Novel isolates of *Cydia pomonella* granulovirus (CpGV): deciphering the molecular mechanism for overcoming CpGV resistance in codling moth (*Cydia pomonella*)

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Karolin Elisabeth Eberle

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Ever tried. Ever failed. No matter.

Try again. Fail again. Fail better.

(Samuel Beckett)

Contents

List of abbreviations

%	percent
× g	multiple of g
°C	degree Centigrade
µg	microgram
µm	micrometer
A	adenine
aa	amino acid
bp	base pair
C	cytosine
CM	codling moth
cp	compare
CpGV	*Cydia pomonella* Granulovirus
ddH_2O	bidistilled water
DNA	deoxyribonucleicacid
ds	double stranded
e.g.	for example
et al.	and others
etc.	et cetera
Fig.	figure
g	gram
G	guanine
GV	granulovirus
h	hour
ha	hectare
kb	kilobases
kV	kilovolt

L	liter
LB	Luria-Bertani
LC_{50}	median lethal concentration
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ms	millisecond
N°	number
ng	nanogram
NPV	nucleopolyhedrovirus
nt	nucleotide
OB	occlusion body
OD	optical densitiy
PCR	polymerase chain reaction
rpm	rotations per minute
SDS	sodium dodecyl sulfate
sec	seconds
T	thymidin
Tab.	table
V	volt
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

Summary

During the last twenty years, *Cydia pomonolla* granulovirus (CpGV, *Baculoviridae*) has become the most important biological control agent for the codling moth (CM) in organic and integrated apple production. CpGV conforms to all requirements of a biocontrol agent: it has a narrow host range and is highly virulent against CM larvae, it is environmentally safe and does not interfere with non-target organisms. Today, CpGV is applied in Europe on 100-150,000 ha per year. Thereby, all commercial CpGV products in Europe are based on the isolate CpGV-M, which was discovered in Mexico (Tanada, 1964). A serious threat to succesfull application of CpGV products is the occurrence of CpGV resistance of CM observed in the field. From 2003 on, populations with up to 1000-10,000 fold reduced susceptibility were reported from commercial apple orchards in Germany, France, Italy and the Netherlands- the first reported cases of field resistance to baculovirus products ever. In order to provide alternatives to the presently used CpGV-M, novel CpGV isolates of different geographic origion were tested for their efficacy against susceptible and resistant CM larvae. This thesis focuses on the identification and characterisation of resistance overcoming CpGV isolates and the analysis of their molecular difference to CpGV-M.

In total, five out fo sixteen CpGV isolates tested in laboratory bioassays were found to be able to completely overcome resistance to CpGV. All isolates were compared by restriction fragment length polymorphism (RFLP) analysis, in order to identify differences in their genomic content. It was found that isolates corresponding in their restriction profile to CpGV-M showed no efficacy against resistant CM populations, whereas isolates able to overcome resistance in CM differed in their restriction profile from CpGV-M. To pinpoint the differences on molecular level resulting in this improved virulence of some CpGV isolates, major genomic areas of difference were amplified, sequenced and analysed. Insertions of about 0.7 kb were found in the resistance overcoming isolates CpGV-I01, -I12 and -E2, but not in other resistance overcoming isolates. Analysis of the insertions` structure revealed that they might be due to transposition events, but not involved in overcoming resistance.

With regard to a future registration of novel CpGV isolates, it is necessary to identify different isolates reliably in mixtures. RFLP analysis showed that the conventionally used assignment of CpGV isolates to their geographic origin is no longer applicable, as isolates from different regions shared the same profiles, or isolates from the same region differed in their profiles. Hence, a new method for determination based on molecular analysis was established. Partial sequencing of the conserved *polyhedrin/granulin* (*polh/gran)*, *late expression factor-8* (*lef-8)* and *late expression factor-9* (*lef-9)* genes revealed single nucleotide polymorphisms (SNPs). SNP analysis corresponded with the grouping obtained by RFLP analysis, therefore, a phylogenetic classification of CpGV genome types A-E is proposed. Phylogenetic analysis based on these SNPs suggested that CpGV-M was the phylogenetically youngest of the tested CpGV isolates. The resistance overcoming isolates were allocated to several genome types. Therefore, whole genome sequencing of two resistance overcoming isolates CpGV-I12 (type D genome) and -S (type E genome) was performed, in order to identify a common feature which distinguishes these genomes from CpGV-M. CpGV-M was also completely re-sequenced as a reference. Comparison of the three genomes revealed a high sequence identity. Nevertheless, several insertions and deletions ranging from single to hundreds of nucleotides (nt) were found. Comparison on open reading frame (ORF) level revealed that CpGV-I12 and -S shared only one protein alteration when compared to CpGV-M: a stretch of 24 nt present in ORF cp24 of CpGV-M, which codes for the early gene *pe38*. This stretch was also not found in any of the other resistance overcoming isolates. Combined with the results of the phylogenetic analysis, it is proposed that these 24 nt are a recent insertion into the CpGV-M genome.

The role of *pe38* in overcoming resistance was investigated by knocking out *pe38* of a CpGV-M based bacmid. This construct was not able to induce infection of susceptible (CpS) or resistant (CpRR1) CM larvae. When *pe38* of the resistance overcoming CpGV-I12 was inserted into the knock-out bacmid, the infectivity could not be rescued, neither in CpS nor in CpRR1, suggesting that the genomic portion of *pe38* might also play a role in its function.

Combining bioassay data, RFLPS and phylogenetic analysis, it can be concluded that the recently observed resistance is not a resistance to CpGV in general, but to CpGV-M

and related A-type genomes. In this thesis, several resistance overcoming isolates were identified. RFLP and SNP analysis provide tools for identifying and characterising different CpGV isolates reliably, a pre-condition for a future registration of CpGV products based on novel CpGV isolates.

1 Introduction

"Pest insects do not develop resistance or tolerance" to naturally occurring pathogens indicated Jaques (1983) as advantage for their use instead of chemical pesticides in insect pest control. Emergence of mass resistance of insects to baculoviruses was considered unlikely for several years, due to the long time of co-evolution of insects and their viruses (Kirschbaum, 1985; Hawtin, 1992). Indeed, observations of increase in baculovirus field resistance were extremely rare (for review see Cory and Myers, 2003), and resistance to baculovirus infection was rather reported as a reversible reaction selected for in the laboratory. Briese (1986a), in contrast, claimed "to consider the evolutionary consequences of artificially manipulating the balance between pathogen and host in the field" by increased use of microbial control agents. He suggested to keep control program management and the selection of more virulent pathogen strains in view.

These considerations were promptly of actuality when in 2005 the first cases of baculovirus field resistance were reported, concerning codling moth populations in Germany (Fritsch et al., 2005). Later, resistant populations were also reported from France, Italy, Austria, Switzerland and the Netherlands (Sauphanor et al., 2006; Asser-Kaiser et al., 2007; Jehle et al., 2008). This was the first documented case of field resistance against a baculovirus biocontrol product. Resistance to the biological control agent *Cydia pomonella* granulovirus (CpGV) is a severe threat to the efficient control of codling moth in organic and integrated farming. For designing sustainable strategies for future application, it is crucial to search for alternatives, such as further CpGV isolates which are able to overcome this resistance. The molecular mechanism of developing and overcoming resistance needs investigation, in order to evaluate future resistance management strategies. This thesis addresses the characterisation and methods for the identification of novel, resistance overcoming CpGV isolates, as well as investigations on the molecular mechanism involved in overcoming codling moth resistance to CpGV.

1.1 Baculoviruses

1.1.1 Baculovirus structure and infection cycle

More than 20 families of insect viruses are described, covering DNA and RNA viruses (Fauquet and Mayo, 2005). In three virus families, occluded viruses can be observed: the *Baculoviridae*, cypoviruses (CPVs, *Reoviridae*) and entomopoxviruses (EPVs, *Poxviridae*). Development of insect viruses as microbial control agents is based predominantly on baculoviruses, which are double stranded DNA viruses with a circular closed genome of 80-180 kb (Rohrmann, 2008). Baculovirus DNA is packed into rod shaped virions, which are embedded in a proteinacous matrix called occlusion body (OB) (Granados, 1980) (Fig. 1.1, Fig. 1.2). The OB does not only protect the virions from physical damage by environmental agents (e.g. UV light), it also delivers the virus to the alkaline midgut of the host (Hu et al., 2003). More than 600 viruses belong to the family of *Baculoviridae*. A typical feature of baculoviruses is the appearance of two virion phenotypes during their replication cycle. Beyond the occluded form, which mediates oral infection from insect to insect, the spreading of infection from cell to cell is transmitted by the non-occluded budded virus (BV) (Federici, 1997). Genetically, these two morphological forms are identical.

Two major groups of baculoviruses can be observed based on OB morphology: the Nucleopolyhdrovirus (NPV) and Granuloviruses (GV). For NPVs, the occlusion bodies of 0.15-15 µm in size are called polyhedra and contain several virions. Occlusion bodies of GV (granules) with a size of 120-300 nm are significantly smaller and contain typically one (rarely two or more) virions (Hu et al., 2003; Rohrmann, 2008) (Fig. 1.1 A, B). Among NPVs, virions with single nucleocapsids (SNPV) or multiple nucleocapsids (MNPV) are distinguished (Granados, 1980) (Fig. 1.1 A).

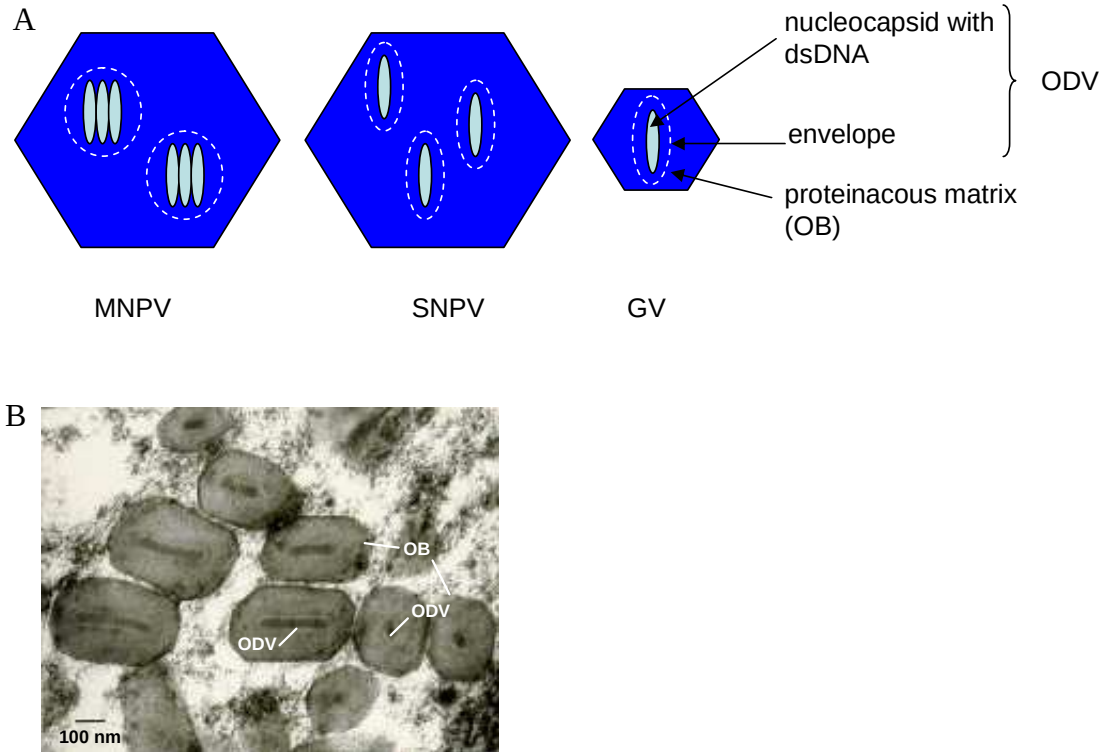


Fig. 1.1. (A) Schematic drawing of occlusion bodies (OBs) of MNPVs, SNPVs and GVs. MNPVs contain multiple nucleocapsids, SNPVs and GVs only one per virion. MNPVs and SNPVs contain several virions, GVs only one. (B) Electron micrograph of *Cydia pomonella* granulovirus (CpGV). Viral occlusion bodies (OB) and virions (ODV) are clearly visible. Photo: Dr. A. Huger, Julius Kühn-Institut, Darmstadt.

Baculoviruses infect only larval instars, and the natural way of infection is the oral infection by uptaking OBs. Primary infection is initiated when virus OBs are dissolved in the alcaline conditions (pH > 10) of the insect midgut (Fig. 1.2). The virions then released from the OBs are called occlusion-derived viruses (ODVs). The next barrier to virus infection in the insect midgut is the peritrophic membrane (PM). For the disintegration of the PM two mechanisms are discussed, one by virus-coded metalloproteinases (Wang et al., 1997) or by bacterial proteinases associated with the OB (Rubinstein and Polson, 1982; Rohrmann, 2008). Infection of the midgut epithelial cells is then mediated by four genes, the three *per os infectivity factors (pif)* and *p74*. These genes have conserved homologues in all sequenced baculovirus genomes (Faulkner et al., 1997; Kikhno et al., 2002; Pijlman, 2003; Fang et al., 2006). P74 was shown to be essential for primary infection of larval midgut cells by mediating ODV binding to

cellular targets (Faulkner et al., 1997). PIF-1 and PIF-2 are also essential for mediating receptor binding to midgut cells, whereas PIF-3 is involved in another crucial, but yet unidentified event in primary infection (Ohkawa, 2005). After entering the cell, the ODVs are transported to the nucleus, a process, which most likely involves actin polymerization (Lanier and Volkman, 1998). Here, transcription of viral genes and genome replication is induced. Viral replication leads to the development of a virogenic stroma and hypertrophy of the nucleus (Walker et al., 1982). Typical for the granulovirus initial phase of replication is the enlargement of the nucleus, which is attended by the disintegration of its membrane (Federici, 1997). Following the production of nucleocapsids, the newly synthesised genomes are packaged with viral proteins. The nucleocapsids bud out of the basal plasmalemma and acquire an envelope derived from the cell membrane during this process (Granados and Lawler, 1981). This virion phenotype is now called budded virus (BV) and spreads via tracheal and/or hemolymph in the insect causing the secondary infection. The major BV fusion protein is the F protein, which is involved in entering into and budding from cells. The F protein is found in all lepidopteran baculoviruses (Pearson and Rohrmann, 2002). However, in some lepidopteran NPVs, the so-called group I NPVs, the function of the F protein is replaced by GP64 (Lung et al., 2002). Insect tissues are entered by either direct penetration trough the basal lamina, or by tracheal junctions. Some GVs, e.g. CpGV, infect the tracheal matrix it enters the tissue by direct penetration of BV from the midgut cells into tracheoblasts (Federici, 1997). Later in secondary infection, the nucleus enlarges and ruptures; cytopslam and nucleoplasm mix. Nucleocapsids assemble and are enveloped by membranes outside the stroma. The OB protein Granulin is highly expressed in the very late phase of infection, and virions are occluded and the cell becomes filled with granules (Federici, 1997). In the final stage of infection, tissues and cells of the insects disintegrate and the OBs are released into the environment, ready to start a new infection cycle when they are ingested by other insects.

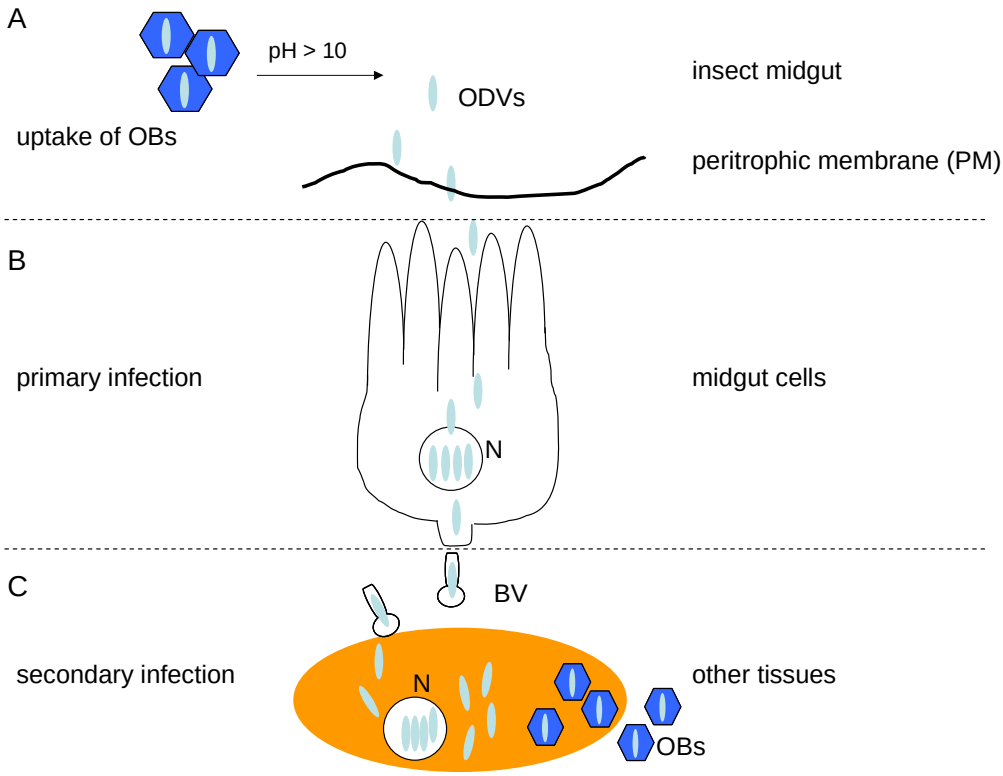


Fig. 1.2. Schematic CpGV infection cycle. (A) After uptake of occlusion bodies (OBs) *per os*, the OBs are solubilised in the alkaline milieu of the insect midgut, releasing the occlusion derived viruses (ODVs). ODVs pass the peritrophic membrane (PM). (B) Primary infection starts with fusion of ODVs with the midgut epithelial cells. They enter the nucleus (N), where replication is started. The newly synthesised nucleocapsids bud through the membrane of the epithelial cell. (C) Secondary infection starts with release of budded virus (BV) and the infection of other tissue cells. After replication in the nucleus (N), nucleocapsids are embedded in proteinaceous occlusion bodies (OBs) very late in infection. Upon liquefaction of the host cells, OBs are released into environment to start the next infection cycle.

1.1.2 Classification of baculoviruses

Until recently, classification was mainly based on the morphological features of OBs. With increasing availability of genomic data, phylogenetic analyses using conserved baculovirus genes provided further insight into baculovirus diversity and evolution. Based on gene content, NPVs can be divided into two groups. Group I NPVs for example possess as their BV fusion protein GP64, group II NPVs lack *gp64*, using the F protein (Zanotto et al., 1993; Pearson et al., 2002). Based on phylogenetic comparison

of 29 baculovirus genomes, it was found that baculovirus phylogeny does not follow the OB morphology, rather than host phylogeny (Jehle et al., 2006c; Herniou et al., 2003). Accordingly, a new classification containing four genera of the baculovirus family was established: Alphabaculoviruses include all lepidopteran-specific NPVs, whereas Betabaculoviruses comprise the lepidopteran-specific GVs; Gammabaculoviruses and Deltabaculoviruses include hymenopteran-specific NPVs and dipteran-specific NPVs, respectively. Baculoviruses are named according to the host from which they were isolated. Their names are abbreviated using the first two letters of the genus and species. Only viruses with numerous citations in literature and originally named using only the first letter of genus and species of their hosts, retain their original abbreviations (Rohrmann, 2008).

1.1.3 Baculovirus genome and gene expression

By biochemical analysis and electron microscopy, baculovirus genomes were found already more than 30 years ago to appear as circular, covalently closed DNAs with wide divergence in size within different viruses (Summers, 1972). Restriction endonuclease analysis (REN) has been available since the 1970s for characterisation of baculovirus genomes. It provides a powerful tool for the estimation of total genome sizes, identification of different baculoviruses or for establishing physical maps of the genomes (Possee and Rohrmann, 1997). The next step to revolutionise genome analysis was the possibility of whole genome sequencing. Since the first publication of the completely sequenced genome of *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) in 1994 (Ayres et al., 1994), the number of sequenced baculovirus genomes increased rapidly over three in 1997 (Possee and Rohrmann, 1997) to more than 50 today (Harrison, 2009). Genome sizes of baculoviruses described so far range from 80 to 180 kb, encoding between 89 open reading frames (ORFs) in *Neodiprion sertifer* (Nese) NPV to 181 ORFs in *Xestia c-nigrum* (Xecn) GV (Van Oers and Vlak, 2007). Comparative analysis of 13 baculovirus genomes revealed 30 core genes, which are present in all baculoviruses analysed. The products of these genes are involved in different stages of the infection cycle and could be assigned to RNA transcription, DNA

replication, to structural and auxiliary proteins and proteins which function have not been determined yet (Herniou et al., 2003). The wide range of genome sizes indicates that some baculoviruses lack genes present in other baculoviruses (Possee and Rohrmann, 1997). Indeed, differences in gene content were not only found between different viruses, but even in isolates of one virus. Comparison of the genomes of two genotypes of *Mamestra configurata* nucleopolyhedrovirus-A (MacoNPV-A) isolated from the same host population revealed one additional ORF in one of the genotypes (Li et al., 2005). Comparison of four variants of AcMNPV showed that these field isolates contained an additional gene compaired to the previously determined sequence (Yanase et al., 2000), showing that genomic heterogeneity in baculoviruses can also be found between isolates of one species.

Several sources of genetic variability have been located during the last decades. Genetic differences are primarily found in the region of *bro (baculovirus repeated open reading frame)* genes, suggesting that they are involved in providing baculovirus genome variation (Li et al., 2005). Recombination events (for example when a host is infected with more than one virus isolate) and the uptake of transposons play a role in the acquirement of new genes (Van Oers and Vlak, 2007). Horizontal transfer of transposable elements has also been described for some baculoviruses, including CpGV (Jehle et al., 1995; 1998). Beyond that, regions containing *hr (homologous region)* repeats are hotspots of intragenomic recombination (Van Oers and Vlak, 2007), contributing to the genomic plasticity described for baculoviruses. These regions consist of both direct repeats and imperfect palindromes with similar counterparts spread over the genome (Possee and Rohrmann, 1997). *Hr* regions were also shown to function as *cis*-acting transcriptional enhancers of early gene expression in AcMNPV (Lu and Miller, 1997) and may act as origins of replication (*ori*) (Kool et al., 1994).

Baculovirus ORFs are present on both DNA strands in clockwise and anti-clockwise orientation. They start with ATG and generally do not contain introns, an exception is the AcMNPV *ie0 transcriptional unit* with an intron of 4.5 kb. Baculovirus gene expression is temporarily regulated by four classes of genes: Immediate early genes (IE), delayed early genes (DE), late (L) and very late (VL) genes (Van Oers and Vlak, 2007). By definition, the early genes are transcribed before virus replication. Therefore, early

transcription is independent of viral DNA replication and late gene expression (Friesen, 1997). Early genes are transcribed by host RNA polymerase II and are thus not affected by inhibitors of DNA synthesis. Their gene products like the transactivator *ie-1* activate delayed-early genes. Proteins needed for viral DNA replication or for reprograming hosts cells towards virus propagation are encoded by early genes.

Late genes are transcribed after the beginning of viral DNA replication and are encoded by viral RNA polymerase, insensitive to α-amanitin. They code for structural proteins; for example *p6.9*, *vp39*, *vp1054* and *vp91* are involved in nucleocapsid structure or assembly and are conserved among baculovirus genomes (Herniou et al., 2003). In the very late phase, genes for structural proteins like polyhedrin/granulin for OB synthesis are transcribed. Baculovirus late gene expression is linked to DNA replication. For expression of late genes, the so-called late expression factors (LEFs) are needed (Friesen, 1997; van Oers and Vlak, 2007). All but one genes associated with AcMNPV late gene expression and DNA replication are also involved in host range determination (Thiem, 1997).

1.1.4 Baculovirus host range determination

In general, baculoviruses have a very narrow host range. The determining factor of their host specificity is the ability to enter cells and tissues of the host organism, to replicate there and to produce new virus particles (Thiem, 1997). The occlusion body is the first limitation for host entry. Lepidopteran larvae have an alkaline midgut milieu, where the proteinacous matrix of the OB is dissolved for release of the virions. Organisms lacking these alkaline conditions should therefore not be susceptible to baculovirus infection. Indeed, studies on non-target organisms like birds, mammals and wasps revealed no effect of OB uptake (Miller and Lu, 1997). As the occluded form of virus is the form usually applied in the field, this kind of host range limitation is a natural advantage for the use of baculoviruses as biocontrol agent.

