellow top virus

Table 2: Species in the family *Luteoviridae* (D'Arcy and Mayo, 1997)

Genus *Luteovirus*:
Barley yellow dwarf virus – MAV
Barley yellow dwarf virus – PAV
Genus *Polerovirus*:
Beet mild yellowing virus
Beet western yellows virus
Cereal yellow dwarf virus – RPV, formerly Barley yellow dwarf virus – RPV
Cucurbit aphid-borne yellows virus
Potato leafroll virus
Genus *Enamovirus*:
Pea enation mosaic virus-1

Luteoviruses are solely transmitted by aphids (Harrison, 1984; Sylvester, 1980). However, transmission through grafting or vegetative propagation of an infected host plant is possible. The relationship of a luteovirus and an aphid is highly specific; a given luteovirus is efficiently transmitted by only one, or a few different aphid species (Rochow, 1969). It is generally accepted that luteoviruses do not replicate in aphids, although some earlier reports contradict this (Ponsen, 1972; Eskandari *et al.*, 1979; Weidemann, 1982). Luteoviruses are highly persistent in which the hemolymph acts as a reservoir for retaining virus particles during the whole lifespan of aphids.

Replication of viruses is mainly restricted to the phloem tissue of plants in which viruses are believed to replicate in companion cells of the sieve tubes. Electron microscope studies on potato plants infected with PLRV have revealed the presence of virus-like particles in mature sieve elements and companion cells, and in plasmodesmata between this cells of the phloem tissue (Shepardson *et al.*, 1980), but also in mesophyll cells of neighboring phloem vessels (van den Heuvel *et al.*, 1995). The spread of PLRV from cell to cell in the mesophyll was not observed.

Luteoviruses consist of small icosahedrical particles of 24 nm to 30 nm. Virus particles contain one single-stranded messenger-sense RNA molecule of approximately 6 kilobasepair (kbp) (Brakke and Rochow, 1974; Hewings and D'Arcy, 1983; Rowhani and Stace-Smith, 1979; Takanami and Kubo, 1979). A small protein (VPg) is covalently attached to the 5'-end of the RNA molecule, which is not polyadenylated at the 3'-end (Mayo *et al.*, 1982; Murphy *et al.*, 1987). The RNA genome is enclosed in 180 copies of M_r 23,000 protein (P23: coat protein) (Rowhani and Stace-Smith, 1979) and that of PLRV contains 7 ORFs (Fig. 1). Except for ORF 0, all putative products of the other ORFs of PLRV show high homologies with those of other members of the subgroup II of the genus *Luteovirus*. It is suggested that the ORF 0 encoding protein plays a crucial role in host determination (van der Wilk *et al.*, 1997).

The 3'end of the RNA genome of subgroup I and II luteoviruses differ and probably originate from different virus genera, whereas the 5' ends, which contain the ORFs encoding the coat proteins, are very similar among all luteoviruses. The capsid proteins contain the determinants required for virus transmission by aphids (Jolly and Mayo, 1994; Brault *et al.*, 1995; Chay *et al.*, 1996; Filichkin *et al.*, 1997).

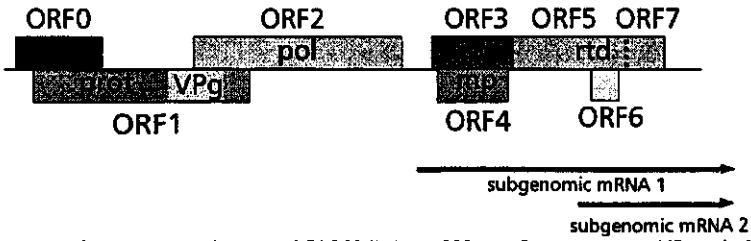

Fig. 1. Genome organization of PLRV RNA (5882 nt). Prot, protease; VPg, viral protein genome-linked; pol, polymerase; cp, coat protein; mp, movement protein; rtd, readthrough domain.

The coat proteins are translated from a subgenomic mRNA (Veidt *et al.*, 1988; Dinesh-Kumar *et al.*, 1992; Reutenauer *et al.*, 1993; Mayo and Ziegler-Graff, 1996) (Fig. 2a). The M_r 23,000 coat protein is produced by translation of ORF 3 due to the presence of a leaky amber stop codon at the end of ORF 3, translation occasionally continues till the next stop at the end of ORF 5 (Bahner *et al.*, 1990; Dinesh-Kumar *et al.*, 1992; Reutenauer *et al.*, 1993). The latter results in a small amount of a M_r 80,000 protein (P80) and consists of the 57kDa-readthrough domain (RTD), joined to the C-terminal end of the 23 kDa CP. However, in purified virus particles the P80 protein is reduced in size and exists as a truncated ~57 kDa form which lacks the C-terminal regions of the RTD (Massalski and Harrison, 1987; Bahner *et al.*, 1990; Brault *et al.*, 1995; Wang *et al.*, 1995; Filichkin *et al.*, 1997). The truncated RTD is exposed on the surface of the virus particle (Fig. 2c) and contains the determinants necessary for virus transmission by aphids (Jolly and Mayo, 1994; Brault *et al.*, 1995; Chay *et al.*, 1996; Filichkin *et al.*, 1997).

An additional ORF is observed at the C-terminus of ORF5, ORF7 (Ashoub *et al.*, 1998). Proteins encoded by ORF6 and ORF7 are translated from a second subgenomic mRNA (Fig 1).

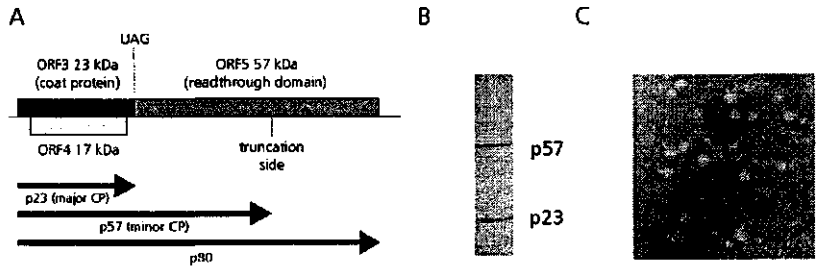

Fig. 2. The major and minor coatproteins of luteovirus particles. (a) Schematic illustration of translation of the 5'end of the luteovirus genome encoding the coat proteins; (b) Western blot showing the capsid proteins of PLRV particles, immunodetection with anti-PLRV antibodies and goat antibody secondary antibodies; (c) Detection of the RTD in virions with immunogold labeling using anti-RTD IgGs conjugated to colloidal gold beads.

TRANSMISSION OF LUTEOVIRUSES BY APHIDS

The sieve elements are the major nutritional source for aphids, and as a consequence, aphids acquire virus particles along with phloem sap while feeding on infected plants. Luteoviruses are transmitted by aphids in a circulative manner whereby luteovirus particles have to cross several barriers; the epithelium cells of the gut, degradive agents in the hemolymph, the epithelium cells of the salivary gland, and basal lamina's of the gut and salivary glands (Fig. 3).

The alimentary canal of the aphid consists of the foregut, the anterior midgut (stomach), posterior midgut (intestine) and the hindgut (Ponsen, 1977). The chitin-lined foregut opens into the large stomach. The stomach is not chitin-lined and consists of a single layer of epithelial cells, which extend into the gut lumen. The elongated posterior midgut is coiled and folded throughout the aphid abdomen. Cells of the posterior midgut are generally characterized by a dense cytoplasm rich in rough endoplasmatic reticulum, mitochondria, Golgi bodies, coated vesicles, and lysosomal vesicles. Furthermore, the apical and basal plasmamembranes are highly invaginated forming many membrane-lined channels, which extend deeply into the cytoplasm of the cell. The hindgut consists of a single epithelial cell layer lacking microvilli or membrane-lined channels.

PLRV acquisition most likely occurs by receptor-mediated endocytosis of midgut cells (Garret *et al.*, 1996), whereas other luteoviruses such as *Barley yellow dwarf virus* (BYDV) and *Soybean dwarf virus* (SDV), were found in the cell cytoplasm of the hindgut (Gildow, 1985; Gildow, 1993; Gildow *et al.*, 1994). All luteoviruses initiate coated-vesicle formation and are contained within larger cytoplasmic vesicle (endosome) (Gildow, 1993; Garret *et al.*, 1993). After maturation of the vesicle into a lysosome, particles are released into the hemocoel. PLRV was observed in coated vesicles 12 h after initiation of membrane feeding of aphids and 4 hours later virions were present in the hemolymph (Garret *et al.*, 1996).

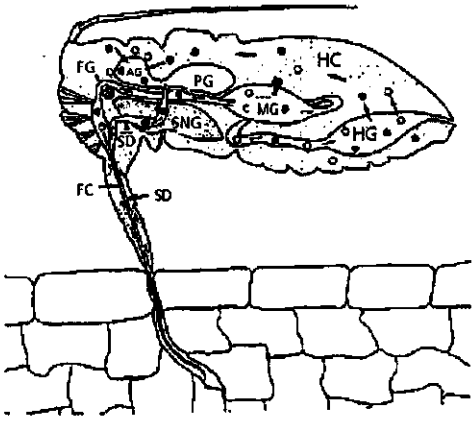

Fig. 3. Schematic presentation of the circulative transmission of luteoviruses by aphids. HC, aphid hemocoel; MG, midgut; HG, hindgut epithelium; AG, accessory salivary gland; PG, principal salaviary gland; SD, salivary duct; FC, food canal; FG, foregut; SNG, suboesophageal nerve ganglion; PSG, principal salivary gland (PSG).

The hemolymph acts as a reservoir for luteoviruses (Eskandari *et al.*, 1979). By injection of luteoviruses into the hemolymph of aphids it was revealed that solely a small decline in virus titer occurred shortly after injection and then a basic level was maintained (Massalski and Harrison, 1987). The persistent nature of non-replicative viruses like luteoviruses is rather unique, since other proteins or agents artificially injected into the haemocoel are quickly

degraded (personal communication, van den Heuvel). Luteovirus particles are thought to circulate in the hemolymph passively until they reach the salivary gland; there is no evidence for an active transport system that guides a luteovirus particle. However, as is described by van den Heuvel *et al.* (1994) and in this thesis (chapter 2 and 5), a protein (GroEL or symbionin) is involved in the persistence nature of the luteovirus particle. Interestingly, GroEL is abundantly produced by the bacterial endosymbionts (*Buchnera* sp.) and is thus not directly synthesized by aphids. All aphid species harbor these bacteria in specialized cells, mycetocytes (chapter 2). High amounts of *Buchnera* GroEL are present in the hemolymph of aphids because of active or passive secretion by *Buchnera* spp. Luteoviruses bind to GroEL in the hemolymph and, consequently, are protected against degradation (chapter 2 and 5).

Luteoviruses, which are present in the hemolymph, associate with the basal lamina of the accessory salivary gland (ASG) and are not attached to the basal lamina of other tissues, like the principal salivary gland (Gildow, 1982; Gildow and Gray, 1993; Gildow *et al.*, 1994; Pfeiffer *et al.*, 1997). The basal lamina covers the hemocoel-facing surfaces of the gland, the basal plasmamembrane, and is composed of collagen and laminin glycoproteins combined with smaller molecules (Fessler and Fessler, 1989; Timpl, 1989; Yurchenco and Schittny, 1990; Pedersen, 1991). Virus particles penetrate through the basal lamina and come into contact with the ASG basal plasmamembrane. The ability for a luteovirus particle to penetrate the basal lamina is a prerequisite for transmission and this event is highly specific for each aphid-luteovirus combination (Gildow and Gray, 1993; Gildow *et al.*, 1994; Pfeiffer *et al.*, 1997).

Once the luteovirus particles have penetrated the basal lamina, a second recognition site is encountered at the basal plasmamembrane of the ASG. The ASG is composed of 4 secretory cells with high concentrations of mitochondria, secretory vesicles, lysosomes, and multivesicular bodies. The basal plasmamembrane consists of membrane-lined channels invaginating into the cytoplasm and provide the cell an increased surface area exposed to the hemolymph (Ponsen, 1977). Virus particles associate with the membrane invaginations and are likely to be transported by receptor-mediated endocytosis (Gildow, 1982; Gildow, 1987; Garret *et al.*, 1991; Gildow and Gray, 1993). The association of a given luteovirus particle with the basal plasmamembrane of an aphid is also highly specific for each aphid-luteovirus interaction. Virus particles are observed in coated pits and accumulate in tubular vesicles followed by transportation to the other side of the cell, the apical plasmamembrane. This occurs by formation of coated vesicles containing individual virus particles. The apical plasmamembrane is directly connected to the lumen of the salivary duct. By fusion of the coated vesicles with the apical plasmamembrane virions are released into the canal lumen of the salivary duct containing the saliva. Evidently, virions are introduced into plants with the salivary secretions.

SCOPE OF THE THESIS

Reduction of the vectorial capacity of aphids may be achieved by interfering with the specific interaction between luteoviruses and putative receptors of the gut and salivary gland, and/or by decreasing the persistent nature of luteoviruses in the hemolymph. To develop strategies and to find agents that compete with or inhibit luteovirus binding, these receptors or hemolymph-derived components should be identified and characterized. Experiments were designed to identify and isolate aphid-derived proteins that influence luteovirus transmission (Chapter 2). A 60 kDa protein (p63) that specifically binds to luteoviruses was identified by a virus overlay assay of protein blots containing aphid whole-body homogenates. Isolation and characterization of this protein demonstrated that p63 is a GroEL homologue, which is

abundantly produced by the primary bacterial endosymbionts (*Buchnera* sp.). *In vivo* studies revealed that virions possibly interact with *Buchnera* GroEL in the hemolymph of aphids for protection against degradation.

To further improve knowledge of the association between PLRV and *Buchnera* GroEL, the *groE* operon of the primary endosymbiont of *M. persicae* was characterized (Chapter 3). Moreover, the PLRV-binding domain of *Buchnera* GroEL was identified by mutant analysis. Site-directed mutagenesis of single amino acids in the subunit of GroEL revealed that specific regions of the GroEL protein are involved in binding virus particles (Chapter 4). The experiments in chapter 5 were performed for the determining whether the major or minor luteovirus capsid proteins are involved in binding to *Buchnera* GroEL and whether deletion of the *Buchnera* GroEL-binding domain coincides with increased particle degradation in the aphid's hemolymph. By single amino acid replacements of conserved residues in one of the virus capsid proteins attempts were made to define the exact binding position of GroEL (Chapter 6).

In chapter 7 the effects of neem seed kernel extracts and azadirachtin on the primary endosymbiotic bacteria of *M. persicae* has been studied and it is investigated whether treatment inhibits the transmission of PLRV as well. As discussed in chapter 8, the knowledge derived of the binding sites and the interaction of *Buchnera* GroEL and luteovirus particles may contribute to the control of luteovirus transmission by aphids in the future.

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Chapter 2

Molecular bases of the interactions between luteoviruses and aphids

Saskia A Hogenhout, Martin Verbeek, Fabienne Hans, Petra M Houterman, Mohammed Fortass, Frank van der Wilk, Harm Huttinga, Johannes FJM van den Heuvel

DLO Research Institute for Plant Protection (IPO-DLO), PO Box 9060, NL-6700 GW Wageningen, the Netherlands

Summary- Many viruses infecting vertebrates or plants are transmitted by arthropod vectors in a circulative manner. This requires the virus to cross epithelial cells of the gut and salivary gland, and to resist the potentially hostile environment of the vector. Fundamental knowledge regarding the strategies adopted by these persistent viruses to overcome the transmission barriers mentioned is surprisingly meager. Here we describe the involvement of endosymbiotic bacteria in the transmission of potato leafroll virus by its aphid vector, *Myzus persicae*. Symbionin, the major protein synthesized and released into the haemolymph by the bacterial endosymbiont, was found to determine the persistent nature of the luteovirus in its vector. The virus displays a strong affinity to this protein (M_r = 63,000) which has high homology with the *Escherichia coli* heat shock protein GroEL. The absence of symbionin in the haemolymph of aphids treated with antibiotics leads to rapid degradation of the major capsid protein of the virus and concomitant loss of infectivity.

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INTRODUCTION

Potato leafroll virus (PLRV) is a single-stranded RNA virus that belongs to the genus *Luteovirus* (Randles and Rathjen, 1995). Species of this genus infect a wide range of mono- and dicotyledonous plants in which they replicate almost exclusively in the phloem tissue (van den Heuvel *et al.*, 1995). Luteoviruses are transmitted by aphids in a circulative manner (Harrison, 1958; Sylvester, 1980). Briefly, this implies that virus particles are ingested along with phloem sap from infected host plants and transcellularly transported through the hindgut into the haemocoel. The acquired virus particles are retained in an infective form in the haemolymph for the aphid's lifespan, apparently without replication (Eskandari *et al.*, 1979). Upon contacting the accessory salivary glands, they may be transported through this gland, eventually arriving in the salivary duct from which they are released with the saliva during feeding of the aphid (Gildow and Gray, 1993). Luteoviruses display a high degree of vector specificity among aphid species. These well-developed specificities suggest an intimate association between a luteovirus and its vectors in which both surface domains of the viral capsid and sites or substances in the aphid are involved (Gildow, 1987). The role of the viral capsid proteins in conferring aphid transmissibility to a luteovirus has been convincingly demonstrated (Rochow, 1970, 1982; Brault *et al*, 1995). Studies on the identification on aphid-derived components interacting with luteoviruses have recently been initiated (van den Heuvel *et al.*, 1994). Here we report on the development of ligand assays to ascertain whether particles of PLRV bind proteinaceous components from its major aphid vector, *Myzus persicae* (Sulz.). Moreover, we elaborate on the role of symbionin, an aphid endosymbiont-derived protein for which the virus shows a high affinity.

MATERIAL AND METHODS

Viruses, aphids and antibodies

PLRV-Wageningen (van der Wilk *et al.*, 1989) was maintained on *Physalis floridana* (van den Heuvel and Peters, 1990) and purified (van den Heuvel *et al.*, 1990). Other plant viruses used were kindly provided by colleagues at IPO-DLO, and CNRS-IBMP (Strasbourg). *M. persicae* biotype WMp2 was reared on *Brassica napus* L. subspecies *oleifera* (oilseed rape) in a greenhouse compartment at 20 ± 3 °C, with a 16 h photoperiod. Cohorts of nymphs differing in age by 24 h were produced by daily transfer of mature apterae to fresh host plants. Clones of field-collected *Aphis fabae*, *A. craccivora* (from India), *Acyrtosiphon pisum*, *Macrosiphum euphorbiae*, *Rhopalosiphum padi*, *Metopolophium dirhodum* and *Sitobion avenae* were maintained at IPO-DLO. *Bemisia tabaci* and *Frankliniella occidentalis* derived from Plant Protection Service (Wageningen) and the WAU Department of Virology, respectively.

Anti-idiotypic antibodies (AiAbs) were raised to PLRV-specific monoclonal antibodies (Mabs) (van den Heuvel *et al.*, 1990).

Protein blots

Isoelectric focusing (IEF) was carried out essentially as described before (O'Farrell, 1975) using 2% (v/v) pH 3.5-10, 1809 Ampholine (Pharmacia) in both tube-gel monomer solution and IEF-sample buffer. One-day-old *M. persicae* nymphs, homogenized in IEF-sample buffer (35 mg wet weight/ml) were spun (10,000 *x g*, 15 min, 4 °C) and 50-µl samples of the

supernatant were loaded onto tube gels. In the second dimension, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose.

Virus overlay assay and immunodetection

Aphid proteins were tested for specific affinity for PLRV by incubating blots with purified PLRV particles (10 µg/ml) or PLRV-specific AiAbs (1 µg/ml) in PBS-Tween containing 2% polyvinylpyrrolidone (w/v) and 0.2% ovalbumin (w/v). This was followed by adding alkaline phosphatase-conjugated anti-PLRV antibodies or goat anti-rabbit secondary antibodies, respectively. Immobilized conjugates were visualized by the addition of 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride in 0.1 M ethanolamine-HCl, pH 9.6, containing 4 mM $MgCl_2$.

Immunogold labelling

One-day-old *M. persicae* nymphs were fixed overnight in 0.1 M cacodylate buffer, pH 7.2, containing 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde, dehydrated, and embedded in LR Gold (The London resin Co Ltd). Ultra-thin sections mounted on nickel grids were labeled with 2.5 µg AiAbs per mL of PBS for 3 h at RT followed by a 1.5 h exposure to goat antirabbit antibodies linked to gold particles (10 nm diameter). Sections were stained with 2% uranyl acetate and lead citrate and examined using Philips CM12 electron microscope.

RESULTS AND DISCUSSION

Binding of PLRV to aphid-derived components

To test whether PLRV showed affinity to protein components from its aphid vector, *M. persicae*, we separated whole-body extracts of the aphid (Fig. 1A) by two-dimensional SDS-polyacrylamide gel electrophoresis, transferred the proteins to nitrocellulose and probed them with purified virus. In this way five proteins were resolved that displayed a virus-binding capacity (Fig. 1 B). The protein with the highest affinity for the virus was identified and its role in virus transmission investigated. This protein, called P63 had a M_r of 63,000 and an isoelectric point between 5.8 and 6.0. P63 was also selected because it reacted with AiAbs (AiAb #5 [Fig. 1C] and AiAb #6 [data not shown]) raised to the Mabs WAU-A5 and -A6 which have been shown to recognize topologically-related surface epitopes of the viral capsid of PLRV (van den Heuvel *et al.*, 1990).

PLRV-binding proteins were not found in extracts of whiteflies (*B. tabaci*) or thrips (*F. occidentalis*) which transmit species of the genera 'subgroup III geminivirus' and *Tospovirus*, respectively, in a circulative manner. P63 did not exhibit affinity toward several other vector-borne plant viruses, tobacco mosaic virus, on PLRV-specific anti-idiotypic antibodies other than #5 and #6 P63 (van den Heuvel *et al.*, 1994).

Symbionin readily binds to purified PLRV and other luteoviruses, beet western yellows, bean leafroll, and barley yellow dwarf virus (Fig. 2) in an enzyme-linked immunosorbent-based assay. In this assay, native symbionin was trapped by a polyclonal antibody to symbionin on the solid phase. Other vector-borne plant viruses previously tested (van den Heuvel *et al.*, 1994), as well as carnation ringspot virus and tobacco mosaic virus did not bind.

The PLRV-binding capacity was not restricted solely to symbionin from *M. persicae*. Symbionin-like proteins from a number of aphid species of the *Aphidinae* also showed binding to PLRV in immunoblots (Fig. 3). Although most species of this subfamily have not been reported to transmit PLRV, our finding is consistent with the observation that non-vector aphids also can acquire and retain luteoviruses in their haemolymph (Rochow and Pang, 1961; Massalski and Harrison, 1987). Vector specificity seems to be determined at the level of the accessory salivary gland (Gildow and Gray, 1993).

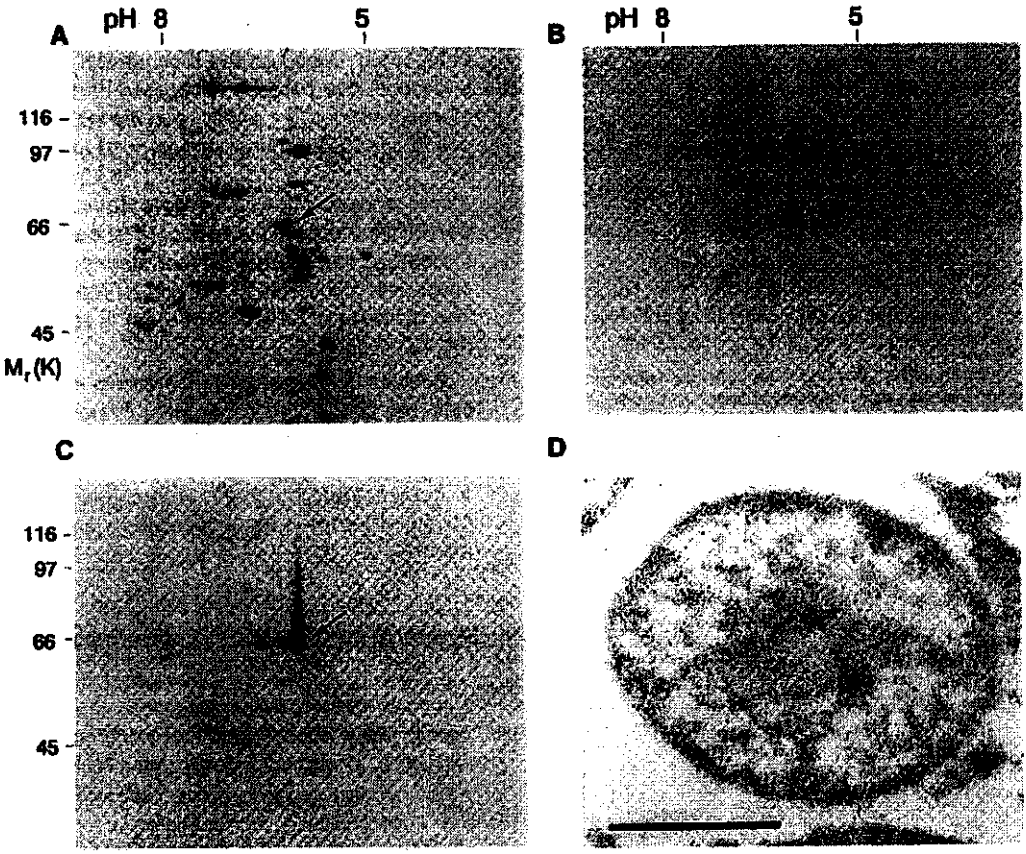

Fig. 1. Identification of PLRV-binding proteins in homogenates of *Myzus persicae* and the localization of symbionin (P63) in the primary endosymbiont of the aphid. **(A)** Two-dimensional (2D) profile of whole-body proteins of *M. persicae* stained with Coomassie brilliant blue R250. **(B)** Immunoblots of 2D separated whole-body proteins of *M. persicae* decorated with purified PLRV, and **(C)** anti-idiotypic antibody #5 (AiAb #5), respectively. The position of symbionin is marked by an arrow. **(D)** Electron micrograph showing immunogold labelling of the primary endosymbionts in the mycetocyte of *M. persicae* using AiAb #5. Bar represents 1 μm.

Identification and localization of symbionin

Immunogold-labelling experiments on ultra-thin sections of *M. persicae* using AiAb #5 showed that the antibody specifically tagged the cytoplasm of the primary endosymbiont of

the aphid (Fig. 1D). These Gram-negative bacteria belong to the genus *Buchnera* which diverged from *Escherichia coli* about 420 million years ago (Unterman *et al*, 1989), and are found in the haemocoel of the aphid. The endosymbionts are harbored in specialized host-derived polyploid cells, called mycetocytes (Buchner, 1965). *In vivo* radiolabelling studies of endosymbiont proteins in the pea aphid, *A. pisum*, showed that synthesis is almost exclusively directed to one protein with a M_r of 63,000, which is tentatively named symbionin (Ishikawa, 1982). Symbionin-like molecules are found in all major aphid groups except the Phylloxeridae, are immunologically closely related, and are highly homologous to the *E. coli* heat shock protein GroEL, a member of the chaperonin-60 family of molecular chaperons (Ohtaka *et al.*, 1992). Based on the biochemical characterization of the protein, the abundance in the endosymbiont of the aphid, and the 100% homology of the 35 N-terminal amino acids to symbionin of *A. pisum* we concluded that P63 from *M. persicae* is symbionin (van den Heuvel *et al.*, 1994). Symbionins are, like GroEL, 14-subunit homo-oligomers composed of two stacked rings of seven subunits each (Hara and Ishikawa, 1990). The diameter of native symbionin is about 7 nm (Fig. 4).

Besides the presence of symbionin in the cytoplasm of the symbiotic bacteria, it is also readily detected in the haemolymph of the aphid. It probably is released by exclusion of degenerating endosymbionts by the mycetocytes (Ponsen, 1972) although active secretion can not be excluded.

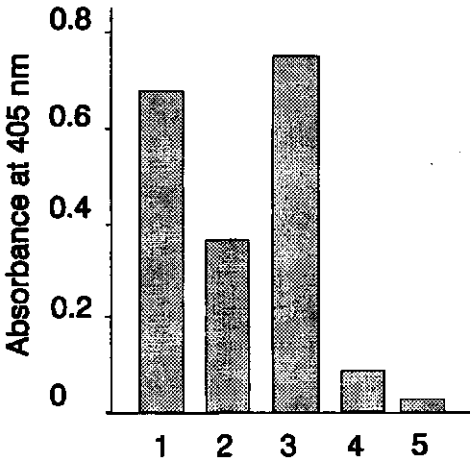

Fig. 2. Enzyme-linked immunosorbent-based *in vitro* binding assay of luteoviruses to native symbionin of *M. persicae*. 1, potato leafroll virus; 2, beet western yellows virus; 3, bean leafroll virus; 4, barley yellow dwarf virus; 5, carnation ringspot virus (negative control). The absorbency at 405 nm (A_{405} [ELISA value]) are given (Y-axis).

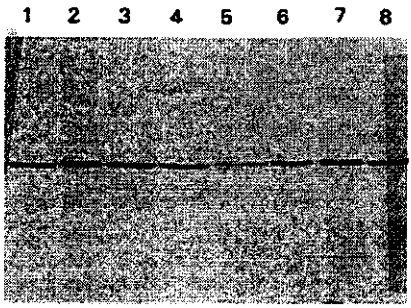

Fig. 3. Virus overlay assay. Binding of purified PLRV to symbionin-like molecules of *Myzus persicae* (1), *Macrosiphum euphorbiae* (2), *Aphis fabae* (3), *A. craccivora* (4), *Rhopalosiphum padi* (5), *Sitobion avanae* (6), *Metopolophium dirhodum* (7), and *Acyrthosiphon pisum* (8).

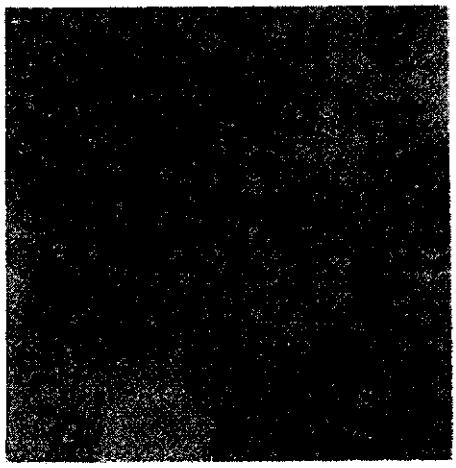

Fig. 4. Electron micrograph showing purified symbionin.

Role of symbionin in luteovirus transmission

It is likely that PLRV particles suspended in the haemolymph interact with symbionin, since purified PLRV readily binds to native symbionin in vitro. In order to investigate the role of P63 in PLRV transmission we subjected one-day-old *M. persicae* nymphs to a tetracycline treatment (50 µg/ml of artificial diet) 24 h prior to virus acquisition. Inhibition of prokaryotic protein synthesis by the antibiotic selectively eliminated this protein from the haemolymph (Fig. 5A) without markedly affecting aphid feeding behaviour. Virus transmission by the antibiotic-treated aphids was reduced by more than 70% (Fig. 5B). The major coat protein species of PLRV of M_r 23,000 was prone to degradation in the antibiotic-treated aphids (Fig. 1C), which would result in an increased exposure of viral RNA to enzymatic breakdown and concomitant loss of infectivity. Strikingly, the other virion-associated structural protein (M_r 56,000) was still present in amounts similar to the control group of aphids not treated with the antibiotic.

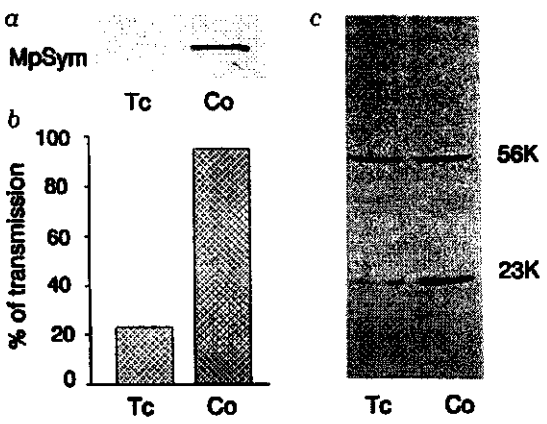

Fig. 5. The effect of tetracyclin treatment of *Myzus persicae* on the presence of symbionin (P63) in the haemolymph of the aphid (**A**), the transmission of PLRV (**B**), and capsid integrity of acquired virus (**C**). Tc: Tetracyclin treated; Co: control (no antibiotic added).

Transovarial transmission

Symbiotic bacteria have only been implicated in the transmission of rice dwarf virus (RDV; genus Phytoreovirus) by leafhoppers (Nasu, 1965). RDV is transmitted in a circulative, replicative manner by leafhoppers. Electron microscopy (EM) studies revealed that the virus binds to the surface of the L-symbiont of the leafhopper. Since these bacterial symbionts are transovarially transmitted, RDV was also carried to the next generation

Symbiotic bacteria of aphids are transmitted transovarially to their offspring as well. EM observations of ultra-thin sections of *M. persicae* neonates showed a number of embryos in different stages of development. In some of these embryos, endosymbiotic bacteria were clearly visible in the blastocoel; the majority was still uninfected (not shown). The bacteria enter the embryo in the blastula stage (Fig. 6). A few cells in the blastoderm develop into conical structures forming the blastopore, a canal that facilitates the influx of endosymbiotic bacteria from maternal mycetocyte contiguous with the follicular epithelium (Fig. 6). Transovarial transmission of luteoviruses has never been observed. A direct interaction between the endosymbiotic bacteria of aphids and a luteovirus, as seen for RDV and leafhoppers, is therefore unlikely to occur.

Fig. 6. Transovarial transmission of endosymbiotic bacteria in one-day-old nymphs *of Myzus persicae*. con: conical cell; e: embryo; m: muscle; myc: mycetocyte; s: symbiont.

Concluding remarks and prospects

The presence of the endosymbiotic protein symbionin in the haemolymph of aphids is of eminent importance to the persistent nature of PLRV. It is envisaged that virus particles in the haemolymph associate (transiently) with this protein which retards proteolytic breakdown of the virus in the aphid. Although the coexistence of symbiotic organisms in arthropods is well documented and widespread, it is surprising that there is very little information regarding their ability to influence vector competence (Hardy et al., 1983). What is more, such knowledge may open up novel ways of preventing circulative transmission of a wide range of arthropod-borne viruses and parasites – not only by disturbing the interactions between endosymbiotic bacteria or proteins and disease agents, but also through the expression of recombinant proteins which may interfere with particular phases of the infection cycle of viruses and parasites in their vectors. In closing, we believe that the application of binding assays similar to the ones recently described (Schmidt *et al*, 1994; van den Heuvel *et al.*, 1994) will contribute significantly to understanding the molecular basis of virus transmission by invertebrate vectors.

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Chapter 3

Potato leafroll virus binds to the equatorial domain of the aphid endosymbiotic GroEL homolog

Saskia A Hogenhout[1], Frank van der Wilk[1], Martin Verbeek[1], Rob W Goldbach[2] and Johannes FJM van den Heuvel[1]

[1]Department of Virology, DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, and [2]Section of Virology, Department of Plant Breeding and Crop Protection, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, the Netherlands

Summary - A GroEL homolog with a molecular mass of 60 kDa, produced by the primary endosymbiotic bacterium (a *Buchnera* sp.) of *Myzus persicae* and released into the hemolymph, has previously been shown to be a key protein in the transmission of potato leafroll virus (PLRV). Like other luteoviruses and pea enation mosaic virus, PLRV readily binds to extracellular *Buchnera* GroEL, and *in vivo* interference in this interaction coincides with reduced capsid integrity and loss of infectivity. To gain more knowledge of the nature of the association between PLRV and *Buchnera* GroEL, the *groE* operon of the primary endosymbiont of *M. persicae* (MpB *groE*) and its flanking sequences were characterized and the PLRV-binding domain on *Buchnera* GroEL was identified by deletion mutant analysis. MpB GroEL has extensive sequence similarity (92%) with *Escherichia coli* GroEL and other members of the chaperonin-60 family. The genomic organization of the *Buchnera groE* operon is similar to that of the *groE* operon of *E. coli* except that a constitutive promoter sequence could not be identified; only the heat shock promoter was present. By a virus overlay assay of protein blots, it was shown that purified PLRV bound as efficiently to recombinant MpB GroEL (expressed in *E. coli*) as it did to wild-type MpB GroEL. Mutational analysis of the gene encoding MpB GroEL revealed that the PLRV-binding site was located in the so-called equatorial domain and not in the apical domain which is generally involved in polypeptide binding and folding. *Buchnera* GroEL mutants lacking the entire equatorial domain or parts of it lost their ability to bind PLRV. The equatorial domain is made up of two regions at the N and C termini that are not contiguous in the amino acid sequence, but which are in spatial proximity after folding of the GroEL polypeptide. Both the N- and C-terminal regions of the equatorial domain were implicated in virus binding.