On molecular level, host range is limited by the ability of the viral protein products to interact with host cell components. Numerous studies have been made using insect cell

culture transfected with *Bombyx mori* nuclear polyhedrosis virus (BmNPV) and AcMNPV. These two baculoviruses show a high sequence identity of about 93% on predicted protein level, but they differ significantly in their infectivity for host cells. BmNPV replicates in *Bombyx mori* (BmN) cells, but not in *Spodoptera frugiperda* (Sf) cells; AcMNPV replicates in Sf (and many other cell lines), but not in BmN cells. Despite the fact that these two viruses do not infect their heterologous cell line, the patterns of gene expression differed in the non-permissive cell lines (for review see Rohrmann, 2008).

Further limitations to viral infection can occur at the level of virus entry into cells or by a block of replication in the selected cells (reviewed in Lu and Miller, 1997). Regarding virus entry into cell, the role of BV is crucial for host range considerations, because they spread the systemic infection in the insect tissues. BV enter cells by endocytosis (Volkman et al., 1986). Studies on AcMNPV BV entry into non-permissive insect cells showed that the BV were able to enter the cells and to deliver the viral DNA to the nucleus (Miller and Lu, 1997). The expression of reporter genes under the control of different promoters revealed that expression could be detected from host and early viral promoters, while it was not present in all cell lines from late or very late promotors. The finding that AcMNPV was able to enter into the nucleus of several cell lines suggested that the baculovirus host range is not determined by the presence of appropriate receptors for virus attachment and entry into the host cell, but that the block of infection in semi- or nonpermissive insect cells takes place during or after gene transcription, expression or viral DNA replication. This was corroborated by the fact that AcMNPV is also able to enter mammalian cells (Miller and Lu, 1997; Thiem, 1997).

During the last two decades, several genes which seem to be involved in host range determination could be identified. Following homologous recombination by co-infection of Sf21 cells with BmNPV and AcMNPV, a recombinant virus eh-AcMNPV with wider host range could be isolated (Maeda et al., 1993). This recombinant virus was able to replicate in both Sf21 and BmN cells. A 572 bp fragment could be identified in eh-AcMNPV which originated from BmNPV and was responsible for this altered host range. This fragment derived from the coding region of the putative DNA helicase gene, implicating that DNA helicase plays a role in baculovirus host range definition

(Maeda et al., 1993). Further studies performed on the minimal essential nucleotides (nt) involved in expanding the AcMNPV host range showed that substitution of the two nucleotides G and C in AcMNPV to A and T in the recombinant virus were sufficient to expand its host range (Croizier et al, 1994). This substitution changed a single amino acid from Ser (AcMNPV) to Asp (BmNPV). Coinfection of wild-type BmNPV and AcMNPV in BmN cells led to a complete inhibition of BmNPV replication and a stop of both viral and host protein synthesis. This effect was not found when co-infection was done using the eh-AcMNPV mutant carrying the 572 bp fragment originating from BmNPV. Therefore, it was suggested that the AcMNPV wild-type DNA helicase inhibits directly or indirectly the translational machinery of BmN cells (Kamita and Maeda, 1993).

Transient expression assays in Sf21 revealed that 18 genes of AcMNPV contribute to the expression of a reporter gene under late viral promotor control, the so-called late expression factor (*lef*) genes (Lu and Miller, 1995). Testing of this LEF library in TN-368 cells (deriving from *Trichoplusia ni*) indicated that in this cell line, an additional factor was necessary for reporter gene expression. By co-transfection of the LEF library with an AcMNPV clone library, ORF 70 could be identified as beeing involved in late gene expression and was therefore termed host cell-specific factor-1 (*hcf-1*) (Lu and Miller, 1995). Another host range factor-1 (*hrf-1*) was identified as a *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) gene which enabled AcMNPV to infect Ld652Y cells (deriving from *L. dispar*) after co-transfection with the two viruses (Thiem et al., 1996). *Hrf-1* is not associated with DNA replication or late gene expression (Thiem, 1997). Among the baculoviruses, only one *hrf-1* homologue is found in *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV), raising the question of the evolutionary origin of *hrf-1* (Ikeda et al., 2005).

One reaction of insect cells to counteract viral infection is the initiation of host cell apoptosis. To overcome this block of infection, baculoviruses are able to interfere with this process using different genes and mechanisms (reviewed in Rohrmann, 1998). The AcMNPV *p35* overcomes the host cellular defense against infection by expression of an inhibitor. P35 blocks apoptosis, which can be induced in some insect cells as a repsonse to viral infection, by binding to and inactivation of caspase 1 (Bump et al., 1995). In

CpGV, a gene with similar function was identified and termed *iap* (inhibitor of apoptosis). The predicted polypeptide sequence of IAP, containing a zinc-finger motif, had no homology to AcMNPV P35, indicating that these genes are unrelated in their mode of action and evolutionary history.

1.1.5 *Cydia pomonella* Granulovirus (CpGV)

CpGV was first isolated from infected codling moth (CM) larvae in 1963 in Mexico (CpGV-M) (Tanada, 1964). Due to its high virulence against codling moth, its detection was promising for the development of CM control strategies (Tanada, 1968). Since 1979, research on biological control using baculoviruses was concerned with production, standardisation, formulation and application of virus products. In France, an apple orchard IPM (integrated pest management) program was then based on the application of CpGV, and the product Carpovirusine was developed and tested in several orchards. In 1987, the CpGV-product Madex was registerd in Switzerland by Andermatt Biocontrol. Two years later, Höchst registered Granupom in Germany (Huber, 1998). Since then, CpGV products have been applied in most of the European countries, in South Africa, North and South America and in New Zealand. All products registered in Europe contain the isolate CpGV-M as active ingredient. Based on restriction fragment length polymorphism (RFLP) analysis, further isolates from England (CpGV-E) and Russia (CpGV-R) have been identified (Crook et al., 1985; Crook et al., 1997), but not applied in the field, because they did not show any superior effect to CpGV-M in laboratory bioassays.

The isolate CpGV-M1, an *in vivo* cloned genotype of CpGV-M, was completely sequenced (Luque et al., 2001). The circular closed genome contained 143 putative ORFs and had a length of 123,500 nt. Eleven of these ORFs are so far found to be unique to CpGV (Lange, 2003). 118 ORFs were homologous to other baculoviruses. Coding sequences represented 88.4% of the genome. In CpGV, the homologous repeat sequences (*hrs*), present in most baculovirus genomes (Van Oers and Vlak, 2007), were not present. Instead, one major repeat region and 13 copies of a 73-77 bp imperfect palindrome dispersed over the genome were found (Luque et al., 2001). These 13 repeat

regions were shown to replicate in infection-dependent assays and are therefore putative *origins of replication* (*ori*) in CpGV (Hilton and Winstanley, 2007).

1.2 *Cydia pomonella*: a pest in apple orchards

The geographical origin of the codling moth *Cydia pomonella* (L.) is Eurasia, where it was associated with apples and walnuts. From there, it spread around the world with growing production of apples and pears and became a major pest in plantings. Further hosts are quinces and apricots. The damage on the fruits is caused by the feeding larvae (Barnes, 1991). Today, CM is an important pest in Europe, North and South America, South Africa, New Zealand and Australia (Vickers and Rothschild, 1991). CM infestation can result in consequences as drastic as a complete loss of marketable fruits, causing severe economic damage to the growers.

1.2.1 Codling moth life cycle and damage

Full-grown codling moth larvae (L5) are overwintering in a cocoon hidden under the bark of sterns or branches, in a stage called diapause. Between April and June, they start to pupate, most likely controlled by temperature (Barnes, 1991). End of May, the moths of the first generation hatch. Their flight time is during dusk at temperatures of about 15°C (Alford, 1984). Eggs are laid on fruits and leaves, with egg development being temperature-dependent and taking 80-85 degree days with temperatures over 10°C (Harzer, 2006). After 10 to 14 days, L1 larvae start to hatch (Alford, 1984). Between several hours and a few days later, the neonate larvae start to bore into the fruit, often through the calyx to the core, where they also feed (Harzer, 2006). The following larval stages (L2-L5) develop in the fruit. After four to five weeks, the L5-larvae leave the fruit. The length of the single larval stages is mainly temperature-dependent. L5 larvae then either hide under rough bark for the next round of diapause, or, beginning of July until August, they start to pupate again and develop a second generation. From end of July to beginning of September, the second generation of moth flys. In Southern Europe, two to three generations per year are common, whereas in Northern Europe only one generation appears. Depending on climatic conditions, up to four generations per year can be observed (Lacey et al., 2007). Usually, only one larva develops in one

apple. Infested fruits are not marketable and can only be used for juice production. Inside the fruit, the larva feeds on the pulp and also on the developing seeds. The openings fill with feces and the color of the entry hole changes to red. Damaged fruits ripen and fall down from the tree earlier than healthy fruits. The second generation can cause damage even more severe as the first generation, because eggs are laid on already ripening fruits. High infestation is especially observed close to fruit storehouses or to the storage of fruit boxes from the preceding year (Alford, 1984). Even after heavy frost periods, when all fruit sets are destroyed and no fruits develop, CM does not die out, the larvae then feed on leaves and sprouts (Heinze, 1978).

1.2.2 Codling moth control strategies

Codling moth is the most devastating pest of apple (Barnes, 1991). If CM control fails in an orchard, further pest control (mites, aphids, leafrollers etc.) is invalid, because production of high quality fruits is not possible (Croft and Riedel, 1991). Assailable for biological control are the larval stages L1 and L5, the other larval stages L2 to L4 develop in the fruits. Neonates are only amenable until they bore into the fruit. Diapausing or L5 larvae are accessible to control agents for a longer period, but they do not feed anymore and therefore can not uptake pathogens *per os* (Lacey et al., 2007). The application of chemical insecticides to control neonate larvae underwent several “eras” (Croft and Riedel, 1991). Until the 1950s, control of L1 larvae was done with lead arsenate as gut insecticide or using DDT, because of its long effectivity (Kotte, 1958). However, CM populations developped resistance in the field to both chemicals (Croft and Riedel, 1991). Therefore, new compounds like the organophosphate parathion or several pyrethroids were introduced, but the possibility of further resistance development had to be kept in mind. Chemical insecticides can also have severe effects on non-target organisms. Besides, the consumer today does not accept fruits with residues of chemical pesticides. Therefore, biological control agents became more and more important. On one hand, natural enemies of insects like parasitoids, nematodes, protozoa and bacteria haven been tested in the field (reviewed in Falcon and Huber, 1991). But they showed only little decrease of fruit damage or were limited to distinct climatic conditions. Reduction of codling moth damage was reported when using entomopathogenic

fungi (Falcon and Huber, 1991). On the other hand, an attempt to control CM on the level of moth is mating disruption using pheromone dispensers. However, success of this so-called mating disruption depends on population densitiy and the number of generations per season and is therefore only usefull for monitoring. Usually, fruit damage must not exceed 1-2% to be economically maintenable for the grower. Therefore, mating disruption is combined with the use of insecticides when the larval population exceeds 1000 per hectare (Vickers and Rothschild, 1991). Against the background of these control attempts, it is apparent that the discovery of CpGV solved several problems of the conventional insecticides. It is highly specific to CM and therefore does not interfere with non-target organisms in the orchard. This is combined with a very high virulence to CM; its LD_{50} for neonate larvae was determined to 1.2 granules/larva (Huber, 1986). Transmission of the virus from insect to insect may spread infection in a CM population. On the other hand, there are some concerns of the growers regarding CpGV application: its inactivation by UV radiation, a slow speed of kill and the need for multiple applications, which is attended by higher costs. Today, CpGV is applied on more than 100,000 ha in Europe and North America, according to informations from CpGV producers.

1.2.3 Occurrence of codling moth resistance to CpGV

It was often assumed that the development of resistance in codling moth against CpGV was unlikely, due to the complex replication cycle of CpGV (Rohrmann, 1998). Resistance was only induced in lab experiments when populations were kept under selection pressure, and insects returned to their initial level of susceptibility, when selection pressure was ended. Threefold resistance was observed in a laboratory strain of the fall armyworm, *Spodoptera frugiperda*, under selection pressure, which was lost when selection was stopped (Fuxa and Richter, 1989). A laboratory strain of the velvetbean caterpillar *Anticarsia gemmatalis* developed five fold resistance to its nucleopolyhedrovirus (AgMNPV) within four generations, but returned to its original level of susceptibility after stopping virus exposure. Two further cycles of selection for resistance and its reversion could be performed, suggesting that a control agent which induced resistance in a population can regain its usefulness when virus treatment is terminated (Fuxa

and Richter, 1998). After selection with NPV for over 13 generations, Brazilian colonies of *A. gemmatalis* became 1000 fold more resistant, whereas colonies from the United States revealed only five times higher resistance (Abot et al., 1996).

Codling moth populations which showed a reduced susceptibility to CpGV application in the field were first observed in two orchards in 2003. F1 offspring of these field populations was tested in bioassay and showed a 500-1000 fold reduced susceptibility to CpGV than the sensitive lab population (Fritsch et al., 2005). At the same time, bioassay tests on a CM field population in France revealed a 13,000 fold reduced susceptibility compared to the internal lab population (Sauphanor et al., 2006). Until 2006, CM populations of 13 German organic apple orchards were tested systematically in bioassay for their susceptibility to CpGV-M. Resistance ratios from 1000 to 10,000 fold were reported (Asser-Kaiser et al., 2007). Initially, mass crossing experiments of a laboratory reared field strain (BW-FI-03, Süd-Baden) suggested that CpGV resistance is inherited in an autosomal, semidominant manner (Eberle and Jehle, 2006). However, when single pair crosses of a strain CpRR1, which derived from BW-FI-03 were performed, clear evidence for a sex-linkage to the chromosome Z and a concentration dependent dominance could be provided (Asser-Kaiser et al, 2007; 2010). This was consistent with the observation of rapid emergence of CpGV resistance. Selection for both homozygous and heterozygous resistant insects was fostered by the fact that all commercially available products in Europe were all based on one isolate CpGV-M, which was applied several times during a season for several years in the orchards.

1.3 Aim of this thesis

Since the first occurrence of codling moth resistance a few years ago, an intense search for future alternatives to CpGV-M was initiated (Jehle et al., 2006a; Jehle, 2006b). When the mode of inheritance of resistance to CpGV-M was determined, it was clear that resistance management could not be based on further CpGV-M application. Sex-linked inheritance provides a rapid selection, and it was suggested that the resistance gene is selected for in the field even at low virus concentrations (Asser-Kaiser et al., 2007).

This situation was the basis of questions to be solved in this theses:

- Are there other CpGV isolates more virulent to CM than CpGV-M?
- If so, what is the difference to conventional CpGV-M?
- Is it due to an additional gene present in resistance overcoming isolates or is it based on a single nucleotide mutation?
- What is the molecular mechanism of overcoming resistance?
- And what does this mean for the future application of CpGV in biological control of codling moth?

In Chapter 3, 16 CpGV isolates originating from different geographic regions were tested for their virulence in bioassays. For determining differences in their genomic content or organisation and for their characterisation, CpGV isolates were compared to CpGV-M1/-M by RFLP analysis.

Genomic differences found between resistance overcoming isolates and CpGV-M1 by RFLP analysis were investigated by partial genome sequencing in described Chapter 4. By phylogenetic analysis of single nucleotide polymorphisms (SNPs) in conserved genes, a new grouping of isolates which is not based on the geographic origin was aimed for.

Whole genome sequencing (Chapter 5) of two resistance overcoming isolates and CpGV-M as internal reference was done to investigate differences between the isolates on genomic level.

In Chapter 6, the difference found between resistance overcoming isolates and CpGV-M1 was tested for its role in overcoming resistance. The role of the gene *pe38* was investigated by knock out of *pe38* of a CpGV-M bacmid and swapping of CpGV-I12 *pe38* into the knock out bacmid.

The results of all chapters are combined in Chapter 7 and discussed for a general conclusion.

2 Materials

2.1 Chemicals

The following chemicals (Table 2.1a) and antibiotics (Table 2.1b) were used:

Table 2.1a. The chemicals used were purchased from the following suppliers.

1 kb DNA ladder	GeneRuler™
10 × NH_4 reaction buffer	Axon Labortechnik
50 mM $MgCl_2$ solution	Axon Labortechnik
Antibiotics	Roth
dNTP-Mix 12.5 µml	Axon Labortechnik
Fast Digest restriction enzymes	New England Biolabs
iProof™ High-Fidelity DNA Polymerase 2 U/µl	Bio-Rad
IPTG	Roth
other chemicals	Roth
PCR Primer	Eurofins mwg operon
Proteinase K	Roth
QuantiTect SYBRGreen I PCR Kit	Qiagen
Restriction enzymes	Fermentas Life Sciences
RNAse A	Roth
T4 DNA Ligase 1U/µl	Fermentas Life Sciences
Taq DNA Polymerase 1000 units, 5 U/µl	Axon Labortechnik
X-Gal	Roth
λ/*Hin*dIII DNA ladder	Fermentas Life Sciences

Table 2.1b. Antibiotics for selecting the different bacmids constructed.

Antibiotics	Stock solution
Chloramphenicol	30 mg/ml in EtOH
Ampicillin	100 mg/ml in 50% EtOH
Tetracycline	10 mg/ml in 75% EtOH
Kanamycin	30mg/ml in ddH_2O

2.2 Bacterial strains and plasmids

For cloning of plasmid and bacmids, following materials (Table 2.2) were used.

Table 2.2. Cells and plasmids used for the cloning of different bacmid constructs.

***E. coli* strain**	**Description**	**Supplier**
DH5α	F^-Φ80d*lac*ZΔM15Δ(*lac*ZYA)U169, *rec*A1, *end*A1, *hsd*R17, (r_k^-,m_k^+), *pho*A, *sup*E44, λ^-, *thi*$^-$ *gyr*A96 *rel*A1	Invitrogen
TransforMax™ EPI300™	F^-,mcrA, Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15, Δ*lac*X74, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ^-, *rps*L, *nup*G, *trf*A, *dhfr*	Epicentre
Plasmid	**Description**	**Supplier**
pUC19	0.5 µg/µl	Fermentas Life Sciences
CpBac	Elmenofy (2008)	-

2.3 Media and buffers

All solutions, buffers and media were prepared in ddH_2O and autoclaved (121°C, 1bar, 20 min) or sterilised by filtration (0.11 µm Millipore-Filter).

LB-Agar (Luria Bertani Medium)

5 g Bacto Tryptone

10 g Bacto Yeast Extract

5 g NaCl

15 g Agar were added when preparing solid LB media. The pH value was adjusted to 7.2-7.5 by titrating with 1 N NaOH or HCl before filling up to a volume of 1 L.

SOC-Medium

20 g Bacto Tryptone

5 g Bacto Yeast Extract

0.5 g NaCl

0.2 g KCl

2 g $MgSO_4 \times 7\ H_2O$

After filling up to a volume of 1 L and sterilisation by autoclaving, 20 ml sterile filtered 1 M glucose were added.

TE- Puffer pH 8

10 mM Tris-HCl pH 8.0

1 mM EDTA pH 8.0

10 × TAE- Puffer

48.4 g Tris

11.4 ml acetic acid

10 ml 1 M EDTA pH 8.0

Sterilisation was done by autoclaving after filling up to a volume of 1 L.

Semi- synthetic insect medium (Ivaldi-Sender)

For 500 ml semi-synthetic medium (Ivaldi-Sender, 1974) the following components were weighed in:

32 g corn meal

5 g agar agar

33 g wheat germ

15 g brewers yeast

2.85 g ascorbic acid

1.15 g 4-Hydroxybenzoesäuremethylester (nipagin)

Agar and corn meal were disolved in 500 ml water and autoclaved. Ascorbic acid was dissolved in 25-30 ml distilled water, nipagin in a small volume of 96% EtOH (v/v). Corn meal, agar, wheat germ, brewer`s yeast and nipagin were mixed directly after autoclaving. In order to avoid decomposition, ascorbic acid was added when the mixture had cooled down to 60°C.

2.4 Insect strains

Susceptible *Cydia pomonella* larvae (CpS) used as reference strain in the bioassays were derived from the insect rearing at the DLR Rheinpfalz, Neustadt/Weinstrasse. This population originated from Andermatt Biocontrol (Switzerland) and has been reared

continuously virus free for more than nine years. The generation time of CpS was about one month. After the population became CpGV contaminated at the end of 2008, a new population was established from codling moth eggs delivered from Andermatt Biocontrol AG (Switzerland).

The resistant strain CpR (BW-FI-03) originated from a field population from an orchard in South Baden, Germany, where CpGV products failed to control the codling moth population (Fritsch et al., 2005). The population CpR has been reared at the DLR Rheinpfalz since 2005.

Establishment of a homogenous resistant population CpRR1 based on the insect strain CpR had become necessary in 2006. Testing of the offspring of single pair crosses of CpR had shown that this strain still contained susceptible insects (Asser-Kaiser et al., 2007). The offspring of single pair crosses were tested at a discriminative concentration of 5.8×10^4 OB/ml (Asser-Kaiser et al., 2009). Families with resistant offspring were subjected to two further rounds of selection before establishing the homogenous strain CpRR1 (Asser-Kaiser et al., 2007).

2.5 Origin of CpGV Isolates

The so-called “Mexican Isolate” CpGV-M (Tanada, 1964) is identical to the virus used in the commercial products registered in Europe (Huber, 1998). It was already established as a virus stock at the DLR Rheinpfalz. By propagating this strain, the virus stock CpGV-M (EU) was produced as a reference for the EU project “SustainCpGV”.

The isolates originating from Iran, Georgia and Azerbaijan were collected and kindly provided by Dr. Mohammadreza Rezapanah (PPDRI Tehran, Iran) (Table 2.3). The *in vivo* cloned isolate CpGV-E2 derived from the so-called “English Isolate” CpGV-E (Crook et al., 1985) and was kindly provided by Dr. Doreen Winstanley, Horticulture Research International (HRI), Wellesbourne, UK.

Two isolates from Georgia, CpGV-G01 and -G02, were kindly provided by Dr. T. Chkhubianishvili (University of Tiflis, Georgia).

Table 2.3. Designation and origion of CpGV isolates examined in this work.

Isolate	Origin
CpGV-M	virus stock, DLR Rheinpfalz
CpGV-M (EU)	virus stock, propagated from CpGV-M Batch-N° TPCpGVBTPS_02
CpGV-I12	Tabriz, Iran Stock DLR Rheinpfalz, Batch- N° TPCpGVI12_01
CpGV-I01	Ghazvin, Iran (Laborstamm), larvae collected in September 1999 (Rezapanah et al., 2008)
CpGV-I08	Orumiyeh, Iran, larvae collected in September 1999 (Rezapanah et al., 2008)
CpGV-I66	Masshad, Iran, larvae collected in September 1999 (Rezapanah et al., 2008)
CpGV-I68	Orumiyeh, Iran, larvae collected in September 1997 (Rezapanah et al., 2008)
CpGV-I07	Ghazvin, Iran (lab strain) (Rezapanah et al., 2008)
CpGV-G01	Georgia
CpGV-G02	Georgia
CpGV-E2	isolated by *in vivo* cloning of CpGV-E (Reading) (Crook et al., 1997)
CpGV-S	isolation of Virosoft™
CpGV-AZ1	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-AZ2	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-AZ3	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-AZ4	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-AZ5	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-AZ6	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-AZ7	Guba, Azerbaijan, collected August 2007-March 2008
CpGV-G03	Gori, Azerbaijan, collected August 2007-March 2008
CpGV-G04	Gori, Azerbaijan, collected August 2007-March 2008

2.6 Software

For assembling the contigs obtained from whole genome sequencing to a consensus sequence, the program Seqman of the DNAStar Lasergene (version 7.1.0, DNASTAR Inc.) package was used. For open reading frame (ORF) prediction, the consensus sequence was exported into Genequest. Primerdesign was performed using PrimerSelect.

Sequence alignments were performed using Clustal W implemented in Bioedit (BioEdit Sequence Alignment Editor 7.0.5.3). Phylogenetic analysis based on these alignments was done using MEGA 3.1 (Kumar et al., 2004).

The statistical analysis of bioassays was performed with ToxRat Solutions Standard Version 2.09 (ToxRat Solutions GmbH).

Documentation of agarose electrophoresis gels was done using Opticon Monitor 3.1.12 (BioRad, 2005).

2.7 Oligonucleotides

Primer walking strategy was used to fill the remaining gaps after CpGV-I12 whole genome sequencing (Table 2.4).

Table 2.4. Oligonucleotides designed for filling the gaps after sequencing the CpGV-I12 genome. Given are the internal name, primer sequence in direction 5`-3` and the corresponding genome position in CpGV-M1 (Luque et al., 2001) which was used as a template for sequence assembly.

primer	sequence 5`- 3`	nt CpGV-M1
55115_for	GTCTCTGAGGGGTCGTCTTT	54,351-54,370
55785_rev	AATCCAACCCGCTCTGAATC	55,002-55,021
27839_for	TATCTGGGCGGCGAGGTTATCT	27,124-27,145
28275_rev	CCGAGGAAGTAGTGGTAGTGGTGA	27,560-27,583

primer	sequence 5`- 3`	nt CpGV-M1
12586_for	GGATCCTCACCCAGTTTCAGT	12,586-12,606
13131_rev	AGTGTCGTGGGTTGTAGTATTTTG	13,131-13,154
#3Karolin_for	AGGAGGAGGTTAGTGGAGGGTGTT	46,682-46,705
#3Karolin_rev	AGGAGAGTAAATCGTGGCAGTTGA	47,208-47,231
66568_upper	ACTTGTTCCTTGACCGCCTACGA	65,562-65,584
66568_lower	TCACCCCTTTTGCACCCTTGTT	66,381-66,402
64665_upper	TTGTTCCTTGACCGCCTACG	65,564-65,538
65091_lower	GACCCCTTGTTCACCACTCG	66,372-66,388
100712_upper	GAGACGACAAAGGGCGACGAC	99,525-99,545
100955_lower	CATCAGCGACTCCAGTTCAATCAG	100,146-100,169
119999_upper	CACGGTGTTGACAATGATGCTGA	119,056-119,078
119999_lower	AACAGTGCCACCACGCTCACA	119,630-119,650
64618_upper	CCACCCACATACATCATCAAATCT	63,506-63,529
65043_lower	CGCACCCACCAAATCGTTC	64,285-64,303
88245_upper	CGGCCGTGCATCGTTTTAG	86,684-86,702
89345_lower	GGGGCAGACTCGGTGTTACTG	86,6118-88,631
88587_upper	GAGGGTGGCGCGGAATAAGA	87,736-87,755
88587_lower	ACAATGCATCAAAGGCGTCGTT	88,684-88,705
27145_upper	TTTTGACGCCGCAGAGCCAGTA	26,295-26,316
27145_lower	CGGACACAAACACGCCACATA	27,233-27,253
36980_upper	GGCGGGTCCAGAAAATGTTA	36,143-36,162
36980_lower	GGTGGTGGTGGTAGTGATGATAGT	37,036-37,059
42600_upper	CGAGTGCCAAGCTGAAGGTGATAG	41,818-41,841
42600_lower	TGGTTGCGCTGGTTCTTGTTTTAG	42,206-42,229
3195_upper	GTGAGCGAAAACGGAAAATGACA	2951-2973
3195_lower	CGAAGCGGACGTGGAGGAGTT	3752-3772

For localisation of deletions, insertions or comparison of mutations in different CpGV isolates, specific PCR primers were designed (Table 2.5). These primers were also used for sequencing (Genterprise Genomics, Mainz). CpGV-M1 (Luque et al., 2001), was used as template sequence.

Table 2.5. Oligonucleotides designed for localisation of insertions and deletions in different CpGV isolates. Given are the internal name, primer sequence in direction 5`-3` and the corresponding genome position in CpGV-M1 (Luque et al., 2001).

primer	sequence 5`- 3`	nt CpGV-M1
ODV-e66_upper	TTGCCGGCCGTGCGTGTC	32,600-32,617
ODV-e66_lower	GAGGAACCCCCAACCGAACCATT	33,231-33,253
84000_upper	ACCGCCTCACCGTCCATAAACT	83,192-83,213
84000_lower	ACCCATCCCACGGCCTACATAC	84,285-84,306
82000_upper	CTTTCGCCGTCAACCTCTTCTGCT	81,634-81,657
82000_lower	GCGTTCCACCGATTACCGTCTCC	82,228-82,250
86000_upper	GGCGCACACTAAAATTACGACGAG	84,829-84,852
86000_lower	GGCGAGATACACCTGGGCAAAGT	85,645-58,667
93000_upper	GCGGCGGTGTCCAGTTTGAG	92,834-92,853
93000_lower	GCTATCGGTCCGTTGGGTAATGT	93,407-93,429
107000_upper	CGCGTATCAAAACAGCCCCTCATT	106,956-106,979
107000_lower	GTACCCGCCGATCCCGAAACAAA	107,728-107,750
110000_upper	AGCCCGTTTCACCTGTTCATAGTA	109,028-109,051
110000_lower	ATTTTAGAGCGAGCCGATTCAG	109,542-109,563
113000_upper	TGCGGCGAGGACAAGTTCAAT	113,698-113,718
113000_lower	GGTGGTGGAGCGGGTGGATAG	111,942-111,962
p74_upper	GCCCCGTGCACCAATCTCAAAA	49,721-49,742
p74_lower	GCTCGCGACGGCAACATTCAGT	50,266-50,287
pk1_upper	CTGATGCGCAACAACCCCAACT	1449-1470
pk1_lower	TAAGATTTCATACGCCACCACTCC	1874-1851
ORF94_upper	ATTGTCGCCGGCATTATTA	76,456-76,474
ORF94_lower	TTGGTTAGTGGTGGTTTATTTAGA	76,932-76,909

primer	sequence 5`- 3`	nt CpGV-M1
VS_for	GAATCTTCTCCCCCTCCAC	51,925-51,943
VS_rev	TCATCATTATTGCGACTGTTTC	52,214-52,193
18-20_for	ACCCTTATATTCCTACTCTTCTCT	17,968- 18,005
18-20_rev	TGTCCTTGGCTAGCTTATCTTA	20,003-20,024

For amplification of the highly conserved genes *late expression factor (lef)*-8 and *granulin (polh/gran)* degenerated primers were used (Lange and Jehle, 2003; Jehle et al., 2006) (Table 2.6). Sequencing was performed using the standard primers M13 und T7 (Genterprise Genomics, Mainz).

Table 2.6. Oligonucleotides for the amplification of the conserved genes *lef-8* and *polh/gran*.

primer	sequence 5`- 3`	target gene
PolH-M13 Forward	TGTAAAACGACGGCCAGTNRCNGAR GAYCCNTT	
PolH-M13-29	CAGGAAACAGCTA-GACCDGGNGCRAAYTC YTT	*polh/gran*
8Rmod-M13-29	CAGGAAACAGCTATGACCA(CT)(AG) TA(GC)GG(AG)TC(CT)TC(GC)GC	
T7-5F	TAATACGACTCACTA-TAGGGCA(CT)GG(ACT)GA(AG)ATGA C	*lef-8*

Three oligonucleotide pairs covering the region between 16 and 26 kb (CpGV-M1) were designed to localise the 0.7 kb insertion in CpGV-E2 (Table 2.7). The overlapping fragments had a size of about 4 kb each. For the area between 18 and 22 kb of the CpGV genome, it was not possible to find a specific primer pair. This region contains a *hr*-like region: repeat structures which are spread 13 times over the whole genome (Hilton and Winstanley, 2008).

Table 2.7. Primer for the localisation of the 0.7 kb insertion in CpGV-I12 and -E2.

primer	sequence 5`- 3`	nt CpGV-M1
16-20_lower	TAATCGCCGCCGCTTATCAA	19,907-19,926
16-20_upper	TGCGCCCTACCTATCAACATACG	15,911-15,933
20-24_lower	TCCTCAAACGAACCCTCAATAAG	23,996-24,018
20-24_upper	AAACCGCCTAATCAACTGATACAA	19,945-19,968
22-26_lower	GTCCGCCACACTGTCCTTGATG	25,945-25,966
22-26_upper	AGCGCCGGCCTGATAGCA	22,036-22,053

Oligonucleotides were designed to knock out *pe38* by homologous recombination (Table 2.8). They corresponded in their 5`- 3` nucleotides 1 to 50 to the CpGV-M1 sequence. The adjacent 25 and 24 bp, respectively, were specific to amplify the Tn5-neomycin-cassette of the Tn5-neo template DNA (Quick and Easy BAC Modification Kit, Gene Bridges). Neomycin was used as marker gene for the selection of knock out mutants. The expected size of the PCR product of the neomycin-cassette Tn5-neo was 1082 bp.

Table 2.8. Oligonucleotides for the construction of the *pe38* knock out bacmid CpBacΔpe38. The 50 nt corresponding to the CpGV-M1 sequence are given in italics.

primer	sequence 5`- 3`	target gene
left arm pe38 k.o.	*TTTTAACAATGTACTGGTGGCTGATGAA CTTCTGCTTGAGGTTCAATAGT*GGACA GCAAGCGAACCGGAATTGC	nt1-50: 18,624-18,673
right arm pe38 k.o.	*TTGGAGTATGAACCGATCGCGACCACT ACACCGGCGAGTGCCATA*TGTCGAA-GAACTCGTCAAGAAGGCG	nt1-50: 19,671-19,618
k.o.-check_lower	CCAAGCGGCCGGAGAACCTG	18,124-18,143
k.o.-check_upper	CGCCCTGCCCGACTGTGAAA	neomycin-cassette

The primer pair pe38_au_upper and pe38_au_lower2 flanked the *pe38*-gene in CpGV-I12 (Table 2.9). The upper primer contained a *Bam*HI and the lower primer an *Eco*RI restriction site to allow directed cloning of the PCR product into a vector. The expected PCR product size was 1317 bp.

Table 2.9. Oligonucleotides for the amplification of *pe38* of CpGV-I12. Underlined: inserted recognition sites for *Bam*HI and *Eco*RI respectively.

primer	sequence 5`- 3`	nt CpGV-I12
pe38_au_upper	GCCCAGGATCCAAAATAACAGCAATAATA	19,233-19,261
pe38_au_lower2	TCTAGAATTCGATAAACTTGTTTGTGCCAC	20,519-20550

Primer pair BacAmpCp15-rep_F and BacAmpCp15-rep_F was designed by Dr. Wael Elmenofy to swap a target sequence cloned in pUC19 into the GFP locus of the bacmid CpBac by homologous recombination (Tables 2.10-2.11). For detailed description of the sequences see 6.2.4.

Table 2.10. Oligonucleotides for construction of a bacmid CpBacΔpe38/I12-pe38 by homologous recombination, using the CpGV-I12 *pe38* gene. The nt corresponding to the GFP locus are given in italics. For detailed description see text.

primer	sequence 5`- 3`	target gene
BacAmpCp15-rep_F	*TACAGCCAGTAGTGCTCGCCGCAGTC GAGCGACAGGGCGAAGCCCTCGAG*G TACCTGAAGTTTTAAATCAATCTAAAGT	1-50: GFP-cassette (*italic*) 51-80: pUC19
BacAmpCp15-rep_R	*TAATTGTGTTTAATATTACATTTTTGTTGA GTGCATATCGAGGTCGACG*GTACCTCGTAT GTTGTGTGGAATTGT	1-50: GFP-cassette (*italic*) 51-78: pUC19

Table 2.11. Primer pairs used for screening for successful homologous recombination.

primer	sequence 5`- 3`	target gene
Swap_upper	AAAGCGCGGATCTGGGAAGTGACG	Chl^R-Gen
Swap_lower	ATGTGCGCGGAACCCCTATTTGT	Amp^R-Gen
Pacsite_upper	AGACGCGCCTGTTTACTTGT	101800-101819
Pacsite_lower	TGGCGGCACCTTTCTACG	102406-102423

3 Overcoming CpGV resistance in codling moth with new CpGV isolates

Parts of the results presented in this chapter have been published in: Eberle et al., 2008; Eberle et al., 2009.

3.1 Introduction

Due to its high virulence and host specificity, *Cydia pomonella* granulovirus (CpGV) is known to be one of the most efficient and safe products for the control of the codling moth in pome fruit production (Huber, 1998). For historic reasons, all registered CpGV products in Europe are based so far on the so-called "Mexican isolate" CpGV-M (Tanada, 1964) as active ingredient. Further isolates are known from literature: two deriving from England (CpGV-E) and one from Russia (CpGV-R) (Crook et al., 1985; Harvey, 1983). A product based on a virus isolated in the CP4 region of Quebec, Canada, is CpGV Virosoft™ (Vincent et al., 2007). These isolates differ among each other in terms of their biological activity and in their restriction profiles. Recently, further CpGV isolates originating from Iran and Georgia were identified and characterised by restriction fragment length polymorphism (RFLP) analysis, single nucleotide polymorphism (SNP) analysis and partial genome sequencing (Rezapanah, 2001; Rezapanah et al, 2008).

The resistance to CpGV-M observed in field populations of *Cydia pomonella* (Fritsch et al., 2005; Asser-Kaiser et al., 2007) not only raises the question to the mechanism of this resistance, but also requires new strategies of virus application in biological control programes. In order to test alternatives to the currently used CpGV-M based products, CpGV isolates of different geographical origin were examined in this work with regard to their biological activity and their molecular differences. Most of the isolates analysed in this thesis derived from the Caucasian region (Iran, Georgia), where the origin of

codling moth is supposed to be. The term isolate refers to a virus sample isolated from one or few larvae collected at the same place and at the same time (Cory et al., 1997).

In previous studies, it could be shown that the Iranian isolate CpGV-I12 is able to overcome resistance in the lab population CpR (Jehle et al., 2006, Eberle et al., 2008). The factor leading to this improved efficacy is not known yet.

Characterisation and identification of the different CpGV isolates were done by determining their median lethal concentration (LC_{50}) in bioassays and by DNA endonuclease restriction (REN) analysis followed by comparison with the published REN profiles of CpGV-M1 (Crook et al., 1997). Here, the question raised if different virus genotypes are phenotypically neutral or if they show differences in their virulence.

Differences in the reaction of an insect population to virus infection can be quantified by comparing bioassays of the concerning populations. Variables like instar of the insects, pathogen concentration or environmental factors (temperature, photoperiod) should be constant in order to obtain a reliable concentration-mortality relationship. Usually, the relationship between dose or concentration of a pathogen and mortality of an organism or between period of time and mortality are determined. Viruses like baculoviruses with occlusion bodies (OBs) are applied orally with the diet, either by mixing the diet with known OB concentrations or by applying known OB doses (Evans and Shapiro, 1997).

Differences between populations should be examined at different concentrations, in order to obtain the complete concentration-mortality relationship. This is necessary to be able to distinguish if the reaction to a pathogen is a real shift in the reaction to an infection or if it is only the elimination of susceptible host individuals of a population (Briese, 1986a). The applied concentration should induce mortality levels in the insects between 10 and 90%. At least 20 insects should be applied for every concentration (Jones, 2000). Every bioassay must include its own negative control, which contains all components except the virus. As effects like time of day, variation in temperature or in handling influence the experiment, every bioassay should be repeated at least two times. The replicate should be done at a different day with freshly prepared medium, but preferably under the same conditions to assure a real replicate (Robertson and Preisler, 1992). The

activity of a pathogen is usually measured as median lethal dose (LD_{50}) or median lethal concentration (LC_{50}), inducing a mortality of 50% in the test organisms.

Beyond the biological activity of an isolate, characterisation is done by comparison of DNA restriction endonuclease (REN) profiles (Burgess, 1983). Differences between virus genotypes like insertions, deletions or point mutations, which result in alterations of the REN profile can be identified. Beyond that, REN analysis allows distinguishing between a single genotype and a mixture of genotypes. Naturally occurring baculovirus isolates often consist of a mixture of different genotypes, which can usually be detected by submolar bands in the REN profile (Smith and Crook, 1988). REN analyses also provides the information for constructing physical maps, which assign the position of the fragments along the DNA molecule (Kurstak, 1991).

In order to develop a better insight into the biological and genetic diversity of different CpGV isolates and to identify new isolates able to overcome CpGV resistance, it is necessary to be able to identify different CpGV isolates reliably. The main question addressed to in this work was: which differences lead to an improved efficacy of CpGV isolates against resistant codling moth populations? For that, isolates overcoming resistance needed to be identified and then compared on molecular level to CpGV-M1, to find out, if there is an alteration which they have in common. This chapter describes how various isolates from different origins worldwide were tested for their efficacy to susceptible and resistant codling moth larvae. Their REN profiles were compared among each other and to CpGV-M1 (Crook et al., 1997), in order to identify and to allocate differences in genome content.

3.2 Methods

3.2.1 Virus propagation

Propagation of CpGV isolates was performed in L4 to L5 codling moth larvae, following the so-called diet plug method. Neonate larvae of the susceptible laboratory strain CpS, reared continuously at the DLR Rheinpfalz/Neustadt (see chapter 2.4), were kept on virus free diet for 10 days until they reached instar L4. These larvae were then infected orally at a low virus concentration to allow them to grow and to produce a maximum of virus. The virus suspension was applied in a volume of one µl containing 1000 OBs to the surface of a small piece of diet (about two mm^2). Each larva was put into a well containing one piece of diet. Larvae, which had eaten the piece of diet completely within 24 h were transferred again to virus free diet. Until infection was visible - usually after five to six days - larvae were incubated at 26°C and controlled daily. Infected larvae were collected and frozen at -20°C until OB isolation. Propagation of one virus isolate was usually done using 200 to 250 infected larvae.

For isolating OBs from larvae, the collected larve were pestled in SDS (0.5%, w/v). By filtering the suspension through cottonwool, head capsules were removed from the suspension. After pelleting the OBs by centrifugation (30 min, 17,000 g) the pellet was resuspended in a small volume of ddH_2O. Purification of the OBs was performed by several centrifugation steps over a glycerol layer (60/70/80%, v/v) for 40 min at 12,000 g. The OBs concentrated in the 60% glycerol layer. The OBs were washed with ddH_2O and pelleted by centrifugation for 30 min at 12,000 g. Purified OBs were resuspended in about one ml of H_2O, depending on the size of the pellet.

3.2.2 Estimation of virus concentration

The number of OBs per ml in a virus stock solution was scored using a Petroff-Hauser couting chamber (depth 0.02 mm) in the dark field optics of a Leica light microscope (Leica DMBRE). Dilution of OBs was done in steps 1:10. At least 10 µl of the OB suspension were used for every dilution step. OB concentration was estimated calculating the mean of four countings. Counting was done for each bioassay separately and independently of other countings, in order to avoid day-dependent variations. For long time storage, virus stocks were frozen at -80°C, for short time storage at -20°C.

3.2.3 Bioassays: Estimation of the LC_{50}

Reaction of the susceptible (CpS) and resistant (CpR, CpRR1) codling moth lab strains (see chapter 2.4) to infection with different virus isolates was scored in bioassays. Bioassays were performed in autoclavable 50-well plates containing 45 ml of diet (Ivaldi-Sender, 1974) mixed with five ml OB suspension of different concentrations per plate. For control plates the diet was mixed with five ml H_2O instead of OB suspension. Different from the insect rearing the diet was prepared with half the amount of agar-agar to assure that diet could cool down to 40°C without solidifying. Therewith, thermal inactivation of the virus was avoided. Freshly made diet plates were allowed to dry for at least one day before setting up larvae. In order to exclude those larvae from the experiment which died from handling, larval mortality was determined at the first day following the experimental setup. In order to estimate the median lethal concentrations (LC_{50}), the concentration-mortality relationship was determined at six different concentrations: 3×10^2, 1×10^3, 3×10^3, 1×10^4, 3×10^4 and 1×10^5 OB/ml. Fifty neonate larvae (L1) were used for each concentration, and 100 L1 larvae for the untreated control. Mortality was determined after one, seven and 14 days. Control mortality was general below 15% and in the case of CpGV-M against CpR (14 days) 33.8%. Three independent replicates were applied. Mortality data were corrected for control mortality (Abbott, 1925). Bioassay plates were incubated at 26°C with a relative humidity of 60% and a light-dark period of 16:8h.

3.2.4 Statistical analysis

Estimation of the median lethal concentrations (LC_{50}) and slopes of the concentration-mortality lines were done using probit analysis (ToxRat Solutions Standard Software Version 2.10.05 (see 2.6)). Parallelism of the probit lines implies a constant relative potency at all levels of response. If the slopes are parallel, then the overlap of the 95% confidence intervals for two analyses indicate that no significant difference exists. The LC_{50} values were considered significantly different if their probit lines were parallel and their 95% confidutial limits (CL) did not overlap (Robertson and Preisler, 1992; Jones, 2000). Parallelism of the slope of probit lines was tested using the parallel line assay option (parallel line assay with potency estimation for quantal responses) implemented in ToxRat Standard 2.10.05.

3.2.5 DNA isolation of a virus solution

For isolating DNA from viral OBs, the virus suspension was centrifuged for 15 min at 14,000 rpm and 4°C. The pelleted OBs were resuspended in filter-sterilised 100 mM Na_2CO_3 and incubated for 30 min at 37°C to dissolve the OBs. By adding 1 M HCL, pH value was adjusted to eight. RNAse A was added to the solution in a final concentration of 45 µg/ml and incubated for 10 min at 37°C. By adding SDS to a final concentration of 1% and Proteinase K (final concentration 250 µg/µl), virion particles were disintegrated while incubating the solution for 1 h at 37°C. After extracting with phenol/chloroform/isoamylalcohol (25:24:1) the solution was centrifuged for two min at 14,000 rpm. The upper phase was removed and the step was repeated. When no protein interface was visible between the two layers, a washing step using only chloroform/isoamylalcohol was performed. DNA was precipitated by adding 1/10 of the volume of Na-Acetat (3 M, pH 5.2) and the 2.5 volumes of 96% EtOH. The DNA was allowed to precipitate over night at -20°C or at -80°C for 30 min. DNA was pelleted for 15 min at 14,000 rpm and room temperature. The DNA pellet was washed with 70% EtOH and dissolved in about 100 µl TE buffer (1 mM EDTA, 10 mM Tris, pH 8).

3.2.6 Restriction enzyme analysis (REN)

Viral DNA of different CpGV isolates was digested using the restriction enzymes *Sal*I, *Eco*RI, *Bam*HI, *Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Apa*I and *Hin*dIII for comparison with the previously published DNA restriction endonuclease analysis of CpGV-M1 (Crook et al., 1997). For each restriction profile, 0.6 µg DNA were digested for 3 h. The reaction volume was usually 20 µl using 17 µl virus DNA, 2 µl 10 x buffer and 1 µl restriction enzyme. The enzymatic reaction was stopped by freezing the sample at -20°C or by addition of loading buffer.

3.2.7 Gelelectrophoresis

Digested DNA was electrophoresed at 25 V (24 mA) for 17 h (0.8% agarose, 1 × TAE). The λ/*Hin*dIII DNA ladder and 1kb DNA ladder (GeneRuler™) were used as molecular size standards. The gels were stained in an ethidium bromide bath (0.5 µg/ml) for about 15 min and photographed using the INTAS Documentation System Software.

3.3 Results: Bioassays with CpS, CpR and CpRR1

3.3.1 Activity testing of different CpGV isolates at a given OB concentration

As a first determination of the virulence of the CpGV isolates CpGV-G02, -I66, -I12, -I08, -I01, -I07, -I68 and -G01 deriving from Iran (I) and Georgia (G) and in stock at the DLR Rheinpfalz/Neustadt, the response of sensitive (CpS) L1 larvae was determined at a virus concentration of 2000 OB/ml (Fig. 3.1). This concentration was chosen as the LC_{50} of CpS larvae after seven days in bioassay with CpGV-M was expected to be in this range (Table 3.1). Mortality was always corrected for control mortality (Abbott, 1925).