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INTRODUCTION

Potato leafroll virus (PLRV; genus *Luteovirus*), a positive-stranded RNA virus, mainly replicates in the phloem tissue of a plants and is transmitted by aphids in a persistent and circulative manner (Sylvester, 1980; Gupta, 1995; Randles and Rathjen, 1995). When they feed on the phloem sap, aphids ingest virus particles, which are subsequently transported from the digestive tube into the hemolymph (Garret *et al.*, 1993) and from there across the basal lamina that surrounds the accessory salivary cells into the salivary gland (Gildow and Gray, 1993). Virus particles that reach the salivary gland are eventually released in the phloem sap of the plant as the aphid feeds (Gildow and Gray, 1993). The hemolymph acts as a reservoir in which PLRV is retained in an infective form during the aphid's lifespan without replication (Eskandari *et al.*, 1979).

It has previously been demonstrated that the primary endosymbiotic bacterium (a *Buchnera* sp.) of *Myzus persicae*, the principal vector of PLRV, plays a crucial role in determining the persistent nature of PLRV in the aphid hemolymph (van den Heuvel *et al.*, 1994). *Buchnera* spp. abundantly produce a protein which is highly homologous to the *Escherichia coli* chaperonin, GroEL (Morioka and Ishikawa, 1992; Van den Heuvel *et al.*, 1994; Baumann *et al.*, 1996; Filichkin *et al.*, 1997). GroEL of the *Buchnera* sp. of *M. persicae* (MpB GroEL) was found to be released in the hemolymph, most likely as a result of the lysis of endosymbiotic bacteria (van den Heuvel *et al.*, 1994). After antibiotic treatment of the aphid, MpB GroEL could no longer be detected in the hemolymph and PLRV transmission was greatly reduced due to degradation of virus capsid proteins (Van den Heuvel *et al.*, 1994). Since *in vitro* studies have previously shown that PLRV exhibits specific affinity for MpB GroEL, it was suggested that virus particles associate with MpB GroEL in the hemolymph of the aphid to retard proteolytic breakdown of virus particles (van den Heuvel *et al.*, 1994).

Buchnera spp. are common to all major aphid groups but the Phylloxeridae (Buchner, 1965). These intracellular bacteria are gram-negative and closely related to members of the *Enterobacteriaceae* family (Unterman *et al.*, 1989; Munson *et al.*, 1991). *Buchnera* spp. are harbored in specialized cells, mycetocytes, localized in the abdomen of the aphid (Van den Heuvel *et al.*, 1994) and are maternally inherited (Buchner, 1965). Comparisons of rRNA sequences of *Buchnera* spp. and morphological features of aphid hosts provide strong evidence that a single aphid ancestor was infected by the bacterium about 250 million years ago (Munson *et al.*, 1991).

GroEL of *E. coli* is a heat shock protein (Hsp60) with 60-kDa subunits; it is involved in intracellular folding and assembly of nonnative proteins in an ATP-dependent manner (Ellis and Van der Vies, 1991). Hsp60s are common to prokaryotes, mitochondria, and chloroplasts (Ellis and Van der Vies, 1991; Gupta, 1995). Crystallography of *E. coli* GroEL demonstrated that the protein forms a homo-oligomer of 14 subunits, which are arranged in two heptameric rings stacked back to back, and that each subunit consists of the following three domains: the equatorial domain, the apical domain, and the small intermediate domain (Braig *et al.*, 1994). In general, the apical domain of GroEL has previously been implicated in polypeptide binding (Fenton *et al.*, 1994), a process which may require ATP hydrolysis. The ATPase activity of GroEL is regulated by GroES (Viitanen *et al.*, 1990; Martin *et al.*, 1991), a single heptameric ring of 10-kDa subunits also encoded by the *groE* operon (Tilly *et al.*, 1981; Chandrasekhar *et al.*, 1986). The structural and functional characteristics of *Buchnera* GroELs are highly similar to those of *E. coli* GroEL (Hara and Ishikawa, 1990; Ohtaka *et al.*, 1992; Filichkin *et al.*, 1997). However, unlike *E. coli* GroEL, *Buchnera* GroEL is not restricted to the cytosol of the bacterium; it also occurs extracellularly in the hemolymph of an aphid (Filichkin *et al.*, 1997; van den Heuvel *et al.*, 1997).

In this study, the nucleotide sequence of the gene encoding MpB GroEL was determined and structural and functional domains were identified by sequence comparison to other GroELs. In addition, the regions upstream and downstream of this gene were sequenced and compared with the corresponding regions of *E. coli*. To gain a better understanding of the molecular basis of the association between PLRV and MpB GroEL, the protein was expressed in *E. coli* and mutational analysis was carried out to identify the domain of MpB GroEL implicated in PLRV binding.

MATERIALS AND METHODS

Isolation of genomic DNA from the *Buchnera* sp. of *M. persicae*

Approximately 1 g of *M. persicae* aphids was collected and surface sterilized with 70% ethanol containing 0.5% Tween 20 and 0.5% hypochlorite. Sterilized aphids were rinsed with water and homogenized in 3 ml of isolation medium (Bruening *et al.*, 1971). Subsequently, the homogenate was filtered through cheesecloth and centrifuged at 5,000 x *g* for 15 min. Bacterial genomic DNA was either isolated directly from the resulting pellet (lysis buffer method) or further purification steps were undertaken to enrich for bacterial cells (Ficoll procedure). In the lysis buffer method, the pellet was incubated for 1 h at 56 °C in 0.7 ml of lysis buffer (150 mM Tris-HCl [pH 8.0], containing 150 mM EDTA, 3% sodium dodecyl sulfate [SDS], and 1.5 to 2% sodium lauroyl sarcosine). After 5 min of incubation on ice, 0.5 ml of Tris-EDTA buffer was added, the suspension was gently mixed, and the debris was allowed to precipitate. Genomic DNA was extracted with phenol-chloroform from the supernatant. In the Ficoll method, the pellet was resuspended in 2 ml of 100-fold-diluted isolation medium and layered on a 2 to 10% Ficoll gradient in 0.01 M phosphate buffer (pH 7.2). After centrifugation at 400 x *g* for 10 min, the fraction containing bacterial cells was collected. To this fraction, five volumes of saline-EDTA (0.15 M sodium chloride, 0.1 M EDTA [pH 8.0]) was added and the mixture was centrifuged at 1,000 x *g* for 12 min. The pellet was resuspended in 1 ml of saline-EDTA containing 8% SDS and incubated at 60°C for 10 min, and DNA was extracted as mentioned above.

PCR amplification procedure

PCR amplification was performed in a final volume of 100 µl of 10 mM Tris-HCl (pH 8.3), containing 0.4 mM (total) deoxynucleoside triphosphates, 3 mM $MgCl_2$, 50 mM KCl, 1 µg of DNA, 0.25 µM (each) primers, and 2.5 U of *Taq* polymerase (Boehringer Mannheim). Mixtures were incubated for 2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final incubation of 10 min at 72°C. Samples were stored at 4°C until used. PCR products were analyzed on agarose gels.

Sequencing strategy

Clones containing the MpB *groEL* sequence were generated by PCR with primers F1 and R1 (Table 1). The primer sequences were based on the N-terminal amino acid sequence of MpB GroEL (van den Heuvel *et al.*, 1994) and the 3'-terminal nucleotide sequence of the *Buchnera groEL* gene of *Acyrthosiphon pisum* (Ohtaka *et al.*, 1992). The resulting 1,732 bp PCR product was cloned by using a TA cloning kit (Invitrogen), yielding plasmid pCR[*Buchnera* GroEL].

Overlapping restriction fragments from cDNA clones were subcloned into pBluescript KS (Stratagene), and their nucleotide sequences were determined at the sequence facilities of the Department of Molecular Biology, Wageningen Agricultural University, with a sequencing kit and AmpliTaq DNA polymerase (Applied Biosystems), universal and sequence-specific primers, and an automated sequencer (model 373; Applied Biosystems).

To determine the sequence of the entire MpB *groE* operon, a genomic DNA library was constructed by using a λ ZAP II cloning kit and Gigapack III Gold packaging extract (Stratagene) according to the manufacturer's instructions. Genomic DNA from the *Buchnera* sp. of *M. persicae* was isolated by the lysis buffer method and digested with *Xba*I. Fragments were ligated into *Xba*I-digested λ ZAP II vector arms. Two radiolabeled probes of 569 and 521 bp, corresponding to the 5' and 3' ends of the open reading frame (ORF) coding for MpB GroEL, respectively, were used to screen for recombinant clones. After the excision of positive plaques, the nucleotide sequences of phagemids pSK2500 and pSK3500 (see Fig. 2) were determined.

Southern blot analysis

Genomic DNA from the *Buchnera* sp. of *M. persicae* was isolated by the Ficoll method (see above), and 5 µg of DNA was digested with either *Pst*I, *Xba*I, or *Xho*I. Samples were run on a 1% agarose gel and transferred to HybondN (Amersham). The 1,732 bp PCR product containing the MpB *groEL* gene mentioned above was radiolabeled and used as a probe for hybridization.

GroEL isolation from the *Buchnera* sp. of *M. persicae* and from *E. coli*

GroEL was isolated from the endosymbiotic bacteria of 6-day-old *M. persicae* nymphs and from heat-shocked *E. coli* cells as described before (van den Heuvel *et al.*, 1997).

Cloning and expression of *Buchnera* GroEL deletion mutants

Full-length MpB GroEL and deletion mutants of MpB GroEL in fusion with glutathione *S*-transferase (GST) were expressed in *E. coli* with plasmid pGEX-2T (Pharmacia). GST fusion proteins were affinity purified with glutathione-Sepharose (Pharmacia) according to the manufacturer's recommendations. To remove the GST moiety, fusion proteins were incubated with thrombin for 3 h at 10^0C. Cleaved products were analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) gels and by Western blot analysis with anti-MpB GroEL immunoglobulin G (IgG). To ensure that similar quantities of deletion mutants were tested for their virus-binding capacities (described below), they were diluted to yield bands of similar intensities as assessed by amido black staining after electroblotting. Each mutant was named after the positions of the first and last amino acids bordering the included fragment.

Full-length MpB GroEL was obtained by digesting pCR[*Buchnera* GroEL] with *Bam*HI and cloning the *Bam*HI fragment containing the MpB *groEL* gene into the *Bam*HI sites of pGEX-2T, resulting in pGEX[*Buchnera* GroEL]. Constructs for the expression of MpB GroEL(1-121) and MpB GroEL(1-314) were derived by digesting plasmid pGEX[*Buchnera* GroEL] with *Sma*I (located downstream of the *Bam*HI site in the multiple cloning site of pGEX-2T), and *Cla*I or *Xba*I. Protruding 5' ends were filled in with the Klenow fragment of DNA polymerase I and by religation of constructs. pGEX-2T constructs for the expression of all other truncated mutants of GroEL were generated by PCR. The primers used were complementary or identical to the

border sequences of the three domains recognized in MpB GroEL and included additional restriction sites (*Bam*HI, *Eco*RI or *Hin*dIII sites) for cloning purposes (Table 1). Plasmid pCR[*Buchnera* GroEL] served as the template. All PCR products were first cloned into the pCRII vector (TA cloning kit; Invitrogen), digested with *Bam*HI or *Bam*HI/*Eco*RI, and subsequently religated into the *Bam*HI or *Bam*HI/*Eco*RI sites of pGEX-2T. For the expression of MpB GroEL[122-408/475-548], a pGEX-2T construct was synthesized with primer pair F2 and R3 and primer pair F6 and R1 (Table 1). The amplified fragments of 850 (F2 and R3) and 225 (F6 and R1) bp were cloned into pCRII and digested with *Bam*HI/*Hin*dIII or *Hin*dIII/*Eco*RI, respectively. The *Hin*dIII-cleaved ends of both fragments were ligated, and the ligated product was cloned into the *Bam*HI/*Eco*RI sites of pGEX-2T. All constructs were verified by nucleotide sequence analysis.

TABLE 1. Oligonucleotides used for the construction of MpB GroEL deletion mutants

Oligo-nucleotide	Oligonucleotide sequence (5'-3')[a]	Corresponding positions[b]
F1	cc**ggatcc**ATGGCCGCTAAAGATGTA	1-6
F2	**ggatcc**atgAAAGCTGTTATTAGTGCG	122-127
F3	**ccatgg**atcCGTTAAAGGTATGCAG	189-194
F4	**ggatcc**atgGTTGCAGTACTTAAAGTAG	376-385
F5	**ggatcc**atgGAAGGTGTAGTTGCTGG	409-413
F6	ggtg**aagctt**AACTATGGTTATAATGCAGC	475-480
R1	ac**ggatcc**TTACATCATTCCaCCC	545-548
R2	**gaattc**ttaACCTTTTCCATCTTTTACG	470-474
R3	caata**agctt**TTCAACAGCTGCACCAGT	404-408
R4	**gaattc**ttaTTCAACAGCTGCACGAG	404-408
R5	**gaattc**tagatcaCCTCCTGATAATTTAGC	370-374
R6	**ggatcc**ttaAACGACTTCTAGTTCATTTTG	184-190

[a] Uppercase letters indicate *Buchnera groEL* sequences, and lowercase letters indicate sequences which are not part of the gene. Restriction sites (*Eco*RI, *Bam*HI, and *Hin*dIII) are in boldface. Start codons are double underlined, and termination codons are single underlined.

[b] Numbering refers to the corresponding positions of the amino acids residues of MpB GroEL.

The pGEX constructs mentioned above were introduced into *E. coli* JM101, DH5α, or protease-deficient BL21 (Stratagene). For expression, overnight cultures were diluted 1:10 in Luria broth containing ampicillin (100 μg/ml) and incubated at 37°C for 3 h. Subsequently, 1 mM isopropyl-ß-D-thiogalactosidase was added to induce protein synthesis of the pGEX plasmid and cultures were allowed to grow at room temperature. After 7 h, cells were pelleted at 4,000 x *g* for 10 min and resuspended in 50 mM Tris-HCl (pH 7.5), containing 10 mM $MgCl_2$. Cells were lysed by one cycle of freeze-thaw and sonication. Insoluble debris was removed by centrifugation, and the supernatant containing the soluble protein was collected.

Virus overlay assay

PLRV (van der Wilk *et al.*, 1989) was maintained on *Physalis floridana* as previously described and purified from leaf material by a modified enzyme-assisted procedure (van den Heuvel *et al.*, 1991). The virus overlay was performed essentially as described before (van den Heuvel *et al.*, 1994). Similar amounts of various MpB GroEL polypeptides were run on denaturing polyacrylamide gels for SDS-PAGE. After electrophoresis, gels were conditioned in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0), containing 10% methanol for 1 h and proteins were electrotransferred onto nitrocellulose. Protein blots were incubated overnight with purified PLRV (10 μg per ml), after which immunodetection with anti-PLRV IgG and alkaline phosphatase-conjugated anti-rabbit IgG was carried out (Van den Heuvel *et al.*, 1994).

Nucleotide sequence accession number

The sequence data of the *groEL* operon and its flanking regions have been submitted to the GenBank database under accession no. AF003957.

RESULTS

Characterization of the *groE* operon of the *Buchnera* sp. of *M. persicae*

The nucleotide sequence of the *groE* operon of the primary endosymbiont (a *Buchnera* sp.) of *M. persicae* (MpB *groE*) and its flanking sequences were determined both by PCR and with clones derived from a genomic library. Southern blot analysis revealed that one copy of the MpB *groEL* gene was present on the genome (Fig. 1). The genomic organization of the MpB *groE* operon (Fig. 2) is similar to that of the *groE* and *sym* operons of *E. coli* and the intracellular symbiont of *A. pisum*, respectively (Hemmingsen *et al.*, 1988; Ohtaka *et al.*, 1992). The operon accomodates two ORFs encoding 10- and 60-kDa proteins which have 72 and 73% homologies at the nucleotide level with *E. coli groES* and *groEL*, respectively. The MpB *groE* genes are also highly homologous to *symS* (89%) and *symL* (91%) from *A. pisum*. However, sequence comparisons of the promoter regions of the *groE* operons of various *Buchnera* spp. with that of *E. coli* revealed the only conserved element to be the heat shock promoter. A constitutive promoter similar to the one in the *E. coli groE* operon (and reported to be present on the *A. pisum groE* operon) could not be identified. A terminator sequence comparable to the one in the *E. coli groE* operon was not present either. Most likely, the GC-rich inverted repeat at the end of a *Buchnera groE* operon performs this function.

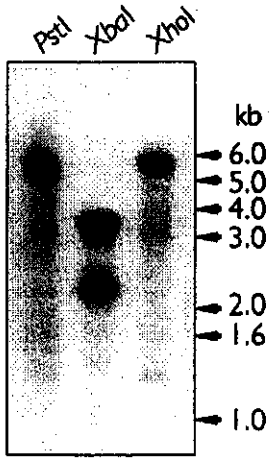

Fig. 1. Southern blot analysis of the *Buchnera* sp. of *M. persicae* to determine the copy number of the *groEL* gene. DNA from the *Buchnera* sp. of *M. persicae* was digested with *Xba*I, *Pst*I or *Xho*I. *Xba*I recognizes a single restriction site within the MpB *groEL* gene, whereas *Pst*I and *Xho*I restriction sites are present only outside this gene. A radiolabeled PCR fragment comprising the gene encoding MpB GroEL was used as the probe.

To determine the degree of conservation of the genomic region flanking the MpB *groE* operon, the regions upstream and downstream were compared with those of *E. coli* (Fig. 2), the only well-characterized free-living relative of *Buchnera* spp. (Munson *et al.*, 1991). Upstream of the MpB *groE* operon three ORFs which show similarities to genes on the *E. coli* genome were identified. The ORF immediately adjacent to the *groE* operon shows homology to the $tRNA^{phe}$ gene (Burland *et al.*, 1993). The other ORFs display 69% similarity to the *E. coli* 50 kDa thiophene and furan oxidation protein (ThdF) and 83% similarity to the C-terminal part of the gene that encodes the 60 kDa inner membrane protein of *E. coli* (Alam and Clark, 1991; Burland *et al.*, 1993). These genes are present at similar sites on the genome of *Buchnera aphidicola*, the primary endosymbiont of *Schizaphis graminum* (Baumann *et al.*, 1995), although the $tRNA^{phe}$ gene has not been previously reported. Interestingly, on the *E. coli* genome, the *groE* operon is separated by approximately 500 kbp from the genes encoding ThdF and the inner membrane protein (Burland *et al.*, 1993; Burland *et al.*, 1995). Downstream of the MpB *groE* operon, two genes which display 70% similarity with a 37.8-kDa protein of *E. coli* of unknown function, and 78% similarity with the N-terminal sequence of elongation factor P of *E. coli* were identified (Aoki *et al.*, 1991; Burland *et al.*, 1995). In *E. coli*, an additional segment of approximately 2 kbp harboring two ORFs with unknown functions is located between the terminator sequence of the *groE* operon and the gene encoding the 37.8 kDa protein (Burland *et al.*, 1995).

Analysis of the *groEL* gene of the *Buchnera* sp. of *M. persicae*

To ascertain whether MpB GroEL has structural and functional similarities to *E. coli* GroEL, the deduced amino acid sequence of the MpB *groEL* product was compared with those of *E. coli* and other *Buchnera* spp. GroELs (Fig. 3). This disclosed that MpB GroEL is 98 to 99% similar to the GroELs of *Buchnera* spp. of the aphids *Sitobion avenae*, *Rhopalosiphum padi*, and *A. pisum* and 92% similar to *E. coli* GroEL. A comparison of the MpB GroEL sequence with conserved residues in 50 prokaryotic Hsp60/GroEL homologs (Fenton *et al.*, 1994) showed that all of these residues except for the alanine at position 294 (Ala294) are identical. In all of the *Buchnera* spp. analyzed, Ala294 is replaced by serine (Fig. 3). Since the *Buchnera* GroEL of *A. pisum* has previously been demonstrated to fully complement *E. coli* GroEL in *groE* mutants of *E. coli* (Ohtaka *et al.*, 1992), this substitution seems to be of minor importance to GroEL's functioning

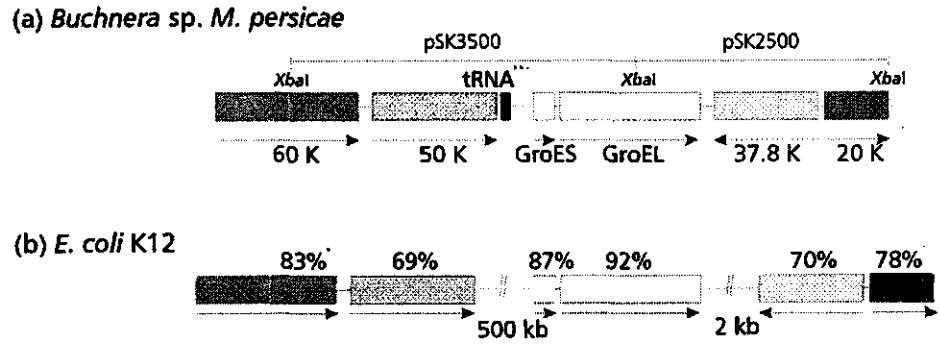

Fig. 2. Schematic representation of the MpB *groE* operon and comparison of the chromosomal arrangements of the regions flanking the *groE* operons of the *Buchnera* sp. of *M. persicae* (a) and *E. coli* (b) (Bukau, 1993; Burland *et al.*, 1995). Identical shading indicates high amino acid sequence similarity of gene products. The percentages noted above a box indicates the similarity of the MpB ORF product to the *E. coli* homologe. #, percentage of similarity of the C-terminal 295 amino acids of the 60-kDa (60K) gene; *, percentage of similarity of the N-terminal 186 amino acids of the 20-kDa (20K) protein. The arrow below each gene indicates the direction of translation.

as a molecular chaperon *in vivo*. Moreover, *in vitro* experiments showed that the replacement of Ala294 by glutamic acid in *E. coli* GroEL did not affect polypeptide binding, folding, or its ATPase activity (Fenton *et al.*, 1994). The highly conserved amino acid residues of *E. coli* and *Buchnera* spp. GroELs are shown in Fig. 3. They are evenly distributed over the three domains of GroEL and are involved in polypeptide binding and folding (mainly located in the apical domain), ATP binding and hydrolysis (equatorial domain), maintaining inter- and intrasubunit interactions, and movement of the GroEL domains relative to each other (Horovitz *et al.*, 1993; Burnett *et al.*, 1994; Fenton *et al.*, 1994). Amino acid residues in less-conserved regions which are known to mediate polypeptide binding (Leu238 and Val264) (Fenton *et al.*, 1994) or which have previously been reported to be essential for ATP binding in *E. coli* GroEL (Ala482 and Asp497) (Boisvert *et al.*, 1996) are also identical in MpB GroEL.

Binding of PLRV to *Buchnera* GroEL deletion mutants

To determine which of the three domains of the MpB GroEL molecule are implicated in the interaction with PLRV *in vitro*, MpB GroEL was expressed in fusion with GST and affinity purified. After the GST moiety was removed by thrombin, the recombinant protein was tested for its PLRV-binding capacity by a virus overlay assay of protein blots which had previously been used to show that PLRV displayed a high and specific affinity for the 60-kDa subunit of MpB GroEL (van den Heuvel *et al.*, 1994). The *in vitro* binding assay clearly established that full-length recombinant MpB GroEL bound PLRV as readily as wild-type MpB GroEL did (data not shown).

By utilizing the sequence similarity between *Buchnera* and *E. coli* GroELs in areas that are relevant for intrasubunit interactions, the first set of deletion mutants, based on the primary structure of the different domains on the GroEL molecule, was generated (Fig. 4a). The crystal structure of GroEL shows that the individual subunits are folded into three distinct domains (Braig *et al.*, 1994) of which only the apical domain is continuous on the primary structure. The equatorial and intermediate domains are discontinuous, with regions located in both N- and C-terminal halves of the molecule (Fig. 4a). Testing similar amounts of MpB GroEL deletion

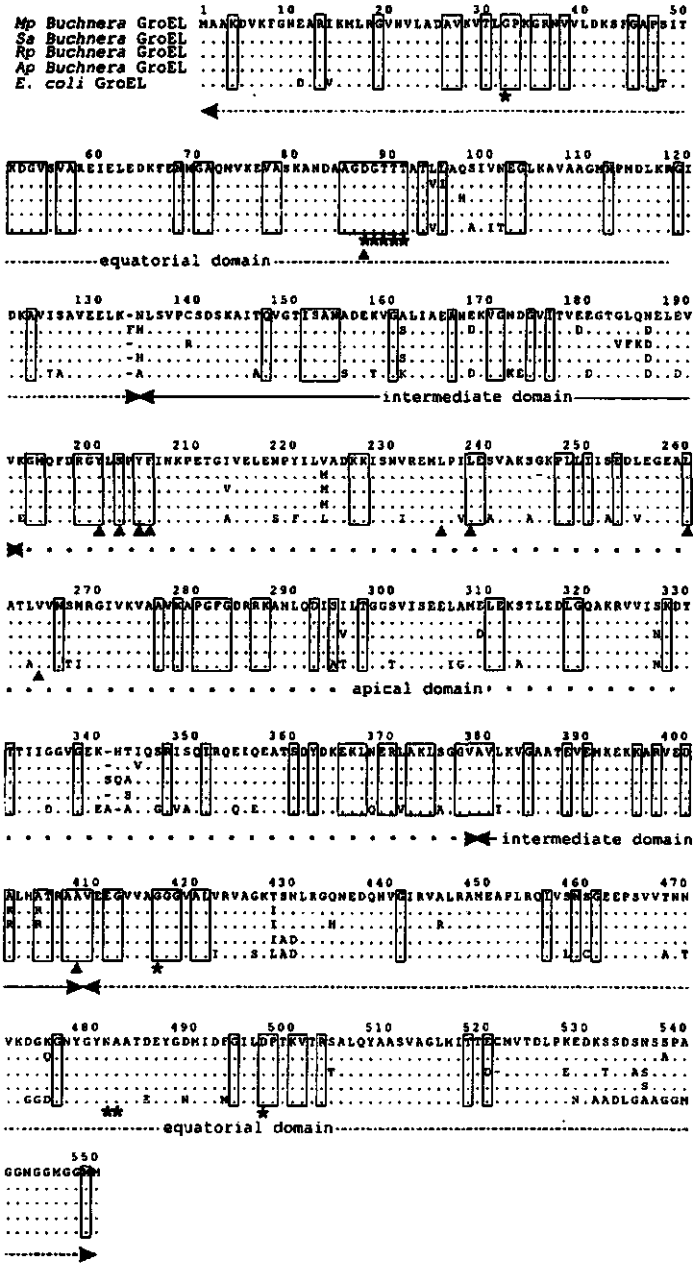

Fig. 3. Amino acid sequence alignment of the GroELs of *E. coli* (Hemmingsen *et al.*, 1988) and *Buchnera* spp. from *M. persicae* (*Mp*), *S. avenae* (*Sa*) (Filichkin *et al.*, 1997), *R. padi* (*Rp*) (Filichkin *et al.*, 1997), and *A. pisum* (*Ap*) (Ohtaka *et al.*, 1992). Conserved regions in other chaperonin/Hsp60 chaperonins (Braig *et al.*, 1994) are boxed. Amino acids that are involved in polypeptide binding are indicated by arrow heads, and those involved in ATP binding are indicated by asterisks. Identical residues and gaps are indicated by periods and dashes, respectively. The equatorial domain is indicated by a dashed line, the intermediate domain is indicated by a continuous line, and the apical domain is indicated by dots. The sequence alignment was carried out using the program PILEUP (Genetics Computer Group, Madison, Wis.) (Devereux *et al.*, 1984).

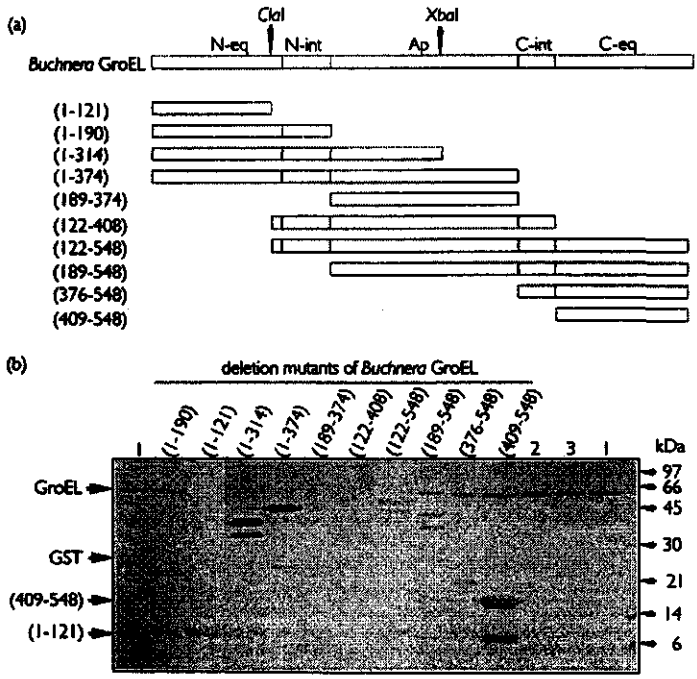

Fig. 4. PLRV binding to deletion derivatives of MpB GroEL. (a) Schematic representations of MpB GroEL deletion mutants. The numbers in parenthesis correspond to the positions of amino acid residues of MpB GroEL (Fig. 3) and mark the borders of the deletion mutants. The *Cla*I and *Xba*I restriction sites are indicated by arrowheads. N-eq, N-terminal region of the equatorial domain; N-int, N-terminal region of the intermediate domain; Ap, apical domain; C-int, C-terminal region of the intermediate domain; C-eq, C-terminal region of the equatorial domain. (b) Virus overlay assays. Lanes: 1, wild-type MpB GroEL; 2, GroEL of *E. coli* treated with thrombin; 3, GroEL of *E. coli*. All other lanes contain the indicated deletion mutant of MpB GroEL, as depicted in panel a. The positions of GroEL (60 kDa), GST (28 kDa), and the smallest truncated MpB GroEL fragments that bind PLRV [(409-548) and (1-121)], are indicated by arrowheads.

mutants in virus overlay assays revealed that purified PLRV displayed affinities for all mutants containing the N- or C-terminal region of the equatorial domain (Fig. 4). Extending the N-terminal equatorial domain [MpB GroEL(1-121)], but not the C-terminal equatorial domain, with sequences of the intermediate and apical domains [MpB GroEL(1-314) and MpB GroEL(1-374)] improved the efficiency of virus binding (Fig. 4). Strikingly, PLRV binding to polypeptides containing the apical domain alone [MpB GroEL(189-374)], or the entire region between the *Cla*I site (amino acid residue 122) and the C terminus of the intermediate domain (amino acid residue 408) did not occur (Fig. 4). The smallest deletion mutants that showed binding to PLRV harbored the N-terminal 121 amino acid residues [MpB GroEL(1-121)], or the C-terminal 139 residues [MpB GroEL(409-548)] (Fig. 4). The presence of at least one of these regions is required for the virus-binding capacities of the MpB GroEL deletion mutants. The virus overlay assay also showed that PLRV interacted with *E. coli* GroEL. *E. coli* GroEL was copurified with mutants expressed in the protease-deficient strain *E. coli* BL21. As *E. coli* GroEL was insensitive to the thrombin treatment (Fig. 4b; compare lanes 2 and 3), and its presence did not interfere with the migrations of MpB GroEL mutant polypeptides during SDS-PAGE, no steps were undertaken to remove endogenous GroEL from suspensions.

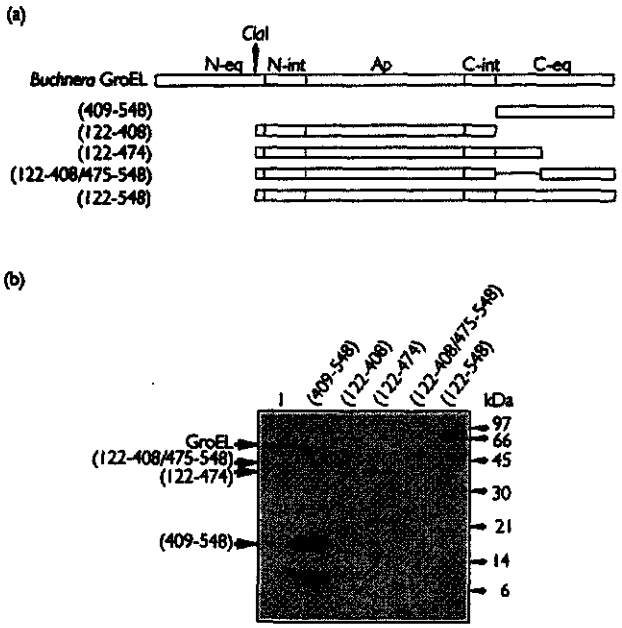

Fig. 5. Localization of the PLRV-binding site in the C-terminal part of the equatorial domain of MpB GroEL. (a) Schematic representations of the C-terminal deletion mutants of MpB GroEL. The numbering and abbreviations used are explained in the legend to Fig. 4. (b) Virus overlay assay of MpB GroEL deletion mutants. Lane 1, wild-type MpB GroEL; all other lanes contain the indicated MpB GroEL mutants as depicted in panel a. The positions of GroEL (60 kDa), *Buchnera* GroEL(122-408/475-548), MpB GroEL(122-474), and MpB GroEL(409-548) are indicated by arrowheads.

It is noteworthy that all MpB GroEL constructs harboring the C-terminal region of the equatorial domain produced smaller fragments for which PLRV showed affinities [MpB GroEL(122-548), MpB GroEL(189-548), MpB GroEL(376-548) and MpB GroEL(409-548)] (Fig. 4). These truncated products were approximately 8.5 kDa smaller than the corresponding mutants. Protein microsequencing by automated Edman degradation of the truncated products MpB GroEL(376-548) and MpB GroEL(409-548) revealed that the N-terminal residues are identical to those expected in the corresponding full-length polypeptides. This implies that the 8.5-kDa fragment was cleaved from the C terminus and that this fragment is dispensable for PLRV binding. Based on the relative molecular masses of the truncated products, the approximate position of the truncation mapped between amino acid residues 471 and 476 of MpB GroEL. The region N terminal of the truncation site, which is involved in PLRV binding, is characterized by the presence of three α-helices (Braig *et al.*, 1994). To investigate more firmly the role of these structural elements in PLRV binding, the following two additional mutants were constructed: MpB GroEL(122-474) which contained the α-helices (between residues 408 and 475), and MpB GroEL(122-408/475-548), from which these elements were deleted (Fig. 5a). Purified PLRV clearly demonstrated an *in vitro* binding affinity for MpB GroEL(122-474) but not for MpB GroEL(122-408/475-548). Thus, the determinant for PLRV binding is located between amino acids 408 and 475 (Fig. 5b) of the C-terminal region of the equatorial domain.

DISCUSSION

In the present study, the *groE* operon of the *Buchnera* sp. from the major aphid vector of PLRV, *M. persicae,* was characterized and the PLRV-binding domain of MpB GroEL was identified by deletion mutant analysis. PLRV-binding studies revealed that virus particles exhibited *in vitro* affinities for all deletion mutants of MpB GroEL containing parts of the N-terminal (amino acid residue 1 to 121) (Fig. 3) or C-terminal (amino acids 409 to 474) (Fig. 3) regions of the equatorial domain (Fig. 4 through 6). Computer-generated structural predictions of the monomer of MpB GroEL (Peitsch, 1996) showed that these two regions assemble in the tertiary structure. It is therefore suggested that the residues involved in PLRV binding from either region join to compose a single PLRV-binding site. These results are remarkable, as previous single amino acid replacement studies of *E. coli* GroEL have demonstrated that residues in the apical domain are generally involved in polypeptide binding and folding (Braig *et al.*, 1994; Fenton *et al.*, 1994). Thus far, the equatorial domain has been implicated only in the *in vitro* binding of two multimeric proteins, ribulose-1,5-biphosphate carboxylase-oxygenase and malate dehydrogenase (Weiss and Goloubinoff, 1995). Apparently, protein-binding sites are not necessarily located in the apical region of the central cavity of the GroEL cylinder but may be located in the equatorial domain as well. Large multimeric proteins and luteoviruses may employ these sites to overcome the size limitations (50 to 80 Å wide; [Chen *et al.*, 1994]) imposed by the central cavity of the GroEL molecule. The equatorial domain also accommodates the ATP-binding site on its external envelope (Braig *et al.*, 1994; Fenton *et al.*, 1994; Roseman *et al.*, 1996). Like the putative PLRV-binding site, this site is composed of amino acid residues from both C- and N-terminal regions of the discontinuous equatorial domain (Fig. 3) (Boisvert *et al.* 1996). All of the MpB GroEL mutants in this study contain deletions known to impair intersubunit interactions (Horovitz *et al.*, 1993; Burnett *et al.*, 1994; Fenton *et al.*, 1994) and are unable to assemble into the multimeric form of GroEL which prevails in the aphids' hemolymph (Van den Heuvel *et al.*, 1997). Single amino acid replacements in the PLRV-binding regions which do not affect GroEL assembly are required to verify the role of the equatorial domain of the native molecule in the interaction with PLRV.

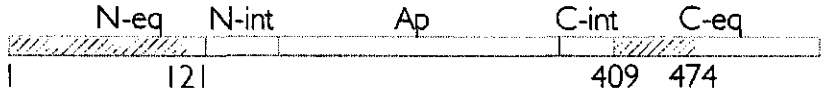

Fig. 6. Summary of the PLRV-binding regions in MpB GroEL. The PLRV-binding regions in the N- (amino acids 1 to 121) and the C- (amino acid 409 to 475) terminal parts of the equatorial domain (N-eq and C-eq, respectively) are shaded. N-int, N-terminal region of the intermediate domain; Ap, apical domain; C-int, C-terminal region of the intermediate domain.

Based on the differential binding of subgroup I and II luteoviruses and pea enation mosaic virus to *Buchnera* GroELs from vector and nonvector species and to GroEL of *E. coli* (Filichkin *et al.*, 1997; van den Heuvel *et al.*, 1997), it was concluded that the basic virus-binding capacity resides in a conserved part of the GroEL molecule (van den Heuvel *et al.*, 1997). Indeed, the regions in the equatorial domain of MpB GroEL which mediate PLRV binding are highly conserved among *Buchnera* GroEL homologs. However, regions of variability in these or other parts of the GroEL molecule may potentially influence the efficiency of binding thus explaining the observed differences in the affinity of luteoviruses for GroEL homologs (Filichkin *et al.*, 1994; van den Heuvel *et al.*, 1997).

While GroEL is abundantly produced by *Buchnera* spp. in aphids (Hara and Ishikawa, 1990;

Baumann *et al.*, 1996), *Buchnera* GroES is difficult to detect (Kakeda and Ishikawa, 1991; Baumann *et al.*, 1996). Undoubtedly, *Buchnera* GroES is an important cofactor for cellular protein folding (Ohtaka *et al.*, 1992), but its potential role in extracellular protein interactions in the hemolymph of an aphid is yet to be investigated. Bacterial symbionts and pathogens, like *Rhizobium meliloti*, *Pseudomonas aeruginosa*, the X-bacteria of *Amoeba proteus*, and *Agrobacterium tumefaciens*, have adopted different strategies at the transcriptional and translational levels to overproduce GroEL homologs relative to GroES production (Choi *et al.*, 1991; Rusanganwa and Gupta, 1993; Ahn *et al.*, 1994; Segal and Ron, 1995; Farinha *et al.*, 1996). *Psuedomonas aeruginosa* and *Rhizobium meliloti* employ three *groEL* genes, of which there are one and two copies, respectively, on operons that also encode GroES (Rusanganwa and Gupta, 1993). In the symbiotic bacteria of *Amoeba proteus*, GroEL is overproduced by an additional promoter in the GroES encoding part in front of the *groEL* gene (Ahn *et al.*, 1994), and in *Agrobacterium tumefaciens* two mRNA fragments are produced and the mRNA fragment containing the gene encoding GroES is rapidly degraded (Segal and Ron, 1995). We detected only one copy of the MpB GroEL encoding gene (Fig. 1), located on the same operon that harbors the *groES* gene (Fig. 2). This suggests that overproduction of MpB GroEL occurs through the mechanisms found in the X-bacteria of *Amoeba proteus* or *Agrobactererium tumefaciens*. Alternatively, it may well be that *Buchnera* GroEL is more stable than is GroES and readily accumulates while GroES is rapidly degraded.

To further identify potential genetic elements that may explain the high level of GroEL accumulation, sequences upstream of the coding regions were compared with the consensus sequences involved in transcription and translation of the *groE* operons of *E. coli* and the *Buchnera* sp. of *A. pisum* (Zhou *et al.*, 1988; Ohtaka *et al.*, 1992; Sato and Ishikawa, 1997). This comparison disclosed the presence of sequences highly homologous to the *E. coli* heat shock promoter and Shine-Dalgarno sequences (Fig. 7). Although the MpB *groE* operon sequence is nearly identical to that of the *Buchnera* sp. of *A. pisum* in this region, we were not able to identify the constitutive promoter sequence of the *groE* operon of *E. coli*, which was previously reported to be present on the *groE* operon of the *Buchnera* from *A. pisum* (Ohtaka *et al.*, 1992). Our observation corroborates recent findings that the only conserved promoter sequences of the *groE* operons of the *Buchnera* spp. of *A. pisum* and *Schizaphis graminum* are those recognized by σ^{32}, a factor involved in the heat shock response (Baumann *et al.*, 1996), and that the heat shock promoter alone is responsible for transcription of the *Buchnera* sp. from *A. pisum* (Sato and Ishikawa, 1997). An AT-rich nucleotide sequence upstream of this promoter, which may enhance promoter activity (Bukau, 1993), is present in the *groE* operons of both *E. coli* and *Buchnera* spp. (Fig. 7). These observations are of interest, since GroEL expression in *Buchnera* spp. is similar to that in *E. coli* cells growing under heat stress (Baumann *et al.*, 1996).

In vivo interference with the interactions among extracellular MpB GroEL, PLRV and beet western yellows virus led to the suggestion that a transient association is required to protect luteoviruses in the hemolymph of an aphid from proteolysis (van den Heuvel *et al.*, 1994; van den Heuvel *et al.*, 1997). Clearly, these interactions differ from the usual intracellular polypeptide-GroEL interactions; the mechanisms and potential roles of cofactors including that of GroES have not yet been revealed. It should be noted, however, that a functional extracellular GroEL was also observed in *Helicobacter pylori*, a gram-negative bacterium, which causes chronic gastritis. It produces a GroEL homolog (HspB) which protects against inactivation of urease outside the bacterial cell in the hostile environment of the stomach of a vertebrate host (Evans et al., 1992; Phadnis *et al.*, 1996). Urease and HspB are released, probably by cell autolysis, and adhere to the surfaces of intact bacteria (Phadnis *et al.*, 1996). Moreover, surface-associated Hsp60 fractions were also found in *P. aeruginosa* and *Legionella pneumophila* (Jensen *et al.*, 1993; Lema and Brown, 1995). In this respect, it is interesting that GroEL proteins

of *Buchnera* spp. were found to be more related to Hsp60s of pathogenic bacteria, such as *L. pneumophila*, than to *E. coli* GroEL (Gupta, 1995).

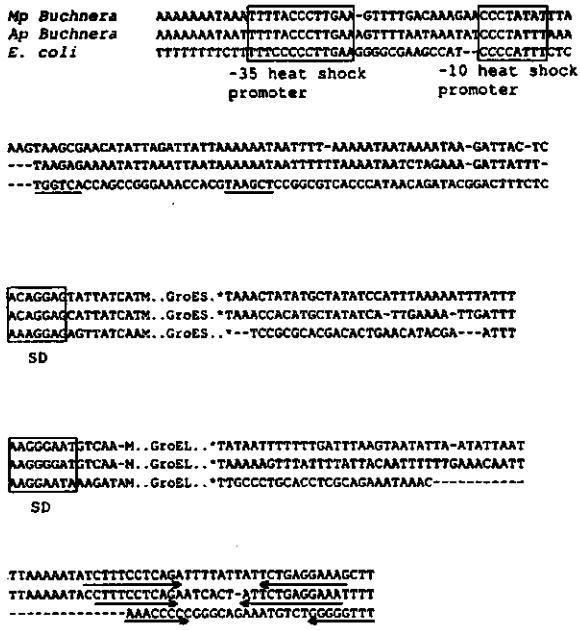

Fig. 7. Sequence comparison of regions involved in transcription and translation of the *groE* operons of *E. coli* (Hemmingsen *et al.*, 1988; Zhou *et al.*, 1988) and the *Buchnera* spp. of *A. pisum* (*Ap*) (Ohtaka *et al.*, 1992) and *M. persicae* (*Mp*). Conserved regions of the putative heat shock promoter and Shine-Dalgarno (SD) sequences are boxed, and the inverted repeats of putative transcription terminator sites are indicated by arrows. The localization of the constitutive promoter within the *groE* operon of *E. coli* is underlined. Gaps are indicated by dashes, stop codons are indicated by asterisks, and start codons are indicated by M.

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Chapter 4

Mapping the amino acids in the equatorial domain of *Buchnera* GroEL involved in *Potato leafroll virus* binding

Saskia A Hogenhout[1], Frank van der Wilk[1], Martin Verbeek[1], Rob W Goldbach[2] and Johannes FJM van den Heuvel[1]

[1]Department of Virology, DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands and [2]Laboratory of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

Summary – GroEL which is abundantly produced by the primary bacterial endosymbiont (*Buchnera* sp.) of aphids, and released in the haemolymph has previously been shown to be implicated in the persistent nature of luteoviruses. While usually hydrophobic residues of the apical domain of GroEL 14-mers are involved in binding of nonnative proteins in the bacterial cytosol, regions containing the determinants required for binding of *Potato leafroll virus* (PLRV) particles are located in the equatorial domain of *Buchnera* GroEL of *Myzus persicae* (MpB GroEL) (Hogenhout *et al.*, 1998). In this study the determinants required for PLRV-binding were characterized. By utilizing the secondary structure elements of the equatorial domain of MpB GroEL, mutants were designed and tested for virus binding in virus overlay assays. This revealed that the amino acids mediating PLRV-binding are located within residues 9 to 19 and 427 to 457 of the N- and C-terminal regions of the equatorial domain, respectively. To assist site-directed mutagenesis, decameric peptides corresponding to the PLRV-binding regions were synthesized. Virus overlay assays of these peptides and alanine replacement studies revealed that residues R13, K15, L17 and R18 of the N-terminus, and R441 and R445 of the C-terminus are required to maintain PLRV affinity of peptides.

Alanine replacement of R441 and R445 simultaneously resulted in loss of PLRV-binding of decameric peptides. In contrary, replacement of these amino acids in full-length and deletion mutants of MpB GroEL reduced but did not abolish PLRV affinity. This indicates that structural components of the α-helix in between residue 427 and 457 residues are important for the composition of the PLRV-binding site of the C-terminal region of the equatorial domain. R441 and R445 may either interact directly with PLRV particles or be important for maintaining the PLRV-binding structure. Site-directed mutagenesis of full-length MpB GroEL and deletion mutants thereof demonstrated that replacement of R13 and K15 mutually eliminate each other and that of L17 and R18. This may indicate that replacement of single residues caused structural rearrangements resulting in loss of PLRV affinity. Therefore, the α-helical structure within residues 9 till 19 is likely to be required for PLRV-binding.

INTRODUCTION

Potato leafroll virus (PLRV; family: *Luteoviridae*), a positive-stranded RNA virus, mainly replicates in the phloem tissue of its host plant and is transmitted by aphids in a persistent and circulative manner (Sylvester, 1980; Van den Heuvel *et al.*, 1995). It has previously been demonstrated that a GroEL homologue synthesized by the primary bacterial endosymbionts of aphids (*Buchnera* spp.) plays a crucial role in determining the persistent nature of luteoviruses in the aphid hemolymph (van den Heuvel *et al.*, 1994 and chapter 2). Recently, it was also revealed that a GroEL homologue of bacterial symbionts of the whitefly *Bemisia tabaci* is involved in the circulative transmission of *Tomato yellow leaf curl virus* (TYLCV; *Geminiviridae*) (Morin *et al.*, 1999). Like *Buchnera* GroEL of aphids, the whitefly GroEL homologue appears to protect TYLCV from destruction during its passage through the hostile environment of the haemolymph of its insect vector.

GroEL proteins are highly conserved and belong to the chaperonin-60 family of proteins, which are involved in intracellular folding and assembly of nonnative proteins in an ATP-dependent manner (Ellis and van der Vies, 1991). Crystallography of *Escherichia coli* GroEL has demonstrated that the protein forms a cylinder-shaped homo-oligomer of 14 subunits arranged in two heptameric rings stacked back to back. *Buchnera* GroEL 14-mers are abundantly present in the haemolymph of aphids (van den Heuvel *et al.*, 1997) and *in vitro* binding studies demonstrated that luteovirus particles bind GroEL 14-mers (Filichkin *et al.*, 1997), suggesting that luteovirus particles interact with GroEL 14-mers in the aphid's haemolymph as well. In addition, the readthrough domain (RTD), which is protruding from the surface of a luteovirus particle, binds GroEL 14-mers, and deletion of the RTD results in rapid degradation of virus particles in the aphid (van den Heuvel *et al.*, 1997).

Hydrophobic residues of the apical domains, which are located on both sides of the GroEL cylinder, are generally involved in binding of nonnative proteins in the bacterial cytosol (Fenton *et al.*, 1994; Braig *et al.*, 1994). However, mutational analysis of the gene encoding *Buchnera* GroEL of *Myzus persicae* (MpB GroEL) revealed that the determinants required for PLRV-binding are located in the equatorial domain (Hogenhout *et al.*, 1998). The equatorial domain forms the waist of the GroEL 14-mer and holds the cylinder together (Braig *et al.*, 1994). The equatorial domain of the GroEL monomer is composed of two regions at the N- and C-terminus that are not contiguous in the amino acid sequence, but which are probably in spatial proximity after folding of the GroEL polypeptide analogous to *E. coli* GroEL. The determinants required for PLRV-binding were located between residues 1 to 122 and 408 to 476 of the N- and C-terminal regions of the equatorial domain, respectively (Hogenhout *et al.*, 1998). In this study the determinants required for PLRV-binding of MpB GroEL were studied in more detail. Individual amino acid residues involved in PLRV binding are characterized using pepscan analyses and single amino acid replacement studies. The amino acids thus identified are conserved among other proteins of the chaperone-60 family.

MATERIAL AND METHODS

PCR amplification procedure

PCR amplification was performed in a final volume of 50 µl of 10 mM Tris-HCl (pH 8.3), containing 0.4 mM deoxynucleoside triphosphates, 3 mM $MgCl_2$, 50 mM KCl, 10 ng of template DNA pCR[*Buchnera* GroEL] (Hogenhout *et al.*, 1998) or 10 ng of pGEX MpB GroEL[1-408], 0.25 µM of each primer, and 2.5 U of *Taq* polymerase (Boehringer Mannheim). Mixtures were incubated for 2 min at 94°C, followed by 35 cycles of 1 min at

94°C, 1 min at 50°C, and 2 min at 72°C, with a final incubation of 10 min at 72°C. Samples were stored at 4°C until used. PCR products were analyzed on agarose gels.

Synthesis and cloning of *Buchnera* GroEL mutants

pGEX-2T constructs for the expression of truncated mutants of GroEL were generated by PCR. Primers used included additional restriction sites (*Bam*HI, *Eco*RI or *Hin*dIII sites) for cloning purposes (Table 1). Plasmid pCR[*Buchnera* GroEL] served as the template (Hogenhout *et al.*, 1998). All PCR products were first cloned into the pCRII vector (TA cloning kit; Invitrogen), digested with *Bam*HI or *Bam*HI/*Eco*RI, and subsequently religated into the *Bam*HI or *Bam*HI/*Eco*RI sites of pGEX-2T. For the expression of MpB GroEL[122-408/475-548], a pGEX-2T construct was synthesized with primer pair F2 and R3 and primer pair F6 and R1 (Table 1). The amplified fragments of 850 (F2 and R3) and 225 (F6 and R1) bp were cloned into pCRII and digested with *Bam*HI/*Hin*dIII or *Hin*dIII/*Eco*RI, respectively. The *Hin*dIII-cleaved ends of both fragments were ligated, and the ligated product was cloned into the *Bam*HI/*Eco*RI sites of pGEX-2T. To express MpB GroEL[1-57/134-408] a construct was synthesized using primer set F10 and R7. Construct pGEX MpB GroEL[1-408] was used as template for PCR. After amplification the PCR fragment was digested with *Hin*dIII and self ligated.

TABLE 1
Oligonucleotides used for the construction of MpB GroEL deletion mutants

Oligo-nucleotide	Sequence (5'-3')[a]	Corresponding positions[b]
F1	ccggatccATGGCCGCTAAAGATGTA	1-6
F2	ggatccatgAAAGCTGTTATTAGTGCTG	122-127
F6	ggtgaagcttAACTATGGTTATAATGCAGC	475-480
F7	ccggatccGGTAATGAAGCCCGAATT	9-14
F8	ccggatccGGAGTTAATGTATTAGCAG	19-24
F9	gaggatccGTAACTTTAGGTCCAAAAG	29-34
F10	ttccaagcttTTATCTGTACCATGTTCAG	134-139
R1	acggatccTTACATCATTCCaCCC	545-548
R2	gaattcttaACCTTTTCCATCTTTTACG	470-474
R3	caataagcttTTCAACAGCTGCACCAGT	404-408
R4	gaattcttaTTCAACAGCTGCACGAG	404-408
R7	caataagcttGATTTCACGAGCTACTGA	53-57
R8	gaattcttaATTGGAAACAATTTGACGTAA	452-457
R9	gaattcttaAGATGTTTTTCCAGCCACTC	422-427

[a] Uppercase letters indicate *Buchnera groEL* sequences, and lowercase letters indicate sequences which are not part of the gene. Restriction sites (*Eco*RI, *Bam*HI, and *Hin*dIII) are in boldface. Start codons are double inderlined, and termination codons are single underlined.

[b] Numbering refers to the corresponding positions of the amino acid residues of MpB GroEL.

Single amino acid mutations were made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Primers were designed and PCR was performed according to the manufacturer's recommendations. Constructs pGEX MpB GroEL[1-408] and pGEX MpB GroEL[122-548] were used as templates for the synthesis of point mutations at the N-terminus and C-terminus, respectively.

To obtain full-length constructs of MpB GroEL containing the point mutations at the N-terminus, the C-terminal *XbaI*/*Eco*RI restriction fragment of pGEX MpBGroEL[122-548] was cloned into the *Xba*I and *Eco*RI sites of construct pGEX MpBGroEL[1-408]R13A, pGEX MpBGroEL[1-408]K15A or pGEX MpB GroEL[1-408]L17AR18A. The *Xba*I restriction site is located in the middle of the region encoding the apical domain of MpB GroEL (Hogenhout et al., 1998). For expression of a full-length construct of MpB GroEL containing the point mutations at positions R13, R441 and R445, the C-terminal *XbaI*/*Eco*RI restriction fragment of pGEX MpBGroEL[122-548]R441AR445A was cloned into the *Xba*I and *Eco*RI sites of construct pGEX MpBGroEL[1-408]R13A. The full-length constructs of MpB GroEL containing the C-terminal point mutations at position R441 and R445, were obtained by ligation of the N-terminal *Bam*HI/*Xba*I fragment of pGEX MpB GroEL[1-408] to the C-terminal *XbaI*/*Eco*RI fragment of pGEX MpBGroEL[122-548]R441AR445A. The ligation product was cloned into the *BamHI*/*EcoRI* sites of pGEX-2T.
All constructs were verified by nucleotide sequence analysis.

Nucleotide sequence analysis

The nucleotide sequence of pGEX constructs was determined at the sequence facilities of the Wageningen Agricultural University, Department Biomolecular Sciences with a sequencing kit and AmpliTaq DNA polymerase (Applied Biosystems), sequence-specific primers, and an automated sequencer (model 373; Applied Biosystems).

Expression and isolation of *Buchnera* GroEL mutants

The pGEX constructs containing GroEL sequences were introduced into *E. coli* DH5α (Stratagene). For expression, overnight cultures were diluted 1:10 in LB-containing ampicillin (100 μg/ml) and incubated at 37^{0}C for 3 h. Subsequently, 1 mM isopropyl-ß-D-thiogalactosidase (IPTG) was added to induce protein synthesis of the pGEX plasmids, and cultures were allowed to grow at room temperature. After 7 h, cells were pelleted at 4,000 x *g* for 10 min and resuspended in 50 mM Tris-HCl (pH 7.5), containing 10 mM $MgCl_2$. Cells were lysed by one cycle of freeze-thaw and sonication. Insoluble debris was removed by centrifugation, and the supernatant containing the soluble protein was collected. Glutathione S-transferase (GST) fusion proteins were affinity purified with glutathione-sepharose (Pharmacia) according to the manufacturer's recommendations. To remove the GST moiety, fusion proteins were incubated with thrombin for 3 h at 10^{0}C. Cleaved products were analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) and by Western blot analysis with anti-MpB GroEL immunoglobulin G (IgG). To ensure that similar quantities of deletion mutants were tested for their virus-binding capacities (described below), they were diluted to yield bands of similar intensities as assessed by amido black staining after electroblotting. Each mutant was named after the positions of the first and last amino acids bordering the included fragment.

Pepscan analysis

Decameric peptides were synthesized on cellulose membranes using Fmoc-amino acid active esters according to the manufacturer's instructions (Genosys biotechnologies). Subsequently, membranes were incubated with blocking buffer (Genosys biotechnologies) for 16 hours at room temperature followed by 10 μg/ml virus per 5 cm^2 in the same buffer for 16 hours at room temperature. Bound virus particles were detected with anti-PLRV IgG (IPO-DLO, Wageningen) followed by goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at a concentration of 1 μg/ml in blocking buffer for three hours. Duplicate membranes, treated in the same manner as above but without the virus, served as negative controls.

Virus overlay assay

PLRV (van der Wilk *et al.*, 1989) was maintained on *Physalis floridana* as previously described and purified from leaf material by a modified enzyme-assisted procedure (van den Heuvel *et al.*, 1991). Virus overlay assays were performed essentially as described before (van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1998). Similar amounts of MpB GroEL polypeptides were separated by SDS-PAGE. After electrophoresis, gels were conditioned in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0), containing 10% methanol for 1 h and proteins were electrotransferred onto nitrocellulose. All experiments were performed in duplicate. One protein blot was incubated overnight with purified PLRV (10 μg per ml), after which immunodetection with anti-PLRV IgG and alkaline phosphatase-conjugated anti-rabbit IgG was carried out (van den Heuvel *et al.*, 1994). The other blot was stained with amido black to confirm whether similar amounts of the proteins were transferred to the membrane.

RESULTS

Identification of PLRV-binding amino acids in the N-terminal part of the equatorial domain of MpB GroEL

For the localization of the N-terminal PLRV-binding residues in the N-terminal part of the equatorial domain of MpB GroEL, a series of deletion mutants was synthesized. All deletion mutants lacked the C-terminal equatorial domain. Deletions in the N-terminal equatorial domain were designed making use of the secondary structure of MpB GroEL (Fig. 1a). The deletion mutants were expressed in *E. coli*, and after removal of the GST moiety similar amounts of MpB GroEL deletion mutants were tested for PLRV binding in a virus overlay assay of protein blots. This assay was previously instrumental in demonstrating that PLRV displayed a high and specific affinity for the 60-kDa subunit of MpB GroEL (van den Heuvel *et al.*, 1994) and mutants thereof (Hogenhout *et al.*, 1998). The virus overlay assay demonstrated that deletion of the first 57 amino acids of the N-terminal equatorial region (MpB GroEL (58-408)) eliminated PLRV binding, whereas PLRV binding was restored when these 57 amino acids were included (MpB GroEL (1-408)) (Fig. 1b). Deletion mutant MpB GroEL(1-57/134-408) lacking the region between the first 57 amino acids and the N-terminal intermediate domain retained its affinity of PLRV. Secondary structural elements of the first 57 amino acids consist of a β-sheet, an α-helix and two β-sheets, adjacently. Additional deletion mutants were designed according to these secondary structural elements (Fig. 1a) and mutants were tested for virus binding in a virus overlay assay (Fig. 1c). Mutant MpB GroEL

(9-408), containing the N-terminal α-helix still bound PLRV, whereas MpB GroEL mutants lacking the first part of the N-terminal α-helix (MpB GroEL(19-408)) or full-length α-helix (MpB GroEL(29-408)) lost their binding capacity (Fig. 1c). This suggests that the PLRV-binding residues are located in between residues 9 and 19.

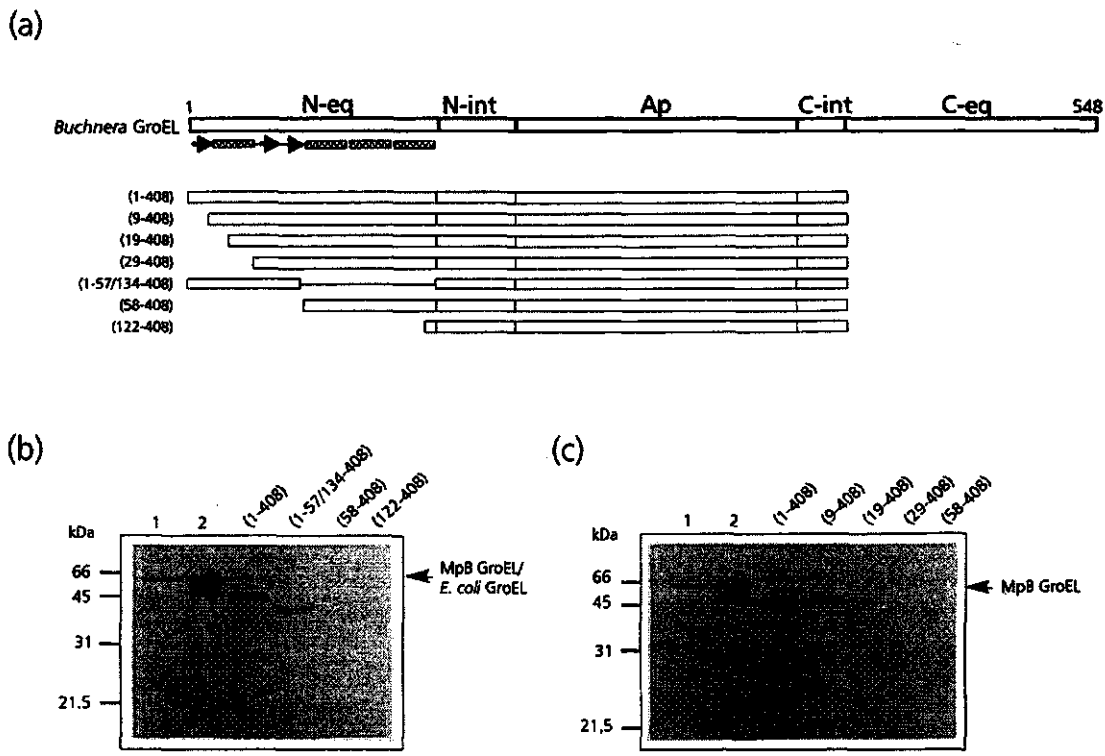

Fig. 1. Mapping of the N-terminal PLRV-binding site by virus overlay assays of deletion derivatives of MpB GroEL. (a) Schematic representation of MpB GroEL deletion mutants. The numbers in parentheses correspond to the positions of amino acid residues of MpB GroEL (accession number: AF003957; Hogenhout *et al.*, 1998), and mark the borders of the deletion mutants. N-eq, N-terminal region of the equatorial domain; N-int, N-terminal region of the intermediate domain; Ap, apical domain; C-int, C-terminal region of the intermediate domain. Secondary structural elements (defined by homology to GroEL of *E. coli*), are indicated by sine wave in rectangles (α-helices) or by a arrows (β-strands). (b) Virus overlay assay result showing that the first 57 amino acid residues are involved in PLRV binding. (c) Virus overlay results showing that residues 10 to 18 are involved in virus binding. Lanes: 1, wild type MpB GroEL isolated from *M. persicae*; 2, recombinant MpB GroEL. All other lanes contain the indicated deletion mutants of MpB GroEL as depicted in panel 'a'. The positions of MpB GroEL and *E. coli* GroEL are indicated by an arrow head.

To assist site-directed mutagenesis of the region of the N-terminal region involved in PLRV-binding, a pepscan analysis was performed. Decameric peptides prepared to the first 57 amino acids of MpB GroEL were tested for PLRV binding in a virus overlay based experiment (Fig. 2a). PLRV particles bound solely to peptides corresponding to the region in between residues 5 and 24. Peptide KFGNEARIKM exhibited the highest affinity for PLRV and peptide RIKMLRGVNV had a higher affinity to PLRV than the peptides containing flanking residues. Therefore, these PLRV overlay assay results using decameric peptides

support the deletion mutant analysis (Fig. 1) and indicate that residues corresponding to the onset of the first N-terminal α-helix are involved in PLRV-binding.

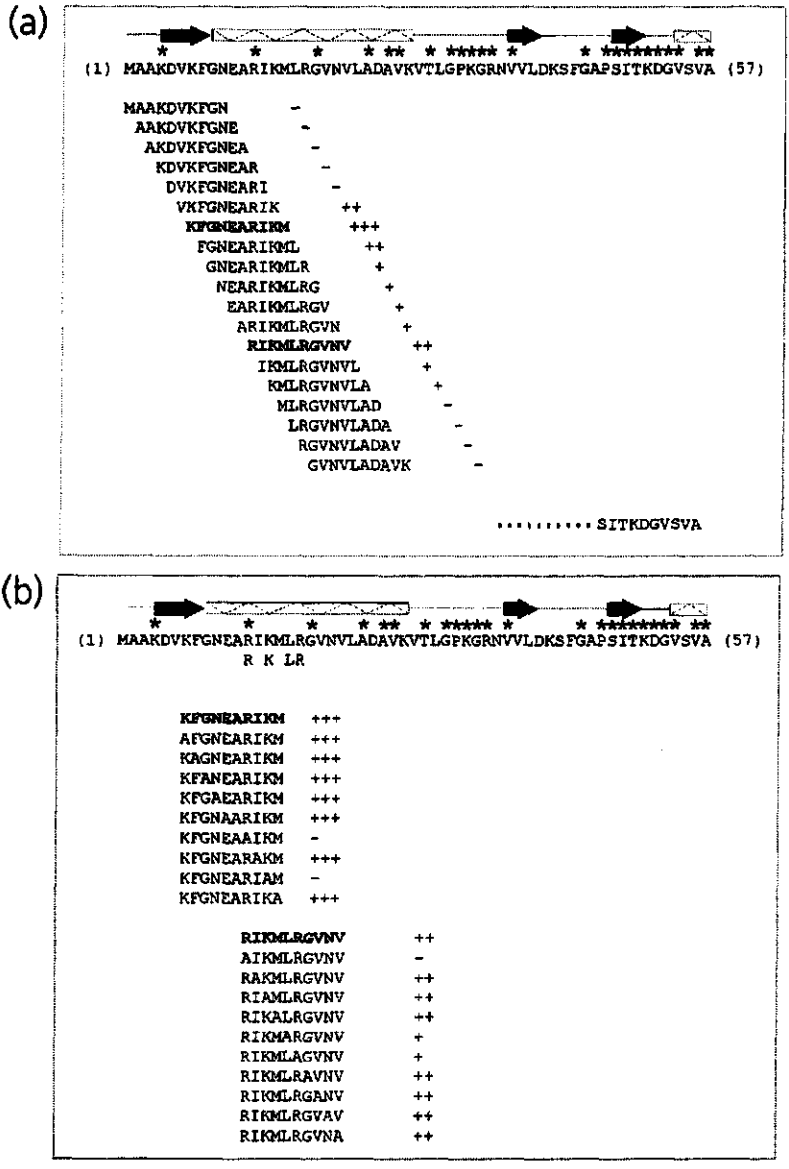

Fig. 2. PLRV-binding activity of synthetic decameric peptides corresponding to the amino acid sequence of MpB GroEL. (a) PLRV binding to decameric peptides corresponding to amino acids 1 till 57 of the N-terminal region of the equatorial domain of MpB GroEL. (b) Alanine replacement study of two decameric peptides with strongest binding capacities as indicated in a (bold characters). The differences in affinity of the peptides to PLRV are indicated; +++, high affinity to PLRV till +, low affinity to PLRV, or -, no PLRV binding detected. Secondary structural elements (defined by homology to GroEL of *E. coli*) are indicated by arrows (β-strands) or sine wave in rectangles (α-helices). Conserved sequences in GroEL/Hsp60 sequences are indicated with an asterisk (Braig *et al.*, 1994).

In order to identify single residues that are responsible for the PLRV-binding, each residue of peptides KFGNEARIKM and RIKMLRGVNV was replaced by an alanine (Fig. 2b). These replacement studies demonstrated that the arginine corresponding to position 13 (R13) of the MpB GroEL sequence eliminated affinity to PLRV of both peptides KFGNEARIKM and RIKMLRGVNV. Alanine replacement of the lysine corresponding to position 15 (K15) of the MpB GroEL sequence eliminated PLRV binding of peptide KFGNEARIKM. However, replacement of K15 by alanine did not change the PLRV affinity of peptide RIKMLRGVNV. Alanine replacement of the leucine or arginine residues corresponding to positions 17 (L17) and 18 (R18) of the MpB GroEL sequence, respectively, slightly reduced PLRV affinity of peptide RIKMLRGVNV. None of the other amino acid residues affected PLRV binding after alanine replacement.

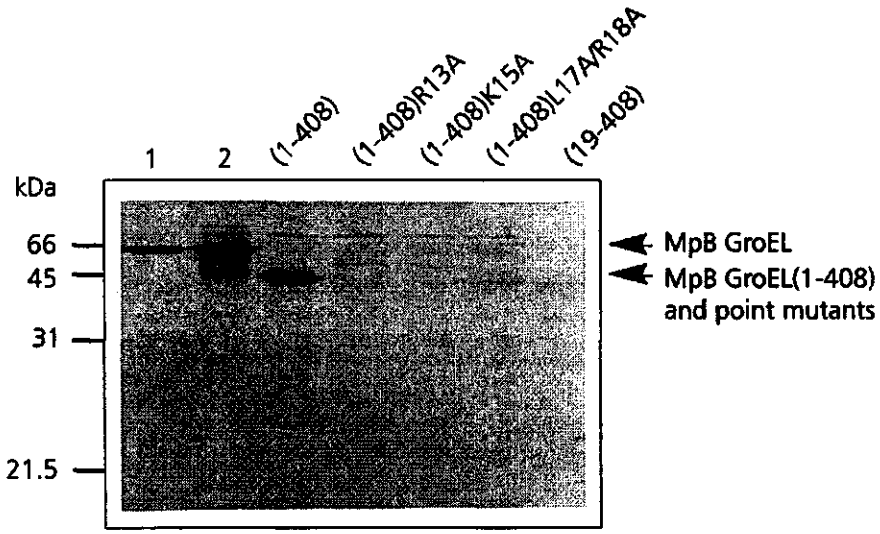

Fig. 3. Virus overlay assay of alanine replacement mutants of MpB GroEL (1-408) to identify individual amino acids involved in PLRV binding. Lanes: 1, wild type MpB GroEL isolated from *M. persicae*; 2, recombinant MpBGroEL. All other lanes contain MpB GroEL (1-408) wild type, and point mutants of MpB GroEL(1-408) as indicated. The positions of full-length MpB GroEL, MpB GroEL(1-408), and alanine replacement mutants of MpBGroEL(1-408) are indicated by arrow heads.

To further confirm the importance of R13 and K15 in virus binding, these residues were also replaced by alanines in the context of the MpB GroEL(1-408) sequence, resulting in MpB GroEL(1-408)R13A and MpB GroEL(1-408)K15A, respectively. Since alanine replacement of L17 and R18 of peptide RIKMLRGVNV did not eliminate the affinity of this peptide completely, it was decided to replace both residues simultaneously in the MpB GroEL(1-408) sequence, resulting in mutant MpB GroEL(1-408)L17A/R18A. The virus overlay assay showed that replacement of R13 by an alanine completely eliminated PLRV binding of mutant MpB GroEL(1-408) (Fig. 3). PLRV-binding to MpB GroEL(1-408) was also completely eliminated when K15 was replaced by an alanine. Replacement of L17 and R18 reduced binding of MpB GroEL (1-408). These findings are in agreement with results obtained from virus overlay assays with alanine replacement studies of decameric peptides (Fig. 2b). The finding that alanine replacement of single amino acids R13 and K15 mutually

eliminates each other's PLRV-binding capacity as well as that of L17 and R18, indicates that a structural component may determine the affinity of PLRV in decameric peptides and MpB GroEL deletion mutants. This would also explain that alanine replacement of K15 of peptide RIKMLRGVNV did not change its PLRV affinity (fig. 2b). Apparently, residue K15 does not influence the PLRV-binding structure of the peptide.