Five out of eight isolates showed after seven days a lower efficacy against CpS larvae as CpGV-M (Fig. 3.1). The isolates CpGV-G02 and -I66 induced mortalities of 55.7 and 25.1%, respectively. Their efficacy was therefore less than the efficacy of CpGV-M (86.5%) (Fig. 3.1). A low efficacy against CpS larvae was also found for the isolates CpGV-I07, -I68 and -G01; they induced only 17.7% (CpGV-I07) mortality at maximum. CpGV-I68 and -G01 were therefore not tested at other concentrations or against the resistant codling moth populations CpR or CpRR1. Three isolates were comparable in their efficacy to CpGV-M: mortality induced by the isolates CpGV-I12, -I08 and -I01 reached values between 80.5 and 84.2 %, respectively (Fig. 3.1). Therefore, these isolates were chosen for full-range bioassays. CpGV-M was used as reference and -I07 was also included for testing one of the low efficient isolates against the different CM populations.

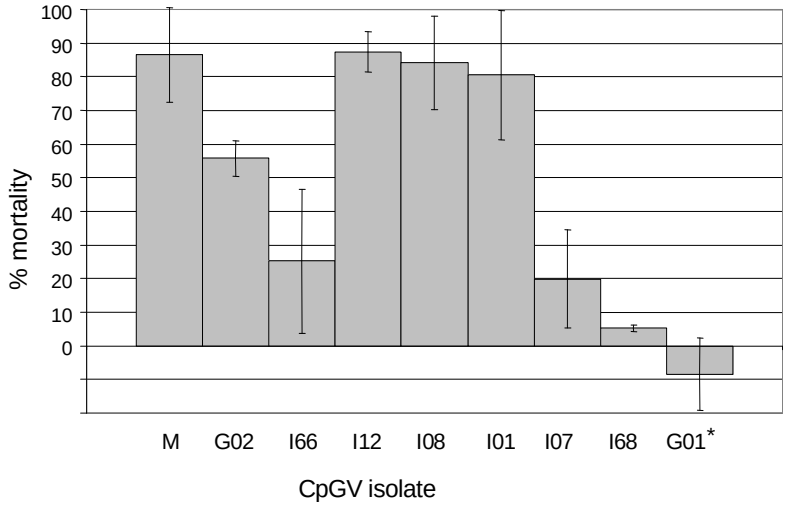


Fig. 3.1. Mortality induced in neonate *C. pomonella* larvae after seven days of incubation on 2000 OB/ml diet with different CpGV isolates. Data shown are means of three independent replicates. For the isolate G01, mortality had a mathematically value of -8.4% because the control mortality was higher than the mortality in the virus treatment. Bars indicate the standard deviation.

3.3.2 Activity testing of different CpGV isolates in full range bioassays

To determine the complete concentration-mortality relationship in CpS using the different isolates and to determine their LC_{50} values, full range bioassays with virus OB concentrations ranging from 3×10^2 to 1×10^5 OB/ml were performed (Fig. 3.2 A, B). Comparison of the obtained LC_{50} values and its 95% confidutial limits is possible when the slope of the probit lines are parallel. Therefore, the slopes of probit lines of the different CpGV isolates were compared by parallel line assay to the included CpGV-M standard. The measure is X^2P, the X^2 for parallelism. If the propability of X^2P ($p(X^2P)$ is equal or lower than the selected significane level (0.050), than the validity criterion of parallelism is not fulfilled.

Comparison of LC_{50} values and the slopes of probit lines of 14 tested CpGV isolates to CpGV-M (EU) (Table 3.1, Table 3.2) revealed that, except for CpGV-S, their LC_{50} values after seven days were slightly lower than that of CpGV-M (EU). For CpGV-I01, -E2, -AZ1, -AZ4 and -G04, no statement could be made about the significance of LC_{50}

differences, because the slopes of probit lines of the testes isolates and CpGV-M (EU) were not parallel (Table 3.2). The isolates CpGV-I08, -I12, -AZ2, -AZ3, -AZ5, -AZ6, -AZ7 and -G03 worked significantly better against CpS compared to CpGV-M (EU) after seven days based on the LC_{50} values and the non-overlapping of their 95% confidutial limits (CL). For CpGV-S, the LC_{50} was with 9.3 x 10^2 OB/ml higher compared to CpGV-M (EU) (1.87 x 10^3), but not significantly different from it based on the overlapping of 95% CLs. After 14 days, LC_{50} values were very similar to each other (Table 3.1), except for CpGV-S (2.5 x 10^3 OB/ml), which was about 10 times higher compared to the other LC_{50} values. The probit lines of CpGV-S, -I08, and -AZ4 were not parallel to the probit line of CpGV-M (EU) (Table 3.2), but their efficacy was similar to CpGV-M. CpGV-AZ5 and -AZ6 had a significant better efficancy than CpGV-M (EU) based on the non-overlapping of 95% CLs, the other isolates did not differ significantly in their efficacy against CpS from CpGV-M (EU) after 14 days of exposure.

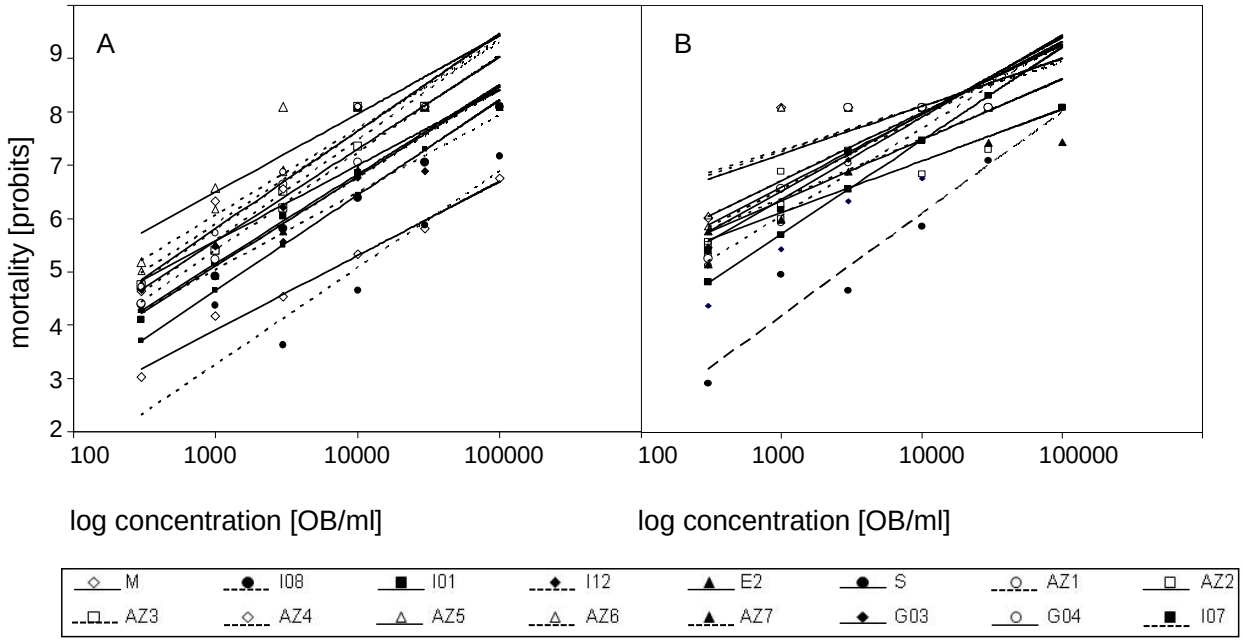


Fig. 3.2. Concentration-mortality response (probits) of the susceptible CM lab strain CpS after (A) seven and (B) 14 days of incubation with 16 CpGV isolates.

Table 3.1. LC_{50} values (CpS) after seven days in bioassay and testing of parallelism of slope of probit lines using CpGV-M (EU) as reference. § = only one replicate. S = slope of the probit line. CL = confidutial limits. n.d. = not determined: no value determined due to mathematical reasons. $X^2P = X^2$ for parallelism, $p(X^2)$ = associated probability- in case p > significane level (0.050), the observations are in agreement with the parallelism hypothesis. n = number of test insects.* indicate statistical differences based on non-overlapping CL. RP = Relative potency (ratio LC_{50} CpGV-M/LC_{50} CpGV isolate).

	7 days							
CpGV	LC_{50} (95% CL) [OB/ml] x 10^3	n	χ^2	S	X^2P	$p(X^2P)$	validity criterion of parallelism	RP
I08	0.93 (0.72- 1.17)	817	2.21	1.39	0.16	0.68	fulfilled	2.0*
I01	0.85 (0.7- 1.30)	815	0.78	1.87	6.73	0.009	not fulfilled	2.2
I12	0.89 (0.73- 1.07)	1082	8.92	1.54	1.51	0.21	fulfilled	2.1*
E2	0.95 (0.79- 1.14)	1173	4.03	1.67	5.98	0.01	not fulfilled	1.9
S	9.30 (1.4- 9.4)	538	49.0	1.42	0.35	0.55	fulfilled	0.2
I07	1.58 (1.3- 1.8)	837	3.29	1.78	10.8	<0.001	not fullfilled	1.1
AZ1	0.58 (0.35- 0.84)	822	9.71	1.69	7.55	0.006	not fulfilled	3.2
AZ2	0.41 (0.33- 0.51)	834	4.98	1.54	3.16	0.07	fulfilled	4.5*
AZ3§	0.53 (0.38- 0.73)	275	2.03	1.78	3.64	0.05	fulfilled	3.5*
AZ4§	0.38 (0.28- 0.50)	279	3,92	2.18	7.04	0.008	not fulfilled	4.9
AZ5§	0.17 (0.11- 0.24)	276	2.48	1.92	3.51	0.06	fulfilled	11*
AZ6§	0.25 (0.17- 0.34)	279	0.59	1.81	2.80	0.09	fulfilled	7.48*
AZ7§	0.37 (0.25- 0.51)	276	2.36	1.73	3.23	0.07	fulfilled	5.0*
G03§	0.48 (0.32- 0.69)	279	5.64	1.45	0.74	0.38	fulfilled	3.8*
G04§	0.39 (0.28- 0.53)	275	0.41	1.98	5.61	0.01	not fulfilled	4.7
M (EU)	1.87 (1.52-2.32)	827	6.34	1.32	-	-	-	-

Table 3.2. LC_{50} values (CpS) after 14 days in bioassay and testing of parallelism of slope of probit lines using CpGV-M (EU) as reference. § = only one replicate. S = slope of the probit line. CL = confidutial limits. n.d. = not determined: no value determined due to mathematical reasons. X^2P= X^2 for parallelism, p(X^2)= associated probability- in case p > significane level (0.050), the observations are in agreement with the parallelism hypothesis. n = number of test insects. .* indicate statistical differences based on non-overlapping CL. RP = Relative potency (ratio LC_{50} CpGV-M/LC_{50} CpGV isolate).

	14 days							
CpGV	LC_{50} (95% CL) [OB/ml] x 10^3	n	χ^2	S	X^2P	p(X^2P)	validity criterion of parallelism	RP
I08	0.93 (0.72- 1.17)	817	2.21	1.39	14.72	<0.001	not fulfilled	0.2
I01	0.85 (0.7- 1.30)	815	0.78	1.87	0.07	0.78	fulfilled	0.2*
I12	0.89 (0.73- 1.07)	1082	8.92	1.54	0.33	0.56	fulfilled	0.2*
E2	0.95 (0.79- 1.14)	1173	4.03	1.67	3.57	0.05	fulfilled	0.2*
S	2.5 (0.6- 7.5)	538	49.0	1.42	5.98	0.01	not fulfilled	0.08
I07	1.58 (1.3- 1.8)	837	3.29	1.78	0.01	0.89	fullfilled	0.1*
AZ1	0.58 (0.35- 0.84)	822	9.71	1.69	0.43	0.51	fulfilled	0.1*
AZ2	0.41 (0.33- 0.51)	834	4.98	1.54	0.008	0.92	fulfilled	0.4*
AZ3§	0.53 (0.38- 0.73)	275	2.03	1.78	2.08	0.14	fulfilled	0.3*
AZ4§	0.38 (0.28- 0.50)	279	3.92	2.18	3.99	0.04	not fulfilled	0.5
AZ5§	0.17 (0.11- 0.24)	276	2.48	1.92	0.16	0.68	fulfilled	1.1
AZ6§	0.25 (0.17- 0.34)	279	0.59	1.81	0.52	0.46	fulfilled	0.8
AZ7§	0.37 (0.25- 0.51)	276	2.36	1.73	0.16	0.68	fulfilled	0.5*
G03§	0.48 (0.32- 0.69)	279	5.64	1.45	0.002	0.96	fulfilled	0.4*
G04§	0.18 (0.12- 0.24)	275	1.30	2.00	0.53	0.46	fulfilled	1.1*
M (EU)	0.20 (0.15- 0.24)	827	4.31	1.76	-	-	-	-

After determining the efficacy of the different CpGV isolates against CpS, the aim was to select isolates which were able to overcome CpGV resistance. Therefore, the isolates CpGV-I12, -I08 and -I01 were tested against the CM strain CpR which showed a 100 times decreased susceptibility, but was genetically not homogenous (Asser-Kaiser et al., 2010). The LC_{50} values for CpGV-I12 tested against CpS and CpR were determined previously in the framework of a diploma thesis (Eberle, 2006).

The isolates CpGV-I12, -I08 and -I01 were able to overcome resistance in the population CpR (Fig. 3.3). Whereas the LC_{50} after seven days was 1.66×10^5 OB/ml in bioassay with CpGV-M, the LC_{50} was about 10 (CpGV-I12) to 30 times (-I01, -I08) lower (Table 3.3) using the so-called Iranian isolates. Based on the overlap of 95% CL, it could be shown that CpGV-I12 had a significant better efficacy against CpR after seven days of incubation as CpGV-M. For CpGV-I01 and -I08, the slopes of probit lines were not parallel to CpGV-M (Table 3.4), but the mortality induced was comparable to CpGV-I12 (Fig. 3.3 A).

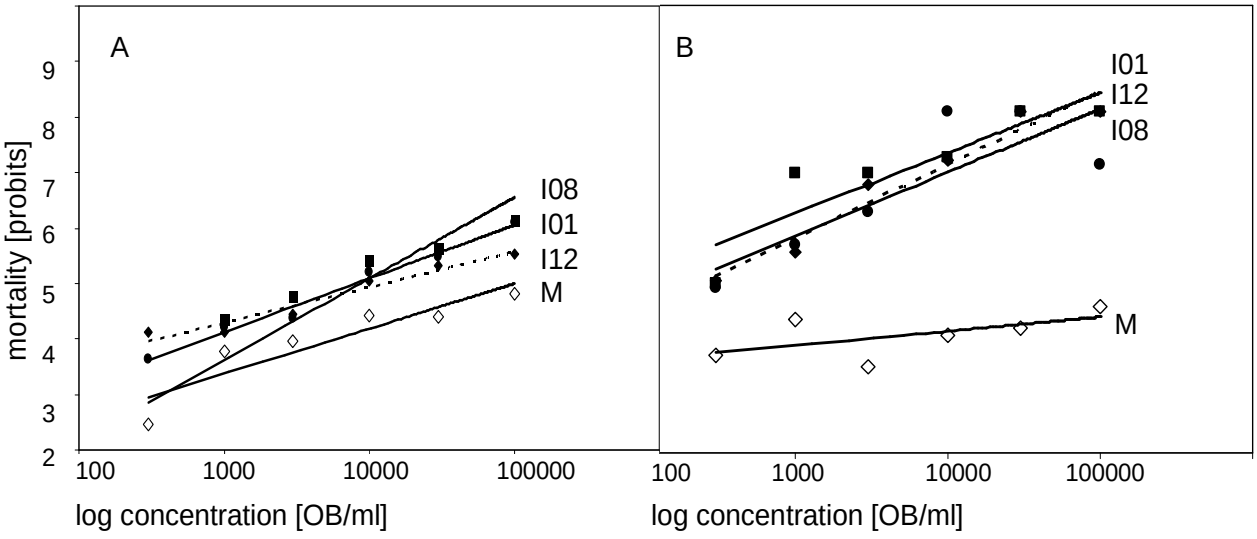


Fig. 3.3. Concentration-mortality response of the resistant CM lab strain CpR after seven (A) and 14 (B) days in bioassay with resistance overcoming isolates CpGV-I12, -I01, -I08 and -M as reference.

After 14 days of incubation, the LC_{50} values for CpGV-I12, I01 and -I08 against CpR were in the range of the LC_{50} values obtained for susceptible larvae (compare Table 3.2). In contrast, it was not possible to obtain a LC_{50} when using CpGV-M. As control mortality was increasing but not the mortality on the virus plates, the Abbott corrected mortality was lower than 50%. Estimation of the 14 day LC_{50} by interpolation leaded therefore to a LC_{50} higher than the value obtained for seven days. Parallelism of slopes of probit lines compared to CpGV-M was not to expect, because the values for CpGV-M did not show a sigmoid concentration-response curve, but were scattering between 10 and 35% mortality.

Table 3.3. LC_{50} values after 7 days in bioassay with different CpGV isolates against CpR. S= slope of the probit line. CL = confidutial limits. n.d. = not determined, no value determined due to mathematical reasons. Testing of parallelism of slope of probit lines was done using CpGV-M (TPCpGVBTPS_02) as reference. X^2 P = X^2 for parallelism, p(X^2) = associated probability- in case p > significane level (0.050), the observations are in agreement with the parallelism hypothesis. n = number of test insects. * indicate statistical differences based on non-overlapping CL. RP = Relative potency (ratio LC_{50} CpGV-M/LC_{50} CpGV-isolate).

		7 days						
CpGV	n	LC_{50} (95% CL) [OB/ml] x 10^3	S	χ^2	X^2P	p(X^2P)	validity criterion of parallelism	RP
I12	1093	12.7 (9.2- 18.5)	0.64	7.52	2.40	0.12	fulfilled	13.0
I08	818	8.2 (6.3- 10.9)	0.93	3.50	17.1	<0.001	not fulfilled	20.2
I01	815	6.1 (2.9- 12.7)	1.09	15.4	45.61	<0.001	not fulfilled	27.2
M (TPCpGVBTPS_02)	1095	166 (34- 816)	0.62	10.7	-	-	-	-

Table 3.4. LC_{50} values after 14 days in bioassay with different CpGV isolates against CpR. S= slope of the probit line. CL = confidutial limits. n.d. = not determined, no value determined due to mathematical reasons. Testing of parallelism of slope of probit lines was done using CpGV-M (TPCpGVBTPS_02) as reference. X^2 P = X^2 for parallelism, p(X^2) = associated probability- in case p > significane level (0.050), the observations are in agreement with the parallelism hypothesis. n = number of test insects. .* indicate statistical differences based on non-overlapping CL. RP = Relative potency (ratio LC_{50} CpGV isolate/LC_{50} CpGV-M).

		14 days						
CpGV	n	LC_{50} (95% CL) [OB/ml] x 10^3	S	χ^2	X^2P	p(X^2P)	validity criterion of parallelism	RP
I12	1093	0.30 (0.22- 0.39)	1.53	6.33	55.9	< 0.001	not fulfilled	5263
I08	818	0.29 (n.d.- 1.0)	1.27	43.0	49.2	< 0.001	not fulfilled	5444
I01	815	0.24 (n.d.)	2.03	33.2	60.0	< 0.001	not fulfilled	6579
M (TPCpGVBTPS_02)	1095	1579 (n.d.)	0.24	30.9	-	-	-	

When comparing the efficacy of these isolates against CpS and CpR, it was found that the full efficacy of the resistance overcoming isolates CpGV-I12, -I01 and -I08 was visible after 14 days of exposure (Fig. 3.3 B). The responses of CpS and CpR to the virus isolates CpGV-M and -I12 are compared in Fig. 3.4. After seven days, CpGV-M caused high mortality of CpS. In contrast, CpR showed a highly reduced susceptibility to CpGV-M, which did not reach 50% mortality, even at the highest concentration of 1×10^5 OB/ml. The isolate CpGV-I12 showed a $LC_{50} = 0.89 \times 10^3$ OB/ml for CpS. In CpR, CpGV-I12 showed a LC_{50} of 12.7×10^3 OB/ml indicating an increased virulence compared to CpGV-M. After 14 days of exposure, there was no further increase in mortality for CpR incubated with CpGV-M. In contrast, CpGV-I12 showed a LC_{50} of 0.3×10^3 OB/ml on CpR, which was similar to that of CpGV-M against CpS. Moreover, its efficiency on CpS ($LC_{50}= 0.89 \times 10^3$ OB/ml) was similar to that of CpGV-M (Fig. 3.4, 4.3 B).

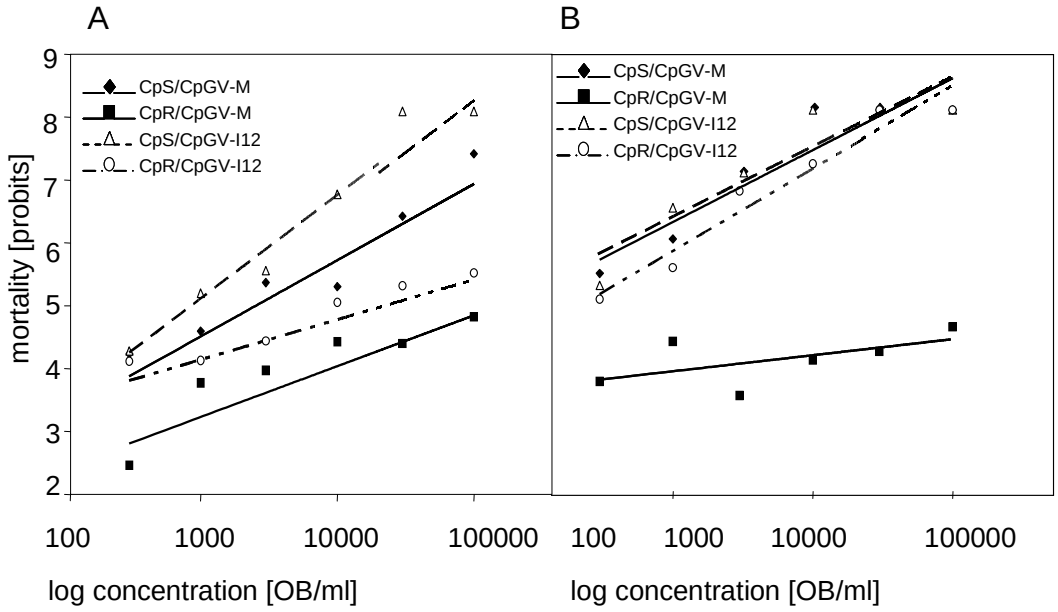


Fig. 3.4. Concentration-mortality response (probits) of codling moth strains CpS and CpR after (A) 7 days and (B) 14 days of incubation with CpGV-M or CpGV-I12.

When the genetically homogenous resistant codling moth strain CpRR1 (Asser-Kaiser et al., 2007) was available, the isolates CpGV-E2, -S and also -I07 and -AZ1 were tested against this strain (Fig. 3.5). CpGV-M was tested against CpRR1 previously (Asser-Kaiser, 2010), but due to the high resistance level in CpRR1, it was not possible to obtain a LC_{50} value after seven or 14 days. Even when increasing the virus concentrations of CpGV-M up to 10^8 OB/ml, mortality never exceeded 4% in any of the treatments (Sabine Asser-Kaiser, personal communication). Therefore, no probit analysis with comparison of slopes of probit lines to CpGV-M (EU) was possible. For CpGV-AZ1, it was also not possible to obtain a LC_{50} value, as mortality did not increase to more than 5%. With CpGV-I07, mortality reached 2.9% after seven and increased to 33.3% after 14 d at the highest concentration of 3 x 10^4 OB/ml. The probit lines of CpGV-E2 and -S were compared to each other, in order to obtain their efficacy against CpRR1. The LC_{50} of CpGV-S at day seven was 7.6 times higher compared to CpGV-E2. After 14 days of exposure, the LC_{50} values of CpGV-E2 and -S against CpRR1 did not differ significantly (Table 3.5). Both isolates were able to overcome resistance in CpRR1, the efficacy was comparable to that of CpGV-M against CpS larvae.