Identification of amino acids involved in the C-terminal binding site of MpB GroEL

With regard to the C-terminal domain, it was previously shown that mutant MpB GroEL(122-474) bound PLRV, whereas MpB GroEL(122-408/475-548) did not (Hogenhout *et al.*, 1998). In order to localize amino acids that are involved in binding PLRV in the C-terminal equatorial domain, additional mutants were synthesized. All mutants lacked the N-terminal equatorial domain, while deletions in the C-terminal equatorial domain were designed using the secondary structural elements of MpB GroEL (Fig. 4a). Virus overlay assays revealed that mutant MpB GroEL(122-427) did not bind PLRV, whereas mutants MpB GroEL(122-457) and MpB GroEL(122-474) both did (Fig. 4b). Since PLRV affinity of MpB GroEL(122-474) was not improved compared to mutant MpB GroEL(122-457), it was concluded that the PLRV-binding residues are located in the region between amino acid 427-457.

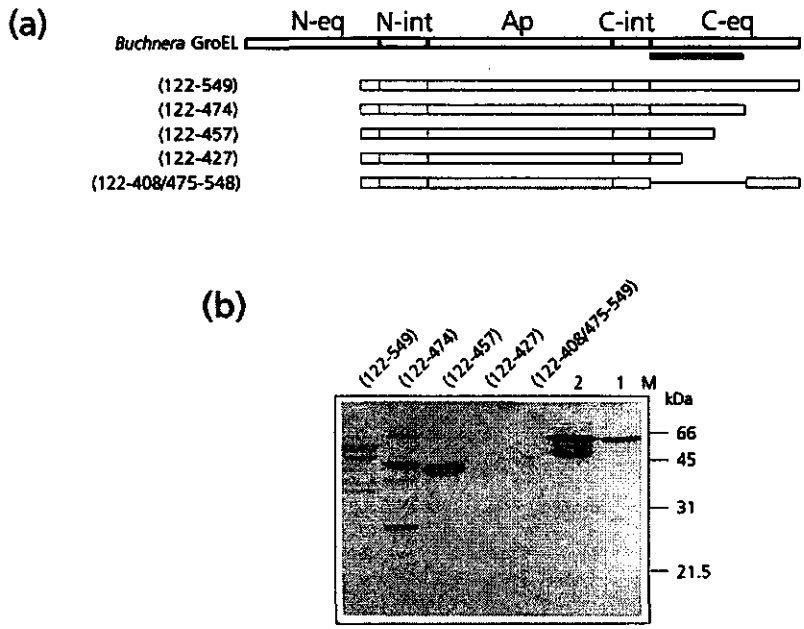

Fig. 4. Mapping of the C-terminal PLRV-binding site by virus overlay assays of deletion derivatives of MpB GroEL. (a) Schematic representation of MpB GroEL deletion mutants. The numbers in parentheses correspond to the positions of amino acid residues of MpB GroEL (accession number: AF003957; Hogenhout *et al.*, 1998), and mark the borders of the deletion mutants. N-eq, N-terminal region of the equatorial domain; N-int, N-terminal region of the intermediate domain; Ap, apical domain; C-int, C-terminal region of the intermediate domain; C-eq, C-terminal region of the equatorial domain. Secondary structural elements (defined by homology to GroEL of *E. coli*), are indicated by sine wave in rectangles (α-helices). (b) Virus overlay assay showing that the α-helix in between residues 427 and 457 is part of the PLRV-binding site. Lanes: 1, wild type MpB GroEL isolated from *M. persicae*; 2, recombinant MpB GroEL. All other lanes contain the indicated deletion mutants of MpB GroEL as depicted in panel 'a'.

To identify the individual amino acids involved in binding PLRV, decameric peptides were prepared from the sequence 423-476 and tested for PLRV affinity. A series of peptides corresponding to region 432-449 bound PLRV (Fig. 5a). Peptide VGIRVALRAM had the strongest affinity for PLRV, and was therefore selected for the characterization of PLRV-binding residues. Single amino acid replacement of the two arginines by an alanine slightly reduced the affinity of PLRV relative to the wild type peptide (Fig. 5b). When both arginines were simultaneously replaced, the peptide lost its affinity for PLRV completely. The arginines correspond to position 441 and 445 of the MpB GroEL amino acid sequence and are part of the region that was identified by deletion mutant analysis to be involved in PLRV-binding (Fig. 4).

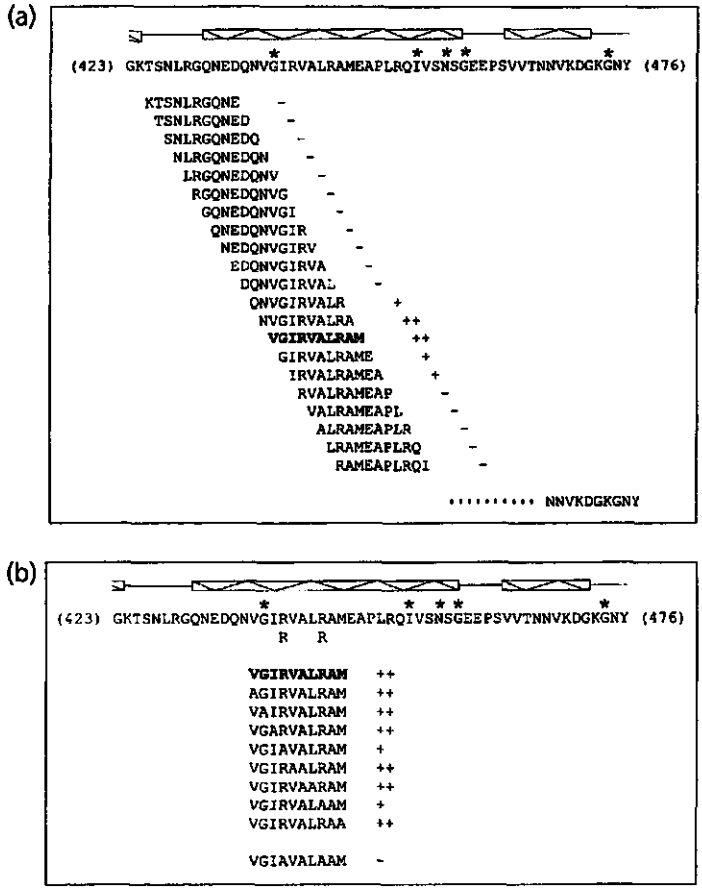

Fig. 5. Schematic presentations of the virus overlay assays of decameric peptides corresponding to the amino acid sequence of MpB GroEL. (a) PLRV binding to decameric peptides corresponding to amino acids 423 till 476 of the C-terminal region of the equatorial domain of MpB GroEL. (b) Alanine replacement study of the decameric peptide with the strongest binding as indicated in a (bold characters). The differences in affinity of the peptides to PLRV are indicated with ++, obvious binding, till +, low affinity to PLRV, or -, no PLRV binding detected. Secondary structural elements (defined by homology to GroEL of *E. coli*), are indicated by sine wave in rectangles (α-helices). Conserved sequences in GroEL/Hsp60 sequences are indicated with an asterisk.

The significance of PLRV-binding of the C-terminal arginines R441 and R445 was further investigated by site-directed mutagenesis. Each residue, R441 and R445, and both residues simultaneously were replaced by alanines in MpB GroEL(122-548), resulting in mutants MpB GroEL(122-548)R441A, MpB GroEL(122-548)R445A and MpB GroEL(122-548) R441A/R445A. Virus overlay assays of protein blots containing MpB GroEL deletion mutants showed that PLRV binding to the double amino acid replacement mutant (MpB GroEL(122-548)R441A/R445A) was slightly reduced compared to that of the wild type construct (MpB GroEL(122-548) but maintained affinity to PLRV (Fig. 6). Alanine replacement of R441 reduced the PLRV affinity of MpB GroEL(122-548) more than replacement of R445. These results indicate that additional residues besides R441 and R445 are required for the formation of the complete PLRV-binding site at the C-terminal equatorial domain of MpB GroEL. Since additional residues were not characterized in the pepscan analyses of the C-terminus, it is likely that these residues are important for the structural conformation of the PLRV-binding site of the α-helix in between residues 427 till 457.

PLRV also interacted with endogenous *E. coli* GroEL, which is copurified with some of the GST-fusion deletion mutants. This phenomenon was observed before (Hogenhout *et al.*, 1998). Consequently, some lanes show higher molecular weight proteins corresponding to *E. coli* GroEL that bind PLRV (Fig. 4b and 6). Comparison of the MpB GroEL amino acid sequence with that *E. coli* GroEL revealed that the PLRV-binding of the N-terminal equatorial domain (R13, K15, L17 and R18) were identical. However, the arginine at position 441 of the PLRV-binding site of the C-terminal equatorial domain of MpB GroEL is a lysine in GroEL of *E. coli* GroEL. It was investigated whether replacement of R441 and R445 by lysines of deletion mutant MpB GroEL(122-548) affected PLRV binding. Virus overlay assays demonstrated that PLRV affinity to the lysine replacement mutant, MpB GroEL(122-548)R441K/R445K, did not differ from that of MpB GroEL(122-548) (data not shown). This indicates that the exchange of arginines by lysines does not influence the PLRV-binding capacity of GroEL proteins.

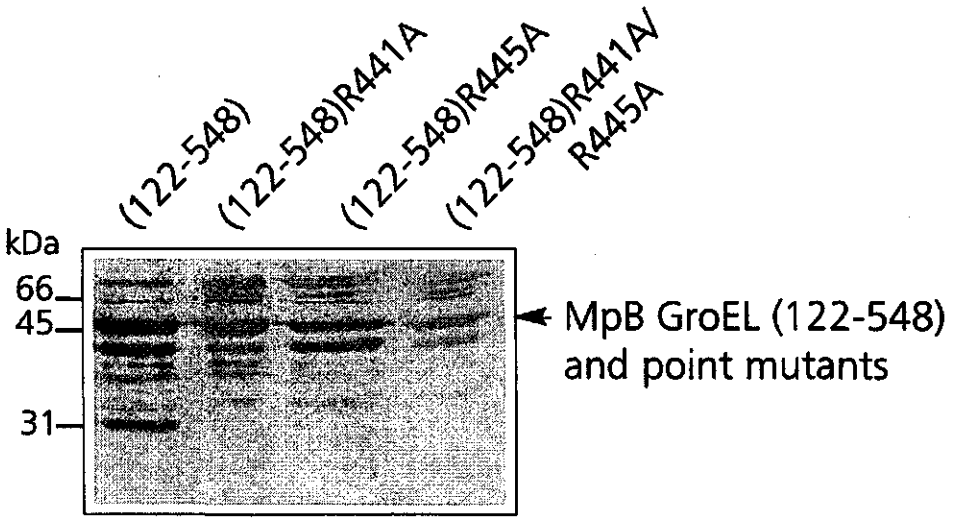

Fig. 6. Virus overlay assay of alanine-replacement mutants of MpB GroEL (122-548) to identify individual amino acids involved in PLRV binding. Lanes contain MpB GroEL (122-548) wild-type, and point mutants of MpB GroEL(122-548) as indicated. The positions of MpB GroEL(122-548), and alanine replacement mutants of MpBGroEL(122-548) are indicated by arrow heads.

Replacement of PLRV-binding amino acids in full-length MpB GroEL

The finding that amino acid residues of the N-terminal and C-terminal equatorial domain influence binding of PLRV particles to MpB GroEL deletion mutants was verified using full-length MpB GroEL. Therefore, MpB GroEL alanine replacement mutants R13A, K15A, L17A/R18A, R13A/R441A/R445A, and R441A/R445A were synthesized and tested in virus overlay assays. This experiment demonstrated that replacements of R13, K15, or L17 and R18 by alanines equally reduced PLRV affinity of full-length MpB GroEL (Fig. 7). PLRV affinity of MpB GroEL mutants in which R13, R441 and R445 were simultaneously replaced by alanines (R13A/R441A/R445A MpB GroEL) was even more reduced compared to that of R13A, K15A and L17A/R18A mutants, but binding was not completely abolished. Replacement of both R441 and R445 residues by alanines reduced PLRV affinity of MpB GroEL, but this mutant still had a better affinity for PLRV than the other alanine replacement mutants tested in this assay.

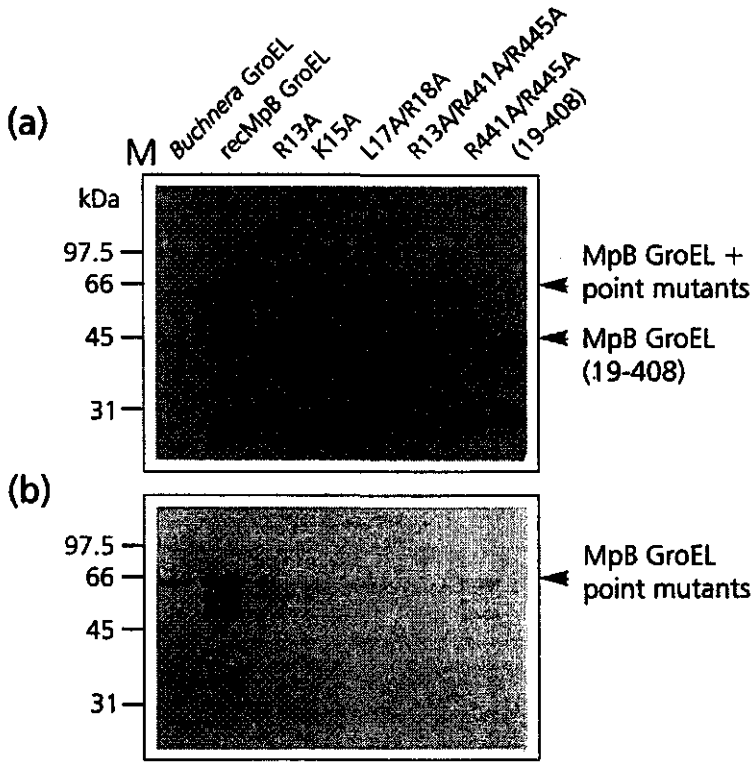

Fig. 7. Virus overlay assay of recombinant MpB GroEL, alanine replacement mutants of MpB GroEL and MpB GroEL (19-408). (a) Amido black-stained Western blot. (b) Virus overlay assay of a duplicate Western blot as shown in 'a'. The positions of MpB GroEL, MpB GroEL point mutants, and MpB GroEL (19-408) are indicated by arrow heads.

The alanine replacement studies with full-length MpB GroEL indicate that residues of the N-terminal equatorial domain (R13, K15, L17 and R18) have a stronger impact on PLRV-binding of MpB GroEL than residues of the C-terminal equatorial domain (R441 and R445).

Similar to findings of the site-directed mutational analysis of MpB GroEL deletion mutants, it is likely that replacement of R13 or K15 eliminates the N-terminal PLRV-binding site of MpB GroEL completely so that PLRV particles only bind to the C-terminal residues of MpB GroEL. Accordingly, alanine-replacement of R441 and R445 by alanines in the mutant that already contained an alanine on position 13 reduced PLRV-binding even more (Fig. 7). However, PLRV affinity of R13A/R441A/R445A MpB GroEL will not be completely eliminated because of the residual binding capacity of C-terminal residues, which was not identified in the pepscan analysis. Therefore, the virus overlay assays with site-directed mutants of full-length MpB GroEL mutants confirm those obtained with MpB GroEL deletion mutants, and indicate that residues R13, K15, L17, R18, R441 and R445 of MpB GroEL are all implicated in the interaction with PLRV.

DISCUSSION

The experiments presented in this chapter reveal that only a limited number of residues significantly influence the affinity of MpB GroEL for PLRV. These residues are located between the amino acid residues 9-19 and 427-457 of the N- and C-terminal equatorial regions, respectively. In the tertiairy structure, the residues 9-19 of the N-terminal equatorial domain form an α-helix (Fig.1 and 2). Virus-binding studies with alanine-replacement mutants of decameric peptides, MpB GroEL and deletion mutants of MpB GroEL as well as with decameric peptides all demonstrated that residues R13, K15 or L17 and R18 are important for maintaining a structure required for PLRV-binding to the N-terminal equatorial domain. It is therefore quite likely that the helical structure plays a crucial role.

The decameric peptide VGIRVALRAM, which bound PLRV particles in the virus overlay assay (Fig. 4a) and contains residues R441 and R445 of the C-terminal PLRV-binding site of MpB GroEL (Fig. 4b), is also part of a helical structure. This peptide likely forms a α-helical structure by itself. Therefore, alanine replacement of the two arginines could have disturbed the helical structure of the peptide and thereby eliminate PLRV affinity. Alternatively, the peptide may loose its hydrophilic character because of the replacement of the hydrophilic arginine residues by the more neutral alanine residues and, concomitantly, loose the affinity for PLRV. The finding that alanine replacement of these arginines in MpB GroEL(122-548) or full-length MpB GroEL reduced but did not abolish PLRV binding suggests that the replacement did not affect the structure or the hydrophilicity of the full-length α-helix as present in MpB GroEL as much as it affected the peptide. Probably, this effect of the secondary structure of the PLRV-binding site was not present in the decameric peptides and, consequently, the conformational characteristics of the full-length α-helix required to bind PLRV absent. These observations suggest that the helical structure located in between residues 427 and 457 is important for PLRV-binding of which residues R441 and R445 may or may not interact directly with PLRV particles. The finding that replacement of arginines with lysines did not affect PLRV-affinity suggests that the hydrophilicity of the α-helix is important for PLRV-binding rather than the presence of particular residues.

The finding that the amino acids involved in the conformation of the PLRV-binding site are located in the equatorial domain of MpB GroEL confirms previous results (Hogenhout *et al.*, 1998). Computer-generated structural predictions of the quarternary structure of *E. coli* GroEL demonstrates that the N- and C-terminal equatorial domain assemble to form one equatorial domain in the tertiary structure of the MpB GroEL monomer (Braig *et al.*, 1994). *Buchnera* and *E. coli* GroEL are highly comparable in their sequence, structural and functional characteristics (Ohtaka *et al.*, 1992; Filichkin *et al.*, 1997; Hogenhout *et al.*, 1998), suggesting that within *Buchnera* GroEL the N- and C-terminal regions of the equatorial

domain of MpB GroEL assemble into a single equatorial domain as well. Virus particles bind to MpB GroEL 14-mers and the RTD located on the surface of luteoviruses is involved in GroEL-binding (Van den Heuvel *et al.*, 1997). Therefore, the PLRV-binding site of MpB GroEL 14-mers should be accessible for the RTD of luteovirus particles. The α-helix containing the residues R441 and R445 is located to the exterior of the GroEL 14-mer, whereas the α-helix containing residues R13, K15, L17 and R18 is located towards the cavity of the GroEL cylinder (Braig *et al.*, 1994). The residues are positioned behind each other inside a cavity, which is visible from the outside of the 14-mer and thus may be accessible for the luteovirus RTD. The PLRV-binding site is not the only example in which amino acids of the N- and C-terminal part of the equatorial domain join to form a complex binding site. The ATP-binding site of *E. coli* GroEL is also composed of amino acids of both the N- and C-terminal part of the equatorial domain (Boisvert *et al.*, 1996).

Residues of MpB GroEL that are likely to be important for PLRV-binding are mainly hydrophilic in character, whereas usually hydrophobic residues of GroEL are implicated in binding proteins (Fenton *et al.*, 1994; Braig *et al.*, 1994). Moreover, PLRV-binding residues are located in the equatorial domain of MpB GroEL and hydrophobic residues involved in binding of nonnative proteins in the cytosol of bacteria are located in the apical domain of GroEL (Braig *et al.*, 1994). These differences indicate that PLRV binding to MpB GroEL is not similar to other interactions of GroEL identified thus far, and suggests that *Buchnera* GroEL does not act as a foldase in the interaction with a luteovirus in the aphid's haemolymph. The involvement of hydrophilic residues in protein-protein interaction has been found for other systems (Singh *et al.*, 1998; Davis et al., 1998), and it is demonstrated that interactions between pairs of hydrophilic residues are very strong compared to those of hydrophobic-hydrophobic or hydrophobic-hydrophilic pairs (Xu *et al.*, 1997). Therefore, it does not seem unlikely that hydrophilic residues of the readthrough domain of PLRV particles are involved in MpB GroEL binding as well.

Chaperonins have been classified into two groups (Kim *et al.*, 1994; Gupta, 1995). One group contains chaperonins of bacterial origin (like GroEL) and of eukaryotic organelles such as the mitochondrial Hsp60 or the Rubisco binding protein from chloroplasts, all of which exhibit at least 50% sequence identity (Gupta *et al.*, 1989; Gupta, 1995). The second group contains chaperonins from thermophilic bacteria such as the two-subunit-comprising TF55 from *Sulfolobus shibatae* or *S. solfataricus*, and the 9-subunits-comprising eukaryotic cytosolic TCP-1 and are 32-39 % identical (Trent *et al.*, 1991; Kubota *et al.*, 1994). The two groups are weakly related but carry out similar functions, which is folding of proteins in the cell cytosol, and have structural similarities as well (Mummert *et al.*, 1993; Creutz *et al.*, 1994; Kim *et al.*, 1994; Marco *et al.*, 1994; Melki and Cowan, 1994). Since PLRV binds to *Buchnera* GroEL from several aphid species (Hogenhout *et al.*, 1995) and to *E. coli* GroEL (Hogenhout *et al.*, 1998), it indicates that the amino acids implicated in virus binding should be highly conserved among GroEL homologues. Alignment of amino acid sequences of *Buchnera* GroEL and *E. coli* GroEL indeed demonstrate that most amino acids involved in binding PLRV (R13, K15, L17, R18, and R445) are conserved within *Buchnera* and *E. coli* GroEL. The arginine at position 441 of *Buchnera* GroEL proteins is a lysine in GroEL of *E. coli* (Fig. 8b). But replacements of arginines by lysines have been shown not to influence PLRV-binding capacity of MpB GroEL(122-548) in virus overlay assays. Interestingly, R13 of MpB GroEL is conserved among all Hsp60 sequences (Fig. 8a) and is also found in two subunits of TCP-1 (Kim *et al.*, 1994; Kubota *et al.*, 1994).

A

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                    1                                                  50                                                  80
      cpnα A.thal   MASANALSSA SVLCSSR... QSKLGGGNQQ QGQRVSYNKR TIRRFSVRAN VKEIAFDQHS PAALQAGIDK LADCVGLTLG
      cpnα B.napus  MATANALSSP SVLCSSR... QGKLSGGSQQ KGQRVSY.RK AWRRFSLRAN VKEIAFDQSS PAALQAGIDK LADAVGLTLG
   cpnα P. sativum  MASTNALSST SILRSPTNQA QTSLSKKVKQ HG.RVNFRQK PN.RFVVKAA AKDIAFDQHS RSAMQAGIDK LADAVGLTLG
B.GroEL A. pisum   .......... .......... .......... .......... ........MA AKDVKFGNEA RDRMLRGVNV LADAVKVTLG
B.GroEL M. pers.   .......... .......... .......... .......... ........MA AKDVKFGNEA RDRMLRGVNV LADAVKVTLG
B.GroEL S. aven.   .......... .......... .......... .......... ........MA AKDVKFGNEA RDRMLRGVNV LADAVKVTLG
B.GroEL R. padi    .......... .......... .......... .......... ........MA AKDVKFGNEA RDRMLRGVNV LADAVKVTLG
  GroEL E. coli    .......... .......... .......... .......... ........MA AKDVKFGNDA RDKMLRGVNV LADAVKVTLG
M. hsp60 A.thal.   .......... .......MYR FASMLASKAR IA.QNARQVS SRNSWSRNYA AKEIKFGVEA RALMLKGVED LADAVKVTMG
M. hsp60 C.ur.     .......... .......MHR FASGLASKAR LARKGAMQIA SRSSWSRNYA AKDVKFGVEA RGLMLKGVED LADAVKVTMG
M. hsp60 B.napus   .......... .......MYR LISSIASKAR VARNCTSQIG SRLSSTRNYA AKDIRFGVEG RALMLRGVEE LADAVKVTIP
M. hsp60 D.mel.    .......... .......MFR LPVSL.ARSS ISRQLA.... .....MRGY. AKDVRFGPEV RAMMLQGVDV LADAVAVTMG
M. hsp60 M.zea     .......... .......MLR LPTVLRQMRP VSRALAPHL. .....TRAY. AKDVKFGADA RALMLQGVDL LADAVAVTMG
                                                                                                  *
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B

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500      511
LGADIVQKALL
LGADIVQKALV
LGADIVQKALV
VGIRVALRAME
VGIRVALRAME
VGIRVALRAME
VGIRVRLRAME
VGIKVALRAME
IGVQIIQNALK
IGVQIIQNALK
IGVQIIQNALK
LGVEIVRRALR
IGIEIIKRALK
```

Fig. 8. Alignment of hsp60/GroEL amino acid sequences of mitochondria, *E. coli* and chloroplasts. cpn α, the α subunit of chloroplast hsp60 of *Arabidopsis thaliana* (P20238), *Brassica napus* (P35480), and *Pea sativum* (P08926). M, mitochondrial hsp60 of *A. thaliana* (P29197), *Curcubita maxima* (*C. ur*) (Q05046), *B. napus* (P35480), *Drosophila melanogaster* (O02649), and *Mais zea* (P29185). *B, Buchnera* GroEL sequences of *Acyrthosiphon pisum* (P25750), *M. persicae* (2827011), *Schizaphis avanae* (2827011), and *Rhopalosiphum padi* (1841530), and GroEL of *E. coli* (536987). Sequences were aligned using the program PILEUP (Genetics Computer Group, Madison, Wis) (Devereux *et al.*, 1984). (a) Alignment of first 80 amino acids of GroEL/Hsp60 N-terminal regions of equatorial domains. (b) Alignment of amino acids 500 till 511 of GroEL/Hsp60 C-terminal regions of equatorial domains. Amino acids shown to be involved in PLRV binding are boxed. The conserved R13 is indicated by an asterisk.

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Chapter 5

The N-terminal region of the luteovirus readthrough domain determines virus binding to *Buchnera* GroEL, and is essential for virus persistence in the aphid

Johannes FJM van den Heuvel[1], Arnaud Bruyère[2], Saskia A Hogenhout[1], Véronique Ziegler-Graff[3], Véronique Brault[3], Martin Verbeek[1], Frank van der Wilk[1], and Ken Richards[3]

[1]Department of Virology, DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, the Netherlands; [2]Station de Recherche 'Grandes Cultures', INRA, 38 rue de Herrlisheim, Colmar 68021 Cédex, France; [3]Institut de Biologie Moléculaire des Plantes du CNRS et de l'Université Louis Pasteur, Strasbourg, France

Summary - Luteoviruses and the luteovirus-like pea enation mosaic virus (PEMV; genus *Enamovirus)* are transmitted by aphids in a circulative, nonreplicative manner. Acquired virus particles persist for several weeks in the aphid hemolymph, in which a GroEL homolog, produced by the primary endosymbiont of the aphid, is abundantly present. Six subgroup II luteoviruses and PEMV displayed a specific but differential affinity for *Escherichia coli* GroEL and GroEL homologs isolated from the endosymbiotic bacteria of both vector and nonvector aphid species. These observations suggest that the basic virus-binding capacity resides in a conserved region of the GroEL molecule, although other GroEL domains may influence the efficiency of binding. Purified luteovirus and enamovirus particles contain a major 22-kDa coat protein (CP), and lesser amounts of an ~54 kDa readthrough protein (RTD), expressed by translational readthrough of the CP into the adjacent open reading frame. Beet western yellows luteovirus (BWYV) mutants devoid of the readthrough protein did not bind to *Buchnera* GroEL, demonstrating that the RTD (and not the highly conserved CP) contains the determinants for GroEL binding. *In vivo* studies showed that virions of these BWYV mutants were significantly less persistent in the aphid hemolymph than were virions containing the readthrough protein. These data suggest that the *Buchnera* GroEL-RTD interaction protects the virus from rapid degradation in the aphid. Sequence comparison analysis of the RTDs of different luteoviruses and PEMV identified conserved residues potentially important in the interaction with *Buchnera* GroEL.

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INTRODUCTION

Species of the genus *Luteovirus* occur worldwide and infect a wide range of mono- and dicotyledonous plants, in which they replicate almost exclusively in the phloem tissue (Martin *et al.*, 1990; van den Heuvel *et al.*, 1995). Two subgroups (I and II) are recognized within the genus based on genome organization and the type of RNA-dependent RNA polymerase (Mayo and Ziegler-Graff, 1996). Luteoviruses are persistently transmitted by aphids in a circulative manner. Briefly, virions are ingested with phloem sap from infected plants and transcellularly transported through the gut into the hemocoel by receptor-mediated endocytosis-exocytosis (Gildow, 1987). The hemolymph acts as a reservoir in which acquired virus particles are retained in an infective form for the aphid's lifespan, without replication (Eskandari, 1979). Upon contacting the basal lamina of the accessory salivary gland, virus particles may be transported through this gland, eventually arriving in the salivary duct from which they are excreted with the saliva when the aphid feeds (Gildow and Gray, 1993). The high degree of vector specificity of luteoviruses among aphid species implies an intimate relationship between the surface domains of the viral capsid and aphid components (Gildow, 1987; van den Heuvel *et al.*, 1994).

Symbionin (Ishikawa, 1984), a protein released by the primary endosymbiotic bacteria (genus *Buchnera*) of aphids into the hemolymph, appears to be essential for luteovirus transmission (Veidt *et al.*, 1992). Symbionin-like molecules are immunologically closely related and share more than 80% sequence identity with the *Escherichia coli* heat shock protein GroEL, a member of the chaperonin 60 family (Ohtaka *et al.*, 1992; van den Heuvel *et al.*, 1994; Filichkin *et al.*, 1997). Chaperonins are essential for cell viability, since they bind and stabilize newly translated or translocated aggregation-prone polypeptides (Buchner *et al.*, 1991) and mediate their functional folding and assembly in an ATP-dependent manner (Goloubinoff *et al.*, 1989; Ellis and van der Vies, 1991; Hartl, 1996). The structural characteristics of *Buchnera* GroEL are highly similar to GroEL of *E. coli* (Hara and Ishikawa, 1990; Ohtaka *et al.*, 1992; Hartl, 1996), and there is extensive amino acid sequence homology in functionally significant regions with *E. coli* GroEL (Ohtaka *et al.*, 1992; Filichkin *et al.*, 1997). Moreover, *Buchnera* GroEL from *Acyrthosiphon pisum* has been shown to be functional as a folding and assembly factor in a GroEL-deficient *E. coli* strain (Ohtaka *et al.*, 1992), to possess ATPase activity, and to be able to reconstitute dimeric ribulose 1,5-biphosphate carboxylase-oxygenase (RuBisCO) from its unfolded subunits *in vitro* (Kakeda and Ishikawa, 1991). However, unlike *E. coli* GroEL, *Buchnera* GroEL is not restricted to the cytosol of the bacteria. It occurs at a high concentration extracellularly in the aphid hemolymph (van den Heuvel *et al.*, 1994; Filichkin *et al.*, 1997).

Ligand binding assays have shown that potato leafroll virus (PLRV; subgroup II) and barley yellow dwarf virus (BYDV; subgroup I) have a high specific affinity for GroEL homologs of both vector and nonvector species (van den Heuvel *et al.*, 1994; Filichkin *et al.*, 1997). Antibiotic treatment of *Myzus persicae* larvae dramatically decreases symbionin levels in the hemolymph, which was accompanied by inhibited transmissibility of PLRV and loss of capsid integrity in the hemolymph (van den Heuvel *et al.*, 1994). These observations have led to the suggestion that luteoviruses associate with *Buchnera* GroEL in the hemolymph to retard proteolytic breakdown (van den Heuvel *et al.*, 1994; Filichkin *et al.*, 1997).

The importance of the viral capsid in determining aphid transmissibility has been convincingly demonstrated (54). Two capsid-associated proteins have been detected: the major capsid protein (CP) with a molecular mass of ~22 kDa, which is encoded by open reading frame (ORF) 3; and a minor polypeptide, the readthrough domain (RTD), which is expressed as a result of translational readthrough of the ORF 3 termination codon into the neighboring ORF 5 (*Veidt et al.*, 1988; Bahner *et al.*, 1990; Dinesh-Kumar *et al.*, 1992; Reutenauer *et al.*, 1993). In extracts of infected plants or protoplasts, the ORF 3-ORF 5 fusion protein of ~74 kDa is readily detected;

in purified virus particles, the readthrough protein exists as a truncated ~54 kDa form which lacks the C-terminal region of the RTD (Bahner *et al.*, 1990; Martin *et al.*, 1990; Wang *et al.*, 1990; Filichkin *et al.*, 1994; Brault *et al.*, 1995). The truncated RTD is exposed on the surface of the virus particle and contains determinants necessary for virus transmission by aphids (Jolly and Mayo, 1994; Brault *et al.*, 1995; Chay *et al.*, 1996; Filichkin *et al.*, 1997).

In this paper, we show that six subgroup II luteoviruses and the luteovirus-like pea enation mosaic virus (PEMV; genus *Enamovirus*) can bind to native GroEL homologs derived from *Buchnera* spp. of vector and nonvector aphids. In contrast to the situation reported for BYDV (20), we have found that the aforesaid viruses also readily bind to *E. coli* GroEL. Using beet western yellows luteovirus (BWYV) mutants with deletions in the RTD, we demonstrate that the presence of the RTD is indispensable for the interaction with GroEL. Finally, we have tested the fate of the BWYV RTD deletion mutants in the hemolymph of *M. persicae* and show that RTD-less virions are less persistent in the aphid.

MATERIALS AND METHODS

Aphids

A parthenogenic line of *M. persicae* biotype WMp2 was reared on *Brassica napus* subsp. *oleifera* at 20 ± 3 °C under a photo period of 16 h/day. Cohorts of nymphs differing in age by less than 24 h were produced by daily transfer of mature apterae, which were confined to leaf cages, to fresh plants. Clones of *A. pisum* and *Rhopalosiphum padi* were maintained under similar conditions on *Pisum sativum* and *Avena sativa*, respectively.

Viruses and antibodies

PLRV and BWYV were maintained on *Physalis floridana*, and bean leafroll virus (BLRV) on *P. sativum* by repeated aphid transfers. The viruses were purified from frozen leaf material by a modified enzyme-assisted (Cellulase R-10 and Macerozyme R-10, Yakult Honsha Co., Tokyo, Japan) procedure (van den Heuvel *et al.*, 1990). Purified virus was stored at –80 °C in 0.1 M sodium citrate (pH 6.0) containing 25% sucrose. Purified beet mild yellowing virus (BMYV), and cucurbit aphid-borne yellows virus (CABYV), soybean dwarf virus (SDV) and their homologous antisera were kindly provided by O. Lemaire (INRA, Colmar, France), H. Lecoq (INRA, Avignon, France), and V. Damsteegt (USDA-ARS, Frederick, Md), respectively. An aphid-transmissible isolate of PEMV was purified from *P. sativum* (Demler *et al.* 1997) and anti-PEMV immunoglobulin (IgG) was obtained from S. Demler (Michigan State University, East Lansing, Mich.). Carnation ringspot dianthovirus (CRSV) was kindly provided by S. Lommel (North Carolina State University, Raleigh, N.C.) and was purified from *Nicotiana clevelandii* (Lommel *et al.*, 1982). Anti-BLRV was a gift of L. Katul (BBA, Braunschweig, Germany). The antiserum to native *Buchnera* GroEL from *M. persicae* was raised according to previously described procedures (van den Heuvel *et al.*, 1994).

Purification of *Buchnera* and *E. coli* GroEL

Native tetradecameric *Buchnera* GroEL was purified from 5- to 6-day old aphids as described before (Kakeda and Ishikawa, 1991) with modifications. Aphids (0.25 g) were homogenized in

10 ml of phosphate-buffered saline (PBS; 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl, 2 mM KCl) containing 0.5% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was sonicated for 30 s with a Vibra Cell (Sonics & Materials, Inc., Danbury), and centrifuged at 10,000 *X g* for 15 min to remove the debris. A 40% polyethylene glycol 8,000 solution was added to the supernatant, to a final concentration of 8%. The suspension was then incubated for 1.5 h on ice, followed by centrifugation at 18,000 *X g* for 20 min. The pellet was resuspended in 50 mM Tris-HCl (pH 7.6) containing 35 mM KCl, 25 mM NH_4Cl, 10 mM MgAc, and 1 mM dithiothreitol, and the suspension was incubated for 1 h on ice, and centrifuged at 18,000 *X g*. The supernatant was sedimented through a 10 to 50% linear sucrose gradient in a Beckman SW41 rotor at 30,000 rpm for 16 h. The GroEL-containing bands were identified by Western blot analysis using an antiserum raised to *Buchnera* GroEL of *M. persicae*. *E. coli* GroEL was purified from DH5α cells grown at 37 °C until an optical density at 600 nm of 0.6 was attained, when they were transferred to 45 °C for 16 h (Khandekar *et al.*, 1993). The cells were pelleted, resuspended in PBS, and sonicated 3 times for 1 min. Further purification was carried out as described for *Buchnera* GroEL. All steps were done at 4 °C. Approximately 200 to 250 µg of the native protein was obtained from 100 mg (wet weight) of aphids or pelleted *E. coli* cells. GroEL suspensions were stored at –80 °C.

BWYV mutants from agroinfected plants

The recombinant binary vectors containing wild-type BWYV full-length cDNA and BWYV RTD deletion mutants have been described previously (Veidt *et al.*, 1992; Brault *et al.*, 1995; Bruyère *et al.*, 1997) and are summarized in Fig. 3. BW_0 represents the wild-type construct (Veidt *et al.*, 1992). In mutant BW6.4, the entire RTD has been eliminated by deletion and frameshifting (Reutenauer *et al.*, 1993). The other constructs used, BW6.51, BW6.106, BW6.104, BW6.ΔTB, BW6.50, BW6.ΔE1, BW6.40, and BW6.41, contained short in-frame deletions at different locations in the RTD (Bruyère *et al.*, 1997). *N. clevelandii* plants were agroinoculated according to previously described procedures (Leiser *et al.*, 1992). Infected plants were identified by enzyme-linked immunosorbent assaying (ELISA) (van den Heuvel *et al.*, 1990) with BWYV-specific IgG, and virus was purified as described above.

GroEL ligand assay

Immunoplates (Maxisorp F96, Nunc, Roskilde, Denmark) were sensitized with 100 µl of 10 µg purified GroEL per ml of 0.05 M sodium carbonate (pH 9.6) (coating buffer) for 16 h at 4 °C and incubated with 100 µl of purified virus at a concentration of 10 µg/ml SEB (PBS containing 0.05% Tween-20, 1% polyvinyl pyrrolidone and 0.1% ovalbumin) for 16 h at 4 °C. Then, the homologous IgGs at 1 µg/ml in SEB were incubated for 3 h at 37 °C. The antigen-bound primary antibodies were detected by goat-anti-rabbit IgG alkaline phosphatase conjugate (Sigma, St. Louis, Mo) in SEB for 3 h at 37 °C. The amount of immobilized alkaline phosphatase was revealed by adding 1 mM *p*-nitrophenyl phosphate (disodium salt) in 10% diethanolamine (pH 9.8). Color development at 405 nm was measured with a Bio-Kinetics Reader EL312 (Bio-Tek Instruments, Inc., Winooski, Vt).

Mg-ATP dissociation of GroEL

Buchnera GroEL was dissociated following procedures previously described for *E. coli* GroEL (Lissin, 1995). Briefly, 2 µg of purified GroEL was incubated in 50 µl of 50 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 5 mM ATP, 5 mM creatinine phosphate (sodium salt), and 2 U creatine phosphokinase for 30 min at 20 °C. Subsequently, 25 µl of 40% sucrose was added and 10-µl samples were loaded onto an sodium dodecyl sulfate (SDS)-free 4% polyacrylamide gel. Following polyacrylamide gel electrophoresis (PAGE), proteins were transferred to nitrocellulose (Toubin *et al.*, 1979), after which immunodetection with anti-*Buchnera* GroEL IgG and alkaline phosphatase-conjugated anti-rabbit IgG was carried out (van den Heuvel *et al.*, 1994).

Aphid microinjection

Seven-day old *M. persicae* nymphs were microinjected (Murayama and Kojima, 1965) with 60 nl of purified virus at 80 µg/ml by using calibrated glass capillaries (Gabay Instruments, Geneva, Switzerland). The microinjected aphids were transferred to healthy potato plants which were maintained at 20 ± 0.1 °C and with 16 h of light per day. After 2, 6, 24, 72, and 120 h, batches of three aphids were collected and stored at –80 °C untill further processing by triple-antibody-sandwich (TAS)-ELISA.

Detection of virus by TAS-ELISA.

Prior to sample incubation, the immunoplates were coated with 150 µl of 1-µg/ml anti-BWYV IgG in coating buffer for 16 h at 4 °C. The samples consisted of three aphids triturated in 150 µl SEB, and were incubated for 16 h at 4 °C. Viral antigen was detected by monoclonal antibody WAU-A12 (van den Heuvel *et al.*, 1990) and goat-anti-mouse IgG linked to alkaline phosphatase.

RESULTS

Purification of GroEL

Native tetradecameric GroEL was isolated from the endosymbiotic bacteria of *M. persicae*, *A. pisum*, *R. padi*, and from *E. coli* cells. SDS-PAGE of GroEL revealed a single ~60 kDa band corresponding to GroEL subunits (Fig. 1A, lane 1). *Buchnera* GroEL, like *E. coli* GroEL, is an oligomer of 14 identical subunits arranged into two stacked heptameric rings (Hara and Ishikawa, 1990; Braig *et al.*, 1994; Filichkin *et al.*, 1997). PAGE of purified GroEL under nondenaturing conditions, followed by western blot analysis with an antiserum to *Buchnera* GroEL, revealed a single band of 14-meric GroEL (Fig. 1B, lane 1). Electron microscopy of the purified *M. persicae* GroEL suspension clearly showed the multimeric nature of the protein (Fig. 1C). As observed for GroEL of *E. coli* (Luo and Horowitz, 1994) and *Buchnera* GroEL of *A. pisum* (Kakeda and Ishikawa, 1991), *Buchnera* GroEL of *M. persicae* underwent partial dissociation into lower-molecular-mass species upon incubation with Mg-ATP (Fig. 1B, lane 2). However, no dissociation was observed when the GroEL 14-mer was incubated overnight in ELISA coating buffer (Fig. 1B, lane 3), indicating that the protein retains its oligomeric state under the conditions used during its immobilization onto immunoplates for tests of luteovirus binding (see

below). The purified GroEL from *A. pisum*, *R. padi*, and *E. coli* behaved similarly in the aforesaid tests (data not shown). Finally, nondenaturing PAGE of hemolymph samples taken directly from *M. persicae* (van den Heuvel *et al.*, 1994), established that the GroEL 14-mer prevails in the aphids' body fluid (Fig. 1B, lanes 1, 2 and 4).

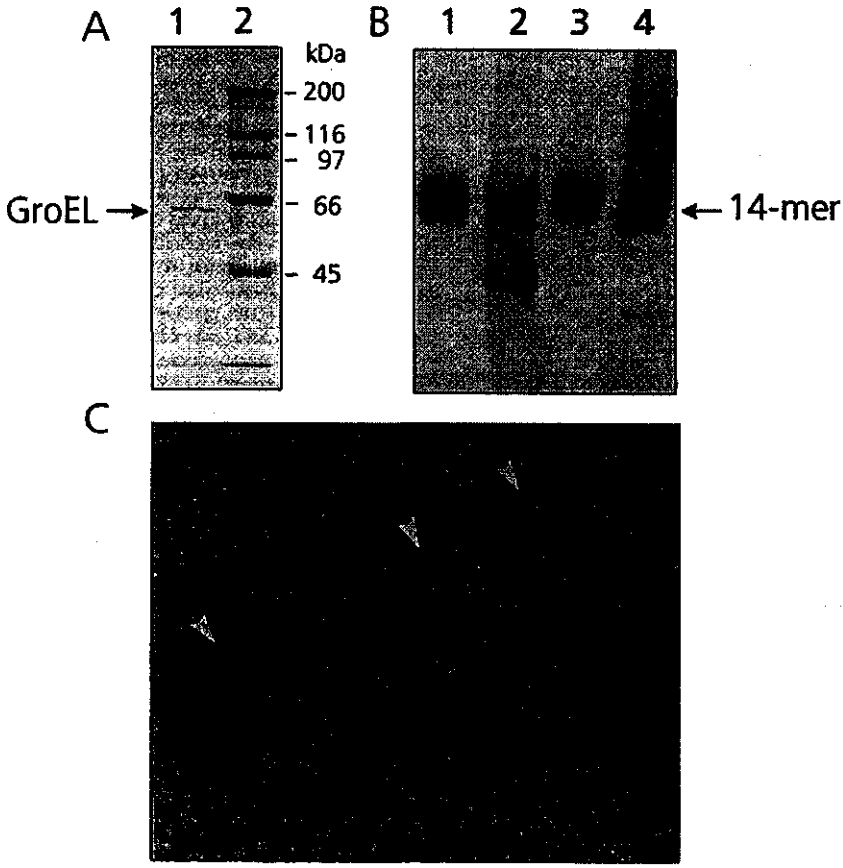


Fig. 1. Characterization of purified *Buchnera* GroEL from *M. persicae*. (A) A 1-µg amount of sucrose density gradient-isolated GroEL on an SDS-8.5 % PAGE stained with Coomassie brilliant blue (lane 1). Lane 2, Molecular markers. (B) Nondenaturing PAGE of native and dissociated GroEL followed by Western blot analysis with anti-*Buchnera* GroEL IgG. Lanes: 1, native GroEL; 2, Mg-ATP-incubated GroEL; 3, GroEL incubated overnight in ELISA coating buffer; 4, hemolymph sample from *M. persicae*. (C) Electron micrograph of GroEL oligomeric complexes stained with 2% (wt/vol) uranyl acetate. Black and white arrows indicate side and top views of GroEL, respectively. Bar, 50 nm.

GroEL-binding of luteoviruses and PEMV

Purified GroEL from *M. persicae*, *A. pisum*, *R. padi*, and *E. coli* were immobilized onto immunoplates and their affinities for six subgroup II luteoviruses (BWYV, BMYV, PLRV, CABYV, BLRV and SDV) and PEMV were tested in the GroEL-ligand assay (Fig. 2). PEMV was included because of the striking similarities with luteoviruses concerning aphid transmissibility, genomic organization of PEMV RNA 1, and incorporation of a 55-kDa coat

protein-RTD polypeptide in the viral capsid (Demler *et al.*, 1996).

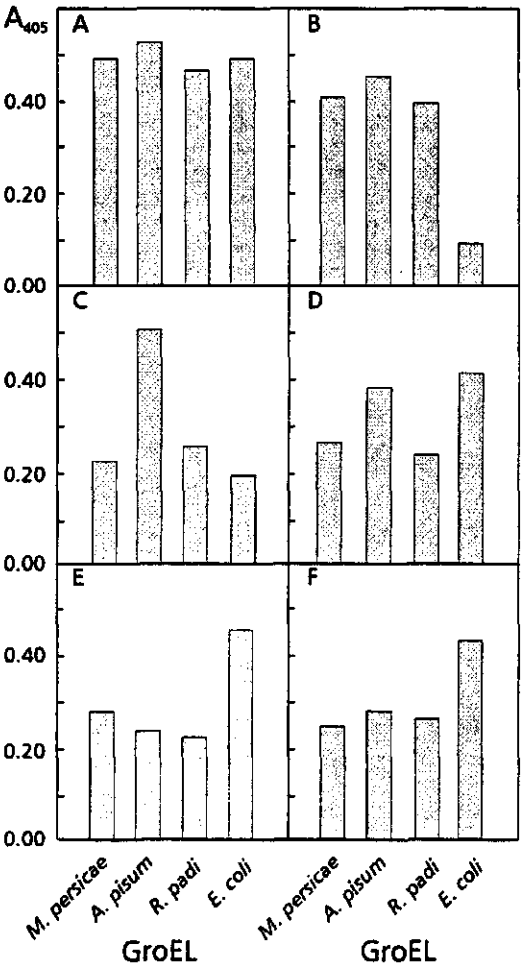


Fig. 2. Affinity binding of PLRV (A), BLRV (B), BWYV (C), CABYV (D), SDV (E), and PEMV (F) to GroEL homologs purified from the endosymbiotic bacteria of *M. persicae*, *A. pisum*, and *R. padi* and from *E. coli*. All samples were tested in duplicate, and the mean absorbency at 405 nm (A_{405} [ELISA value]) are given. Binding data for BMYV are comparable to those for BWYV (not shown). Virus-binding to ovalbumin and CRSV binding to the GroEL homologs (negative controls) gave ELISA values lower than 0.02.

The results show that PLRV bound to *Buchnera* GroEL from the nonvector aphids *A. pisum* and *R. padi* and to *E. coli* GroEL with an avidity similar to that of *M. persicae*, its primary vector (Fig. 2A). All luteoviruses tested and PEMV also bound to the four GroEL homologues (Fig. 2), but with different affinities, which were not related to whether GroEL was derived from a vector or a nonvector aphid. Thus, BWYV and BMYV bound more efficiently to *Buchnera* GroEL of *A. pisum*, a rather poor vector (E. Herrbach, personal communication), than to the GroEL of the efficient vector *M. persicae* (Fig. 2C). Likewise, *A. pisum* is a very efficient vector of BLRV,

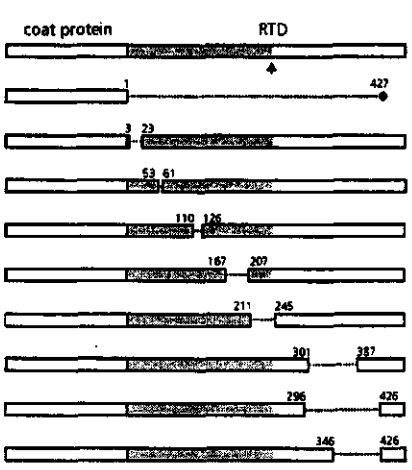
SDV, and PEMV; however these viruses did not bind to *A. pisum* GroEL with an affinity notable higher than that to the GroEL proteins from other sources (Fig. 2B, E, and F). The finding that these viruses have affinity for GroEL homologs derived from aphid species which do not transmit or inefficiently transmit them is entirely consistent with the observations that aphids can acquire and retain luteoviruses they do not transmit (Rochow and Pang, 1961; Massalski and Harrison, 1987). Plant viruses which are not aphid transmitted in a circulative fashion did not show affinity for any of the GroELs in the ligand-binding assay (data not shown). The viruses tested were from the genera *Potyvirus* (blackeye cowpea mosaic virus), *Tospovirus* (tomato spotted wilt virus), *Comovirus* (cowpea mosaic virus), *Furovirus* (beet necrotic yellow vein virus), and *Dianthovirus* (CRSV). Because CRSV is a spherical virus with dimensions similar to those of the luteoviruses, it was used as a negative control in the remaining experiments.

Identifying the region on the BWYV capsid implicated in GroEL-binding

The fact that different luteoviruses all have affinity for *Buchnera* GroEL suggests that conserved feature(s) of the luteovirus capsid are involved. Comparing the derived amino acid sequences of the luteovirus major CPs (Veidt *et al.*, 1988; van der Wilk *et al.*, 1989; Ueng *et al.*, 1992; Rautenauer *et al.*, 1993; Guilley *et al.*, 1994, 1995; Demler *et al.*, 1997) revealed that 25% of the residues are identical. The N-terminal half of the RTD, which is also present in purified luteovirus particles (see introduction), contains 16% of globally identical residues. Global sequence identity in the C-terminal region of the RTDs, which are not present in purified particles, was negligible.

To ascertain which of the two capsid-associated proteins is responsible for the interaction with *Buchnera* GroEL, experiments with particles of BWYV mutants engineered to contain deletions of different portions in the RTD were carried out. In mutant BW6.4 (Rautenauer *et al.*, 1993), the entire RTD had been eliminated by deletion and frameshifting (Fig. 3). In mutants BW6.ΔE1, BW6.40 and BW6.41, in-frame deletions eliminated 85, 128, and 79 amino acid residues from the C-terminal half of the RTD (see reference Bruyère *et al.*, 1997 for descriptions of these and the following mutants). In mutant BW6.50, 33 residues spanning the junction between the conserved and the nonconserved portions of the RTD were eliminated and in mutants BW6.51, BW6.106, BW6.104 and BW6ΔTB, 21, 7, 15 and 39 residues, respectively, of the conserved N-terminal portion of the RTD were deleted. We have shown elsewhere (Bruyère *et al.*, 1997) that no readthrough protein can be detected in virions of any of the conserved domain mutants and that trace amounts were present in only some preparations of BW6.50 virions (Bruyère *et al.*, 1997). By contrast, particles of BW6.ΔE1, BW6.40 and BW6.41 contain C-terminally truncated readthrough protein in amounts similar to those observed in wild-type virions (Bruyère *et al.*, 1997).

When used in the GroEL ligand assay, the mutants with deletions in the C-terminal half of the RTD, which produce particles which are similar in capsid protein composition to wild-type BWYV, bound as efficiently to *Buchnera* GroEL of *M. persicae* as did wild-type virus (Fig. 3). The mutants which produced particles that were deficient in RTD, on the other hand, did not bind (Fig. 3). The fact that BW6.4, whose capsid contains only CP subunits, did not show affinity for *Buchnera* GroEL indicates clearly that this protein is not directly involved in binding. Therefore, we conclude that it is the RTD and, more particularly, the conserved N-terminal half of the RTD which is implicated in the interaction with *Buchnera* GroEL.



	RTD incorporation	Transmissibility	GroEL-binding (A_{405})
BW_0	+++	+	0.501 ± 0.014
BW 6.4	−−−	−	0.017 ± 0.011
BW 6.51	−−−	−	0.012 ± 0.015
BW 6.106	−−−	−	0.018 ± 0.002
BW 6.104	−−−	−	0.009 ± 0.001
BW 6.ΔTB	−−−	−	0.008 ± 0.010
BW 6.50	−−+	−	0.020 ± 0.017
BW 6.ΔE1	+++	+	0.460 ± 0.100
BW 6.40	+++	+	0.462 ± 0.117
BW 6.41	+++	+	0.493 ± 0.080

Fig. 3. Affinity-binding of BWYV RTD mutants to *Buchnera* GroEL of *M. persicae*. The structures of the readthrough proteins of wild-type BWYV (BW_0) and the various deletion mutants (Bruyère *et al.*, 1997) are shown to the left. The conserved portion of the RTD (see text) is shaded; vertical arrow, the approximate site of cleavage to yield the C-terminally truncated form of readthrough protein associated with purified wild-type virus. The deletion in each mutant is indicated by a dotted line and the numbering refers to the amino acid coordinates of the deletion boundaries relative to the beginning of the RTD (see Fig. 5). The deletion in mutant BW6.4 provoked a frameshift and the resulting missense sequence is indicated by a small circle. The GroEL-binding data to the right give the amount of virus (as measured by ELISA) which bound to immobilized *Buchnera* GroEL from *M. persicae* in the GroEL ligand assay. The values are the mean ELISA readings (A_{405} ± standard error) for three samples from different batches of purified virus. CRSV was used as a negative control and yielded a mean A_{405} value of 0.023. Data on the ability of aphids to transmit the mutants and on the incorporation of the RT protein into purified virions are taken from reference Bruyère *et al.*, 1997.

Fate of microinjected BWYV mutants

To investigate the role of the RTD-*Buchnera* GroEL interaction *in vivo*, purified wild-type virus (BW_0) and BW6.E1, which both bind to *Buchnera* GroEL, and two nonbinding mutants, BW 6.4 and BW 6.50, were microinjected directly into the aphid's hemocoel. Although virus particles devoid of the RTD are stable in the intestine of the aphid (Brault *et al.*, 1995), and are able to cross the gut epithelium (Choy *et al.*, 1996), the gut-hemocoel interface was by-passed in these experiments, since it is not clear whether the RTD modulates the efficiency of the passage. The fate of the viruses in the microinjected aphids was monitored by TAS-ELISA with a monoclonal antibody that reacts with quaternary surface epitopes of the virus (van den Heuvel *et al.*, 1990) and thus specifically recognizes intact virus particles. The ELISA-readings (Fig. 4) directly reflect the amount of virus present in the aphid.

Wild-type BWYV and the GroEL-binding mutant BW6.ΔE1 were readily detected in the microinjected aphids, and, although a slight decline in virus titre was visible, the total amount of virus present 120 h post-injection was still ~67% of the amount injected. The virus titre declined slowly and gradually over the entire experimental period: about 16% during the first 2 h and 21% from 2 to 120 h. In contrast, the level of the nonbinding BWYV mutants BW6.4 and BW6.50 declined rapidly immediately after injection and thereafter. During the first two hours a greater than 60% decline in virus content was observed, and from 2 to 120 h the level fell by another 70%. In total, only 10% of the injected virus was detected at 120 h post injection.

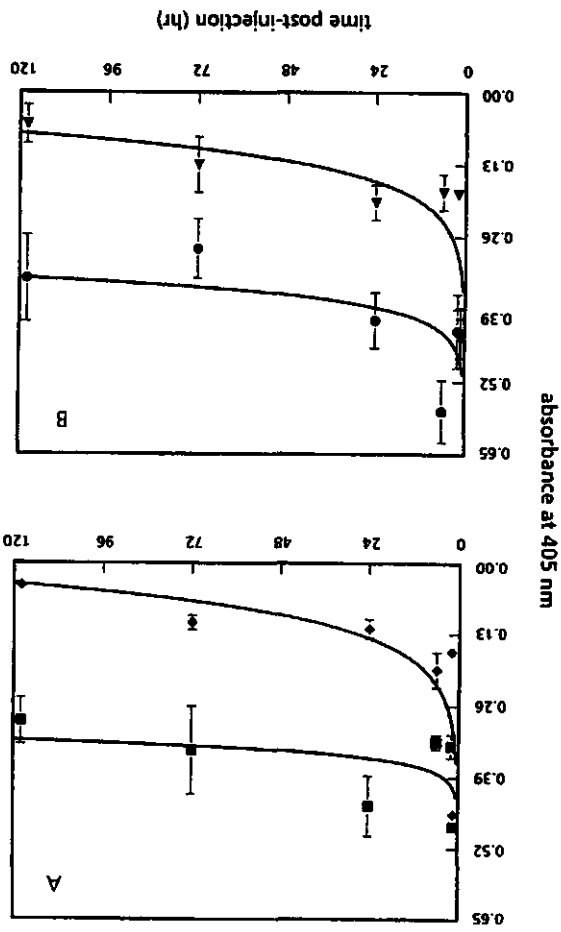


Fig. 4. Retention of BWYV wild-type and readthrough deletion mutants in *M. persicae* tested by ELISA (A_{405}). (A) BW6.ΔE1 (■) and BW6.50 (◆). Each point represents the mean (± standard error) of three samples from three aphids. (B) Wild-type BW_O (●) and BW6.4 (▲). Seven samples from three aphids were tested. The aphids were microinjected with ~5 ng of virus. Due to rapid degradation of the BWYV mutants BW6.4 and BW6.50, time zero of the four viruses was established by adding purified virus directly to aphid homogenates. Mock-microinjected aphids gave ELISA values of less than 0.01 (not shown).

DISCUSSION

The RTD plays an important role in the infection cycle of luteoviruses: it harbors determinants implicated in the accumulation of virus in plants after agroinfection (Brault *et al.*, 1995; Chay *et al.*, 1996; Ziegler-Graff *et al.*, 1996), and in virus transmission by aphids (Jolly and Mayo, 1994; Brault *et al.*, 1995; Wang *et al.*, 1995; Bruyère *et al.*, 1997; Filichkin *et al.*, 1997). With respect to aphid transmission, PEMV resembles a luteovirus, since it requires the RTD to be incorporated in the viral capsid (Demler *et al.*, 1997). Here, we have shown by mutational analysis of a full-length infectious clone of BWYV that the RTD is also important for the

interaction with *Buchnera* GroEL *in vitro* (Fig. 3) and that this interaction might determine virus retention by the aphid *in vivo* (Fig. 4). BWYV mutants deficient in the RTD were quickly degraded in the aphid hemolymph after microinjection. These data corroborate earlier findings on the loss of PLRV capsid integrity in *Buchnera* GroEL-deficient aphids (van den Heuvel *et al.*, 1994).

In vitro binding to *Buchnera* GroEL is a phenomenon common to all plant viruses transmitted by aphids in a circulative nonreplicative manner. Six subgroup II luteoviruses (Fig. 2), BYDV-PAV (subgroup I; Filichkin *et al.*, 1997), and the type species of the genus *Enamovirus*, PEMV (Fig. 2), all displayed a strong affinity for native GroEL homologues from endosymbiotic bacteria of aphids. Thus, it is most likely that highly conserved regions on the RTDs of luteoviruses and PEMV are involved. *E. coli* GroEL binds substrate polypeptides by an apparent hydrophobic interaction (Lin *et al.*, 1995). Structural features recognized by GroEL are predominantly hydrophobic surfaces typically exposed by partially folded polyproteins (Hayer-Hartl *et al.*, 1994) but also certain amino acid sequence patterns (Landry *et al.*, 1992) and specific secondary structures (Schmidt and Buchner, 1992). Comparing the deduced amino acid sequences of the luteovirus and PEMV RTDs revealed that only the N-terminal half of the readthrough proteins are conserved and contain hydrophobic regions (Fig. 5). The C-terminal halves of the luteovirus RTDs, from residue 241 onward (Fig. 5), have no significant sequence identity, nor do they contain regions of a hydrophobic nature. Furthermore, most of the C-terminal region is missing from the PEMV RTD. Therefore, it is concluded that the GroEL binding capacity resides in the N-terminal conserved region of the RTD. This coincides well with the size of the RTD present in purified virus particles. Based on mutational analysis and mass spectroscopy, the calculated C-terminus of the truncated RTD of BYDV-PAV was mapped to amino acid residue 242 (Fig. 5) (Filichkin *et al.*, 1994). The highest overall level of sequence similarity in the RTD extends from position 184 to 223 (Fig. 5), where about 23% of the residues are identical. Moreover, this region is relatively hydrophobic compared to the rest of the RTD. Amino acid replacement studies are required to verify whether the determinants for the interaction with *Buchnera* GroEL reside in this region of the RTD.

In addition to *Buchnera* GroEL binding, all luteoviruses and PEMV showed affinity for GroEL of *E. coli* (Fig. 2), which indicates that the capacity of GroEL to interact with these viruses resides in a conserved part on the GroEL molecule rather than in a variable domain as was previously suggested (Filichkin *et al.*, 1997). Three domains are distinguished on the GroEL subunit: the apical and the equatorial domains, in which polypeptide and nucleotide binding sites are located, and the intermediate domain, which harbors ATPase activity and potential hinge functions, allowing allosteric movement of the other domains relative to each other (Braig *et al.*, 1994; Chen *et al.*, 1994). In *E. coli*, GroEL facilitates productive folding through cycles of protein binding and release, which is a process which may require ATP-hydrolysis. The ATPase activity of the GroEL subunit is regulated by GroES (Viitanen *et al.*, 1990; Martin *et al.*, 1991), a single heptameric ring of 10-kDa subunits also encoded by the *GroE* operon. Functional chaperonins may involve symmetrical or asymmetrical GroEL-GroES complexes (Engel *et al.*, 1995), and different binding sites on the GroEL molecule are involved. In the asymmetrical complex, with GroES blocking one end of the GroEL cylinder, the monomeric substrate binds within the cylinder at the level of the apical domains (Langer *et al.*, 1992) which expose a putative binding surface toward the cavity (Braig *et al.*, 1993; Chen *et al.*, 1994; Fenton *et al.*, 1994; Mayhew *et al.*, 1996). Although it was previously suggested that luteoviruses may bind to the apical domain of *Buchnera* GroEL or to its flexible C termini which are projected into the cylinder (Filichkin *et al.*, 1997), this is highly unlikely to occur in the aforesaid manner, since the dimensions of the luteovirus particle (diameter 23 nm) should prohibit virions from entering the central cavity, which is approximately 50-80 Å wide (Chen *et al.*, 1994). Typically, the GroEL

cylinder accommodates substrates ranging from 15-60 kDa (reviewed in Hartl, 1996).

On the other hand, symmetrical GroEL-GroES complexes (with the central cavity capped on both sides by GroES) stably bind and assist the folding and assembly of large multimeric macromolecules like RuBisCO and malate dehydrogenase (MDH) on their external envelope (Azem *et al.*, 1994). The equatorial domain seems to be responsible for binding of these large multimeric macromolecules (Weiss and Goloubinoff, 1995). It may well be that luteoviruses and PEMV employ similar sites on the GroEL subunit similar to those of RuBisCO and malate dehydrogenase, thus overcoming the size limitations imposed by the central cavity. Interestingly, it was shown that in the absence of GroES, GroEL also binds polypeptides with unstable secondary structure and transiently maintains them in a soluble, folding-competent conformation (Bochkareva *et al.*, 1988; Lecher *et al.*, 1989; Langer *et al.*, 1992; Braig *et al.*, 1993; Chen *et al.*, 1994; Fenton *et al.*, 1994). This observation may be of importance in understanding of the luteovirus-*Buchnera* GroEL interaction. Although the *Buchnera GroE* operon accommodates a gene for a 10-kDa protein that is highly homologous to *E. coli* GroES (Ohtaka *et al.*, 1992), it seems to be repressed at the translational level, since *Buchnera* GroES was not detected in the aphid (Kakeda and Ishikawa, 1991).

In conclusion, our data may provide insight into how luteoviruses escape destruction in the hemolymph of the aphid vector. Indeed, the hemolymph of invertebrates constitutes a potentially hostile environment (Ourth and Renis, 1993). In several hematophagous insects and ticks, host serum components including antibodies are readily detectable in the hemolymph after engorgement, although they disappear quickly immediately after cessation of feeding (Chinzei and Minoura, 1987; Vaughan and Azad, 1988; Vaughan *et al.*, 1990). Even isolated *Buchnera* cells directly injected into aposymbiotic aphids lyse rapidly (Wright, 1971). Several hypotheses have been put forward concerning possible mechanisms involved in the survival of pathogens and parasites within compatible invertebrate hosts (70). Among the possibilities evoked are evasion of host recognition either by molecular mimicry or active acquisition of host molecules and interference with the host defense mechanism. Although specific data on aphid immunology are rare, it may well be that association with *Buchnera* GroEL provides the virus with a means of escaping the host's immune response.

ACKNOWLEDGMENTS

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          1                                                    36
BWYV  VDEE..... .PGPSPGPSP SPQ......P TPQK.KYRFI VYTGVPVTRI
BMYV  VDKE..... .PGPSPGPSP SPQ......P TPSK.KYRFI VYTGVPVTRI
PLRV  VDSG..... .PEPGPSPQP TPT......P TPQK.HERFI AYVGIPMLTI
 SDV  VDGE..... .PGPKPGPDP APQPTPTPKP TPAK.HERFI AYTGTLSTLI
CABYV  VDGS..... .SPPPPSPSP TPPPPPPPQP QPQPCAQRFW GYEGNPQNKI
BYDV  VDSSTSEPQP APEPTPTPQP TPAPQPAPEP TPAPVPKRFF EYIGTPTGTI
PEMV  GDD..... .APPSPGPDP GPQPPPPPPP SPTPVGARFW GYEGVPESRM
          *                 * * * * *       *  *     **   * *

          37                                                   86
BWYV  MAQSTDDAIS LYDMPSQRFR YIEDENMNWT NLDSRWYSQN SLKAIPMIIV
BMYV  MAQSTDDAIS LYDMPSQRFR YIEDENMNWT NLDSRWYSQN SLKAIPMIIV
PLRV  QARESDDQII LGSLGSQRMK YIEDENQNYT NVSSEYYSQS SMQAVPMYYF
 SDV  SARQSSDSIS LYSIRNQRIR YIEDENSSWT NIDAKWYSQN SVEAIPMFVI
CABYV  LTAENSRNID SRPLNFVQMY KWEDEKWDKV NLQAGYSRND RRCMETYLTI
BYDV  STRENSDSIS VSKLGGQSMQ YIENEKCETK VIDSFWSTNN NVSAQAAFVY
PEMV  ISERNDHDID VKPLSFITMY KWEDESNTSV KLSASYLQND QVEATPYFLI
                *                 * *

          87                                                  136
BWYV  PVPQGEWTVE ISMEGYQPTS STTDPNKDKQ DGLIAYNDDL SEGMNVGIYN
BMYV  PVPQGEWTVE ISMEGYQPTS STTDPNKDKQ DGLIAYNDDL KEGWNVGVYN
PLRV  NVPKGQWSVD ISCEGYQPTS STSDPNRGRS DGMIAYSNAD SDYWNVGEAD
 SDV  PVPEGTWSIE ISCEGYQAAS STSDPHRGKC DGMIAYDDDS SKVWNVGQQN
CABYV  PADKGKFSVY LEADGEFVVK HIGDELDGSW LGNIAY.DVS QRGWNVGNYK
BYDV  PVPEGSYSVN ISCEGFQSVD HIGGNEDGYW IGLIAYSNSS GDNWGVGNYK
PEMV  PSSKGKFSVY IECEGFQAVK SIGGKSDGCW GGLIAY.NRK KDGWQARAYT
          *         *                    ***         *

          137                                                 183
BWYV  NVEITNNKAD NTLKYGHP.. .DMELNGCHF NQGQCLERDG DLTCHIKTTG
BMYV  NVEITNNKAD NTLKYGHP.. .DMELNSCHF NQGQCLERDG DLTCHVKTTG
PLRV  GVKISKLRND NTYRQGHP.. .ELEINSCHF REGQLLERDA TISFHVEAPT
 SDV  NVTITNNKAD NDWKYGHPDP LDLMINGDRF DQNQVVEKDG IISFHLVTTG
CABYV  GCKITNYQSN TVFVAGHP.. .DATMNGKSF DTARAVEVDW FASFELECDD
BYDV  GCSFKNFLAT NTWRPGHK.. .DLKLNDCQF TDGQIVERDA VMSFHVEATG
PEMV  GTVLSNYRST TTVINGHP.. .DCEVNDCKF KPDRGVESDL ICSFHLEA.E
                     **       *   *       * *

          184                                                 230
BWYV  DNASFFVVGP AVQKQSKYNY AVSYGAWTDR MMEIGMIAIA LDE...QGSS
BMYV  DNASFFVVGP AVQKQSKYNY AVSYGAWTDR MMEIGMIAIA LDE...QGSS
PLRV  D.GRFFLVGP AIQKTAKYNY TISYGDWTDR DMELGLITVV LDE.HLEGTG
 SDV  PNASFFLVAP AVKKTAKYNF CVSYGDWTDR DMEFGMVSVV LDE.HLEGAR
CABYV  EEGSWAIYPP PIQKDSSYNY TVSYGNYTEK YCEWGAISVS IDE.DNNGNE
BYDV  TDACFYLMAP KTMKTDKYNY VVSYGGYTNK RMEFGTISVT CDESDVEAER
PEMV  EDSYWALQAP PIQKSSDYNY VVSYGGYTEK SIEWGSVSIS IDEVNQTASA
          *    *  **    ***       *        **

          231                                                 278
BWYV  GSVKTERPKR VGH.SMAVST WETIKL.PEK GNSEGYETSQ RQDSKTPPTA
BMYV  GSAKIERPKR VGH.SMAVST WETINL.PEK ENSGEFKTDQ RQDLKTPPTS
PLRV  SANRVRRPPR EGH.TYMASP REPEGK.PVG NKPRDETPIQ TQERQPDQTP
 SDV  SSQYVRKSPR PGHFGVNRSH RLQDSFTPVE YVSDDDSSSS SSIVSNRPST
CABYV  .......PRR IPRGVMAWST PEPSFSGDDS QRQDFNTPSL EERGSDALES
BYDV  ITRHAETPIR FKNILVSEQY EQPLPTIIDQ CLCDVQTPEQ EQTLVDE...
PEMV  SP....WRGR ARKLAILQET VVPLPFPPGG AMDYRLGDRE GDQTGTSEKG
                *
```

Fig. 5. Comparison of the N-terminal RTD-deduced amino acid sequences from subgroup I (Schmidt and Buchner, 1992) and II (Veidt *et al.*, 1988; van der Wilk *et al.*, 1989; Guilley *et al.*, 1994; Rathjen *et al.*, 1994; Guilley *et al.*, 1995) luteoviruses and PEMV (Demler *et al.*, 1997). Asterisks, identical amino acid residues; boldface, hydrophobic regions. There is no significant level of sequence homology beyond residue 240. Based on mass spectroscopy and mutational analysis, the C terminus of the truncated full-length CP-RTD of BYDV was suggested to map to residue 242 (Filichkin *et al.*, 1994) which is located in the middle of the RTD. The full-length RTD of PEMV is only 20 amino acids longer than the presented sequence. The numbering refers to the position of the amino acid residues on the BWYV RTD. Hydrophobicity is based on the output of the PeptideStructure program (Genetics Computer Group, Inc., Madision, Wi).

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Chapter 6

Evidence that the conserved region within the luteovirus readthrough domain is involved in *Buchnera* GroEL binding.