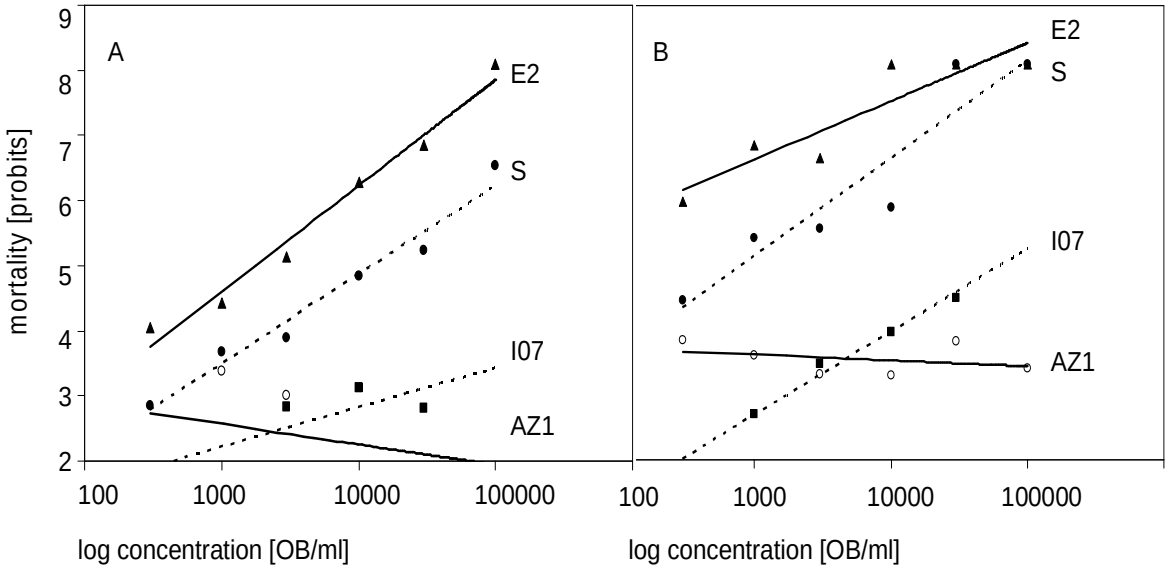


Fig. 3.5. Concentration-mortality response of the homogenous resistant CM lab strain CpRR1 after seven (A) and fourteen (B) days in bioassay with the CpGV isolates CpGV-E2, -S, -AZ1 and -I07.

Table 3.5. LC_{50} values after seven and 14 days in bioassay with CpGV-E2 and -S against CpRR1. For CpGV-M (EU), I07 and -AZ1, it was not possible to obtain LC_{50} values due to the low mortality induced by these isolates. S = slope of the probit line. CL = confidutial limit. n.d. = not determined, no value determined due to mathematical reasons.

	7 days				14 days		
CpGV	LC50 (95% CL) [OB/ml] x 10^3	n	S	χ^2	LC50 (95% CL) [OB/ml] x 10^3	S	χ^2
E2	1.89 (1.53- 2.31)	770	1.50	8.45	0.28 (0.08- 0.96)	1.00	4.06
S	14.4 (11.4- 18.5)	623	1.33	8.83	0.72 (0.11- 1.66)	1.09	16.5
I07	-	970	-	-	-	-	-
AZ1	-	888	-	-	-	-	-
M (EU)	-	977	-	-	-	-	-

Table 3.6. Testing of parallelism of slope of probit lines of CpGV-S and -E2. $X^2P = X^2$ for parallelism, $p(X^2)$ = associated probability- in case p > significane level (0.050), the observations are in agreement with the parallelism hypothesis.

	7 days			14 days		
CpGV	X^2P	$p(X^2P)$	validity criterion of parallelism	X^2P	$p(X^2P)$	validity criterion of parallelism
E2/S	5.8	0.015	not fulfilled	0.0002	0.99	fulfilled

In total, five resistance overcoming isolates (CpGV-I01, -I08, -I12, -E2 and -S) could be identified in bioassays against resistant (CpR, CpRR1) CM strains. These isolates worked against CpS as well as CpGV-M or even slightly better (Table 3.1, 3.2). Against resistant CM populations, the isolates revealed full efficacy after 14 days: their efficacy was then comparable to the efficacy of CpGV-M (EU) against CpS.

Taking together, the following isolates showed an improved efficacy in CpGV resistant CM strains (CpR and CpRR1) when compared to CpGV-M: CpGV-I12, -I01, .-I08, -E2 and -S.

3.4 Results: RFLP analysis of 14 CpGV isolates

As a first step towards a molecular characterisation of the new CpGV isolates, restriction analyses were performed using the enzymes *Sal*I, *Eco*RI, *Bam*HI, *Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Apa*I and *Hin*dIII, in order to compare them with the previously established restriction map of CpGV-M1 (Crook et al., 1997) or CpGV-M in stock at DLR Rheinpfalz/Neustadt. CpGV-M1 and -M show the same restriction profile. Profiles of the isolates CpGV-I07, -I68 and -G01 were previously determined by Dr. Samy Sayed (Eberle et al., 2009).

CpGV-I01, -I08 and -I12

The isolates CpGV-I01, -I08 and -I12 shared identical REN patterns to each other when digested with 10 enzymes (Fig. 3.6-3.8). In six (*Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Hin*dIII) out of 10 digests their profiles did not differ from the previously determined profile of CpGV-M1 (Crook et al., 1997). However, differences were found in the *Sal*I profile: the fragments E and F were visible as a double band of about 7.3 kb in size. In CpGV-M1, *Sal*I-E and -F are visible as two bands of 8.7 and 7.3 kb.

In the *Eco*RI profile, two bands of 18.1 (A1) and 10.5 (A2) kb instead of a single *Eco*RI-A (27.9 kb) band as typical for CpGV-M were found, indicating an additional *Eco*RI site in the genomes of CpGV-I01, -I08 and -I12. As the total sum of these two fragments was greater than the size of the corresponding *Eco*RI fragment A in the other isolates, this region was suggested to harbour an insertion of 0.7 kb. This conclusion was corroborated by the *Bam*HI profile: *Bam*HI-C, corresponding to the same genome region as *Eco*RI-A, was running at a higher molecular weight than in CpGV-M (15.2 kb).

The *Apa*I digest of CpGV-I01, -I08 and -I12 revealed one additional band of 2.5 kb not present in CpGV-M1, indicating an additional *Apa*I site in CpGV-I12.

For none of these profiles submolar bands were visible on the gel, suggesting that the isolates CpGV-I01, -I08 and -I12 are rather homogenous isolates, which do not contain further isolates at a high level.

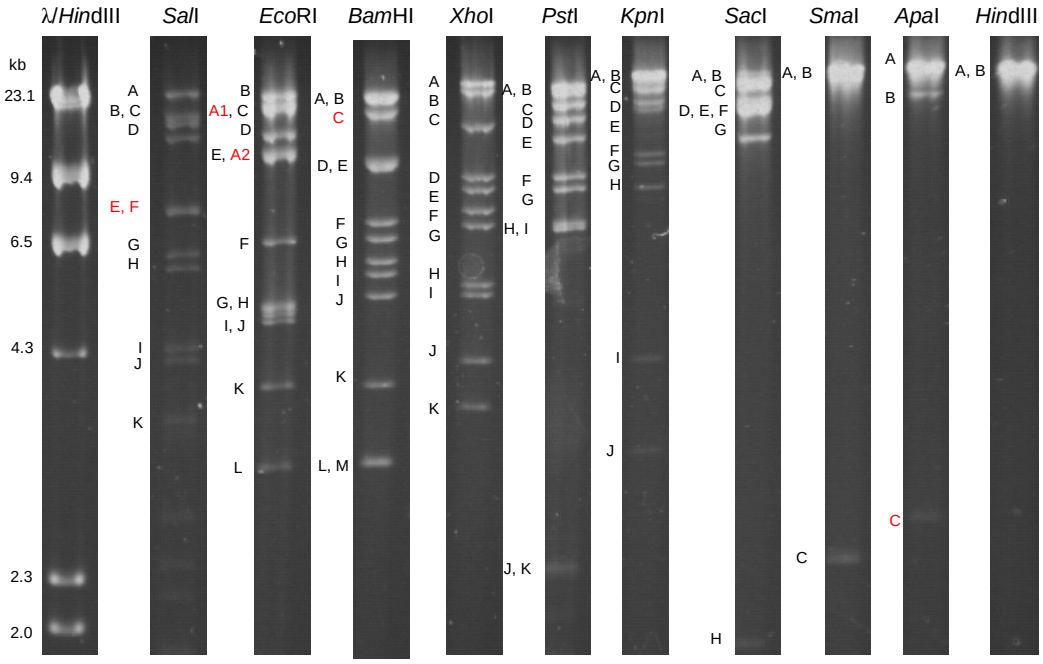


Fig. 3.6. DNA restriction analysis (0.8% agarose, 1 x TAE) of CpGV-I12 for *Sal*I, *Eco*RI, *Bam*HI, *Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Apa*I and *Hin*dIII. Restriction fragments are lettered in sequential order of their size. λ DNA digested with *Hin*dIII is included for molecular size standards. Red: REN fragment differences to CpGV-M1 (Crook et al., 1997).

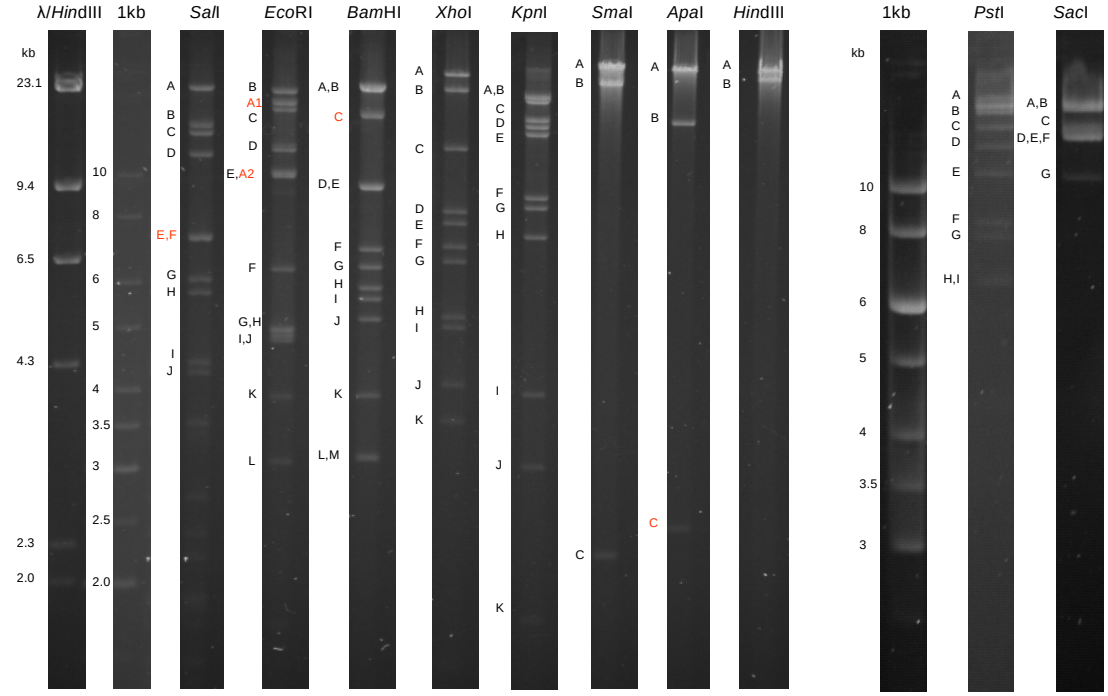


Fig. 3.7. DNA restriction analysis (0.8 % agarose, 1 x TAE) of CpGV-I01 for *Sal*I, *Eco*RI, *Bam*HI, *Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Apa*I and *Hin*dIII. Restriction fragments are lettered in sequential order of their size, λ DNA digested with *Hin*dIII is included for molecular size standards. Red: REN fragment differences to CpGV-M1 (Crook et al., 1997).

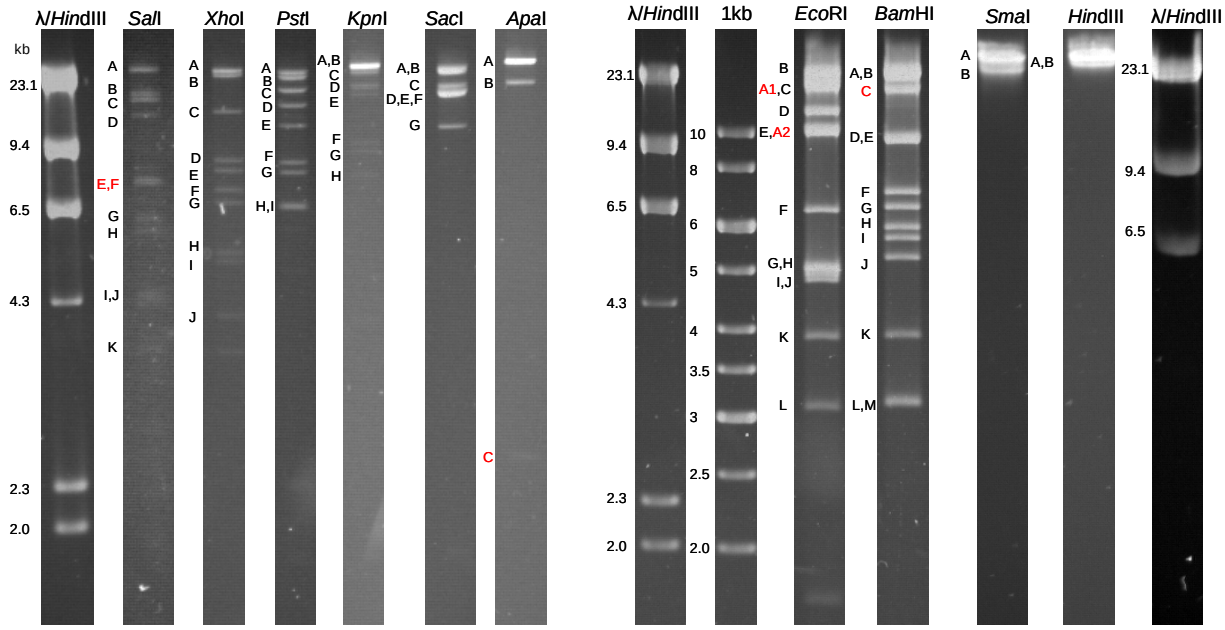


Fig. 3.8. DNA restriction analysis (0.8% agarose, 1 x TAE) of CpGV-I08 for *Sal*I, *Eco*RI, *Bam*HI, *Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Apa*I and *Hin*dIII. Restriction fragments are lettered in sequential order of their size, λ DNA digested with *Hin*dIII and 1 kb ladder (1 kb) are included for molecular size standards. Red: REN fragment differences to CpGV-M1 (Crook et al., 1997).

Since the main differences of these isolates and CpGV-M1 indicating that CpGV-I12 carried a genome insertion were found using the enzymes *Bam*HI and *Eco*RI, these enzymes were chosen for the analysis of CpGV-E2. CpGV-E2 was isolated by *in vivo*-cloning of the English isolate CpGV-E (Crook et al., 1985), in order to identify putative common insertion sites. Furthermore, the enzymes *Xho*I and *Pst*I were included to allow a comparison with the previously performed analysis of several CpGV isolates (Reza-panah, 2001; Rezapanah et al., 2008).

CpGV-E2

The *Xho*I profile of CpGV-E2 showed an additional fragment of 11.2 kb compared to CpGV-M1 (Crook et al., 1997) and CpGV-M (Fig. 3.9). Concurrently the fragments corresponding to *Xho*I-F (7.1 kb) and -J (4.1 kb) of CpGV-M were missing. As these bands are lying adjacent in the CpGV-M restriction map, it is proposed that the 11.2 kb band is due to a missing *Xho*I restriction site in CpGV-E2. Fragment *XhoI*-I (4.7 kb) was slightly smaller compared to that of CpGV-M (4.9 kb). This band corresponds to the area 51.5-56.4 kb in the CpGV-M genome map. Either this area harbours a deletion of about 0.2 kb or there is an additional *Xho*I restriction site, which would give an additional fragment that is too small to be seen on the gel.

The *Bam*HI digest revealed all bands present in CpGV-M. For *Bam*HI-J, one band was present at the same size as in CpGV-M (5.2 kb), a second was slightly smaller with 5.0 kb. Both bands were fainter compared to the following, smaller bands, suggesting a submolarity of each of the two bands. *Bam*HI-J covers the genome region of 12.9-18.1 kb and might harbour a deletion of about 200 bp in CpGV-E2. The 5.0 kb band corresponds to the fragment previously described for CpGV-E2 as a shift in a *Bam*HI site (Crook et al., 1985). As the two fragments of 5.0 and 5.2 kb are both fainter than the other bands, it is proposed that they derive from two genotypes present in the isolate CpGV-E2.

The *Pst*I profile showed a submolar band of 11 kb between the fragments *Pst*I-D (13.4 kb) and -E (10.5 kb). *Pst*I-E was present at the same size as in CpGV-M. As both bands were faint, they are likely submolar bands of two genotypes, with one *Pst*I-E corresponding to CpGV-M. The fragments *Pst*I-F and -G (8.2 and 7.7 kb in CpGV-M) were present as a double band in CpGV-E2. The fragments *Pst*I-H and -I, which are present as a double band in CpGV-M (6.4 kb), were visible as two bands of 6.4 and 6.9 kb in CpGV-E2. As the size difference between the fragments *Pst*I-F and -G and also between *Pst*I-H and -I amounts to 0.5 kb and the fragments *Pst*I-F and -I are adjacent in the genome map, it is likely that there is a shift in a *Pst*I site, resulting in a smaller fragment *Pst*I-F and a lager fragment *Pst*I-I. The fragments *Pst*I-J and -K visible as a double band in CpGV-M

(2.2 kb) were present as two bands in CpGV-E2, fragment *Pst*I-J is about 0.5 kb larger as fragment K. This is likely to be due to an insertion, as this genome region (19.5-21.7 kb) corresponds in parts to the fragments *Eco*RI-A1 and -A2, which were also supposed to harbour an insertion of about 0.7 kb.

Fragment *Eco*RI-A (27.9 kb) was missing in CpGV-E2, instead, two additional fragments A1 and A2 of 15.1 and 13.5 kb, respectively, could be observed. As the total size of these two fragments (28.6 kb) is larger than the size of CpGV-M1 fragment *Eco*RI-A, this area likely harbours an insertion of 0.7 kb, similar to CpGV-I12. However, the A1 and A2 fragment sizes were not identical to those of the isolates CpGV-I12, -I01 and -I08, suggesting that CpGV-E2 differed from these isolates in respect to the insertion or the insertion site (see Chapter 4).

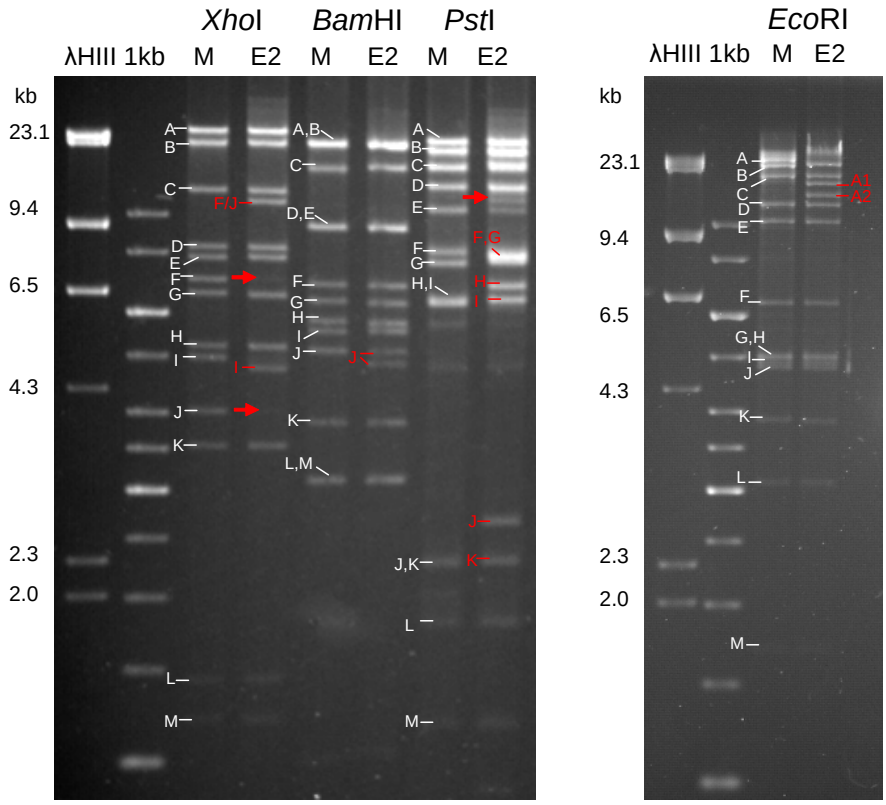


Fig. 3.9. DNA restriction analysis (0.8% agarose, 1 x TAE) of the *in vivo* cloned isolate CpGV-E2 and CpGV-M (EU-Standard) for *Xho*I, *Bam*HI, *Pst*I and *Eco*RI. Restriction fragments are lettered in sequential order of their size. λ/*Hin*dIII DNA ladder and 1 kb DNA ladder were included for molecular size standards. Red: CpGV-E2 fragments differing from CpGV-M. Red arrows indicate additional or missing REN fragments.

CpGV-S

Compared to CpGV-M1 and to the isolates -I12, -I08, -I01 and -E2, the CpGV-S profile revealed several insertion and deletion sites (Fig. 3.10).

The fragments *Bam*HI-C (15.2 kb) and -J (5.2 kb) were missing; instead, a single band of about 20.1 kb was present. As the fragments *Bam*HI-C and -J are lying adjacent in the CpGV-M1 restriction map (Crook et al., 1985), this finding could be due to a missing *Bam*HI restriction site in CpGV-S and a fused fragment C/J.

*Pst*I-E (12.4 kb) was larger compared to the other isolates (10.5-11 kb), indicating that this genome region (54.5-65.0 kb in CpGV-M1) carries an insertion of about 0.2 kb. Fragment *Pst*I-C (16.6 kb in CpGV-M1) was missing; instead, two additional bands *Pst*I-C1 (8 kb) and -C2 (7.7 kb) were visible. As the sum of this two fragment sizes (about 15.7 kb) was smaller as the size of fragment *Pst*I-C in CpGV-M1 (16.6 kb), it is possible that this genome region (79.6-96.2 kb in CpGV-M1) carries one ore more deletions of totally 0.9 kb.

The same genome region corresponds in parts to the fragment *Eco*RI-B (21.6 kb), which was also smaller than those of other isolates (21.9 kb). *Eco*RI-C was not present, but two fragments C1 (11 kb) and C2 (5.8 kb) could be identified.

One difference was found in the *Sac*I digest: *Sac*I-C (16.7 in CpGV-M1) and -F (15 kb in CpGV-M1), adjacent regions in the genome, were most likely fused to a single band of 31.4 kb. All other restriction fragments of CpGV-S corresponded to CpGV-M, supporting the above interpretation of gain and losses of single REN sites.

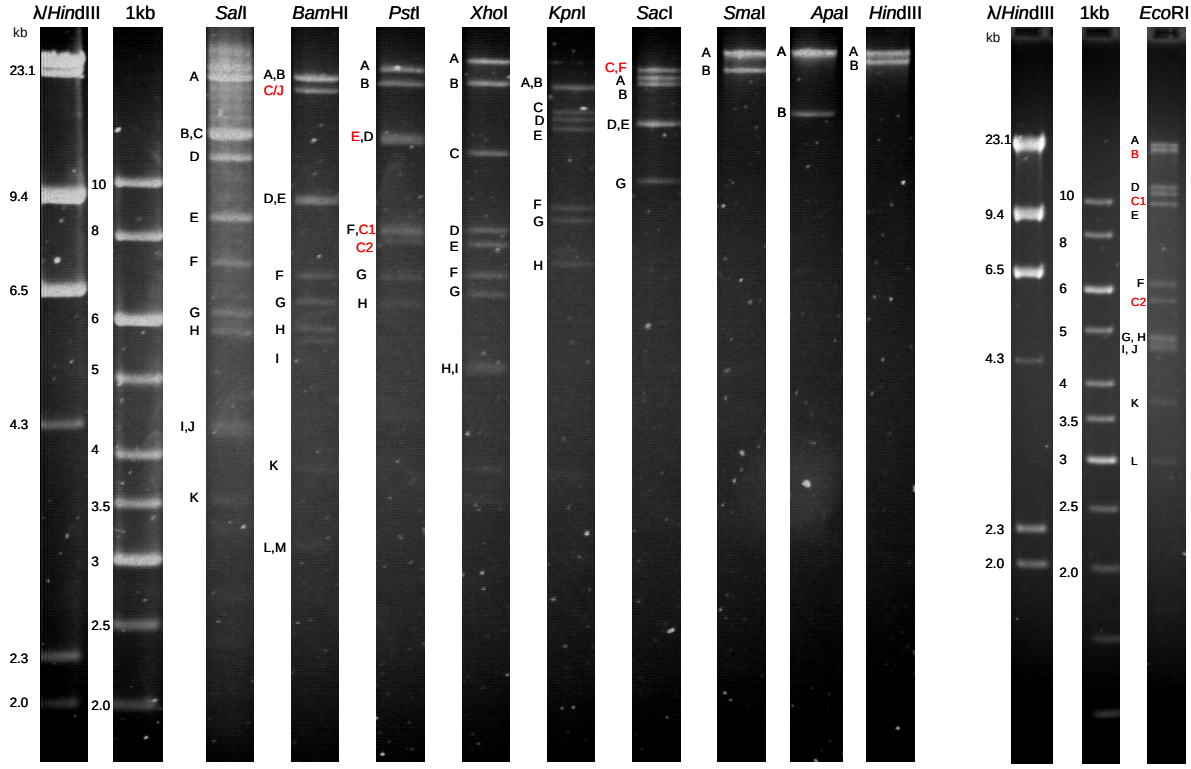


Fig. 3.10. DNA restriction analysis (0.8% agarose, 1 x TAE) of CpGV-S for *Sal*I, *Eco*RI, *Bam*HI, *Xho*I, *Pst*I, *Kpn*I, *Sac*I, *Sma*I, *Apa*I and *Hin*dIII. Fragments are lettered in sequential order of their size. λ/*Hin*dIII DNA ladder and 1 kb DNA ladder are included for molecular size standards. Red: fragments differing from the CpGV-M1 profile (Crook et al., 1997).