Saskia A Hogenhout, Frank van der Wilk, Martin Verbeek, Rob W Goldbach and Johannes FJM van den Heuvel

Summary- To investigate which region of the readthrough domain (RTD) of the *Potato leafroll virus* (PLRV) minor capsid protein is responsible for *Buchnera* GroEL binding, a series of deletion mutants of this protein was generated. The mutants were expressed in *Escherichia coli* and tested for their affinity for GroEL *in vitro*. A RTD deletion mutant protein lacking the conserved region in the RTD domain, located between amino acid residues 184 and 225, showed significantly reduced affinity, suggesting that this region harbours the determinants for binding to *Buchnera* GroEL. Analysis of an additional set of mutant RTD polypeptides revealed that deletion of the C-terminal stretch of hydrophobic residues within this conserved region significantly reduced MpB GroEL affinity, implying that this region is involved in the binding to MpB GroEL. The MpB GroEL affinity was also lost after denaturation of mutant RTD proteins, indicating that the structure of the RTD is important for the binding as well.

INTRODUCTION

Potato leafroll virus (PLRV) has been shown to bind GroEL proteins produced by the primary bacterial endosymbionts (*Buchnera* spp.) of the aphid *Myzus persicae* (MpB GroEL) *in vitro* (van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1998). This binding was proposed to be crucial for the persistence of PLRV in the haemolymph of aphids (van den Heuvel *et al.*, 1994; 1997). PLRV and all other luteoviruses are isometric particles consisting of a 23 kDa major coat protein (CP) and a few copies of a 57kDa (minor) capsid protein expressed by translational readthrough of the CP gene. The readthrough domain (RTD) has been shown to protect the virus particle (Jolly and Mayo, 1994; Brault *et al.*, 1995) and contains the MpB GroEL-binding site (Filichkin *et al.*, 1997; van den Heuvel *et al.*, 1997). In this paper it is attempted to localize more precisely the GroEL-binding site within the RTD moiety. Among different luteoviruses the highest overall level of sequence identity (23%) is located in the region between the amino acid residues 184 to 225 of the RTD (van den Heuvel *et al.*, 1997). In order to investigate whether this conserved region of the RTD is involved in binding MpB GroEL, recombinant RTD and mutations thereof were expressed in *Escherichia coli* and tested for binding using a GroEL-ligand assay.

MATERIALS AND METHODS

PCR amplification procedure

PCR amplification was performed in a final volume of 50 µl of 10 mM Tris-HCl (pH 8.3) containing 0.4 mM (total) deoxynucleoside triphosphates, 3 mM MgCl2, 50 mM KCl, 10 ng of template DNA, 0.25 µM of each primer, and 2.5 U of *Taq* polymerase (Boehringer Mannheim). Mixtures were incubated for 30 s at 94 °C, followed by 25 cycles of 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C, with a final incubation of 10 min at 72 °C. Samples were stored at 4 °C until used. PCR products were analyzed on agarose gels.

Nucleotide sequence analyses

The nucleotide sequence of pGEX clones were determined at the sequencing facilities of the Wageningen Agricultural University, Department Biomolecular Sciences with a sequencing kit and AmpliTaq DNA polymerase (Applied Biosystems), universal and sequence-specific primers using an automated sequencer (model 373; Applied Biosystems).

Cloning of PLRV RTD deletion mutants.

A cDNA clone containing the open reading frames (ORFs) 4, 5 and 6 (pSKORF4+6) of PLRV (van der Wilk *et al.*, 1989) was used as template to amplify the RTD fragments as shown in Figures 1 and 3. Each mutant was named after the positions of the first and last amino acids of the truncated protein. Deletion mutants RTD(33-320), RTD(33-247), RTD(33-225) and RTD(33-183) were obtained by PCR amplification using primer combinations pFOR1 and pREV4, pFOR1 and pREV3, pFOR1 and pREV2, and pFOR1 and pREV1 (Table 1), respectively. The amplified products were cloned by using a TA cloning kit (Invitrogen), yielding plasmids pCR[RTD(33-320)], pCR[RTD(33-247)], pCR[RTD(33-225)], and pCR[RTD(33-183)]. The *Bam*HI-*Bam*HI fragment of 233 basepairs of pCR[RTD(33-183)]

was ligated to the *Bam*HI-*Eco*RI fragment of each pCR clone and the ligated products were subsequently ligated into the *Bam*HI-*Eco*RI sites of pGEX-2T (Pharmacia), yielding plasmids pGEX[RTD(33-320)], pGEX[RTD(33-247), pGEX[RTD(33-225)], and pGEX[RTD(33-183)]. RTD fragments encoding deletion mutants RTD(33-193), RTD(33-203), RTD(33-208) and RTD(33-214) were obtained by PCR amplification using primer sets pFOR2 and pREV1a, pFOR2 and pREV1b, pFOR2 and pREV1c, and pFOR2 and pREV1d (Table 1), respectively. Fragments were digested with *Bgl*I and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pGEX-2T. All pGEX-2T clones were verified for mutations and orientation by nucleotide sequence analysis.

TABLE 1
Oligonucleotides used for the construction of recombinant RTD polypeptides

Name	Oligonucleotide sequence (5'-3')[a]	Corresponding positions[b]
pFOR1	caggatccATGCTAACCATTCAGGCCAG	4311-4330
pFOR2	ttatagatctATGCTAACCATTCAGGC	4311-4327
pREV1	gggaattcttaAGTAGGCGCTTCAACGTGG	4763-4745
pREV2	atgaattcttaTTCATCAAGCACCACGGTG	4888-4869
pREV3	ttgaattcttaCATATAGGTGTGGCCCTCC	4955-4937
pREV4	aagaattcttaGGGCCAGTCTGTACCATC	5174-5157
pREV1a	ttatgaattcttaAGCGGGACCAACGAG	4793-4779
pREV1b	tagaattcttaAGTATAGTTATACTTTGCG	4823-4805
pREV1c	tatgaattcttaGTCACCGTATGAGATAG	4822-4838
pREV1d	tatgaattcttaCATGTCTCGGTCCGTC	4841-4856

[a] Uppercase letters indicate PLRV RTD sequences, and lowercase letters indicate sequences which are not part of the RTD sequence. Restriction sites (*Bgl*I, *Bam*HI, and *Eco*RI) are indicated in boldface. Termination codons are underlined.

[b] Numbering refers to the corresponding positions of the nucleotides of the RTD of PLRV.

Expression and isolation of RTD deletion and point mutants

The pGEX constructs mentioned above were introduced into *E. coli* DH5α (Stratagene). For large scale expression, overnight cultures were diluted 1:10 in LB in a final volume of 2 liter containing ampicillin (100 µg/ml) and incubated at 37°C for 3 h. Subsequently, 1 mM isopropyl-ß-D-thiogalactosidase (IPTG) was added to induce protein synthesis from the pGEX plasmid, and cultures were allowed to grow at room temperature. After 7 h, cells were pelleted at 4,000 *x g* for 10 min and resuspended in 40 ml of 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. Cells were lysed by freeze-thawing and sonication. Insoluble debris was removed by centrifugation (4,000 x *g*), and the supernatant containing the soluble protein was collected. Triton X-100 was added to a final concentration of 1 % and the solution was incubated on ice for 1 h. GST fusion proteins were bound to glutathione-Sepharose by addition of 2 ml of 50% glutathione-Sepharose (Pharmacia), prepared according to the manufacturer's recommendations, in phosphate-buffered saline (PBS; 2 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 M NaCl, 2 mM KCl) and 0.05% Tween 20, and incubated for 1 h at room temperature on an orbital shaker. Sepharose was pelleted by centrifugation at 500 *x g* using a

swing-out rotor and the pellet was washed three times with 10 ml of cold PBS. GST-fusion proteins were released by addition of 500 µl of 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0) followed by incubation for 3 h at room temperature while gently shaking. GST-fusion proteins present in the supernatant after centrifugation at 500 x *g* were analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis with anti-GST immunoglobulin G (IgG) (Pharmacia).

To cleave off the GST moiety, GST-fusion proteins bound to glutathione-Sepharose were pelleted at 500 x g using a swing-out rotor and treated with thrombin at a final concentration of 0.024 units/µl in 500 µl PBS, during 3 h at room temperature. The recombinant RTD polypeptides present in the supernatant after centrifugation at 500 *x g* were analyzed on SDS-PAGE gels and by Western blot analysis with anti-RTD IgG. The amount of protein in each isolated batch of a RTD polypeptide was estimated using the BCA Protein Assay Reagent (Pierce) kit.

Antiserum against RTD(33-225)

Mutant protein RTD(33-225) was resolved by SDS-PAGE, the protein electro-eluted from gel slices, and emulsified in 50 µg portions in Freund's incomplete adjuvant. This emulsion was subcutaneously injected into a rabbit at days 1 and 26. The rabbit was bled after 2, 4, 6, 8, and 10 weeks. Antiserum obtained from the fifth bleeding was used for the immuno-analysis.

GroEL ligand assay

Immunoplates were coated with 1 µg of purified MpB GroEL (Van den Heuvel *et al.*, 1994) in coating buffer for 16 hours at 4 °C. Blocking was performed with 2% skimmed milk in PBS containing 0.05% Tween 20 for 1 h at 37 °C. Coated GroEL was incubated with 100 µl of purified PLRV at a concentration of 10 µg/ml in SEB or with similar concentrations of heterologously expressed recombinant RTD proteins (assayed with Pierce kit) in 100 µl of SEB for 16 h at 4 °C. Bound PLRV or recombinant protein was detected by anti-PLRV (DLO-Research Institute for Plant Protection) or anti-RTD at a concentration of 1 µg/ml in SEB and incubated for 3 h at 37 °C. Bound anti-PLRV and anti-RTD was detected by goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in SEB for 3 h at 37 °C.

Denaturation of RTD(33-320)

Denaturation of mutant protein RTD(33-320) was performed in PBS by addition of 2 volumes of ureum to a final concentration of 6 M, or a mixture of ureum and β-mercaptoethanol to a final concentration of 6 M and 2.5 %, respectively. Samples containing β-mercaptoethanol were heated for 5 min at 100 °C. The denaturing agents were removed by step-wise dialysis against 0.3 x PBS containing 4 M ureum, 0.3 x PBS containing 2 M ureum, and 0.3 x PBS adjacently, using a Microdialyzer system (Pierce). Solutions were then 3 times concentrated using a speed vacuum exicator.

GroEL overlay assay and virus overlay assay

The recombinant RTD polypeptides or MpB GroEL isolated from *M. persicae* (van den Heuvel et al., 1994) were run on denaturing polyacrylamide gels. After electrophoreses, gels were conditioned in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0) containing 10 % methanol for 1 h and proteins were electrotransferred onto nitrocellulose. Sheets of nitrocellulose containing the RTD polypeptides were incubated overnight with 10 µg/ml of purified MpB GroEL, and those containing MpB GroEL were incubated with purified 10 µg/ml PLRV particles. Bound MpB GroEL or PLRV particles were detected with anti-63K IgG or anti-PLRV IgGs, respectively, and alkaline phosphatase-conjugated anti-rabbit IgG. Immobilized conjugates were visualized by the addition of 5-bromo-4-chloro-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride in 0.1 M-ethanolamine-HCl, pH 9.6, containing 4 mM-$MgCl_2$. A second Western blot of both experiments was stained with amido black to verify whether equal amounts of the RTD polypeptides or MpB GroEL were transferred to the nitrocellulose membrane.

RESULTS and DISCUSSION

Isolation of recombinant RTD polypeptides

To assess whether the conserved region, corresponding to positions 184 to 225 of the RTD is involved in *Buchnera* GroEL binding, four deletion mutants of the RTD protein were designed (Fig. 1). RTD polypeptides started after the proline-rich region at the beginning of the RTD as for *Barley yellow dwarf virus* (BYDV) this region was previously shown not to be involved in binding (Filichkin *et al.*, 1997). Polypeptide RTD (33-247) ended 3 amino acids after the putative *in vivo* truncation site (Filichkin *et al.* (1997). RTD (33-225) harboured the conserved region but RTD (33-183) lacked this sequence completely. The RTD polypeptides were expressed in fusion with glutathione S-transferase (GST) and the GST moiety was removed from the RTD proteins prior to further analysis (Fig. 2a).

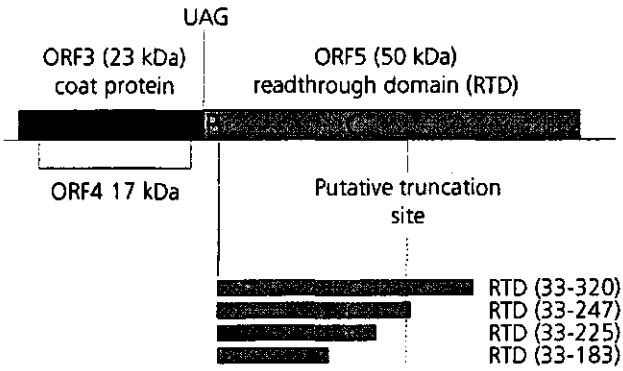

Fig. 1. Schematic representation of the first series of PLRV RTD deletion mutants. The numbers in parentheses correspond to the positions of amino acid residues of the recombinant RTD polypeptides. ORF, open reading frame; UAG, amber stop codon in PLRV RNA; P, proline-rich region; C, conserved region.

Purified RTD (33-225) was used to raise antibodies against the RTD protein and the quality of the newly derived antibody, anti-RTD, was tested on Western blots. Anti-RTD IgG detected all recombinant RTD polypeptides as well as the RTD of natural virus particles (Fig. 2b and 2c), and did not cross-react with MpB GroEL, *E. coli* GroEL or GST, underlining the specificity of the antiserum. The Western blot analysis also showed that expressing of RTD(33-320) resulted in two additional smaller protein products: one of about 40 kDa, and another one of the same size as RTD(33-247). The truncated products of RTD(33-320) might have been the result of proteolytic cleavage or premature termination of translation during expression in *E. coli*. However, it is also possible that the smallest product, which co-migrated with RTD(33-247), was produced by autocatalytic truncation as is generally observed for wild-type RTD *in vivo*.
Similar quantities of RTD (33-183), (33-225), (33-247), and (33-320), and immobilized onto ELISA plates were detected by anti-RTD. No differences in detection level of anti-RTD were found between the different RTD polypeptides.

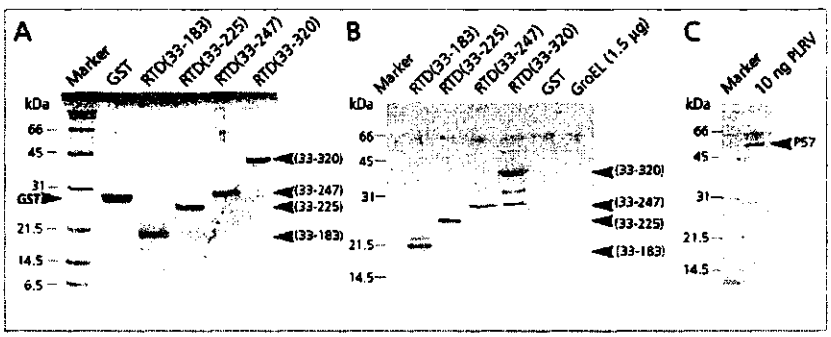

Fig. 2. Purification of RTD deletion mutants and Western blot analysis. (A) Coomassie blue-stained SDS-PAGE gel presenting purified recombinant RTD polypeptides and GST. (B) SDS-PAGE of recombinant RTD peptides, GST and GroEL followed by Western blot analysis with anti-RTD IgG. (C) SDS-PAGE of 10 ng of PLRV followed by Western blot analysis with anti-RTD IgG.

The conserved region in the RTD is involved in MpB GroEL binding

A pilot experiment was performed to investigate whether the recombinant RTD polypeptides as shown in Fig. 1 would bind MpB GroEL in a GroEL-ligand assay. Purified MpB GroEL was immobilized onto immunoplates, and similar amounts of the RTD mutant polypeptides were added and tested for their affinity to MpB GroEL using anti-RTD. As expected from previous results with recombinant RTD from BYDV (Filichkin *et al.*, 1997), mutant RTD(33-320) readily bound to MpB GroEL. Moreover, RTD deletion mutants containing the conserved region (RTD(33-225), RTD(33-247) and RTD(33-320)) had a stronger affinity for MpB GroEL than the polypeptide lacking the conserved region (RTD(33-183)) (Fig. 3). These results indicate that the GroEL-ligand assay was suitable to study differences in GroEL affinity of heterologously expressed RTD polypeptides. Apparently, the conserved region contains the residues that are involved in binding MpB GroEL, although it can not be excluded that residues located in the domain N-terminal of the conserved region contribute to MpB GroEL binding as well. Attempts to express the 23 kDa CP, which is not involved in MpB GroEL binding (van den Heuvel *et al.*, 1997), were not successful. Therefore, appropriate negative controls could not be included in the ligand assays.

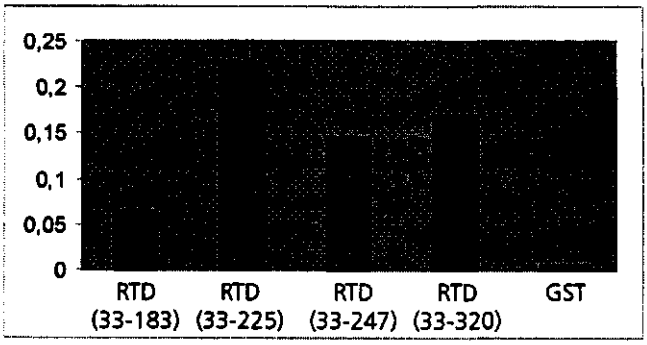

Fig. 3. Affinity binding of PLRV RTD mutants to *Buchnera* GroEL isolated from *M. persicae*. The numbers in parentheses correspond to the positions of amino acid residues of the recombinant RTD polypeptides of PLRV. The absorbance values at 405 nm (A_{405} [ELISA value]) are given.

C-terminal hydrophobic residues of the conserved region are involved in binding to MpB GroEL

The conserved region of the RTD protein consists of a central stretch of 21 amino acids and is flanked at both sites by hydrophobic regions of 8 to 9 residues (Fig. 4). In order to investigate whether one of these regions is responsible for GroEL binding, an additional set of deletion mutants was generated (Fig. 4). Mutant RTD(33-214) lacked the C-terminal hydrophobic region and mutants RTD(33-208), RTD(33-203), and RTD(33-193) lacked parts of the central region harboring the conserved amino acids D, SYG, and YNY, respectively. After removal of the GST moiety, each mutant protein was isolated from several expression batches and tested in equal amounts for affinity to MpB GroEL. Due to its low expression level, mutant polypeptide RTD(33-203) was excluded from further analysis.

The binding studies reveal differences in MpB GroEL affinity between the various mutant RTD polypeptides. Deletion mutants lacking the complete conserved region (RTD(33-183)) or parts of the conserved region (RTD(33-208) and RTD(33-214)) had a lower affinity for MpB GroEL than RTD mutants in which this region was included (Fig. 4). Deletion of the 9 C-terminal hydrophobic residues of the conserved region (RTD(33-214)) is apparently sufficient to significantly reduce MpB GroEL binding. This finding suggests that these residues are responsible for MpB GroEL binding. However, it is also possible that the deletion of the hydrophobic domain affected the structure of the entire region and, subsequently, reduced the affinity for MpB GroEL.

The structural determinants of RTD proteins in MpB GroEL binding

To assess whether the capacity to bind GroEL resides in the primary structure (amino acid sequence) or in the secondary/tertairy structure of RTD, the recombinant polypeptide RTD(33-320) was denatured using β-mercaptoethanol, 6 M ureum and heating at 100 °C, or by treatment with 6 M ureum alone. Subsequently, the denaturing agents were removed by extensive dialysis and the MpB GroEL affinity of the de natured proteins of each treatment (Fig. 5a) were compared with that of untreated RTD(33-320) (Fig. 5b). This demonstrated

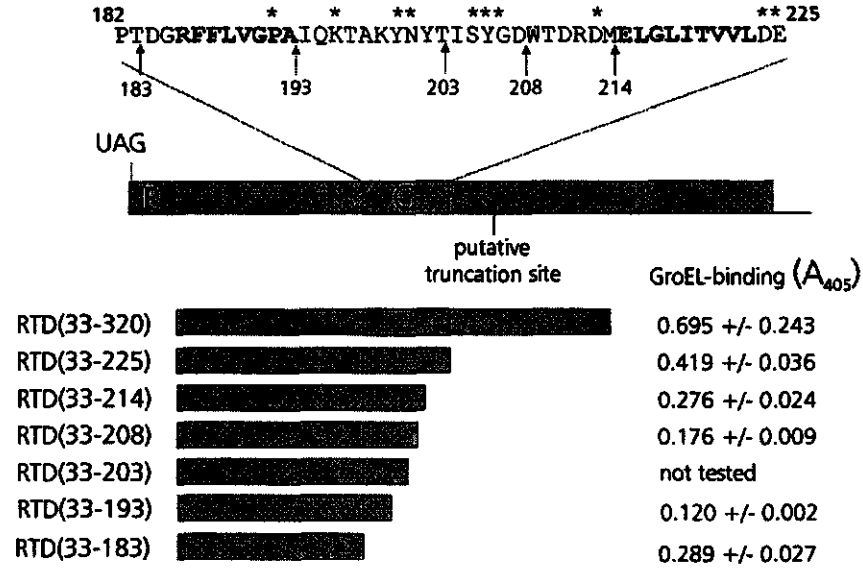

Fig. 4. Schematic presentation of all RTD deletion mutants and affinity binding of these RTD proteins to *Buchnera* GroEL. The numbers in parentheses correspond to the positions of amino acid residues of the RTD of the PLRV minor capsid protein. The GroEL-binding data to the right gives the ELISA readings in a GroEL-ligand assay of recombinant RTD polypeptides Values represents the mean ELISA readings (A_{405} ± standard errors). Mutant proteins RTD(33-193) and RTD(33-208) were tested twice, all other mutants three times. Symbols: UAG, amber stop codon; C, conserved region, P, proline-rich region; amino acids indicated with an asterisk are conserved among all luteoviruses; amino acids in bold are presenting the hydrophobic residues as indicated by van den Heuvel *et al.* (1997); arrows indicate the C-terminal amino acid of the recombinant RTD polypeptides.

that the MpB GroEL affinity of denatured RTD(33-320) was reduced by 75 % compared to non-denatured RTD(33-320), indicating that the folding of RTD(33-320) is important for MpB GroEL binding. No differences were observed between the two denaturation protocols, suggesting that di-sulfide bridges may not be present in the RTD(33-320) mutant.

The importance of the native structure of the RTD in GroEL-binding was confirmed in a GroEL-ligand assay. In this assay mutants RTD(33-183), RTD(33-225) and RTD(33-320) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with purified MpB GroEL. MpB GroEL did not bind to any of these RTD mutants (data not shown). In parallel, a regular Western blot based virus overlay assay, which has previously been demonstrated to be instrumental in studying luteovirus binding to denatured MpB GroEL or other aphid-derived proteins (van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1996 and 1998), was used as control. This showed that purified PLRV particles bound denatured MpB GroEL on Western blot (data not shown).

Both denaturation experiments with polypeptide RTD(33-320) demonstrate that native folding of the RTD sequence is likely to be required for GroEL-binding. Therefore, the reduction in MpB GroEL affinity of the RTD mutant polypeptides, which lack the hydrophobic residues at the C-terminus of the hydrophobic region (RTD(33-214)) (Fig. 4) does not necessarily indicate that this domain contains residues that directly interact with MpB GroEL. It is also possible that deletion of these hydrophobic residues has affected the overall structure of the RTD polypeptide, thus leading to reduction of GroEL binding. Determination of the tertairy structural characteristics of the RTD should aid to reveal the structure or residues which mediate the affinity for GroEL.

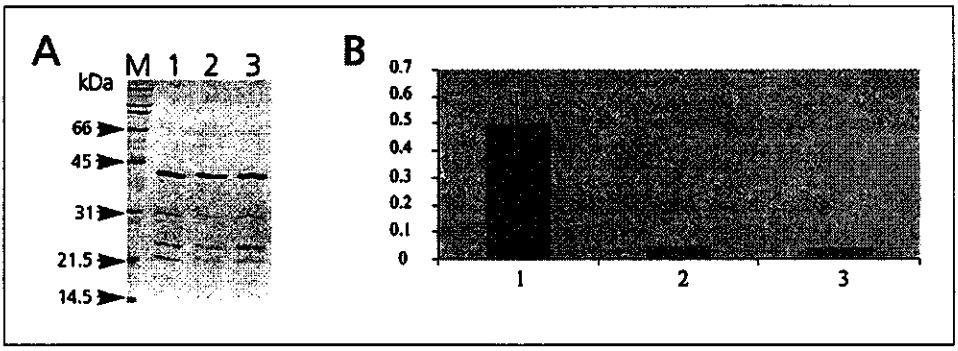

Fig. 5. Affinity-binding of non-denatured and denatured RTD(33-320) to *Buchnera* GroEL from *M. persicae*. (A) Coomassie blue-stained SDS-PAGE gel. (B) Affinity-binding to *Buchnera* GroEL. Lanes 1, non-denatured RTD(33-320); 2, RTD(33-320) treated with 6M ureum, 2.5% β-mercaptoethanol and heating; 3, RTD(33-320) treated with 6 M ureum only; M, marker. The absorbence values at 405 nm are given. The experiment was repeated three times.

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Chapter 7

Azadirachta indica metabolites interfere with the host-endosymbiont relationship and inhibit the transmission of potato leafroll virus by *Myzus persicae*

Johannes FJM van den Heuvel, Saskia A Hogenhout, Martin Verbeek and Frank van der Wilk

Department of Virology, DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, the Netherlands

Summary - The effects of neem (*Azadirachta indica* A. Juss) seed kernel extracts (NSKE) and azadirachtin on the ability of *Myzus persicae* (Sulz.) to transmit potato leafroll luteovirus (PLRV) was studied. Moreover, it was investigated whether treatments with these compounds would exert an effect on larval growth and mortality, and on the aphid intracellular symbionts. Endosymbiotic bacteria play an essential role in the performance of aphids, and in luteovirus transmission by aphids. NSKE and azadirachtin were offered to one-day-old *M. persicae* nymphs via a membrane feeding system. The neem metabolites displayed a 100% mortality at doses higher than 2560 ppm. At intermediate doses, ranging between 320 and 2560 ppm, larval growth and mortality were affected in a dose-dependent manner. The transmission of PLRV by *M. persicae* was inhibited by 55-90%. The endosymbiont population of the aphid was clearly affected by a treatment with neem metabolites as the release of their most abundant protein, *Buchnera* GroEL, into the haemocoel of the aphid was inhibited. Moreover, morphological aberrations on the bacterial endosymbionts were observed in aphids which fed on 2560 ppm of azadirachtin. At doses lower than 160 ppm of NSKE or azadirachtin, the endosymbiont population of *M. persicae*, and mortality, growth and feeding behaviour were similar to that of the untreated groups of aphids. However, PLRV transmission was still inhibited by 40-70%. The possible targets of the neem metabolites in the aphid are discussed.

Entomologica Experimentalis et Applicata 86, 253-260

INTRODUCTION

The transmission of potato leafroll virus (PLRV) by aphids is of major concern to potato growers, worldwide. The virus belongs to the genus *Luteovirus* whose species are obligately transmitted by aphids in a circulative manner (Sylvester, 1980). Virus particles are ingested along with phloem sap from infected host plants and transported transcellularly through the gut into the haemocoel and then through the accessory salivary glands (Gildow and Gray, 1993). The haemolymph acts as a reservoir in which acquired virus particles are retained in an infective form for several weeks, without replication (Eskandari *et al.*, 1979). Interactions of the virus with *Buchnera* GroEL, a chaperonin secreted into the haemocoel by the endosymbiotic bacteria (*Buchnera* spp.) of the aphid, stabilize the virus in the haemolymph (van den Heuvel *et al.* 1994, 1997). PLRV and other species of the genus *Luteovirus* exhibit a high and specific affinity for *Buchnera* GroEL in *in vitro* binding assays (van den Heuvel *et al.*, 1997; Hogenhout *et al.*, 1998; Filichkin *et al.*, 1997). Furthermore, *in vivo* interfering with the luteovirus-*Buchnera* GroEL interaction resulted in loss of particle integrity and concomitant loss of infectivity of the virus (van den Heuvel *et al.*, 1994, 1997).

These observations raised the possibility that interfering with the relationship between endosymbionts and aphids may contribute to the control of luteovirus transmission by aphids. Antibiotics and thermal treatment have been reported to reduce the number of endosymbiotic microorganisms in homopterous insects (Noda and Saito, 1979; Houk and Griffiths, 1980; Chen *et al.*, 1981), and, more recently, Raguraman and Saxena (1994) showed that secondary plant metabolites possess similar activities. The endosymbiont population of the brown planthopper, *Nilaparvata lugens* (Stål) significantly declined after feeding on rice treated with extracts from the neem tree, *Azadirachta indica* A. Juss (Meliaceae). In view of this, we have investigated whether the competence of *M. persicae* to vector PLRV is affected by extracts of neem seed kernels (NSKE) and azadirachtin, its major bio-active compound (Rembold, 1989). We investigated virus transmission and aphid performance (mortality and growth rate) after *M. persicae* nymphs had been fed through membranes on artificial diets containing various dilutions of NSKE and azadirachtin. Western blot analysis was conducted to reveal possible allelopathic effects of NSKE on the endosymbionts of the aphid, and on the presence of *Buchnera* GroEL in the haemolymph, respectively.

MATERIAL AND METHODS

Aphids

Myzus persicae biotype WMp2 was reared on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a greenhouse compartment at 20 ± 3 °C with a photoperiod of 16 h per day. Cohorts of similarly-aged nymphs were produced by daily transfer of mature apterae, confined in leaf cages, to new oilseed rape plants. One-day-old nymphs were used throughout this study as they are the most efficient vectors of PLRV (van den Heuvel *et al.*, 1991).

Virus source and purification

The Wageningen isolate of PLRV (van der Wilk *et al.*, 1989) was maintained by repeated single aphid transfers on seedlings of *Physalis floridana* Rydb., and purified using a modified enzyme-assisted purification procedure (van den Heuvel *et al.*, 1991). Purified virus was stored at -80 °C.

Neem seed kernel extracts and azadirachtin

Seed kernels of *A. indica* were kindly provided by R.C. Saxena (ICIPE, Nairobi, Kenya). An extract of neem seed kernels (NSKE) was prepared by soaking 1 g of ground kernels in 10 ml of water for three days at 4 °C. The debris was then removed by centrifugation, and the supernatant aliquoted and stored at –20 °C. Samples from this NSKE stock were used throughout the study. Azadirachtin (Sigma, St. Louis, MI) was diluted in water to 1 mg/ml, aliquoted, and stored at –20 °C.

Membrane feeding, transmission experiments and aphid performance

One-day-old *M. persicae* nymphs were fed on artificial diet MP148 (Harrewijn, 1983) containing a two-fold dilution series of the NSKE or azadirachtin stocks during a feeding access period of 72 h. The nymphs were then transferred to fresh diets containing NSKE or azadirachtin, and purified PLRV at a concentration of 10 µg/ml for an acquisition access period (AAP) of 24 h. Membrane feeding experiments were done at 20 ± 0.1 °C as described before (van den Heuvel *et al.*, 1991). To determine the percentage of viruliferous *M. persicae*, the nymphs were individually placed on *P. floridana* seedlings for an inoculation access period (IAP) of four days at 20 ± 0.1 °C. Inoculated plants were transferred to a greenhouse compartment at 24 ± 2 °C, L16:D8 for symptom development. The transmission experiments were repeated three times with cohorts of 30 nymphs per treatment. Nymphs feeding on artificial diets without NSKE or azadirachtin served as a reference.

In a parallel experiment, we determined the weight increase of the nymphs over the eight-day period in which virus acquisition and transmission was studied. Per treatment, three groups of 10 one-day-old *M. persicae* nymphs were weighed individually just before membrane feeding, and immediately after the IAP on *P. floridana*. The percentage of mortality of the nymphs was recorded after four days, immediately after completing the feeding and acquisition access periods on artificial diet. Two groups of 30 one-day-old *M. persicae* nymphs were tested for each dose of NSKE and azadirachtin.

The data was subjected to analysis of variance followed by multiple range tests using the computer programme STATGRAPHICS (Statistical Graphics Corporation).

Recording honeydew excretion

The number of honeydew droplets excreted by *M. persicae* nymphs feeding on artificial diets containing 0, 160 and 1280 ppm of NSKE or azadirachtin was recorded using a honeydew clock as described before (van den Heuvel and Peters, 1990). Three cohorts of approximately 20 aphids were tested per dilution of NSKE and azadirachtin.

Embedding of aphids

M. persicae nymphs were fixed in 0.1 M cacodylate buffer (CAB), pH 7.2, containing 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde for 3 h at room temperature, followed by an overnight incubation step in CAB containing 2% paraformaldehyde and 3% glutaraldehyde. The nymphs were then incubated in 1% (w/v) osmiumtetroxide for 4 h, dehydrated, and embedded in LR White Embedding Resin (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin

sections, mounted on single slot copper grids with a 0.7% Formvar support layer, were stained with 2% uranyl acetate and lead citrate (Reynolds, 1963) and examined using a Philips CM12 electron microscope.

Western blot analysis of haemolymph samples

To collect haemolymph from *M. persicae*, nymphs were submerged in 0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, and the tips of their cornicles excised. Samples containing the exudate of five nymphs in 20 µl were prepared and subjected to SDS-PAGE and Western blot analysis to reveal the presence of *Buchnera* GroEL (van den Heuvel *et al.*, 1994) using an antiserum kindly provided by Prof. H. Ishikawa (University of Tokyo, Japan).

RESULTS

Effects of NSKE and azadirachtin on aphid survival and growth

Since data on the mortality response of neem metabolites on larval stages of aphids are lacking, the mortality of one-day-old *M. persicae* nymphs was established. Two-fold dilution series of the NSKE and azadirachtin stocks in artificial diet were offered to these nymphs during a feeding access period of four days after which aphid mortality was recorded. *M. persicae* nymphs showed a dose dependent mortality response to NSKE and azadirachtin (Fig. 1A and B). At concentrations higher than 5000 ppm (v/v) NSKE, or 5000 ppm [w/v]) azadirachtin, 100% mortality was observed within the experimental period. At 2560 ppm of NSKE and azadirachtin, aphid mortality was between 80-90% but this percentage levelled off gradually with decreasing dose. Aphid mortality at 160 ppm or at lower concentrations of NSKE (Fig. 1A) or azadirachtin (Fig. 1B) was comparable to that of the control group of aphids ($P<0.001$).

To assess to what extent azadirachtin and NSKE influence larval growth, it was investigated at which doses of either compound the increase of weight of *M. persicae* nymphs was affected. As shown in Fig. 1C, doses of 320 ppm or more of NSKE significantly reduced weight increase ($P<0.05$). For azadirachtin (Fig. 1D) this parameter was affected only at 640 ppm or higher doses ($P<0.05$).

Effects of NSKE and azadirachtin on the transmission of PLRV

Based on the dose response of NSKE and azadirachtin on growth and mortality of *M. persicae* nymphs, PLRV transmission by *M. persicae* was investigated using doses ranging between 20 and 2560 ppm. Prior to the virus transmission studies, the honeydew excretion of the nymphs was recorded during the AAP. Honeydew excretion is an appropriate measure for diet intake (Sylvester, 1988), and thus a useful parameter for the amount of purified virus ingested by the aphid. Furthermore, the percentage of nymphs eventually transmitting PLRV has been shown to be linearly related to the amount of virus acquired from artificial diets (van den Heuvel *et al.*, 1991). The honeydew excretion of *M. persicae* nymphs was recorded during a six-hour-period in the AAP on different concentrations of NSKE or azadirachtin. Control groups of nymphs which fed on MP148 without neem metabolites added produced on average 1.15 ± 0.45 (mean $\pm$ standard deviation) honeydew droplets per nymph per hour. A significant reduction in honeydew production, and thus in diet ingestions and virus acquisition, was seen at concentra-

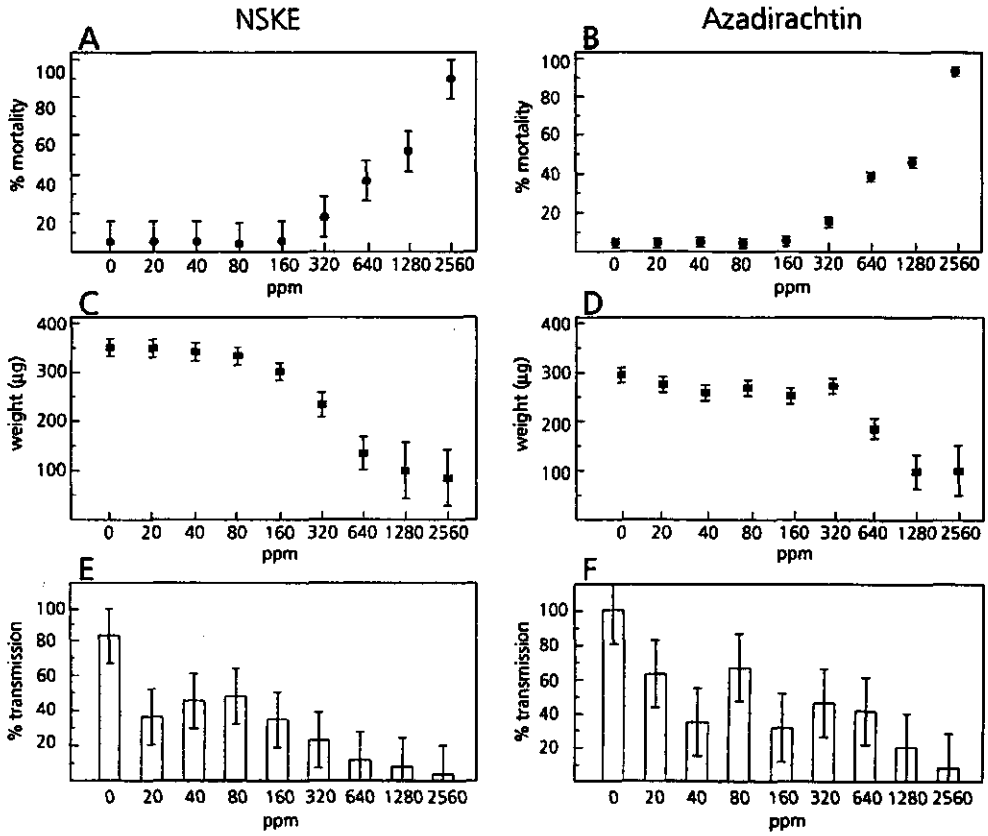

Fig. 1. The effects of NSKE (A, C, E) and azadirachtin (B, D, F) on the mortality and weight of *M. persicae*, and on the aphids' ability to transmit PLRV. One-day-old nymphs were allowed to feed for four days on artificial diets containing the indicated concentrations of azadirachtin and NSKE. Error bars represent the 95% confidence intervals for the means.

tions of NSKE and azadirachtin higher than 160 ppm ($P<0.001$). At 1280 ppm, aphids still produced an average of 0.41 ± 0.25 (azadirachtin), or 0.18 ± 0.18 (NSKE) honeydew droplets per nymph per hour. A phagorepellent effect of the neem metabolites was not observed during our studies. At 160 ppm of azadirachtin and NSKE, nymphs excreted 1.17 ± 0.39 and 0.96 ± 0.30 honeydew droplets per nymph per hour, respectively, which was not significantly different from the amount of droplets produced by the control group ($P>0.05$).

Figures 1E and F show that treatments with both NSKE and azadirachtin significantly affected the transmission of PLRV ($P<0.001$). At the highest doses offered (2560 ppm), the transmission of PLRV was reduced by 90% (azadirachtin) or 95% (NSKE), and at intermediate doses, ranging between 320 and 2560 ppm, by 55-90%. Strikingly, at doses of 160 ppm or lower, NSKE and azadirachtin still inhibited virus transmission, by 40-60% (Fig. 1E), and 40-70% (Fig. 1F), respectively. Even at the lowest dose administered (20 ppm), virus transmission was only 60% (azadirachtin, Fig. 1F) or 45% (NSKE, Fig. 1E) of that of the reference groups of aphids.

Effects of NSKE on the aphid's endosymbiont

To determine whether treatments with neem metabolites affected the intracellular symbiont of *M. persicae*, one-day-old nymphs were fed for four days on MP148 containing 0, 40, 160, and 2560 ppm of azadirachtin or NSKE and prepared for electron microscopy. This revealed that morphological aberrations of the intracellular symbiotic bacteria were visible only at the highest concentration of azadirachtin (Fig. 2) in about 10% of the host cells (mycetocytes) harbouring the endosymbionts. The cytoplasm of the affected mycetocytes contained numerous small vesicles, and the bacteria appeared swollen and strongly deformed. None of these aberrations have been observed in association with age-induced dissociation of the mycetocytes or lysis of the endosymbionts. Bacteria in untreated nymphs were oval-shaped or found in some stage of binary fission. The bacteria in nymphs treated with lower doses of azadirachtin or with NSKE were all similar in appearance to those of the untreated nymphs.

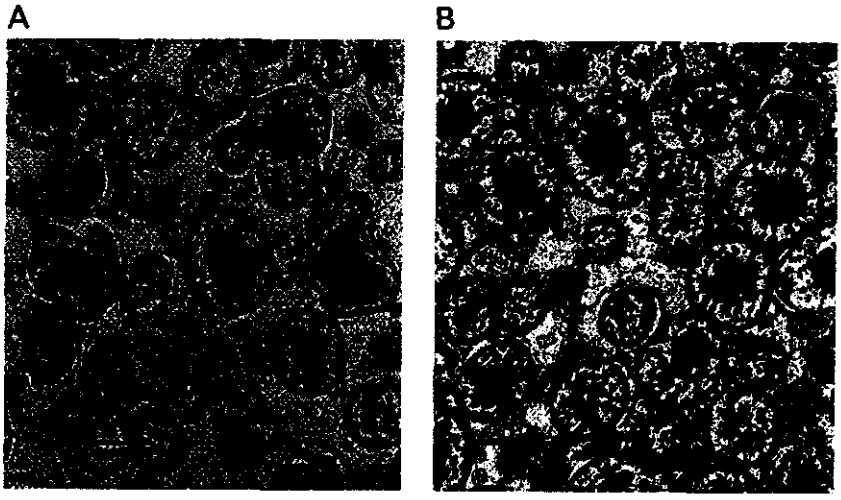

Fig. 2. Electron micrographs of endosymbiotic bacteria of *M. persicae* nymphs which fed for a period of four days on artificial diets containing 2560 ppm (A) or 0 ppm (B) azadirachtin. Bar corresponds to 2 µm.

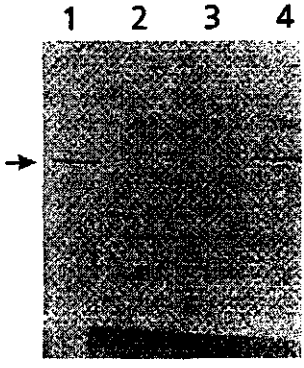

Fig. 3. Western blot analysis using anti-*Buchnera* GroEL IgG of haemolymph samples of *Myzus persicae* nymphs which fed for four days on artificial diets containing 0 (lane 1), 2560 (lane 2), 640 (lane 3) and 160 (lane 4) ppm of NSKE. *Buchnera* GroEL is indicated by an arrow.

Previously, we have demonstrated that *Buchnera* GroEL could not be detected in the haemolymph of aphids fed on antibiotics with a bacteriostatic mode of action, such as (chlor)tetracycline and rifampicine (van den Heuvel *et al.*, 1994). To investigate whether NSKE and azadirachtin are also able to interfere with the protein synthesis and release by the endosymbiont, haemolymph samples of nymphs treated with 0, 160, 640 and 2560 ppm of the neem metabolites were analysed for the presence of *Buchnera* GroEL. Western blot analysis showed that *Buchnera* GroEL was readily detected in the control samples consisting of haemolymph of aphids that fed on artificial diet only (Fig. 3, lane 1). However, at 2560 and 640 ppm the amount of *Buchnera* GroEL was strongly reduced, and poorly (in case of azadirachtin; not shown) or not at all (NSKE; Fig. 3, lane 2 and 3) detectable. At concentrations of 160 ppm, the amount of *Buchnera* GroEL present in the haemolymph of *M. persicae* was comparable to that of the samples taken from untreated nymphs (Fig. 3, lane 4).

DISCUSSION

The use of secondary neem metabolites as natural control agents for about 200 insect species from different orders is well documented (e.g. Schmutterer, 1987; Rembold, 1989; Saxena, 1989). The biological activities of these compounds range from behavioural to physiological effects. The majority of these insects are phytophagous with biting-chewing mouthparts and the compounds are acquired by eating contaminated leaves. Relatively few studies deal with insects with piercing-sucking mouthparts, even though neem metabolites are systemically translocated to all parts of a plant which would make them especially effective against these insects (reviewed in Saxena, 1995). Furthermore, homopterous insects are the major vectors of plant viruses, and interfering with their vector competence may offer interesting opportunities to control the spread of these viruses in a manner that fits well into integrated pest management programmes. Neem metabolites have been shown to influence feeding activities of plant- and leafhoppers and aphids (Nisbet *et al.*, 1993; Mordue *et al.*, 1996), which resulted in reduced transmission of rice tungro associated viruses (Saxena *et al.*, 1987; Narasimhan and Mariappan, 1988; Mariappan *et al.*, 1988; Abdul Kareem *et al.*, 1989), and PLRV (Nisbet *et al.*, 1996), respectively. Neem metabolites have also been shown to affect the endosymbiont population of *N. lugens*. These yeast-like symbiotes are, like those of aphids, intracellular and localized in mycetocytes in the fat body (Noda, 1979; Noda *et al.*, 1995). Endosymbiotic bacteria play a crucial role in aphids' physiology and are essential for the post-embryonic development of aphids (Houk and Griffiths, 1980; Baumann *et al.*, 1995), and are an important factor in the transmission of luteoviruses by aphids (van den Heuvel *et al.*, 1994, 1997; Filichkin *et al.*, 1997).

In this study we have shown that the effects NSKE and azadirachtin exert on young *M. persicae* nymphs can be divided into three distinct phases. At doses of 5000 ppm or higher, neem metabolites are toxic to aphids and kill the insect within a short period of time. At intermediate doses, ranging between 320 and 2560 ppm, a clear response was observed on the endosymbiont population of *M. persicae*: morphological aberrations were observed (Fig. 2), and the release of *Buchnera* GroEL into the haemocoel of the aphid was reduced (Fig. 3). *Buchnera* GroEL, the most abundant protein produced by the aphids' endosymbiont, could not be detected in the haemolymph of aphids treated with the neem-derived compounds in the aforesaid dose range. The neem metabolites induce an identical effect on the presence of *Buchnera* GroEL in the aphid's haemolymph to that previously observed by using antibiotics to inhibit the prokaryotic protein synthesis (van den Heuvel *et al.*, 1994). For both the neem- (Fig. 1) and the antibiotic-treated aphids (van den Heuvel *et al.*, 1994), the reduced levels of *Buchnera* GroEL in the haemolymph of the aphids coincided with a strong reduction of the ability of the aphids to

transmit PLRV. It is yet to be revealed whether the lack of *Buchnera* GroEL destabilized the virus in the haemolymph as was seen for PLRV in antibiotic-treated aphids (van den Heuvel *et al.*, 1994). Whether neem metabolites have a direct bactericidal effect on the aphids' endosymbionts could not be determined unequivocally because the aphid endosymbiont (*Buchnera* sp.) is not culturable due to its adaptation to the intracellular life (Ishikawa, 1989; Baumann *et al.*, 1995). However, the growth of its closest free-living relative, *Escherichia coli* (Munson *et al.*, 1991), was not affected even when it was cultured in the presence of 5000 ppm NSKE or azadirachtin (data not shown). This indicates that these compounds do not possess a direct bactericidal or bacteriostatic mode of action, and suggests that the primary endosymbiont of the aphid is not the prime target for neem metabolites. It has already been demonstrated for a number of insect species that azadirachtin blocks several trophic factors located in the central nervous system resulting in changed hormone titres (Liu, 1974; Schlüter *et al.*, 1985; Sieber and Rembold, 1983; Subrahmanyam *et al.*, 1989). Effects of juvenile hormone on the plasma membrane of symbiotic bacteria have been reported (Liu, 1973, 1974).

Within the 2560-320 ppm range, the malfunctioning of the endosymbiotic bacteria may also have contributed to the inhibited growth of azadirachtin- and NSKE-treated *M. persicae* (Fig. 1B and C). Aphids are highly dependent on their endosymbionts, and it has been reported that their elimination by antibiotics or other treatments leads to changed feeding behaviour, reduced growth, sterility, and eventually death (Wilkinson and Douglas, 1995; Douglas, 1989; Ishikawa, 1989; Sasaki *et al.*, 1991; Ohtaka and Ishikawa, 1991; Prosser and Douglas, 1991).

A very remarkable observation is that at 160 ppm or lower doses of NSKE and azadirachtin, the only parameter affected is the ability of the aphids to transmit PLRV. Virus transmission by *M. persicae* relative to control groups is inhibited by 50-60% and 40-70% for NSKE and azadirachtin, respectively (Fig. 1E and F). Effects on *Buchnera* GroEL production, honeydew excretion, growth and mortality of aphids treated with the neem metabolites at these low concentrations were not apparent. The mode of action of azadirachtin at this low level is not yet understood but will be investigated in more detail as it might provide a clue to a better understanding of the molecular mechanisms involved in luteovirus transmission by aphids at the haemolymph-salivary gland interface. Particularly so, because localization studies showed that azadirachtin associated with basal plasma membranes (Garcia *et al.*, 1989) which led to the suggestion that these membranes contain high-affinity binding sites for the compound. As luteoviruses will have to be recognized by receptors in the basal plasma membrane of the accessory salivary gland of an aphid in order to be successfully transmitted (Gildow and Gray, 1993), it may well be that azadirachtin interferes with this crucial step in PLRV transmission.

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Chapter 8

General Discussion

8.1 Introduction

The experiments described in thesis provide more information on the transmission of luteovirus particles by aphids. Experimental evidence was obtained demonstrating that GroEL, a protein belonging to the chaperonin-60 family of proteins and produced by bacterial endosymbionts (genus *Buchnera*) of aphids, is involved in the persistent nature of luteoviruses (Chapter 2; van den Heuvel *et al.*, 1994). Mutational analyses showed that residues of the equatorial domain of *Buchnera* GroEL are involved in binding of PLRV particles (Chapter 3 and 4). Furthermore, it was revealed that the readthrough domain of the minor capsid protein of a luteovirus particle determines *Buchnera* GroEL binding and not the major capsid protein (Chapter 5). By mutational analysis of the readthrough domain of PLRV it was found that a conserved region of the readthrough domain is likely to house the determinants responsible for binding to GroEL (Chapter 6).

GroEL proteins are chaperonins that are generally functional in the cytosol of bacteria or in cell organelles of eukaryotes. Therefore, it is not clear why and how *Buchnera* GroEL is secreted (actively or passively) into the aphid's haemolymph by *Buchnera* spp. and how *Buchnera* GroEL protects the virus particle against degradation in the haemolymph. However, as is shown in this chapter, secretion of GroEL homologues by bacterial endosymbionts and pathogenic bacteria may be a more common phenomenon. The possible function of GroEL homologues in extacellular environments will be addressed as well as more specifically the role of *Buchnera* GroEL for the aphid. Moreover, some examples of potential agents, which block virus infection, will be analyzed and by using these examples potential applications to inhibit luteovirus transmission by aphids or luteovirus transport in plants will be discussed.

8.2 Possible functions of *Buchnera* GroEL in the aphid

GroEL homologues of *Buchnera* spp. from different aphid species are immunologically closely related and share more than 80% sequence identity with the *Escherichia coli* heat shock protein GroEL, a member of the chaperonin-60 family (Ohtaka *et al.*, 1992; Filichkin *et al.*, 1997; Chapter 3 and 5). In contrary to *E. coli*, *Buchnera* spp. continuously overproduce GroEL; *Buchnera* GroEL production is comparable to GroEL of *E. coli* when under stress (Ishikawa, 1982; Baumann *et al.*, 1996). Moreover, *Buchnera* GroEL 14-mers are found in high concentrations outside bacterial cells in the haemolymph of aphids (Chapter 2 and 5). It is obvious that *Buchnera* GroEL is not only abundantly produced and secreted into the haemolymph solely to assist luteovirus transmission. A more plausible explanation is that luteoviruses use characteristics of *Buchnera* GroEL that are already present for their own benefit.

GroEL of *E. coli* is among the best-characterized bacterial proteins with important functions in the cytosol. *E. coli* GroEL 14-mers are involved in folding of non-native proteins inside the cytosol of a bacterium (Ellis and Hartl, 1996; Lorimer, 1996; Martin and Hartl, 1997; Netzer and Hartl, 1998; Weber *et al.*, 1998). *E. coli* GroEL also interacts with cytoplasmic proteases to degrade unfolded proteins (reviewed in: Sherman and Goldberg, 1996), is involved in export and import of proteins from the bacterial cell (reviewed in: Kumamoto, 1991), stabilizes lipid membranes by binding (Torok *et al.*, 1997), while moreover the monomeric form is involved in mRNA protection (Sohlberg *et al.*, 1993; Georgellis *et al.*, 1995). *Buchnera* GroEL complement *E. coli* GroEL in *groE* mutants of *E. coli* (Ohtaka *et al.*, 1992), indicating that in the cytosol both exhibit similar functions.

Relatively limited studies have been performed on the possible functions of GroEL outside a bacterial cell. GroEL homologues of both symbiotic and pathogenic bacteria are usually highly expressed compared to free-living bacteria and are also found in the extracellular environment of the cell where they appear to have an important function. GroEL is abundantly produced by the endosymbiotic bacteria in five systems investigated so far: *Buchnera* spp. of aphids (Ishikawa, 1982; Van den Heuvel *et al.*, 1994; Baumann *et al.*, 1996), X-bacteria of *Amoeba proteus* (Jeon, 1987; Choi *et al.*, 1991), endosymbiotic bacteria of three species of weevils (*Sitophilus oryzae*, *S. granarius*, and *S. zeamais*) (Charles *et al.*, 1995), symbiotic bacteria of tsetse flies, (Aksoy, 1995), and bacterial endosymbionts of the whitefly *Bemisia tabaci* (Morin *et al.*, 1999). The presence of extracellular GroEL is not studied in in weevils and tsetse flies, but found in the other three systems. Recently, it was demonstrated that a GroEL homologue of bacterial symbionts of the whitefly *B. tabaci* is also involved in the circulative transmission of *Tomato yellow leaf curl virus* (TYLCV; *Geminiviridae*) (Morin *et al.*, 1999). Like *Buchnera* GroEL of aphids, the whitefly GroEL homolog seems to protect TYLCV from destruction during its passage through the hostile environment of the haemolymph of its insect vector. Furthermore, high production of extracellular GroEL homologues have been reported for numerous pathogenic bacteria (Gillis *et al*, 1985; Hunter *et al.*, 1989; Ensgraber and Loos, 1992; Dai *et al.*, 1993; Jensen *et al.*, 1993; Ando *et al.*, 1995; Kirby *et al.*, 1995; Lema and Brown, 1995; Phadnis *et al.*, 1996; Yamaguchi *et al.*, 1996; Dunn *et al.*, 1997; Garduno *et al.*, 1998; Frisk *et al.*, 1998).

Although for symbiotic bacteria the role of GroEL in maintaining symbiosis still remains to be confirmed, for pathogenic bacteria the presence of extracellular GroEL has shown to be crucial for survival intracellularly. GroEL homologues of *Salmonella typhimurium* and *Haemophilus ducreyi* are localized on the cell surface of bacteria and are directly or indirectly involved in attachment to host cells (Ensgraber and Loos, 1992; Frisk *et al.*, 1998). Furthermore, the GroEL homologue of *Helicobacter pylori* binds to urease, a virulence protein produced by the bacterium, outside the bacterial cell and the complex is absorbed onto

the surface of the bacterium (Evans *et al.*, 1992; Phadnis *et al.*, 1996). Urease neutralizes the environment of the bacterium in the stomach of the human host and is therefore essential for survival during pathogenesis of the bacterium (Evans *et al.*, 1992; Phadnis *et al.*, 1996). Thus, GroEL may very well be necessary to chaperone virulence factors in the extracellular environment of bacteria. Moreover, the GroEL homologue of *Legionella pneumophila*, a human pathogen, is found in the endosomal space of the eukaryotic host cell in between replicating intracellular bacteria. Mutants of *L. pneumophila* in which extracellular GroEL is not found are not replicating and survive solely for three hours in the macrophage of the host (Fernandez *et al.*, 1998). It is not known whether GroEL of *L. pneumophila* functions as a chaperone of virulence factors or interacts directly with the host.

Taking into account the functions of extracellular GroEL of pathogenic bacteria, *Buchnera* GroEL may be involved in attachment of *Buchnera* spp. to the cell membrane of mycetocytes or may protect bacterial derived proteins during transport. Investigations have shown that *Buchnera* spp. complement the aphid's diet by synthesizing vitamins, sterols, and aromatic amino acids (Douglas, 1988; Douglas and Prosser, 1992; Munson and Baumann, 1993; Kolibachuk *et al.*, 1995; Lai *et al.*, 1995). *Buchnera* spp. may also synthesize proteins for maintaining symbiosis. The possibility that *Buchnera* GroEL could possibly be involved in transport of a bacterial derived protein, could also explain the association of luteovirus particles and *Buchnera* GroEL in the haemolymph of aphids. Similar to bacterially synthesized products, PLRV particles may use the protective function of *Buchnera* GroEL during transport. The finding that hydrophilic residues of the equatorial domain are binding PLRV and not residues in the apical domain (chapter 3 and 4) supports this hypothesis; MpB GroEL does not function as a foldase but exhibits a protective function in the haemolymph of aphids. So far interactions of extracellular GroEL and bacterial proteins have not been investigated. It is therefore unknown whether this is a general feature or a phenomenon unique to luteoviruses and *Buchnera* GroEL.

8.3 The interaction of *Buchnera* GroEL and luteovirus particles

In vitro studies clearly show that *Buchnera* GroEL has a strong affinity for luteovirus particles, which may suggest that this protein directly interacts with the virus particle in the haemolymph of aphids. It is possible that *Buchnera* GroEL protects virus particles from proteases in the haemolymph, by preventing proteases from reaching the major coat protein by steric hindering. Usually protease synthesis is induced after triggering of the immune system of insects (Dimopoulos *et al.*, 1996; 1997). Therefore, one may hypothesize that specific features of luteoviruses trigger the production of anti-viral agents by the aphid's immune response.

The immune system of aphids is poorly investigated. Insects lack adaptive immune systems, whereas the innate immune system is well developed, which includes cellular and humoral immune responses (reviewed in Gillespie *et al.*, 1997). The cellular response of insects includes hemocytes that attach to invading organisms. A humoral response to invading pathogens is the synthesis of a variety of anti-microbial proteins and peptides, which are secreted into the haemolymph by fat body cells and hemocytes (Iwanaga *et al.*, 1998). In mosquitoes, one of these compounds is a serine protease (Dimopoulos *et al.*, 1996; 1997). A humoral activation mechanism, the Toll pathway, is shown to be conserved among invertebrates, higher vertebrates and plants (reviewed in: Hultmar, 1994; Wilson *et al.*, 1997; Medzhitov *et al.*, 1998; Vogel, 1998), indicating that aphids may have a similar system as well. The Toll pathway consists of the *toll* gene encoding a receptor protein that picks up signals at the cell membrane and sends them to the nucleus by a conserved signalling scheme

(Lemaitre *et al.*, 1996). This will activate Rel-type transcription factors that regulate the transcription of several defense genes.

The initial non-self recognition that activates the innate immune response to viruses of insects is poorly understood. Structures common among invading pathogens, e.g. lipopolysaccharides (LPS) or peptidoglycan (PG), are recognized by proteins in the haemolymph or by the Toll receptor directly which elicit immune reactions specific to bacteria or fungi, respectively (Schumann *et al*, 1990; Kang *et al.*, 1998). It is possible that coat proteins of luteoviruses trigger the production of virus specific proteases by the immune system of aphids. Binding of *Buchnera* GroEL to the readthrough domain may prevent the recognition of virus particles as non-self by aphids (molecular mimicry), and the immune response will not be triggered. *Buchnera* GroEL is likely the most suitable candidate for this function, since cell organelles and the cytosol of eukaryotes contain proteins that are highly homologous to bacterial GroEL. Thus, when aphids would produce degradive enzymes against *Buchnera* GroEL they will also destroy their own proteins. It has been shown that higher vertebrates synthesize antibodies, which specifically recognize bacterial GroEL epitopes thereby preventing the development of auto-immune diseases (Mustafa *et al.*, 1996). Insects do not have an adaptive immune response and, therefore, may not be capable to differentiate between self and non-self in case proteins are highly homologous. *Buchnera* GroEL may bind bacterial derived proteins for the same reason (see section 8.2).

8.4 Possible secretion mechanisms of GroEL by *Buchnera*

The analyses in Chapter 5 reveal that *Buchnera* GroEL is found in the haemolymph in its 14-meric form. GroEL in the haemolymph could originate from degenerating symbionts excluded by the mycetocytes (Ponsen, 1972; Verbeek and Van den Heuvel, 1994). It is also possible that complete mycetocytes degenerate, since during growth of aphids the number of symbiont-containing mycetocytes gradually decreases, whereas the amount of bacteria in the remaining mycetocytes increases (Douglas and Dixon, 1987). Degeneration of *Buchnera* may be directed by the aphid or by the bacterium itself. There are some examples known of bacterial autolysis. Autolysis of *Streptococcus pneumoniae* is regulated genetically (Paton *et al.*, 1993) and virulence of *S. pneumoniae* decreases when the autolysis gene is inactivated (Berry *et al.*, 1992). GroEL, urease and putative virulence factors of *H. pylori* are proposed to be released by autolysis as well; absorption of urease and GroEL onto the surface of intact bacteria then follows (Phadnis *et al.*, 1996).

Secretion mechanisms that could actively secrete GroEL have not been identified so far. Moreover, a GroEL 14-mer is a complex of 13.7 nm in diameter (Hartl, 1994) and bacterial secretion mechanisms able to secrete proteins of such size are neither known. However, studies on *L. pneumophila* have shown that it is very likely that GroEL is actively secreted, since lysis of *Legionella* bacteria in infected macrophages was not observed and extracellular GroEL was found to be crucial for infection (Garduno *et al.*, 1998). There are at least three basic secretion pathways responsible for the export of proteins into the extracellular space of bacteria (reviewed in Salmond and Reeves, 1993; Alfano and Collmer, 1997; Gauthier and Finlay, 1998). The type II *sec*-dependent or general secretory pathway requires that the secreted protein possess a hydrophobic N-terminal signal sequence which is cleaved during secretion (Pugsley, 1993), and the type I or ATP-binding cassette (ABC) export system requires a C-terminal recognition sequence of the secreted protein (Binet *et al.*, 1997). The gene encoding *Buchnera* GroEL of *M.persicae* lacks these signal sequences (Chapter 3). Therefore, the most interesting secretion system regarding to putative GroEL secretion is the type III secretion system. Similarly to extracellular GroEL, the type III secretion system is

unique to animal and plant pathogens and bacterial symbionts, and is rarely found in non-pathogenic organisms (Groisman and Ochman, 1996). It is reponsible for the secretion of virulence proteins or nodulation factors of *Yersinia* sp., *Erwinia* sp., Pseudomonads, Xanthomonads, *Shigella* sp., *Salmonella* sp., and *Rhizobium* sp. (Van Gijsegem *et al.*, 1993; Huang *et al.*, 1995). The type III pathogenicity genes form a pilus-like structure that provides a pore through the inner and outer membrane of the bacterium and through the membranes of the host cell (Cornelis and Wolf-Watz, 1997). In this way the bacterium is capable to transfer proteins directly from the cytoplasma of the bacterium into the host cell. Secreted proteins by the type III system do not need a signal peptide sequence to be secreted. Thick filamentous appendages (invasomes) of 60 nm in diameter have been observed on the surface of *S. typhimurium* upon contact with animal host cells (Ginocchio *et al.*, 1994). As is mentioned above, GroEL of *S. typhimurium* is found at the outside of the bacterial cell and is probably involved in attachment. It may not seem unlikely that *Buchnera* GroEL is actively secreted by an invasome-like structure of *Buchnera* sp. .

8.5 The interaction of luteoviruses and *Buchnera* GroEL

In order to develop agents that prevent PLRV transmission by aphids, it is necessary to identify binding domains of the PLRV particle and *Buchnera* GroEL, and to determine the interaction at the molecular level. It was demonstrated by mutation analysis of MpB GroEL that hydrophylic residues in both the N- and C-terminal equatorial regions are involved in luteovirus particle binding (Chapter 4). Structure analysis of computer-generated *Buchnera* GroEL monomers demonstrate that the equatorial regions assemble into a single equatorial domain. Therefore, the N- and C-terminal luteovirus-binding residues could possibly form one luteovirus-binding site. Since the aphid heamolymph contains mainly *Buchnera* GroEL 14-mers (chapter 5) and *Buchnera* GroEL 14-mers bind luteovirus particles *in vitro* (Filichkin *et al.*, 1997), it is likely that in the haemolymph 14-mers bind luteovirus particles as well. It was also demonstrated that regions in the RTD of luteovirus particles are likely to be involved in the interaction to GroEL. Thus, N- and C-terminal residues of the equatorial domain in the *Buchnera* GroEL 14-mer structure should be accessible for the RTD, which protrudes from the surface of a luteovirus particles.

Localization of the luteovirus-binding residues in the GroEL 14-mer structure showed that the N-terminal residues are located towards the cavity of the 14-mer, whereas C-terminal residues are located on the surface of the GroEL 14-mer (Fig. 1a). A side-view of the GroEL 14-mer demonstrates that the N- and C-terminal luteovirus-binding residues lay behind each-other inside a cavity near the equatorial plain of the GroEL 14-mer (Fig. 1b). Since the structure of the RTD has not been revealed so far, it is not known whether the RTD fits inside the cavity, and will reach the N-terminal residues. It should be taken into account that the structure of GroEL changes extensively in the presence of ATP (Boisvert *et al.*, 1996). The structural change of GroEL may modify the N- and C-terminal luteovirus-binding residues as well, and this may disturb RTD binding or provide other binding opportunities for the RTD. It is not known whether ATP is present in the haemolymph of aphids.

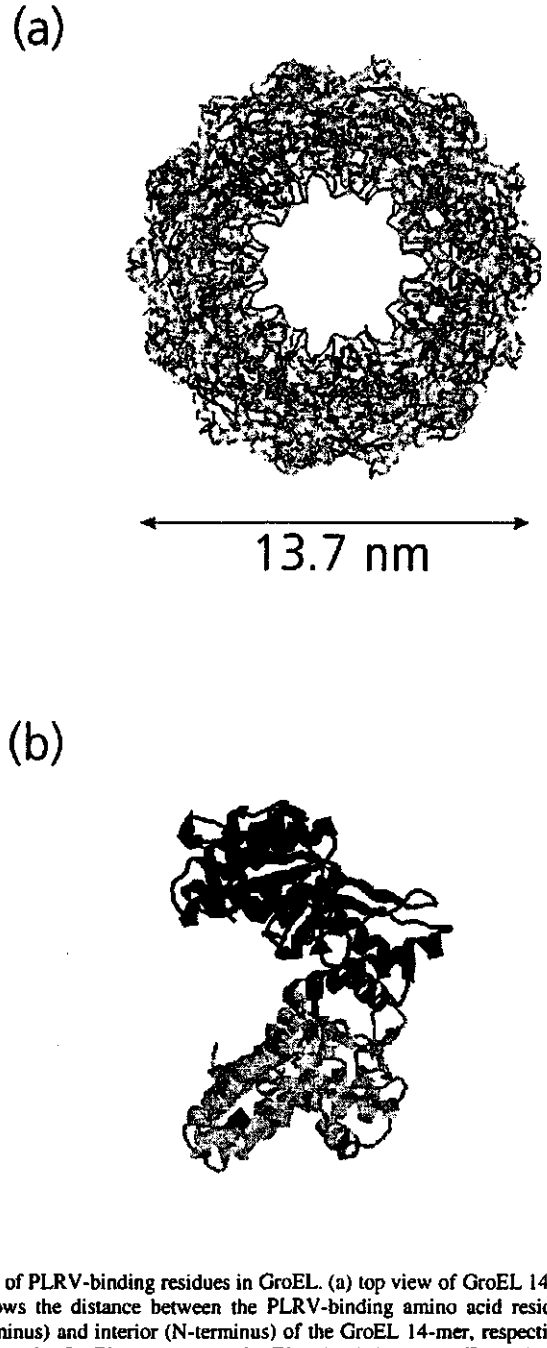

Fig. 1. Position of PLRV-binding residues in GroEL. (a) top view of GroEL 14-mer. The arrow in the 14-mer shows the distance between the PLRV-binding amino acid residues located at the exterior (C-terminus) and interior (N-terminus) of the GroEL 14-mer, respectively. (b) side view from the exterior of a GroEL 14-mer; one GroEL subunit is shown. The apical, intermediate and equatorial domains of the subunit are indicated in different gray scales. The N- and C-terminal PLRV-binding residues of the equatorial domain are indicated in black.

8.6 Future applications to inhibit luteovirus transmission by aphids

The current knowledge of the interaction between *Buchnera* GroEL and luteoviruses provides possibilities for the development of specific control strategies to prevent virus transmission. It has been shown that the feeding of *M. persicae* with neem (*Azadirachta indica* A. Juss) seed kernel extracts (NSKE) and its major active compound, azadirachtin, reduce the ability to transmit PLRV, likely because this secondary plant metabolite has major effects on bacterial symbionts of aphids (Chapter 7) and may therefore affect GroEL synthesis as well. The interaction of *Buchnera* GroEL and luteoviruses in the haemolymph of aphids may also be disturbed directly by using competing antibodies or peptides.

In mammalian systems there are several examples demonstrating that polyclonal antibodies and monoclonal antibodies (Mabs) can interfere with viral attachment. Antibodies against the CD4 molecule, a HIV recognition site and receptor on the surface of T lymphocytes and some other cells inhibit HIV binding and infectivity (Mizukami *et al.*, 1988; Ugolini *et al.*, 1997). Furthermore, Mabs developed to cell receptors of poliovirus and rhinovirus inhibit infection (Minor *et al.*, 1984; Colonno *et al.*, 1986). Mabs mimicking the receptor-binding site of reoviruses (anti-idiotypes) blocked reovirus-binding to its receptor (Kauffman *et al.*, 1983). Synthetic peptides have been used for interfering with biological systems as well. Synthetic peptides that mimic the binding domains of the viral attachment protein are able to inhibit binding of vaccinia virus (Eppstein *et al.*, 1985), HIV (Pert *et al.*, 1986), and foot-and-mouth disease virus (Fox *et al.*, 1989) to their receptors. Synthetic peptides with homology to the leech protein hirudin, an inhibitor of thrombin, are potent and selective inhibitors of coagulation processes thereby preventing blood cloth formation in patients (De Filippis *et al.*, 1998; Cappiello *et al.*, 1998).

Along this line, peptides or antibodies that specifically bind to the equatorial domain of 14-meric *Buchnera* GroEL and/or to the RTD of luteoviruses could serve as competitors disturbing the interaction between *Buchnera* GroEL and luteoviruses. Subsequently, degradation of luteovirus particles will be increased and their transmission reduced. The binding site of antibodies or peptides to GroEL or virus particles can be visualized by scanning transmission electron microscopy (Martin *et al.*, 1994). Future work should reveal whether derived antibodies or peptides indeed are able to interfere in the interaction of *Buchnera* GroEL and luteoviruses *in vivo*.

Buchnera GroEL binds to the readthrough domain of virus particles and the readthrough domain has been found responsible for long distance movement in the plant through the phloem (Brault *et al.*, 1995). By expression of a chimeric protein consisting of two parts of the equatorial domain of *Buchnera* GroEL connected by a loop structure, a similar PLRV-binding site may be mimicked. Expression of this protein induced by a phloem specific promoter (van der Mijnsbrugge *et al.*, 1996; Yin *et al.*, 1997) could possibly result in inhibition of movement of PLRV particles in the plant. Binding of the equatorial domain to virus particles in the phloem may have an influence on virus acquisition of aphids as well. BWYV particles lacking the readthrough domain are recognized by the receptor of the midgut cells and transportation through the epithelium cells is started. However, transportation of virus particles is not completed and secretion to the haemolymph does not occur (Gildow, 1998). Consequently, binding of the equatorial domain to the luteovirus readthrough domain might also prevent virus transport from the gut to the haemolymph.