CpGV-AZ1-7 and CpGV-G03,-G04

The seven isolates CpGV-AZ1-7 collected in Azerbaijanian as well as the isolates CpGV-G03 and -G04 received from Georgia did not differ in their *Pst*I, *Eco*RI, *Bam*HI and *Xho*I profile from CpGV-M (EU) or from the published profile of CpGV-M1 (Crook et al., 1997) (Fig. 3.11-3.14). All bands could be identified at the expected position.

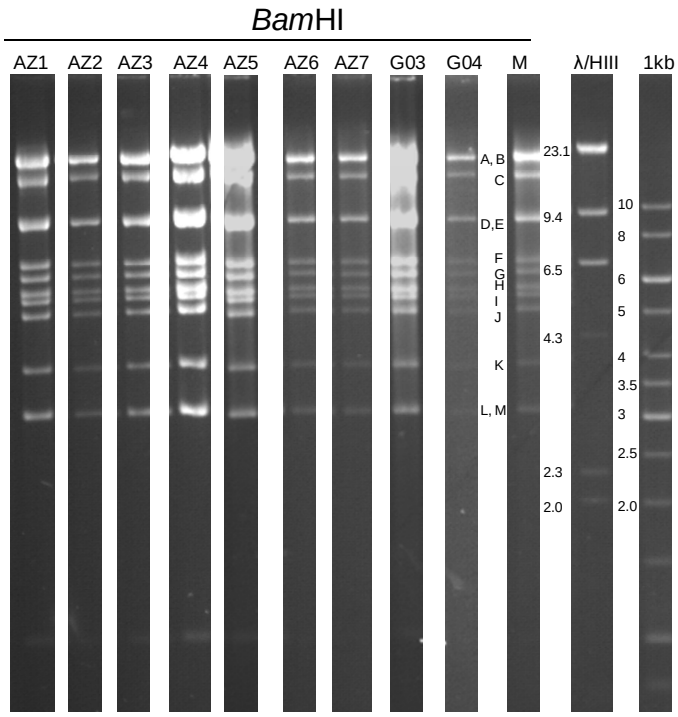


Fig. 3.11. DNA restriction analysis (0.8% agarose, 1 x TAE) of the isolates CpGV-AZ1-7 sowie -G03, -G04 and CpGV-M (EU-Standard) for *Bam*HI. Fragments are lettered for CpGV-M in sequential order of their size. λ/*Hin*dIII DNA ladder (λHIII) and 1kb DNA ladder (1 kb) are included for molecular size standards.

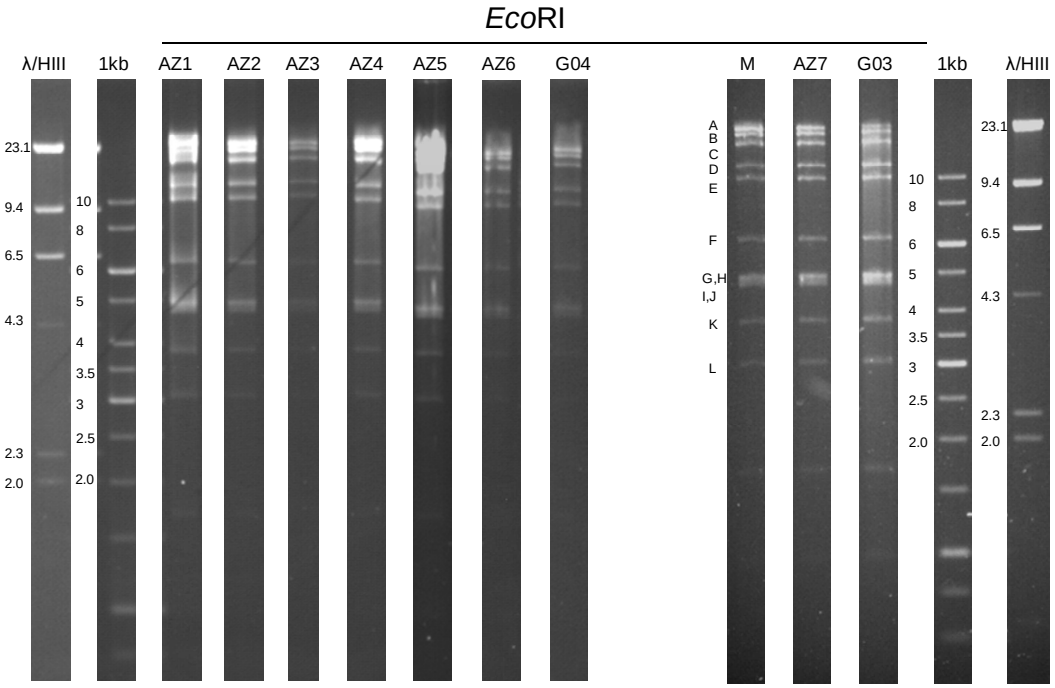


Fig. 3.12. DNA restriction analysis (0.8% agarose, 1 x TAE) for the isolates CpGV-AZ1-7, -G03, -G04 and CpGV-M (EU-Standard) for *Eco*RI. Fragments are lettered for CpGV-M in sequential order of their size. λ/*Hin*dIII DNA ladder (λ/HIII) and 1kb DNA ladder (1 kb) were included for molecular size standards.

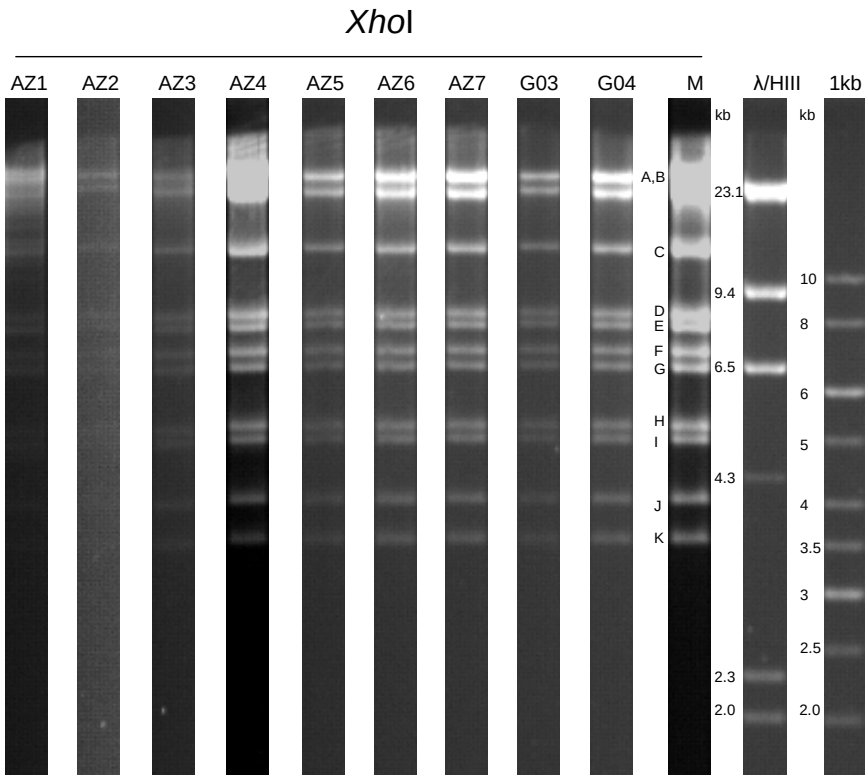


Fig. 3.13. DNA restriction analysis (0.8% agarose, 1 x TAE) of CpGV-AZ1-7, -G03, -G04 and CpGV-M (EU-Standard) for *Xho*I. Fragments are lettered for CpGV-M in sequential order of their size. λ/*Hin*dIII DNA ladder (λ/HIII) and 1kb DNA ladder (1 kb) are included for molecular size standards.

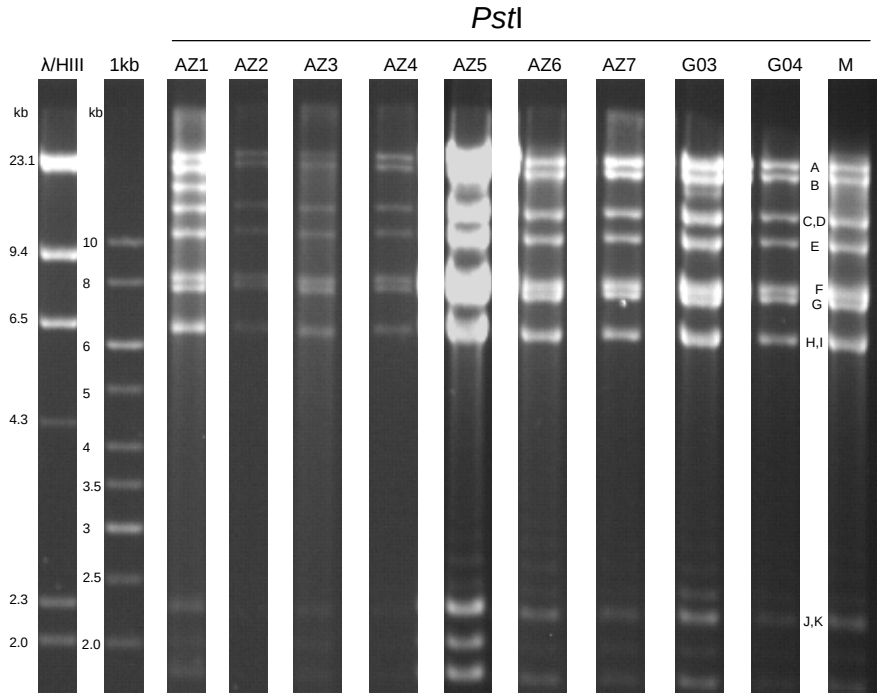


Fig. 3.14. DNA restriction analysis (0.8% agarose, 1 x TAE) of CpGV-AZ1-7, -G03, -G04 and CpGV-M (EU-Standard) for *PstI.* Fragments are lettered for CpGV-M in sequential order of their size. λ/*Hin*dIII DNA ladder (λ/HIII) and 1kb DNA ladder (1 kb) are included for molecular size standards.

Taken togheter, comparison of the restriction profiles with the map of CpGV-M1 established by Crook *et al.* (1985, 1997) revealed that the isolates CpGV-AZ1-7, -G03 and -G04 corresponded to CpGV-M1 and CpGV-M (EU), respectively (Fig. 3.11-3.14). In contrast, the isolates CpGV-I12, -I01 and -I08 contained an approximately 0.7 kb insertion harbouring an additional *Eco*RI site near 19 kb of the genome of CpGV. CpGV-E2 contained an insertion of a similar size, also within *Eco*RI-A but at a slightly different position. Further differences were found in a 0.2 kb deletion in the area of 51.5-56.4 kb and in a missing *Xho*I site. Compared to CpGV-M, the isolate CpGV-S showed several insertion and deletion sites. An insertion of about 0.2 kb was expected in the genome area between 54.5 and 65.0 kb, whereas one ore more deletions of in total 0.9 kb are proposed for the region of 79.6-96.2 kb corresponding to CpGV-M1 (Fig. 3.15).

Table 3.7. DNA restriction fragment sizes (kb) of CpGV isolates different from CpGV-M (CpGV-I12, -I01, -I08, -I12, -E2 and -S) after digestion with *Pst*I, *Eco*RI, *Xho*I and *Bam*HI. *As reference, CpGV-M1 fragments sizes have been included (Crook et al., 1997).

*Pst*I Fragment	I01	I08	I12	E2	S	M1*
A	28.1	28.1	28.1	27.9	27.2	27.9
B	21.5	21.5	21.5	21.0	21.2	21.0
A1	-	-	-	-	-	-
C	16.6	16.6	16.6	16.6	-	16.6
D	13.4	13.4	13.4	13.4	12.7	13.4
A2	-	-	-	-	-	-
E	10.5	10.5	10.5	11	12.4	10.5
F	8.2	8.2	8.2	7.7	8.1	8.2
C1	-	-	-	-	8.0	-
C2	-	-	-	-	7.7	-
G	7.7	7.7	7.7	7.7	6.8	7.7
H	6.4	6.4	6.4	6.9	6.2	6.4
I	6.4	6.4	6.4	6.4		6.4
J	2.2	2.2	2.2	2.7		2.2
K	2.2	2.2	2.2	2.2		2.2
L	1.8	1.8	1.8	1.8		1.8
M	1.3	1.3	1.3	1.3		3.3

*Eco*RI Fragment	I01	I08	I12	E2	S	M1*
A	-	-	-	-	27.3	27.9
B	21.9	21.9	21.9	21.9	21.6	21.9
A1	18.1	18.1	18.1	15.1	-	-
C	16.9	16.9	16.9	16.9	-	16.9
D	12.5	12.5	12.5	12.5	11.7	12.5
C1	-	-	-	-	11.0	-
A2	10.5	10.5	10.5	13.5	-	-
E	10.5	10.5	10.5	10.5	10.1	10.5
F	6.4	6.4	6.4	6.4	6.2	6.4
C2	-	-	-	-	5.8	-
G	5.0	5.0	5.0	5.0	4.9	5.0
H	5.0	5.0	5.0	5.0	4.9	5.0
I	4.9	4.9	4.9	4.9	4.7	4.9
J	4.8	4.8	4.8	4.8	4.7	4.8
K	4.0	4.0	4.0	4.0	3.8	4.0
L	3.0	3.0	3.0	3.0	3.0	3.0
M	1.7	1.7	1.7	1.7		1.7
N	1.1	1.1	1.1	1.1		1.1

*Xho*I Fragment	I01	I08	I12	E2	S	M1*
A	39.2	39.2	39.2	39.2	37.2	39.2
B	24.1	24.1	24.1	23.4	22.7	23.4
C	11.8	11.8	11.8	11.8	11.8	11.8
F+J	-	-	-	11.2	-	-
D	8.4	8.4	8.4	8.4	8.2	8.4
E	7.9	7.9	7.9	7.9	7.7	7.9
F	7.1	7.1	7.1	-	6.9	7.1
G	6.5	6.5	6.5	6.6	6.5	6.6
H	5.3	5.3	5.3	5.2	5.1	5.2
I	4.9	4.9	4.9	4.7	5.1	4.9
J	4.1	4.1	4.1	-		4.1
K	3.6	3.6	3.6	3.6		3.6
L	1.4	1.4	1.4	1.4		1.4
M	1.2	1.2	1.2	1.2		1.2
N	0.8	0.8	0.8	0.8		0.8

*Bam*HI Fragment	I01	I08	I12	E2	S	M1*
A	25.4	25.4	25.4	25.4	24.5	25.4
B	24.5	24.5	24.5	24.5	24.1	24.5
C	15.7	15.7	15.7	15.2	-	15.2
C+J	-	-	-	-	20.1	-
D	9.5	9.5	9.5	9.6	9.4	9.6
E	9.5	9.5	9.5	9.6	9.2	9.6
F	6.9	6.9	6.9	7.0	6.9	7.0
G	6.5	6.5	6.5	6.4	6.3	6.4
H	5.9	5.9	5.9	5.9	5.8	5.9
I	5.7	5.7	5.7	5.7	5.5	5.7
J	5.3	5.3	5.3	5.0	-	5.2
K	3.9	3.9	3.9	3.8	3.8	3.8
L	3.2	3.2	3.2	3.1	3.1	3.1
M	3.2	3.2	3.2	3.1	3.1	3.1
N	1.1	1.1	1.1	1.1		1.1

3.5 Discussion

As some local field populations of codling moth had lost their susceptibility to CpGV-M, a search for alternative CpGV isolates overcoming this resistance phenomenon (Asser-Kaiser et al., 2007) has been initiated. Promising CpGV isolates more effective against CpR and CpRR1 than CpGV-M could be found when testing naturally occurring CpGV isolates originating from different geographic regions: resistance against CpGV could be overcome by five out of 19 examined CpGV isolates: CpGV-I12, -I08, -I01, -E2 and -S.

After seven days in bioassay, efficacy against resistant larvae (CpR, CpRR1) was for CpGV-E2 comparable to the efficacy of CpGV-M against susceptible codling moth larvae (CpS). The LC_{50} of CpGV-I12, -I08, -I01 and -S against CpR or CpRR1 was after seven days 10 to 100 times higher than the LC_{50} obtained for CpS tested with CpGV-M. Full efficacy of the isolates CpGV-I12, -I08, -I01, -E2 and -S became apparent after 14 days in bioassay. Here, the efficacy of all isolates was in the same range as of CpGV-M against susceptible CpS larvae. No statement for the significance of some LC_{50} differences could be made for some isolates after seven or after 14 days, because the slopes of their probit lines were not parallel to the slope of the reference CpGV-M (EU). The bioassays described here were carried out over a period of three years, therefore, influences like the season or variations in the fitness of the reared populations can be reflected in the bioassay data.

The fact that CpGV-M induced a mortality of about 30% in CpR led to the conclusion, that the population CpR still contained susceptible insects. As they died after seven days in bioassay, leaving only the resistant ones, mortality did not increase until day 14. Due to increasing control mortality and its Abbott correcture, mortality after 14 days is mathematically lower than after seven days in Fig. 3.4. For the surviving insects, a LC_{50}> 100,000 than that of CpS can be expected (Asser-Kaiser et al., 2007). Indeed, further studies demonstrated that CpR was a mixture of susceptible and resistant individuals. Based on single pair crosses, it was estimated that 28% of the CpR insects were suscepti-

ble ones (Asser-Kaiser et al., 2009). The 100,000-fold resistance of the other 70% could be overcome completely by the new isolates.

Whether CpGV-I12 or other isolates are suitable for codling moth control in orchards with CpGV resistance needs to be evaluated in the field. In 2007, first field trials with CpGV-I12 had been performed in orchards with CpGV resistance and showed similar good results as another improved CpGV-M (Madex Plus) that had been selected on a resistant codling moth population (Zingg, 2008).

Another requirement for the registration and field application of a new CpGV isolate is the ability to identify all genotypes present in a mixture or to assure if a homogenous isolate is provided. Based on DNA restriction analysis, the resistance overcoming isolates CpGV-S, -I12, -I08 and -I01 were found to be genetically rather homogenous isolates, as they did not show any submolar bands in their restriction profiles. The CpGV-E2 profile revealed submolar bands which sizes correspond to fragments specific for CpGV-M/-M1. CpGV-E2 was isolated by *in vivo*-cloning from the so-called English isolate (CpGV-E) which showed a predominant CpGV-M like profile (Crook et al., 1985). Further rounds of *in vivo*-cloning did not increase the CpGV-E2 genotype, but the CpGV-M like type (Crook et al., 1985).

Characterisation by restriction analysis revealed that isolates that were able to overcome resistance differed from CpGV-M in their profiles (CpGV-I12, -I08, -I01, -E2 and -S). Isolates corresponding in their profiles to CpGV-M (CpGV-AZ1 -AZ7, -G03, -G04) showed a similar biological activity against CpS, CpR and CpRR1, respectively, to CpGV-M. Therefore, resistance to CpGV is not only restricted to one isolate, but seems to be specific to all CpGV-M like isolates.

CpGV-I12, -I08 and -I01 showed very similar restriction profiles to CpGV-M. RFLPs found were later on corroborated by CpGV-I12 whole genome sequencing (Chapter 5). Due to an additional *Sal*I recognition site in CpGV-I12 nt position 112,092, *Sal*I-E of CpGV-I12 was 1.3 kb smaller compared to CpGV-M. This resulted in a similar size of *Sal*I-E as *Sal*I-F, visible as a double band fo *Sal*I-E and -F in the CpGV-I12 profile. The additional *Apa*I restriction site predicted from the RFLP analysis was located in CpGV-I12 nt position 19,121, revealing an additional fragment *Apa*I-C of 2.4 kb.

An insertion of about 0.7 kb was estimated between 18-22 kb in the genome. For CpGV-E2, an insertion of similar size is expected in the same region but at a slightly different position, as *Eco*RI-A1 and -A2, harbouring this insertion site, differed in their sizes from those of CpGV-I12, -I08 and -I01. CpGV-S differed in its restriction profile from CpGV-I12, -I08, -I01 and -E2 as well as from CpGV-M. In contrast to the other examined CpGV isolates, several insertions and deletions are expected based on the restriction profile. The insertion of 0.7 kb could not be detected in CpGV-S. Therefore, a correlation of the 0.7 kb insertion observed in CpGV-I12 and -E2 with overcoming resistance is unlikely.

The restriction profiles of the isolates CpGV-I66, -G01, -G02, -I07 -I68 and -I08 were previously determined by Dr. Samy Sayed (Eberle et al., 2009). It was found that the profiles of CpGV-I66 and -G02 corresponded to CpGV-M. The isolates CpGV-I07, -I68 and -G01 differed from CpGV-M in their *Pst*I profiles. Compared to CpGV-M, a deletion of 2.4 kb in the genome area of 51.6-55.4 kb could be localised for these isolates (Eberle et al., 2009), similar to the previously described so-called Russian isolate (Harvey and Volkman, 1983).

Taken together, the analysis of CpGV isolates from different geographical regions in Iran, Georgia and the *in vivo*-cloned English isolate revealed a considerable variation in their RFLPs. However, by mapping these variations, most isolates can be attributed to genome types which were previously described using isolates from Mexico, England and Russia (Crook et al., 1985; Harvey and Volkman, 1983). The isolates CpGV-I12, -I01 and -I08 showed similar RFLPs compared to the English CpGV-E isolate. CpGV-E was shown to have an additional *Eco*RI site and in total an insertion of about 1 kb compared to CpGV-M (Crook et al., 1985), as it was also observed for CpGV-E2. Hence, it was found that the insertion of CpGV-I12, -I08 and -I01 was at a slightly different position as in CpGV-E2.

The isolates CpGV-AZ1 to -AZ7, -G02 to -G04 corresponded to CpGV-M in their restriction profiles and fragment sizes (Fig. 3.11- 3.14). Also the CpGV isolates CpGV-I66 and -G03 were found to correspond to CpGV-M (Eberle et al., 2009, Fig. 3.14). The isolates CpGV-I07, -G01 and -I68 lacked a 2.58 kb genome region within *Eco*RI-C and

showed a similar restriction profile to CpGV-R (Eberle et al., 2009, Fig. 3.12), for which a deletion of 2.4 kb at the same map position had been proposed previously (Crook et al., 1985).

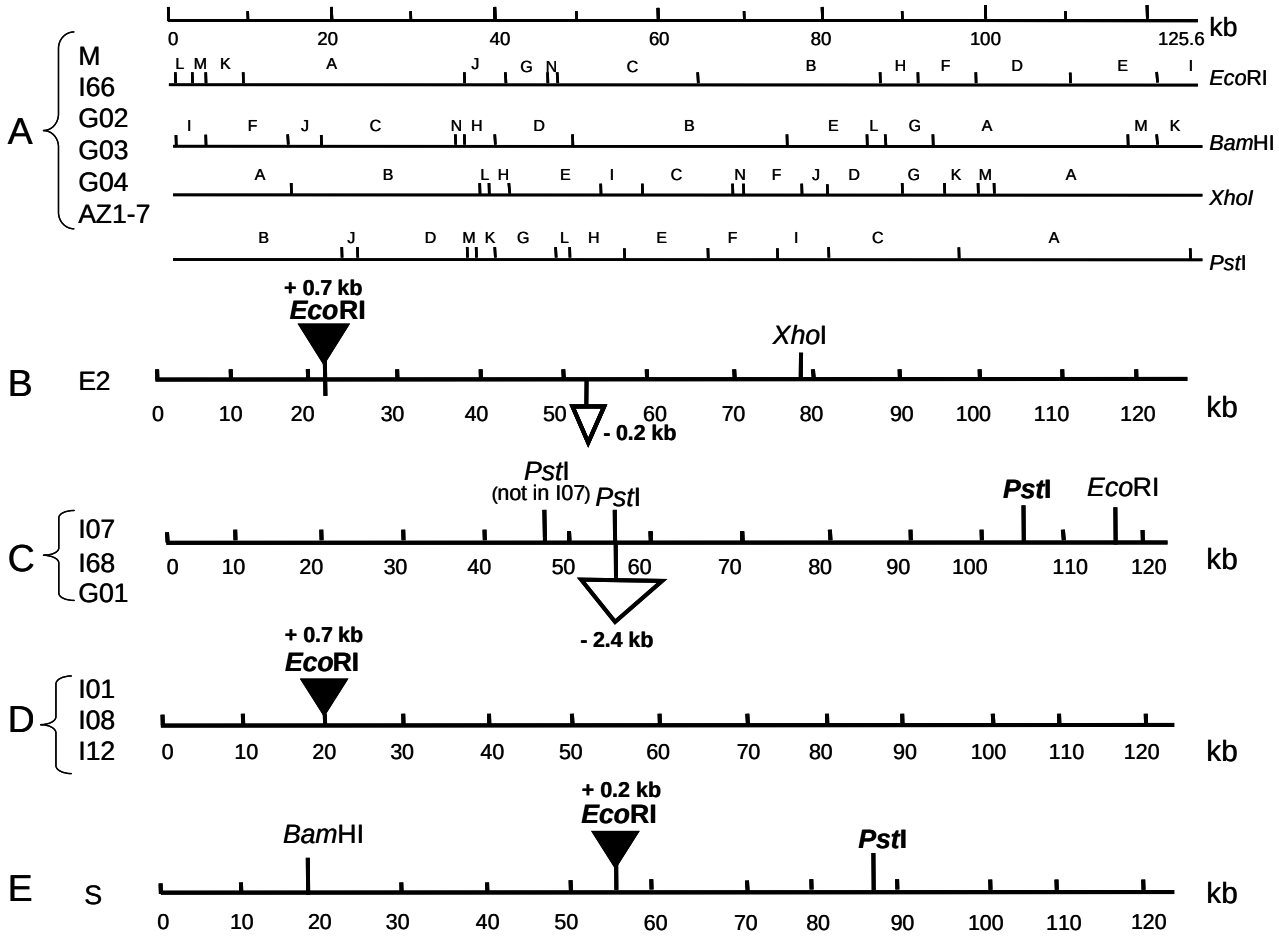


Fig. 3.15. Restriction endonuclease mapping of all isolates compared with the established map of CpGV-M (Crook et al., 1985). Restriction endonucleases in bold type indicate additional endonuclease restriction sites and those in non-bold type indicate missing sites. Mapped sequence insertions and deletions are indicated by black and white triangles, respectively. The proposed genome types are indicated to the left.

It is striking that related genotypes of the previously described isolates could be found in the field in the same geographical region. Conversely, isolates from different geographic regions shared the same restriction profiles. Thus, different CpGV RFLP types cannot be attributed to different geographical origins anymore but have to be considered as putative members of a meta-population of different genotypes of CpGV. This view is corroborated by a previous finding that single genotypes were isolated from CpGV-E by in vivo-cloning, resulting in two genotypes E1 and E2. Whereas E1 was indistinguishable from CpGV-M, E2 had a similar restriction profile to CpGV-M, with modifications, resulting in an additional EcoRI site and about 1 kb more DNA (Crook et al., 1985). Since all analysed RFLPs could be attributed to the dominant genotypes of the previously characterised geographic isolates M, E and R, it is suggested to apply the letters A, B, C, D and so on for identification of the different genome types in CpGV mixtures. According to this, the A type genome corresponds to CpGV-M as well as AZ1-AZ7, I66 and G02-04, whereas the B and C genotype correspond to CpGV-E2 and -R (CpGV-I07, -I68 and -G01), respectively. For the novel isolates CpGV-I12, -I01 and -I08, the D type genome is proposed and for CpGV-S an E type genome.

4 Molecular characterisation of CpGV isolates: partial sequencing of genomic differences

The results presented in this chapter have been published as: Eberle et al., 2009.

4.1 Introduction

Baculovirus genomes are characterised by a high degree of genetic variability, often mirrored by differences in their biological activity (Van Oers and Vlak, 2007). Not only virus isolates originating from different geographical areas show molecular differences, also samples from the same origin may vary in their genetic content. Beyond mutations, intra- and intergenic recombination events and the insertion of transposons play a role in baculovirus natural variation (Van Oers and Vlak, 2007).

Thereby, some genome regions are more often subject to changes than others. For example, the homologous repeat (*hr*) regions, which are interspersed repetitive AT-rich sequences. *Hr* regions can be found at several positions throughout the virus genome and are hotspots of genomic variation (Hilton and Winstanley, 2007; Van Oers and Vlak, 2007). These regions function as *cis* acting enhancing factors for the transcription of early genes by RNA polymerase II (Theilmann and Stewart, 1992). In CpGV, one major repeat region is dispersed in 13 copies of an imperfect palindromic sequence throughout the genome (Luque et al., 2001; Hilton et al., 2008). Beyond the *hr* repeats, the *bro* (baculovirus repeated open reading frame) genes are often target sequences for genomic recombination events (López-Ferber et al., 2003). Analysis of two field isolates of *Mamestra configurata* nucleopolyhedrovirus-A (MacoNPV-A), differing in their DNA restriction profiles and host virulence, revealed that the greatest divergence between the two genomes could be found in the region of three *bro* genes (Li et al., 2005). It was proposed that *bro* genes contribute to baculovirus genome diversity by being involved in recombination events and baculovirus gene loss or acquisition (Li et al., 2005; Harrison, 2009).

Recombination events between baculovirus genomes by homologous recombination also occur with high frequency (Van Oers and Vlak, 2007; Jehle et al., 2003), for example during the infection of a host with two baculovirus genotypes at the same time. Inter-specific recombination events between baculoviruses are less frequent, but observed in case of the *granulin* genes of CpGV and CrleGV (Jehle et al., 2003). Genome comparison of *Xestia c-nigrum* (XecnGV) and *Mamestra configurata* NPV (MacoNPV-B) revealed that MacoNPV-B contained four ORFs which were only present in the distantly related XecnGV, suggesting a lateral gene transfer between both viruses (Li et al., 2002).

The insertion of transposable elements from the host into the virus genome also increases the genetic variability of baculoviruses. Not only when replicating in cell culture, also *in vivo* insertion events could be observed (for review see Friesen, 1993; Jehle et al., 1995; Jehle et al., 1998). Most of these transposons are not able to transpose further. The transposons TCl4.7 and TCp3.2 of the *TC1-like/mariner* family were observed in *in vivo* cloned CpGV variants; the putative transposase gene was also defective (Jehle et al., 1998; Jehle et al., 1995). Transposon TCl4.7, a 4.7 kb insertion sequence, derived from *C. leucotreta*, an alternative CpGV host. The 3.2 kb transposon TCp3.2, characterised by 750 bp of ITRs (inverted terminal repeats), derived from the host *C. pomonella* (Jehle, 1996). Homologous recombination between the ITRs of this transposon was shown to cause *in vivo* inversion of the transposon in the CpGV mutant CpGV-MCp4 (Arends and Jehle, 2002), corroborating the assumption that in baculovirus genomes repeated structures are sources for genetic heterogeneity.

To date, CpGV is the best characterised Betabaculovirus and is therefore often used as a model organism for other GVs. The so far examined CpGV isolates are analysed in the following chapter with regards to their genetic diversity. Partial sequencing of the previously identified RFLPs in different isolates described in Chapter 3 and a phylogenetic analysis should give insight into diversity and evolution of CpGV with regards to their biological activity. The 0.7 kb insertion observed in the *Eco*RI REN profiles of CpGV-I01, -I08, -I12 and -E2 (see Chapter 3) should be localised by PCR, sequenced and analysed for the isolates CpGV-I12 and -E2.

A classification of geographic different isolates on molecular level was aimed for by single nucleotide polymorphism (SNP) detection followed by phylogenetic analysis. For phylogenetic analysis, the *polh/gran*, *lef-8* and *lef-9* genes of isolates of different genome types were selected. These genes are highly conserved in lepidopteran-specific baculoviruses (Lange et al., 2004; Jehle et al., 2006; Herniou et al., 2001). *Polh/gran* codes for *polyhedrin/granulin*; *polyhedrin* is the structural protein of the occlusion body (OB) in Alpha- and Gammabaculoviruses. The homologue gene in Betabaculoviruses is *gran*, coding for *granulin*. Due to their high conservation, the *polh/gran* genes were the first baculovirus genes used for phylogenetic analyses (Rohrmann et al., 1981; Zanotto et al., 1993). The *late expression factor-8* and *-9* (*lef-8* and *lef-9*) genes are coding for subunits of the baculovirus RNA polymerase and were so far identified in all completely sequenced baculovirus genomes (Jehle et al., 2006). The combined data sets of concatenated sequences of *polh/gran*, *lef-8* and *lef-9* genes are useful in species identification (Lange et al., 2004; Jehle et al., 2006). The combination of different genes in phylogenetic analyses gives more robust results than single gene phylogenies (Herniou et al., 2003; Lange et al., 2004). Single gene phylogenies may provide erroneous conclusions, as shown for the *polh* gene of AcMNPV, which is a chimeric gene resulting from a recombination of a group I and a group II NPV (Jehle, 2004).

By partial sequencing and phylogenetic analysis presented in this chapter, evolution of CpGV genomes was elucidated. Partial sequencing of CpGV-I01 and -E2 showed that the two genome types contained an additional non-protein coding insertion of about 0.7 kb. However, this insertion was located at slightly different positions in the genomes. Analysis based on the partial sequences of the *lef-8*, *lef-9* and *polh/gran* genes revealed SNPs which corresponded to the RFLP results and the proposed genome types presented in Chapter 3.

4.2 Methods

4.2.1 PCR amplification

PCR reactions were carried out in an Eppendorf Mastercycler. Annealing temperature and extension time varied according to the size of the amplified PCR product. Standard PCR reactions were performed in 0.2 ml tubes (Eppendorf) with a final volume of 50 µl:

reagent	volume
10 x buffer	5 µl
50 mM $MgCL_2$	2 µl
10 mM dNTPs	1 µl
10 pml primer F	1 µl
10 pmol primer R	1 µl
Taq polymerase	0.5 µl
template DNA	1-3 µl
ddH_2O	to 50 µl

program	temperature [°C]	time [min]
1. Denaturation	95°C	5
2. Denaturation	95°C	1
3. Annealing	45-60 °C	1
4. Extension	72°C	1
5. repetition of step 2- 4, 29 X		
6. Final extension	72°C	7

Prior to sequencing, PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham). DNA concentration was estimated by agarose gel analysis and comparison to a standard with known amount of DNA.

4.2.2 Sequencing of selected marker genes

The partial sequences of the *late expression factor 8* (*lef-8*), *late expression factor 9* (*lef-9*) and *polyhedrin/granulin* (*polh/gran*) genes were amplified using the degenerate primer method described previously (Lange et al., 2004; Jehle et al., 2006). PCR products used for direct sequencing were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham) and both DNA strands were sequenced using M13 universal, M13 reverse and T7 standard primers (MWG), depending on the primers used for amplification.

4.2.3 Partial sequencing of CpGV-I01 and -E2

According to the physical map of all isolates, the 18-20 kb area was amplified by PCR from DNA of isolate CpGV-I01 using the oligonucleotides 18-20_for and 18-20_rev (Table 2.5). To identify the CpGV-E2 insertion site, the region between 16 and 26 kb was amplified in fragments of 4 kb (Table 2.7). Oligonucleotides were designed according to the sequence of CpGV-M1, using the PrimerSelect 5.0 software. The genome area predicted to harbour the insertion was sequenced (Genterprise Genomics) and the resulting sequence was deposited in GenBank under the accession number EU428826. Dot plot analysis of the insertion sequences for repetitive regions was performed using BioEdit 7.0.5.3

4.2.4 Phylogenetic analysis

Partial *polh/gran* and *lef-8* sequences determined for different isolates were concatenated and aligned to each other using Clustal W implemented in BioEdit. The corresponding *polh/gran* and *lef-8* sequences of *Cryptophlebia leucotreta* GV (CrleGV) were included in the analyses as an outgroup (Lange and Jehle, 2003). A phylogenetic analysis using minimum evolution and maximum parsimony algorithms was performed using Mega 3.1 (Tamura et al., 2007).

4.3 Results: partial sequencing of CpGV-I01 and -E2

As suggested from DNA restriction analysis (Chapter 3), insertions of 0.7 kb were expected in the CpGV isolates -I01, -I08, -I12 and -E2 within *Eco*RI-A. The insertion in CpGV-E2 was suggested to be at a slightly different position compared to CpGV-I01, -I08 and -I12. To determine the genome position of the insertion sites in CpGV-I01 and -E2, three pairs of oligonucleotides were designed to amplify overlapping 4 kb fragments of CpGV-M, -I01 and -E2 ranging from 16-26 kb of the CpGV-M genome. Thereby, the insertion could be located between 16-20 kb in the CpGV-I01 genome and between 20-22 kb in CpGV-E2 (Fig. 4.1). These areas were sequenced in order to compare and analyse the complete nucleotide sequences of the insertions.

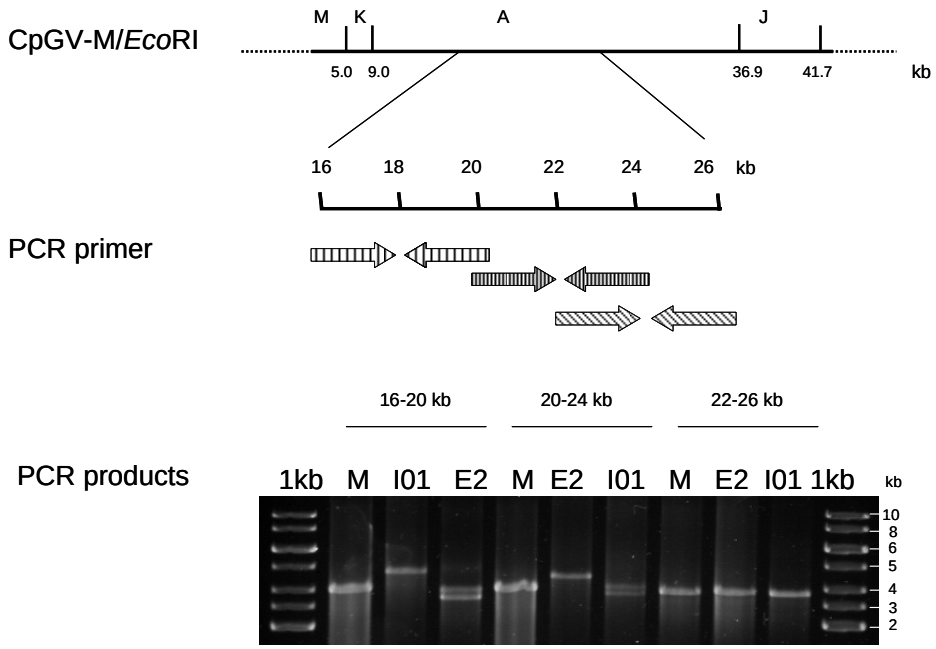


Fig. 4.1. PCR screening for the 0.7 kb insertions in CpGV-I01 and -E2. Three pairs of oligonucleotides were designed to cover the region between 16-26 kb of the CpGV-M genome, as the insertion sites of -I01 and -E2 could be located by REN analysis to this area of *Eco*RI-A. This region was amplified in three overlapping fragments of 4 kb. PCR products without insertion show therefore sizes of 4 kb, PCR products harbouring the 0.7 kb insertion are accordingly larger.

Sequencing of the PCR fragments revealed that CpGV-I01 carried a major insertion of 687 bp in the intergenic region between the ORFs cp23 and cp24 at nt 18,462 of CpGV-M1, while a nearly identical insertion sequence of 723 bp was found between the ORFs cp27 and cp28 in CpGV-E2 (Fig. 4.2). Further smaller insertions and deletions were found in the area of the insertion site of CpGV-I01.

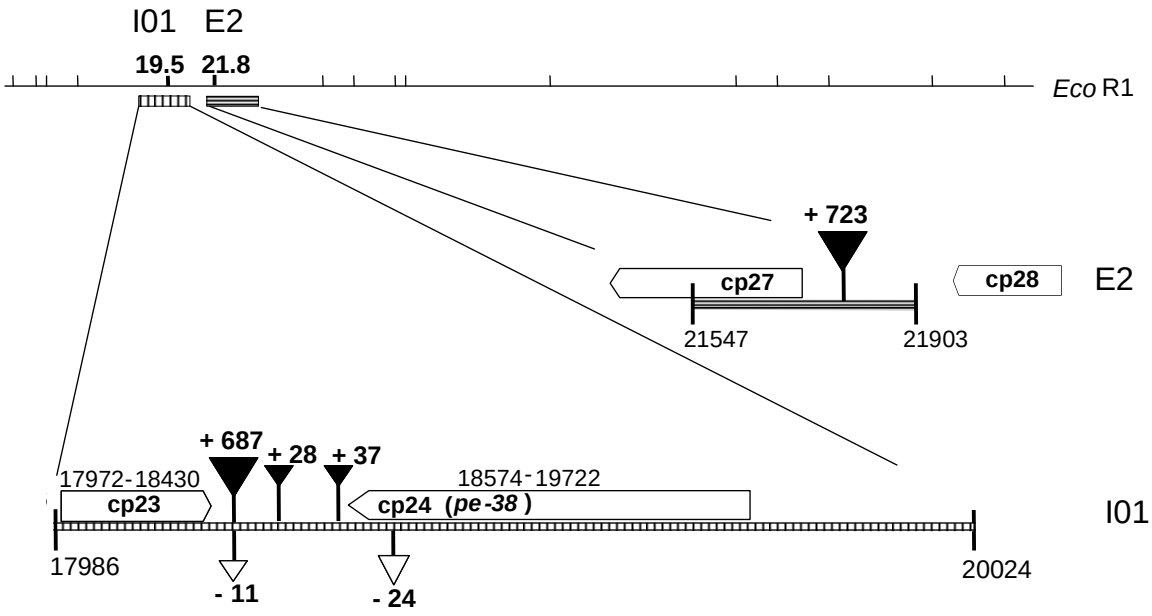
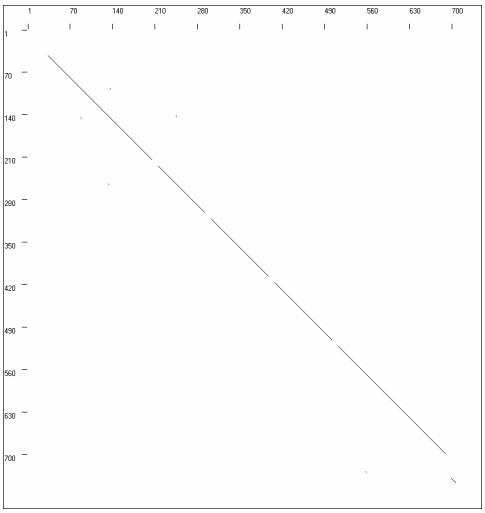


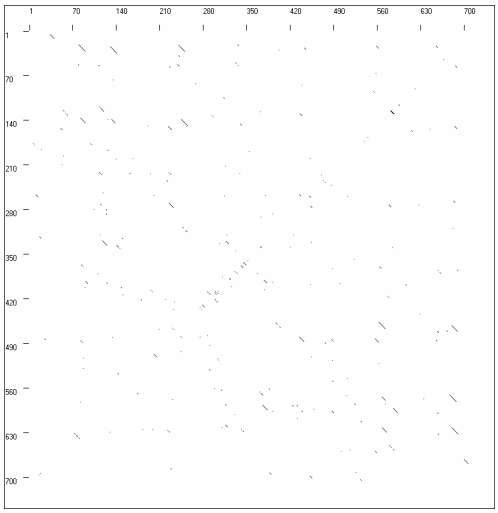
Fig. 4.2. Map of the 18-20 kb area of the genome of CpGV-I01 and the 21 kb area of CpGV-E2 to map the insertion sites. The fragments were amplified by PCR and subjected to sequencing using sequence-specific primers. ORFs are indicated as horizontal arrows. Insertions and deletions are indicated by black and white triangles, respectively. The nucleotide positions are numbered relative to the CpGV-M1 genome sequence (Luque et al., 2001).

Dot plot analysis of the CpGV-I01 and -E2 insertion sequence against each other revealed that the sequences were nearly identical (Fig. 4.3). Repetitive regions were indicated by the patterns on either side of the main diagonals. Dot plot analysis of CpGV-I01 and -E2 against their reverse complements showed that the inserted sequences contained several short inverted repeats, but not longer inverted repeat border sequences as it would be typical for transposable elements.

A CpGV-I01/-E2



B CpGV-I01/-I01 reverse complement



C CpGV-E2/-E2 reverse complement

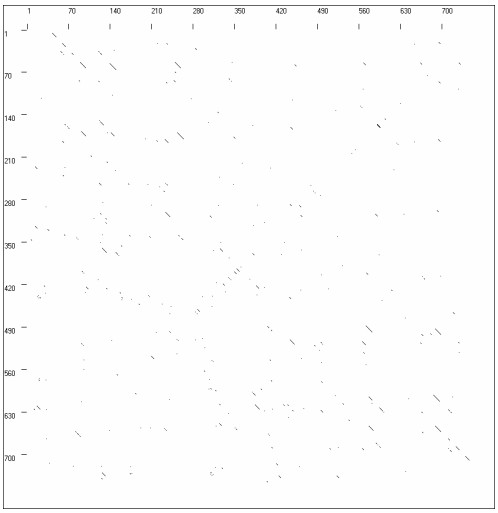


Fig. 4.3. Dot plot analysis of the 0.7 kb insertion sequence of (A): CpGV-I01 (X-axis) against -E2 (Y-axis) (window size: 10, mismatch limit: 0), (B) CpGV-I01 against its reverse complement, (C) CpGV-E2 against its reverse complement (window size: 10, mismatch limit: 2).

Detailed sequence analysis revealed that the insertion sequences were bordered by an inverted terminal repeat of 11 nt (5-(C/T)CTTTAGGCGGG) as well as a duplication of the target site of 5´-AAAATAAA (nt 21,867-21,874 in CpGV-M1) in -E2; no target site duplication was observed in -I01 (Fig. 4.4). The presence of inverted terminal repeats and target site duplications are features similar to insertions of transposable elements. Here, the inverted terminal repeats are very short and the insertion sequence is not protein coding. However, it contained numerous internal inverted repeat structures and revealed a rather symmetrical structure.