Plants engineered to produce antibodies (so called plantibodies) that compete for plant-pathogen binding to vector-derived determinants may inhibit transmission of the pathogen. Promising results were obtained with transgenic tobacco plants expressing plantibodies against stylet secretion of nematodes (Rosso *et al.*, 1996). However, interfering antibodies that block the interaction between GroEL and luteoviruses should be acquired during phloem

feeding of aphids and be transported from the gut to the haemolymph after acquisition. From luteovirus transmission studies it is likely that receptors in the midgut (PLRV and BWYV) and hindgut (BYDV) are involved in transport of luteoviruses to the haemolymph (Gildow, 1985; Gildow, 1993; Gildow *et al.*, 1994; Garret *et al.*, 1996). These putative receptors specifically recognize luteoviruses and could be useful for transportation of antibodies or peptides to the haemolymph of aphids. Besides, characterization of these putative receptors would provide knowledge on transfer pathways necessary to transport proteins from the gut lumen to the haemolymph of aphid. Obviously, characterized receptors in the gut may be used as targets as well.

In conclusion, the molecular studies presented in this thesis not only have shed further light on the interaction between *Buchnera* GroEL and luteoviruses during their persistent circulation in the aphid, but also created new options to block this interaction and thus to aid new control strategies for luteoviruses.

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Chapter 9

Summary

Luteoviruses essentially replicate in the phloem tissue and are transmitted from plant to plant by aphids in a circulative, persistent manner. Virus particles are acquired when aphids feed on phloem sap. Particles are then transported from the midgut or hindgut into the haemolymph and from the haemolymph to the salivary gland, to be eventually released with the saliva to the phloem of uninfected plants. There is no evidence that luteoviruses replicate in the aphid vector. The haemolymph acts as a reservoir in which luteoviruses should persist in an infecting form during the whole lifespan of aphids.

A virus overlay technique was developed for the characterization of aphid-derived proteins involved in the circulative transmission of luteoviruses by aphids (Chapter 2). Proteins from whole-body homogenates of the aphid species *Myzus persicae* were separated with a two-dimensional denaturing poly-acrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes. Subsequently, these membranes were incubated with purified *Potato leafroll virus* (PLRV; genus *Polerovirus*; Family *Luteoviridae*) particles. Bound virus particles were detected by incubating membranes with anti-PLRV IgG and phosphatase conjugated goat anti-rabbit IgG. Thus it was demonstrated that PLRV particles bind to five different proteins. A protein of 63 kilodalton (p63) had the highest affinity for PLRV particles and was characterized by N-terminal amino-acid sequencing and immuno-gold labeling studies. These studies revealed that this protein is a homologue of GroEL and is abundantly synthesized by the primary bacterial endosymbiont (*Buchnera* sp.) of *M. persicae*.

To show whether PLRV particles and *Buchnera* GroEL also interact *in vivo*, aphids were fed on diets containing tetracyclin (Chapter 2). This antibiotic acts as bacteriostatic by inhibiting protein synthesis. After a tetracyclin treatment, *Buchnera* GroEL was not detected in the haemolymph of the aphid, virus transmission was reduced by more than 70%, and the major viral capsid protein was degraded. These observations led to the suggestion that *Buchnera* GroEL is involved in protection of virus particles against proteolytic breakdown during circulation in the haemolymph.

To study the interaction of PLRV and *Buchnera* GroEL of *M. persicae* (MpB GroEL) in more detail, the gene encoding MpB GroEL and its flanking sequences were characterized and compared to those of *Escherichia coli* and *Buchnera* spp. of other aphid species (Chapter 3). The MpB GroEL encoding gene appeared to be part of an operon with a similar organization as the *groE* operon of *E. coli*, containing another gene for a 10-kDa protein with sequence similarities to GroES of *E. coli*. However, a constitutive promoter sequence comparable to that of the *E. coli groE* operon could not be identified; only sequences comparable to the heat shock promoter of the *E. coli groE* operon were observed. Comparison of the deduced amino-acid sequences disclosed that MpB GroEL is approximately 98% similar to GroELs of other *Buchnera* spp. and 92% similar to *E. coli* GroEL. These results demonstrate that MpB GroEL belongs to the family 60-kDa chaperonin or heat shock protein family.

Several functions of GroEL proteins have been described and the most important one is the folding of nonnative proteins inside the cytosol of prokaryotes, mitochondria and chloroplasts. MpB GroEL and other GroEL proteins have typical double-doughnut structures composed of two stacked rings of seven subunits each. Using the crystal structure of *E. coli* GroEL, computer-generated structural predictions of the monomer of MpB GroEL was obtained (Chapter 3). Like *E. coli* GroEL, each subunit of MpB GroEL consists of an apical, an intermediate and an equatorial domain. The apical domain is a continuous domain on the primary MpB GroEL protein structure, whereas the equatorial and intermediate domains are discontinuous with regions located at the N- and C-terminus of the MpB GroEL subunit. The N- and C-terminal regions of the equatorial and intermediate domains assemble in the folded structure of MpB GroEL. Functional studies of *E. coli* GroEL 14-mers have demonstrated that

the apical domains are located at both sides of the cylindrical double-doughnut structure and contains amino acids involved in binding of nonnative proteins. The equatorial domains form the waist of the GroEL 14-mer. Intermediate domains function as hinges for moving the apical domain up and down so that amino acids in the apical domain can bind the unfolded protein. Subsequently, unfolded proteins are kept in the cavity of the GroEL 14-mer where they obtain their native structure without being disturbed by cytosolic compounds.

To investigate which of the domains of MpB GroEL are involved in binding PLRV particles, deletion mutants were designed based on the primary structure of the MpB GroEL protein (Chapter 3). Full-length MpB GroEL and MpB GroEL deletion mutants were expressed in fusion with glutathione-S-transferase (GST) in *E. coli* and affinity-purified. The GST moiety was removed and similar amounts of recombinant protein were tested for PLRV binding in virus overlay assays. This revealed that recombinant full-length MpB GroEL proteins had a similar affinity for PLRV particles as wild type MpB GroEL proteins isolated from *M. persicae*. PLRV particles displayed affinity for MpB GroEL deletion mutants only if they still contained the N- or C-terminal regions of the equatorial domain. Strikingly, PLRV-binding to polypeptides containing the apical domain alone or when extended with flanking sequences did not bind PLRV. Furthermore, virus overlay assays with additional MpB GroEL deletion mutants demonstrated that determinants for PLRV binding at the C-terminal part of the equatorial domain are located between residues 408 and 475 of MpB GroEL (Chapter 4). This region comprises three α-helices. Since the N- and C-terminal regions of the equatorial domain assemble in the folded structure of MpB GroEL, the two PLRV-binding regions may become a single PLRV-binding site. The finding that the equatorial domain was involved in binding PLRV particles and not the apical domain is surprising, since studies of *E. coli* GroEL showed that the apical domain is involved in binding of unfolded proteins in the cytosol of *E. coli* cells. PLRV particles may have different binding characteristics because of the size limitation of the central cavity of the GroEL molecule and the fact that binding occurs extracellularly in the haemolymph.

The interaction between PLRV particles and MpB GroEL was investigated in more detail (Chapter 4). Virus overlay studies with additional MpB GroEL deletion mutants revealed that regions between amino acid residues 1 and 57, and 427 and 457 of the N- and C-terminal regions of the equatorial domain, respectively, contain the determinants for PLRV binding. To determine which amino acids are involved in PLRV binding, overlapping decameric peptides of PLRV-binding regions were synthesized and incubated with virus particles in a virus overlay based experiment (Chapter 4). Alanine replacement studies of binding peptides showed that amino acids R13, K15, L17 and R18 of the N-terminal region of the equatorial domain, and R441 and R445 of the C-terminal region of the equatorial domain are responsible for PLRV binding. Alanine replacement of R13, K15, L17 and R18 eliminated PLRV binding of MpB GroEL(1-408) completely, whereas replacement of R441 and R445 reduced, but not eliminated, virus binding of MpB GroEL(122-548). This suggests that besides R441 and R445 other residues in the C-terminus are part of the PLRV-binding site. These still unknown residues are likely to be located in the region between amino acids 427 till 474, which comprises one α-helix located to the outside of GroEL 14-mers. Residues R13, K15, L17 and R18 are located in a long α-helix that is present more internally of GroEL 14-mers. The N- and C-terminal amino acids are positioned behind each other in a cavity, which might be accessible for the readthrough domain (RTD) which protrudes from the surface of a luteovirus particle.

The luteovirus protein capsid is composed of a major 23-kDa coat protein (CP), and lesser amounts of a ~54-kDa readthrough protein, expressed by translational readthrough of the CP into the adjacent open reading frame encoding the RTD. The RTD is exposed on the surface of the virus particle and contains the determinants necessary for virus transmission by aphids.

To study whether the highly conserved major CP or the RTD of the minor 54-kDa protein are involved in GroEL binding, BWYV mutants devoid of the RTD were synthesized and tested for GroEL affinity in a GroEL-ligand assay (Chapter 5). It was found that the BWYV RTD mutants did not bind GroEL, indicating that the RTD contains the GroEL-binding determinants. BWYV mutants lacking the RTD domain were also injected into the haemolymph of aphids and the persistence of these mutants was compared with those of wild-type virus particles (Chapter 5). These studies clearly showed that BWYV mutants devoid of the RTD were more rapidly degraded than wild-type viruses, indicating that the RTD, containing the GroEL-binding sites, is crucial for the persistency in the aphid.

To reveal whether conserved domains of the RTD are involved in GroEL binding, five luteoviruses belonging to the genus *Polerovirus* and *Pea enation mosaic virus* (PEMV; *Enamovirus*) were tested for binding to *Buchnera* GroEL proteins isolated from several aphid species using GroEL-ligand assays (Chapter 5). All luteoviruses displayed a specific but differential affinity for the GroEL homologues isolated from the endosymbiotic bacteria of both vector and non-vector aphid species, and for *E. coli* GroEL. This indicates that GroEL is not involved in vector specificity. Sequence alignment of the RTDs of different luteoviruses and PEMV revealed that only the N-terminal half of the RTDs is conserved, whereas the C-terminal halves have no global sequence identity. This C-terminal region is also lacking from the PEMV RTD. The highest overall level of sequence similarity in the RTD extends from position 184 to 223 where about 23% of the residues are identical.

To assess whether the viral determinants required for the interaction of luteoviruses with *Buchnera* GroEL reside in the conserved region of the RTD, GST-fusions of the RTD and mutants thereof were expressed in *E. coli* (Chapter 6). After affinity purification, the GST moiety was cleaved and the resulting RTD protein tested for MpB GroEL affinity using a GroEL-ligand assay. This showed that the conserved region of the RTD plays a crucial role in binding GroEL.

The knowledge derived from the binding studies of GroEL and luteoviruses is valuable for the development of specific control methods. The fact that *Buchnera* GroEL and luteoviruses directly interact *in vitro* suggests that this occurs in the haemolymph of aphids as well. Consequently, peptides or antibodies that interfere in this interaction by binding to the equatorial domain of *Buchnera* GroEL or the RTD of luteoviruses reduce specifically the transmission efficiency of luteoviruses by aphids. It is possible to produce these interfering compounds by plants so that aphids acquire them while feeding. Further studies should reveal whether there are possibilities for transporting peptides or antibodies from the gut to the haemolymph.

Chapter 7 of this thesis describes an investigation that may lead to an alternative control strategy. In this chapter the effects of neem (*Azadirachta indica* A. Juss) seed kernel extracts (NSKE) and its major active compound, azadirachtin, on the ability of *M. persicae* to transmit PLRV is studied. This secondary plant metabolite has major effects on bacterial symbionts of leafhoppers. Since endosymbiotic bacteria play a major role in the performance of aphids and luteovirus transmission by aphids, it was investigated whether treatments with these compounds would exert an effect on aphid larval growth and mortality, and on the aphid intracellular symbionts. The neem metabolites displayed a 100% mortality at doses higher than 2560 ppm., and morphological aberrations on the bacterial endosymbionts were observed. At doses lower than 160 ppm of NSKE or azadirachtin, the endosymbiont population of *M. persicae*, and mortality, growth and feeding behavior was similar to that of the untreated groups of aphids. However, PLRV transmission was inhibited by 40-70%. These observations raise the possibility that interfering with the relationship between endosymbionts and aphids may contribute to the control of luteovirus transmission by aphids.

Chapter 10

Samenvatting

10.1 Het probleem

Virussen van de familie *Luteoviridae* worden van plant tot plant overgedragen door bladluizen. De symptomen van luteovirus geïnfecteerde planten zijn onder andere vergeling van de bladeren en vertraagde groei. Luteovirusinfecties veroorzaken vermindering in opbrengst van een groot aantal economisch belangrijke gewassen. Tot nu toe worden luteovirusinfecties voorkomen door bladluizen met pesticiden te bestrijden. Pesticiden zijn schadelijk voor het milieu en sommige bladluizen hebben resistenties ontwikkeld tegen pesticiden. Er is daarom vraag naar andere methoden om luteovirus-infecties te voorkomen en naar alternatieve milieuvriendelijke bestrijdingsmethoden voor bladluizen.

In het kader van dit proefschrift is onderzoek gedaan naar de interactie van luteovirussen en bladluizen. Het onderzoek was erop gericht om kennis te vergaren die kan bijdragen tot de ontwikkeling van een strategie die de overdracht van luteovirussen door bladluizen kan beteugelen. De interacties tussen het aardappelbladrolvirus (*Potato leafroll virus*; PLRV) en de belangrijkste overdrager (vector) van dit virus, de groene perzikbladluis (*Myzus persicae*), is gebruikt als modelsysteem. Naast de opbrengstverliezen, die PLRV in aardappelen veroorzaakt, worden de virusdeeltjes ook naar de knollen getransporteerd, hetgeen de kwaliteit van het pootgoed sterk negatief beïnvloedt.

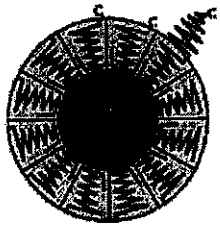
10.2 De circulatie van luteovirusdeeltjes in de bladluis

Om methodes te ontwikkelen die de overdracht van luteovirussen door bladluizen kunnen voorkomen, zal eerst kennis vergaard moeten worden over de circulatie van een luteovirusdeeltje in een bladluis en over welke onderdelen van de plant belangrijk zijn voor het opnemen van virusdeeltjes door de bladluis. Luteovirussen vermeerderen zich alleen in de floëemcellen van de plant en worden getransporteerd naar het floëemsap. Wanneer bladluizen zich met het suikerrijke plantensap voeden, komen de virusdeeltjes de bladluis binnen (Fig. 3, hoofdstuk 1, bladzijde 5). Virusdeeltjes worden dan vanuit de maag of darm opgenomen en getransporteerd naar de hemolymf (lichaamsvloeistof). De virusdeeltjes circuleren in de hemolymf totdat ze de cellen van de speekselklier bereiken en komen vervolgens in het speeksel terecht. Met het speeksel van de bladluis worden virusdeeltjes vervolgens in het floëemsap van een niet-geïnfecteerde plant geïntroduceerd. Hoewel luteovirussen zich niet vermeerderen in bladluizen, worden ze gedurende de gehele levensduur van een bladluis overgedragen. Dit komt doordat de virusdeeltjes langdurig aanwezig blijven (persisteren) in de hemolymf. Gedurende deze circulatie van virusdeeltjes zullen er verscheidene bladluiscomponenten nodig zijn voor het transport van maag of darm naar hemolymf en van hemolymf naar speekselklier. Verder is het waarschijnlijk dat bladluiscomponenten in de hemolymf betrokken zijn bij de bescherming van virusdeeltjes, zodat deze niet afgebroken worden en langdurig infectieus blijven. Aldus kan verondersteld worden dat virusdeeltjes over eigenschappen bezitten om interacties met bladluiscomponenten te kunnen aangaan.

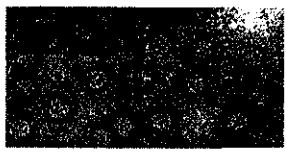
10.3 Het luteovirusdeeltje

Luteovirussen zijn isometrische deeltjes met een diameter van 23 tot 30 nm waarin zich het volledige enkelstrengs RNA-genoom (ongeveer 6000 basenparen lang) van positieve polariteit bevindt. Dit RNA-genoom codeert voor 6 tot 9 verschillende eiwitten. De capside bestaat uit twee eiwitten, een manteleiwit van 24 kDa ("coat protein" of CP) en een verlengde (55 kDa) versie van ditzelfde eiwit dat het "readthrough" eiwit wordt genoemd. Dit "readthrough" eiwit wordt in mindere mate aangemaakt en ingesloten in de mantelstructuur van het virusdeeltje zodanig dat het verlengde gedeelte (RTD) naar buiten steekt (figuur 1). Virusdeeltjes die dit RTD niet hebben, zijn niet overgedraagbaar door bladluizen. Blijkbaar is het RTD belangrijk voor de circulatie in de bladluis en is het wellicht betrokken bij the interactie van het virusdeeltjes met bladluis-componenten.

(a)



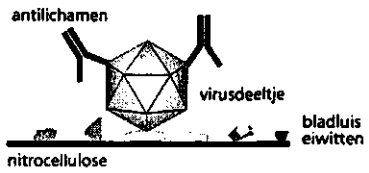
(b)



Figuur 1. Structuur van PLRV-deeltjes. (a) Schematiche weergave van PLRV-deeltjes; het naar buiten stekende gedeelte is het RTD. De carboxi-termini zijn aangegeven met een C. (b) Elektronenmicroscopische opname van PLRV-deeltjes.

10.4 Isolatie van bladluiscomponenten betrokken bij luteovirus overdracht

Voor de identificatie en isolatie van bladluis-componenten die betrokken zijn bij luteovirus-overdracht, werd een speciale techniek ontwikkeld, die "virus overlay assay" wordt genoemd. Deze techniek is schematisch weergegeven in figuur 2 en houdt in dat bladluisextracten gescheiden worden op grootte met behulp van polyacrylamidegel-electrophorese (PAGE), vervolgens overgebracht worden op een nitrocellulosefilter en dan geïncubeerd worden met virusdeeltjes.



Figuur 2. Schematische weergave van de "virus overlay assay".

Deeltjes die eventueel bepaalde bladluiseiwitten herkennen en eraan binden, blijven aan het filter gehecht en kunnen worden gedetecteerd met behulp van specifieke antilichamen. Figuur 1A van hoofdstuk 2 (bladzijde 14) laat een PAGE gel zien, die met Coomassie gekleurd is en waarin alle bladluiseiwitten zichtbaar zijn. Figuur 1B van hoofdstuk 2 (bladzijde 14) laat zien welke bladluiseiwitten binden aan virusdeeltjes.

Met behulp van deze techniek werden vijf bladluiseiwitten gedetecteerd, die virusdeeltjes kunnen binden. Eén van deze eiwitten blijkt in grote mate aanwezig in de bladluis en is aangegeven met een pijltje in figuur 1B van hoofdstuk 2. Dit eiwit (p63) heeft een grootte van 63 kDa en is verder bestudeerd met behulp van eiwitsequentiebepalingen en specifieke antilichamen die het eiwit herkennen. Antilichamen, die aan goudbolletjes gekoppeld zijn, werden gebruikt om het eiwit te lokaliseren in de bladluis met behulp van de

electronenmicroscoop. Eiwit p63 werd aangetroffen in de endosymbiontische bacteriën van de bladluis (figuur 1D, hoofdstuk 2) en wordt niet door het insect zelf gemaakt !

10.5 De bacteriën van de bladluis

Bacteriën, die p63 maken, zijn niet alleen aanwezig in de groene perzikluis *M. persicae*, maar ook in andere bladluissoorten en behoren tot het geslacht *Buchnera*. *Buchnera* spp. zitten in speciale cellen van de bladluis, mycetocyten, en voorzien het insect van belangrijke nutriënten die niet voorkomen in het plantensap. Bladluizen die geen *Buchnera* spp. hebben, blijven klein en kunnen geen nakomelingen krijgen. Omgekeerd kunnen de bacteriën niet buiten de bladluis groeien. Bladluizen en *Buchnera* spp. hebben kennelijk een relatie ontwikkeld waarbij beide partners niet (goed) zonder elkaar kunnen overleven. De bacteriën worden van de moeder naar de embryo's getransporteerd (Figuur 6, hoofdstuk 2, bladzijde 17) en produceren grote hoeveelheden p63 waarbij dit eiwit ook buiten de bacteriecel in de hemolymf van bladluizen gevonden wordt.

10.6 De interactie van p63 en luteovirussen in de bladluis

Om te bestuderen of p63 ook een interact aangaat met PLRV-deeltjes in *M. persicae*, werden bladluizen gevoerd met een sucrosemedium dat het antibioticum tetracycline bevatte. Dit antibioticum remt specifiek de aanmaak van bacteriële eiwitten en daarmee p63. Het bleek inderdaad dat p63 niet meer in de hemolymf van bladluizen aangetoond kon worden. Bovendien werd door tetracycline-behandelde bladluizen 70 % minder virus overdragen in vergelijking met niet-behandelde bladluizen en bleek dat virusdeeltjes afgebroken werden na de tetracyclinebehandeling. Een mogelijke verklaring is dat p63 aan virusdeeltjes bindt waardoor de deeltjes niet als lichaamsvreemd worden herkend door de bladluis (moleculaire mimicry) en niet worden afgebroken. Deze resultaten zouden de persistentie van virusdeeltjes in de hemolymf kunnen verklaren (zie 10.2 "De circulatie van luteovirusdeeltjes in de bladluis.").

10.7 Karakteristieken van p63

Voor verdere analyse van p63, is de nucleotidenvolgorde van het p63-coderende gen, dat gelegen is op het genoom van *Buchnera* opgehelderd (figuur 3). Het p63-coderende gen bleek ongeveer 1500 basenparen lang te zijn en de daaruit af te leiden aminozuurvolgorde van p63 bleek grote overeenkomst ("homologie") te vertonen met het GroEL eiwit van *Escherichia coli*. Het p63-coderende gen (vanaf nu *Buchnera* GroEL genoemd) ligt op een operon dat nog een ander gen bevat dat codeert voor een eiwit (10 kDa) dat grote gelijkenis vertoont met het GroES-eiwit van *E. coli*. De aanmaak van GroEL en GroES van *E. coli* wordt gereguleerd met behulp van twee promotersequenties, de "heat shock promoter" (Phsp) en de constitutieve promoter (Pcon) die vooraan op het operon gelegen zijn (Figuur 3). Promoter Pcon reguleert de expressie van GroES- en GroEL-coderende genen bij standaard groeicondities. Bij extreme groeicondities, bijvoorbeeld bij hogere temperaturen, stijgt de hoeveelheid GroEL in *E. coli* en wordt de expressie gereguleerd door Phsp. Het *Buchnera groE*-operon heeft sequentiehomologie met de Phsp-promoter van het *groE*-operon van *E. coli* maar een sequentie dat gelijkenis vertoont met Pcon werd niet gevonden (Figuur 3). Wellicht heeft

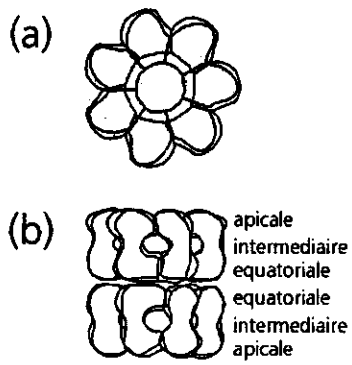
Buchnera van *M. persicae* altijd hoge hoeveelheden GroEL nodig en wordt alleen de Phsp gebruikt voor regulatie van *Buchnera* GroEL- en GroES-expressie.

Buchnera sp. van *Myzus persicae*

Escherichia coli

Figuur 3. Een schematisch overzicht van de *groE* operons van *Buchnera* sp. van *M. persicae* en van *E. coli*. P, promoter; hsp, "heat shock promoter"; con, constitutieve; SD, Shine Dalgarno box; Term, terminator.

GroEL is een eiwit waaraan met name bij *E. coli* al veel onderzoek is gedaan. De meest bestudeerde en waarschijnlijk ook belangrijkste functie van GroEL is het correct doen vouwen van eiwitten. Bacteriën overleven niet zonder GroEL. GroEL bestaat uit twee opeengestapelde doughnuts, die elk zijn opgebouwd uit 7 eenheden (Figuur 4). Elke eenheid heeft drie domeinen, het apicale domein, het intermediaire domein en het equatoriale domein. Deze domeinen hebben ieder een eigen functie tijdens het binden en vouwen van eiwitten in de bacteriecel. Het apicale domein bevat de aminozuren die de ongevouwen eiwitten binden. Het equatoriale domein is betrokken bij het intact houden van de 14-meer structuur. De equatoriale domeinen van alle eenheden liggen dan ook tegen elkaar aan. Het intermediare domein ligt tussen het apicale en equatoriale domein in en is belangrijk voor het naar buiten brengen van de aminozuren in het apicale domein zodanig dat dit domein andere eiwitten kan binden. Gebonden eiwitten worden vervolgens in het gat van de cilinder gevouwen.



Figuur 4. De volledige 14-meer structuur van het GroEL eiwit. (a) bovenaanzicht. (b) zijaanzicht.

In de lineaire structuur van GroEL zijn de equatoriale en intermediaire domeinen beiden uit twee delen opgebouwd (figuur 5). De twee delen van het equatoriale domein liggen aan de linker- (N-terminaal) en rechterkant (C-terminaal) van het GroEL eiwit. De twee intermediaire delen liggen naast de twee equatoriale delen en tussen de twee delen van het intermediaire domein in ligt het gehele apicale domein. In de drie-dimensionale structuur komen de twee stukken van de equatoriale en intermediaire domeinen bij elkaar en het apicale domein steekt naar buiten.

Mogelijk is het apicale domein van *Buchnera* GroEL ook betrokken bij het binden van PLRV. Om dit te onderzoeken hebben we de kennis die al beschikbaar was van het GroEL van *E. coli*, gebruikt om de interactie van PLRV en *Buchnera* GroEL nader te bestuderen.

Figuur 5. De lineaire structuur (548 aminozuren) van één eenheid van het *Buchnera* GroEL. Eq, equatoriale domein; Int, intermediaire domein.

10.8 Welk domein van *Buchnera* GroEL is betrokken bij het binden van PLRV ?

Om te bestuderen welke domeinen van *Buchnera* GroEL betrokken zijn bij binding van PLRV-deeltjes, zijn er ingekorte versies (deletiemutanten) van *Buchnera* GroEL gemaakt. Deze eiwitmutant zijn ontworpen met behulp van de lineaire GroEL-structuur en zodanig gemaakt dat er steeds een bepaald domein ontbreekt (figuren 4 en 5, hoofdstuk 3, bladzijden 30 en 31, respectivelijk en figuren 1 en 4, hoofdstuk 4, bladzijden 42 en 45, respectivelijk). Onderzocht werd welke van de *Buchnera* GroEL-deletiemutanten nog PLRV-deeltjes konden binden met de zogenaamde "virus overlay assay" (figuur 2). Het bleek dat alle *Buchnera* GroEL-deletiemutanten, die één of beide delen van het equatoriale domein bevatten nog steeds in staat zijn om virusdeeltjes te binden. Echter, een deletiemutant die alleen het apicale domein bevatte, bond geen virusdeeltjes meer. Er werden nog meer deletiemutanten gemaakt en uiteindelijk bleek dat de aminozuren 9 tot 19 van het N-terminale en aminozuren 428 tot 457 van het C-terminale equatoriale domein betrokken zijn bij virusbinding (figuur 6). De conclusie dat het equatoriale domein betrokken is bij virusbinding en niet het apicale domein van *Buchnera* GroEL is verrassend, omdat juist in het algemeen het apicale domein van GroEL betrokken is bij binding van ongevouwen eiwitten (10.7 en figuur 4).

Figuur 6. De lineaire structuur van één eenheid van het *Buchnera* GroEL-eiwit (p63) waarin de regio's, die betrokken bij zijn bij PLRV-binding, aangegeven zijn (aminozuren 1 tot 57 en 428 tot 474). Eq, equatoriale domein; Int, intermediaire domein.

10.9 Welke aminozuren van *Buchnera* GroEL zijn betrokken bij het binden van virus ?

De interactie tussen PLRV en *Buchnera* GroEL werd nader onderzocht door te bepalen welke aminozuren binnen de regio's 9-19 en 428-457 (Figuur 6) binden aan virusdeeltjes. Overlappende peptiden van 10 aminozuren met aminozuurvolgorden corresponderend met regio's 1-57 en 428-474 waren getoetst voor virus binding in een "virus overlay assay".

Slechts peptiden die overeenkwamen met regio's 9-19 en 428-457 bleken in staat om virusdeeltjes te binden (figuren 2 en 5, hoofdstuk 4, bladzijden 43 en 46, respectievelijk). Van deze peptiden werden vervolgens de individuele aminozuren vervangen door steeds het aminozuur alanine (A). Wanneer de aminozuren 13 (arginine of R), 15 (lysine of K), 17 (leucine of L), 18 (arginine of R), 441 (R) en 445 (R) door alanines vervangen werden, verloren de peptiden affiniteit tot virusdeeltjes. Deze aminozuren zijn daarom zeer waarschijnlijk verantwoordelijk voor de interactie van PLRV met *Buchnera* GroEL.

Het aandeel van R13, K15, L17, R18, R441 en R445 in de virusbinding werden vervolgens afzonderlijk getoetst in *Buchnera* GroEL. De resultaten wezen uit dat de vier genoemde aminozuren aan de N-terminus van *Buchnera* GroEL allen betrokken zijn en dat de structuur binnen de regio 9-19 daarbij essentieel is. De affiniteit van virusdeeltjes voor de GroEL-mutant waarin R441 en R445 door alanine vervangen werden, was verminderd maar niet volledig uitgeschakeld. Het is deshalve waarschijnlijk dat structurele componenten binnen regio 428-457 bijdragen aan virusbinding.

Met behulp van een computermodel zijn de aminozuren die betrokken zijn bij binding van virusdeeltjes gelokaliseerd in de *Buchnera* GroEL 14-meer. Aminozuren R13, K15, L17 en R18 liggen aan de binnenkant van de GroEL 14-meer. De twee α-helices van regio 427 tot 475 waarbinnen de aminozuren R441 en R445 zich bevinden, liggen aan de buitenkant van *Buchnera* GroEL 14-meer. De twee bindende domeinen liggen achter elkaar en lijken bereikbaar kunnen zijn voor het RTD van luteovirusdeeltjes (figuur 1, Hoofdstuk 8, bladzijde 94).

10.10 Welke manteleiwitten van luteovirusdeeltjes zijn betrokken bij *Buchnera* GroEL-binding ?

Er is bovendien onderzocht of het manteleiwit CP en/of het RTD van het "readthrough"-eiwit betrokken is bij *Buchnera* GroEL binding (figuur 1). Daartoe zijn er mutanten gemaakt van het luteovirus, *Beet western yellows virus* (BWYV), welke het RTD-gedeelte missen (BWYVΔRTD) (figuur 3 van hoofdstuk 5, bladzijde 61). BWYV en BWYVΔRTD werden getoetst voor *Buchnera* GroEL-binding en de resultaten lieten zien dat BWYVΔRTD geen affiniteit meer had voor *Buchnera* GroEL. Het RTD is dus essentieel voor *Buchnera* GroEL-binding.

Met behulp van injectie-experimenten werd onderzocht of BWYV-deeltjes persistenter zou zijn dan BWYVΔRTD virusdeeltjes. Het bleek inderdaad dat na 120 uur nog ongeveer 70% van de geïnjecteerde BWYV-deeltjes over was, terwijl er nog maar 10% van de BWYVΔRTD-deeltjes aangetroffen werd (figuur 4 van hoofdstuk 5, bladzijde 62). Er werd ook aangetoond dat het meeste van de BWYVΔRTD-deeltjes werd afgebroken gedurende de eerste twee uur na injectie. Deze experimenten demonstreren duidelijk dat het RTD betrokken is bij de persistentie van virusdeeltjes in de hemolymf van bladluizen.

10.11 Welk deel van het RTD bindt aan *Buchnera* GroEL ?

Luteovirussen, die tot de drie genera binnen de familie *Luteoviridae* behoren, binden allen aan *Buchnera* GroEL. Dit zou betekenen dat een geconserveerd deel van het RTD betrokken is bij binding aan *Buchnera* GroEL. Vergelijking van de aminozuursequenties van de RTD's van verschillende luteovirussoorten laat zien dat er een aantal regio's in het RTD geconserveerd zijn (figuur 5 van hoofdstuk 5). De regio tussen aminozuren 184 en 223 vertoonde de meeste

gelijkenis tussen de luteovirussen (23% identiek). Aminozuren binnen deze regio zouden mogelijk een rol kunnen spelen in de formatie van de *Buchnera* GroEL bindingsplaats.

De rol van aminozuren 184 tot 223 bij *Buchnera* GroEL-binding werd bekeken aan de hand van RTD-deletiemutanten van PLRV. Het bleek de RTD-mutant, die het geconserveerde stukje tussen aminozuur 184 tot 223 niet bevatte, minder affiniteit hadden voor *Buchnera* GroEL ten opzichte van mutanten die dit geconserveerde stukje wel bevatten. De conclusie is daarom dat de regio tussen 184 en 223 betrokken is bij *Buchnera* GroEL binding. Bovendien bleek dat de structuur van het RTD belangrijk om affiniteit voor *Buchnera* GroEL te behouden.

10.12 Toepassing

De opgedane kennis over de interactie tussen luteovirussen en *Buchnera* GroEL kan gebruikt worden voor de ontwikkeling van alternatieve bestrijdingsmiddelen. Het gegeven dat *Buchnera* GroEL en luteovirusdeeltjes een binding aangaan *in vitro* betekent wellicht dat dit tevens in de hemolymf van bladluizen gebeurd. Peptiden of antilichamen die binden aan het equatoriale domein van *Buchnera* GroEL of aan het RTD van luteovirussen zouden mogelijk de interactie tussen *Buchnera* GroEL en virusdeeltjes kunnen verbreken of voorkomen. Dit zou een vermindering van virusoverdracht tot gevolg hebben. Het is mogelijk om peptiden en antibodies door planten te laten aanmaken. Verdere studies zouden moeten uitwijzen of het tevens mogelijk is om deze peptiden of antibodies van de maag naar de hemolymf van de bladluis te transporteren.

In hoofdstuk 7 is er al een onderzoek beschreven van een mogelijk alternatief bestrijdingsstrategie. Er is bestudeerd of het extract van zaden van de neemboom (NSKE) en azadirachtine een negatieve invloed hebben op *Buchnera* sp. van *M. persicae* en dientengevolge ook een negatief effect hebben op de efficiëntie van PLRV-overdracht. Daartoe werden bladluizen gevoed met verschillende hoeveelheden NSKE of azadirachtine. Alle bladluizen overleden bij een hoge dosis NSKE (2560 ppm). De *Buchnera*-populatie vertoonde duidelijk morfologische verschillen na de behandeling met NSKE ten opzichte van niet-behandelde bladluizen (figuur 2 van hoofdstuk 7, bladzijde 85). Bij behandeling van bladluizen met lagere hoeveelheid azadirachtine of NSKE (160 ppm) vertoonden de bladluizen geen verschil in overleving, groei of voedingsgedrag ten opzichte van niet-behandelde bladluizen. Echter, de efficiency van PLRV-overdracht door neem-behandelde bladluizen was gereduceerd met 40 – 70 %. Deze experimenten demonstreren dat neemextracten gebruikt zouden kunnen worden om PLRV-overdracht te beteugelen.

List of Publications

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Curriculum vitae

Saskia Adriane Hogenhout zag het levenslicht in Haarlem op 10 maart 1969. Na de lagere school werd in 1981 begonnen met het gymnasium aan het toenmalige Haarlemmermeer Lyceum in Badhoevedorp. Vier jaren later werd deze gesloten en heeft zij het Voorbereidend Wetenschappelijk Onderwijs verder afgemaakt op het Hervormd Lyceum West in Amsterdam. Aansluitend werd in 1988 begonnen met de studie Biologie aan de *Vrije* Universiteit van Amsterdam (VUA). Tijdens haar studie heeft zij zich georienteerd in de moleculaire and ecologische richtingen van de biologie en heeft in totaal vier stages gelopen. De eerste stage werd gelopen bij de afdeling genetica van de VUA gedurende een periode van 11 maanden. De tweede stage van 9 maanden werd volbracht aan een plantenziektenkundig instituut te Davis, The Center for Engineering Plant Resistance Against Pathogens (CEPRAP) dat deel uitmaakte van de University of California. Daarna heeft ze een literatuuronderzoek gedaan naar het gebruik van moleculaire technieken in de evolutiebiologie gedurende een periode van 5 maanden bij de afdeling dieroecologie van de VUA. De laatste stage was een 4 maandelijkse veldstudie naar het gedrag en de oecologie van loopkevers in Australië. Na het behalen van het doctoraaldiploma in 1994 werd begonnen aan het promotie onderzoek waarvan de resultaten in dit proefschrift beschreven staan.