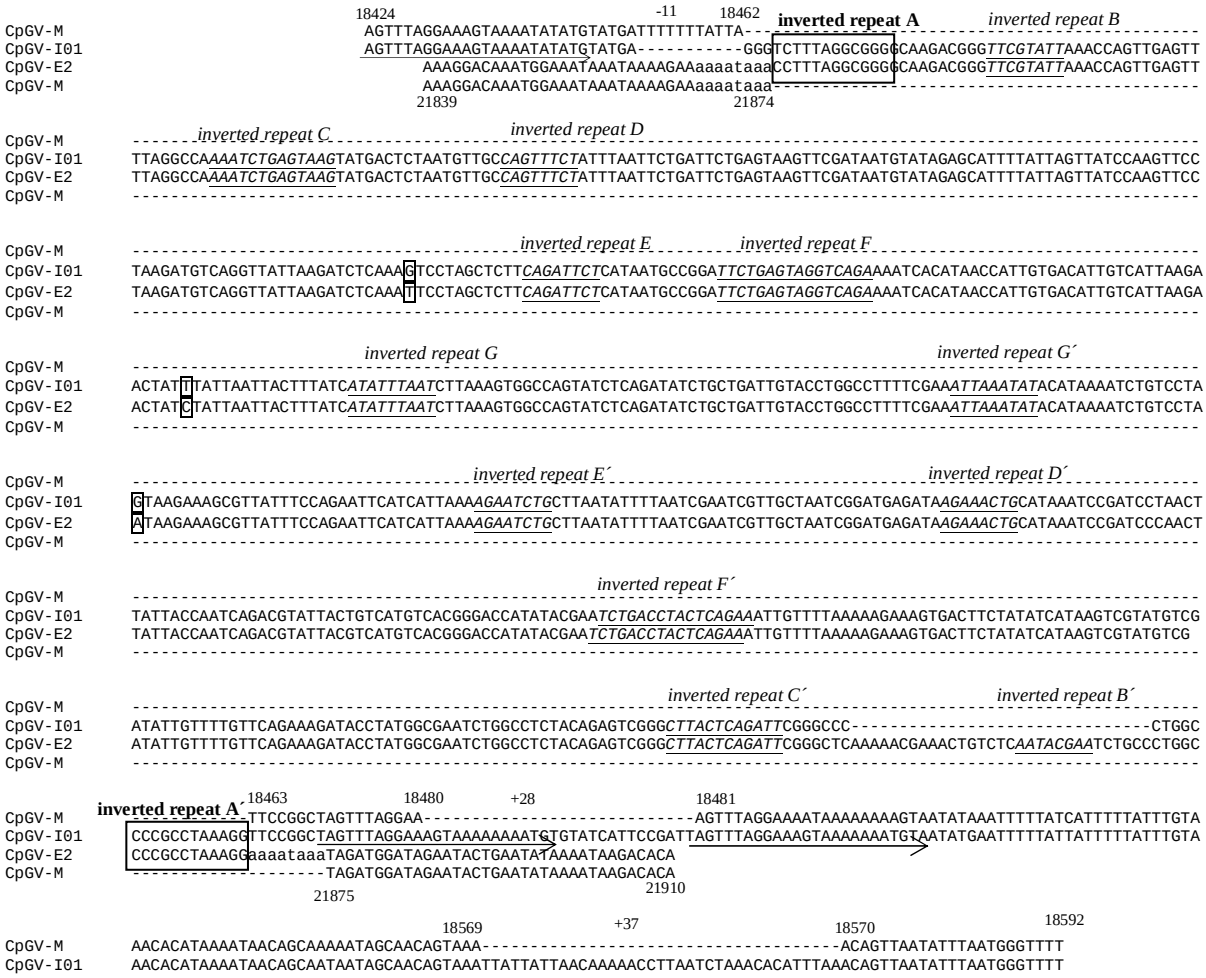


Fig. 4.4. Alignment of the CpGV-I01 and -E2 insertion site. The corresponding parts of the CpGV-M genome are given for both the sequences, numbers indicate the nucleotide position according to the CpGV-M genome and the number of inserted or deleted nucleotides. Arrows indicate direct repeats, inverted repeats are given underlined in italics and lettered alphabetically. The first and the last inverted repeat are shown in boxes. Frames indicate mismatches between the CpGV-I01 and -E2 insertion sequence.

Strikingly, the region containing repeat B, C and D (Fig. 4.5) had sequence similarity to putative *hr* repeats of CpGV, which have been recently identified as origins of replication (Hilton and Winstanley, 2007). Further small indel mutations of 11-28 nucleotides were identified in the neighbourhood of the insertion site in CpGV-I01. Sequencing of the corresponding region of CpGV-I12 (see Chapter 5) revealed the identical insertion at the same position as in CpGV-I01. Based on DNA restriction mapping the same insertion is also predicted for CpGV-I08.

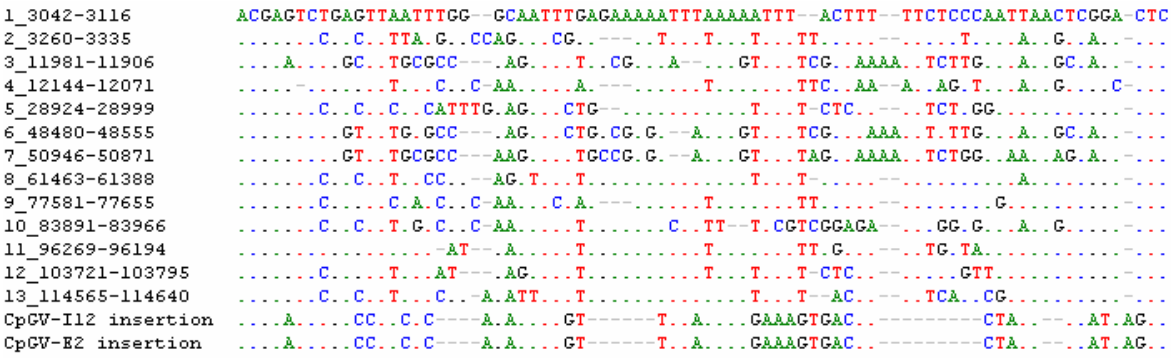


Fig. 4.5. Alignment of a putative *hr* repeat within the 0.7 kb insertion of CpGV-I01 with *hr* repeats identified in the genome of CpGV-M1 (Hilton and Winstanley, 2007).

4.4 Phylogenetic analysis of 11 CpGV isolates

PCR amplification of the partial *polh/gran*, *lef-8* and *lef-9* genes for the isolates CpGV-M1, -G02, -I66, -I01, -I08, -I12, -I07, -I68, -G01, -E2, and -S (Table 4.1) resulted in DNA fragments of about 460, 740 and 260 bp, respectively. Sequencing did not reveal any difference between the gene fragments of CpGV-G02, -I66 and -M1 (Table 4.1).

The isolates CpGV-I01, -I08 and -I12 showed homogenously two single nucleotide polymorphisms (SNPs) in the *lef*-8 sequence at nt 114,274 and 114,693 with two transitions (T to C) (Table 4.1). The SNP at 114,274 nt results in a predicted amino acid change at position 506 of LEF-8 from threonine to methionine, the other SNP in *lef-8* is synonymous. No difference to CpGV-M1 was found in the *polh/gran* sequences.

Compared to CpGV-M1, three SNPs could be detected in the isolates CpGV-I07, -I68 and -G02. Nucleotide 381 in the *polh/gran* gene showed a transition from A to G. Two more transitions from C to T were present at nucleotides 114,267 and 114,693 in the *lef*-8 gene. All transitions were synonymous. No difference was found in the partial *lef*-9 sequence in any of the isolates (Table 4.1).

CpGV-E2 differed in its *polh/gran* sequence from all other isolates, showing one transition from C to T at nt 246 and one transversion from A to C at nt 249. Nucleotide 381 corresponded to CpGV-M1, whereas nt 564 showed a transition to C as observed for the isolates CpGV-I07, -I68 and -G01. None of these changes altered the predicted amino acid sequence of the *polh/gran* genes. The *lef-8* sequence revealed one transition at nt 114,267 from C to T, corresponding to CpGV-I07, -I68 and -G01. A transversion from A to T at nt 114,654 was unique to CpGV-E2 and resulted in an amino acid change from lysine to asparagine.

CpGV-S corresponded in its *polh/gran* SNPs to isolate CpGV-E2. No SNPs were detected for *lef8*; the sequence corresponded to CpGV-M1, -G02 and -I66. The *lef-9* sequence had not been determined for CpGV-E2 and -S, as it was non-informative with regard to SNPs in the other isolates.

Table 4.1. Single nucleotide polymorphisms (SNPs) detected in PCR-amplified fragments of *polh/gran*, *lef-8* and *lef-9* genes. The nucleotide positions of SNPs within the *polh/gran* and *lef-8* genes are given and are numbered according to the nucleotide positions in the published genome of CpGV-M1 (Luque et al., 2001), which was taken as a reference. No SNPs were detected in *lef-9*.

CpGV isolate	proposed genome type	GenBank Accession	*polh/gran* 246	249	381	564	GenBank Accession	*lef-8* 114267	114274	114654	114693	GenBank Accession	*lef-9*
M1	A	NC_002816	C	A	G	T	NC_002816	C	C	A	C	NC_002816	-
G02	A	EU370249	C	A	G	T	EU370242	C	C	A	C	EU370260	-
I66	A	EU370248	C	A	G	T	EU370239	C	C	A	C	EU370257	-
I01	D	EU370243	C	A	G	T	EU370235	C	T	A	T	EU370253	-
I08	D	EU370244	C	A	G	T	EU370236	C	T	A	T	EU370254	-
I12	D	EU370246	C	A	G	T	EU370238	C	T	A	T	EU370256	-
I07	C	EU370245	C	A	A	C	EU370237	T	C	A	T	EU370255	-
I68	C	EU370247	C	A	A	C	EU370240	T	C	A	T	EU370258	-
G01	C	EU370250	C	A	A	C	EU370241	T	C	A	T	EU370259	-
E2	B	EU428824	T	C	G	C	EU428825	T	C	T	C	-	n.d.
S	E	-	T	C	G	C	-	C	C	A	C	-	n.d.

The highly conserved partial *lef-8* and *polh/gran* gene sequences were concatenated and used for phylogenetic analyses. The homologous sequences of the most closely related CpGV neighbour, the *Cryptophlebia leucotreta* granulovirus (CrleGV) were included as outgroup (Lange and Jehle, 2003; Jehle et al., 2006). The phylogenetic tree obtained clearly placed CpGV-I68 and the related isolates -I07, -G01 at the basis of the tree of CpGV isolates (Fig. 4.6), indicating that these genome types are ancestral to those of other isolates.

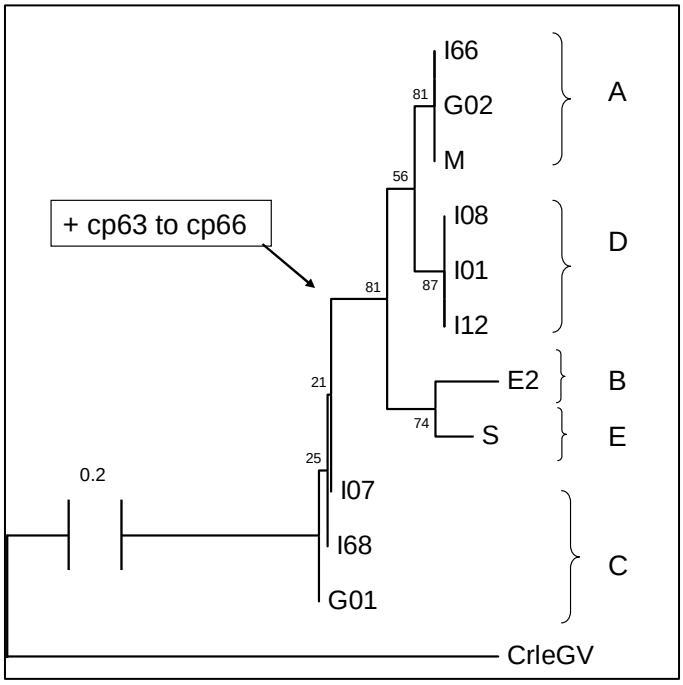


Fig. 4.6. Minimum evolution (ME) tree of 10 CpGV isolates and CpGV-M. The analysis is based on 1032 nt derived from partial *polh/gran* and *lef-8* sequences (Jehle et al., 2006) using CrleGV as an outgroup. Numbers at the nodes indicate bootstrap values (%) of 500 replicates. The optimal tree with the sum of branch length = 0.2 is shown. The ME tree was searched using the close-neighbour-interchange algorithm at a search level of 1; the neighbour-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset; phylogenetic analysis was conducted in Mega 3.1 (Tamura et al., 2007). The proposed insertion of the ORFs cp63-cp66 (see Fig. 4.7) is indicated on the left.

4.5 Discussion

The classification of CpGV isolates into different genome types proposed in Chapter 3 is corroborated by the SNPs detected in the *polh/gran* and *lef-8* genes.

As previously shown, the *polh/gran* and *lef-8* genes are highly conserved among lepidopteran-specific baculovirues and are very well suited for phylogenetic analyis (Herniou et al., 2001; Lange et al., 2004; Jehle et al., 2006).

In the partial *polh/gran* gene, only one position differs between CpGV-M1 and the isolates CpGV-I07, -I68 and -G01. Two SNPs could be detected concerning the *lef-8* sequence in all isolates except the M-type isolates. The isolates CpGV-I12, -I08 and -I01 differ in three nucleotide positions from CpGV-M1, resulting in one amino acid change from threonine to methionine; both are hydrophobic amino acids which therefore probably have a similar function. CpGV-E2 *polh/gran* differs in three positions from CpGV-M1 and in two positions in *lef-8*. CpGV-S corresponds in its *polh/gran* sequence to CpGV-E2, in its *lef-8* sequence to CpGV-M. This poses the question to what extent recombination events are involved in the formation of the different genome types. Homologous recombination of virus variants appears to be a frequent event contributing to the genetic diversity of granuloviruses (Jehle et al., 2003). Recombination within the coding region of the putative DNA helicase gene of *Autographa californica* NPV (AcNPV) and *Bombyx mori* nuclear polyhedrosis virus (BmNPV) resulted in an expanded host range (Maeda et al., 1993). The *polh* gene of AcMNPV shows a mosaic structure of group I and II motifs as a results of a recombination event in its evolutionary history (Jehle, 2004). As such a recombination event can confuse phylogenetic studies based on that single gene, a combined set of conserved genes results in more reliable results to infer phylogenetic trees of baculoviruses (Lange et al., 2004).

Since the SNPs described here are located in highly conserved genome regions, it needs further analyses and comparisons including other isolates to define to what extent these SNPs could be useful for isolate identification or classification. Comparing the SNP analysis to the restriction analyses described in Chapter 3, it is striking that the classification based on the REN profiles corresponds to the grouping based on SNPs: the isolates

CpGV-I66 and -G02 correspond in REN profile and SNPs to CpGV-M. They are therefore grouped together in the phylogenetic analysis (Fig. 4.6). CpGV-I01, -I08 and -I12 do not differ in their restriction profiles from each other, but from all other isolates. Beyond this, they share the same SNPs (Table 4.1) and are therefore grouped together in the phylogenetic tree (Fig. 4.6).

The isolates CpGV-I07, -I68 and -G01 (type C) were grouped as basal branch in the phylogenetic analysis, indicating that they are the phylogenetically eldest isolates of the examined ones. CpGV-M would therefore belong to the phylogenetically youngest. This view is corroborated by further partial sequencing of RFLPs of different genome types (Eberle et al., 2009). The four ORFs cp63-cp66 are not present in type C genomes (Eberle et al., 2009, Fig. 4.7), but in all other examined genome types A, B, D and E. The presence of these four ORFs can be predicted for the isolates analysed in this thesis due to the results of the REN analyses (Chapter 3). The ORF cp63 is a homologue to the baculovirus repeat ORFs (*bro*) and cp66 is homologous to *ptp-2*, whereas cp64 and cp65 are unique to CpGV at this position. The *bro* genes are a multi-gene family in baculoviruses and are thought to have an important function in gene transcription and genome replication (Kang et al., 1999; Zemskov et al., 2000; Bideshi et al., 2003). The loci of *bro* genes have been repeatedly identified in different baculovirus genomes as hotspots of recombination events and genome heterogeneity (López-Ferber et al., 2003). Notably, the ORF cp64 was identified to be homologous to the ORFs crle59 and phop56, suggesting that this gene is widely distributed among GVs specific to *Tortricidae* (Lange and Jehle, 2003). In case of I68 and other type C genomes, however, cp64 is not present. Strikingly, CpGV-M1 has a second homologue of this gene (cp109). Since all known CpGV isolates do not show any RFLP in the genome area of cp109, it is predicted that this ORF is present in all CpGVs including I68 and other C type genomes.

In comparison to other GVs, only A, B, D and E type CpGVs contain the ORFs cp63-cp66 in their genome (Fig. 4.7). The ORF organisation of I68 (C type) is more similar to other GVs than those of CpGV-M1 and -I12, strongly suggesting that the ORFs cp63-cp66 can be considered as recent insertion into the common ancestor of the genomes of A, B, D and E type CpGVs, rather than a deletion in the C type. Based on gene content it

can therefore also be hypothesized that the C type CpGV genome is phylogenetically ancestral, as it was also suggested from the phylogenetic analyses (Fig. 4.6).

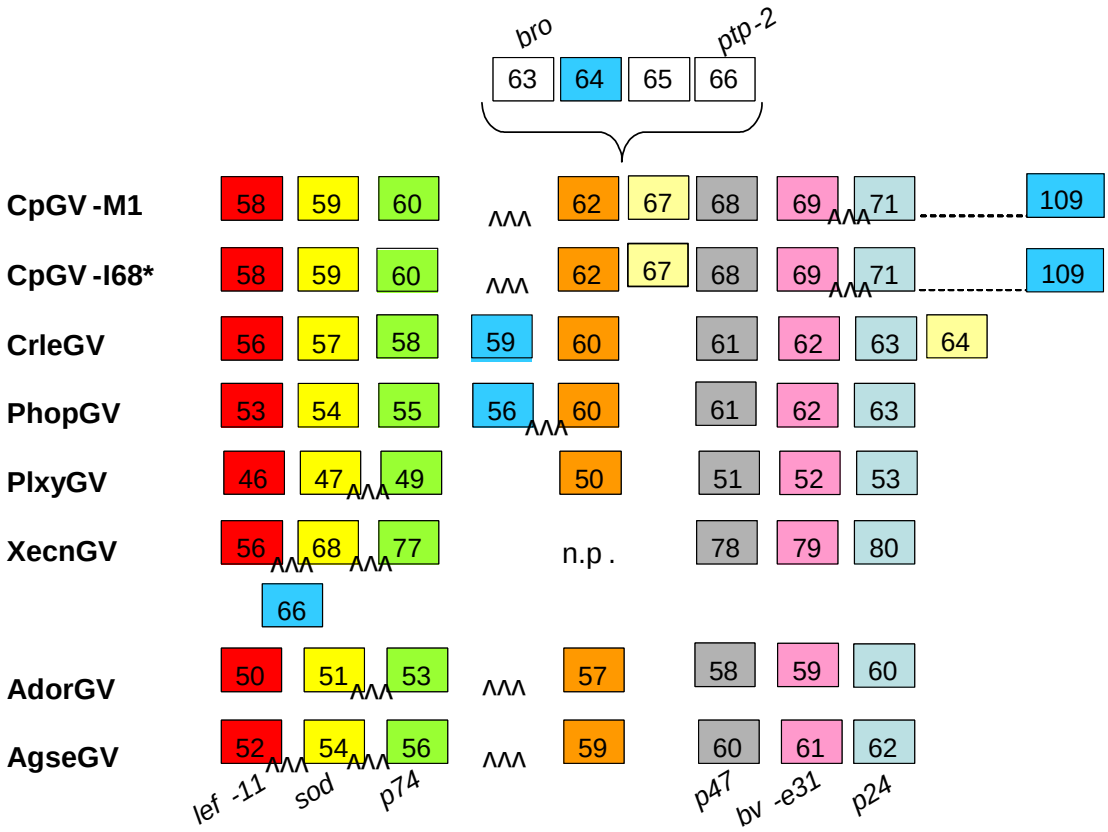


Fig. 4.7. Comparative gene content and gene order analysis of the map unit 50-54 kb of CpGV-M (Luque et al., 2001), including CpGV-I68 (Eberle et al., 2009), CrleGV (Lange and Jehle, 2003), *Phthorimaea operculella* GV (PhopGV) (GenBank accession number NC_004062), *Plutella xylostella* GV (PlxyGV) (Hashimoto et al., 2000), *Xestia c-nigrum* GV (XecnGV) Hayakawa et al., 1999), *Adoxophyes orana* GV (Ador GV) (Wormleaton et al., 2003) and *Agrotis segetum* GV (AgseGV) (NC_005839). Numbers in boxes indicate the ORF numbers in the respective genomes. The same coloured boxes indicate homologous ORFs. ^^^, ORFs not shown; n.p., not present. *, Isolate I68 is numbered according to the CpGV-M1 sequence. The existence of the ORFs is predicted from REN analysis. Redrawn from Eberle et al. (2009).

CpGV-I01, -I12, -I08 and -E2 contain a 0.7 kb non protein coding insertion, which was previously mapped for CpGV-E. The 714 bp insertion found in CpGV-E2 is bordered by the inverted repeat of CTTTAGGCGGG (inverted repeat A and A` in Fig. 4.4) and might be regarded as the original inserted sequence, as this sequence is structured quite symmetrical and is present in CpGV-I01 and -E2. The additional 31 bp in CpGV-E2 10 bp upstream of the last repeat (repeat A`) suggest a deletion in CpGV-I01 rather than an insertion in -E2, because this part contains a sequence of 8 bp which is repeated inverted 10 bp downstream from the first repeat sequence (repeat A). The inverted repeat structure and the target site duplications indicate that the origin of this inserted sequence could derive from a transposable element. One region containing the repeats B, C and D (Fig. 4.) shows some similarity to *hr* regions, which have been recently demonstrated to function as origin of CpGV replication (Hilton and Winstanley, 2007). As this 0.7 kb insertion is not present in all resistance overcoming isolates, it is most likely not involved in overcoming resistance.

In conclusion, the molecular analysis of different CpGV isolates revealed a considerable genetic variability due to point mutations, insertion of ORFs and possibly the insertion of a transposable element. Despite these various differences, it was possible by SNP analysis, in agreement with the RFLP analyses, to group the different isolates to five main genome types. Regarding the question about the resistance overcoming factor, it is indispensable to go more into detail of sequence data: changes on the level of point mutations, which do not lead to RFLPs, need to be determined by whole genome sequencing of resistance overcoming isolates. Then, molecular differences might be related to differences in the biological activity.

Acknowledgements

Analysis of the C type isolates CpGV-I68, -I07 and -G01 by RFLP analysis and partial sequencing was performed by Dr. Samy Sayed.

5 Comparative genomics of different CpGV isolates

5.1 Introduction

As sequencing methods have become more and more straightforward during the last years, the number of completely sequenced baculovirus genomes increased rapidly from the first one in 1994 (Ayres et al., 1994), over three in 1997 (Possee and Rohrmann, 1997) to more than 50 genomes today (Harrison, 2009). Whole genome sequencing of baculoviruses revealed a variety in size and gene content, ranging from 89 ORFs (*Neodiprion sertifer* (Nese) NPV) to 181 ORFs (*Xestia c-nigrum* (Xecn) GV) (Van Oers and Vlak, 2007). Although these genome sequences provided important insight into the diversity of baculoviruses, comparison of different strains of the same virus species by complete genome analysis is limited (Zhang et al., 2005; Li et al., 2005)

For DNA restriction (REN) mapping of baculoviruses, Vlak and Smith (1982) proposed a convention for the zero point as the smallest REN fragment harbouring a *polyhedrin* homologous sequence. Accordingly, the *polyhedrin* or *granulin* ORF is conventionally assigned as number one in the genome, with the adenine of the start codon ATG as base number one. Baculovirus ORFs are located on both DNA strands, with early and late genes distributed over the genome (Van Oers and Vlak, 2007). All ORFs start with an ATG. For ORF prediction, ORFs larger than 50 codons are considered as putative ORFs (Van Oers and Vlak, 2007). In general, baculovirus genomes contain only short intergenic regions and non-overlapping ORFs.

The circular closed genome of the genotype M1, which was *in-vivo* cloned from CpGV-M, was completely sequenced by Luque et al. (2001). It contained 143 putative ORFs with a length of 123,500 nt. The typical homologous repeat sequences (*hrs*), present in most baculovirus genomes (Van Oers and Vlak, 2007), were not found in CpGV. These regions consist of both direct repeats and imperfect palindromes with similar counterparts spread over the genome (Possee and Rohrmann, 1997). Instead of typical *hr* regions, one major repeat region and 13 copies of a 73-77 bp imperfect palindrome dispersed over the genome were identified (Luque et al., 2001; Hilton and Winstanley,

2007). Regions containing *hr* sequences or *baculovirus repeated open reading frame* (*bro*) genes are hotspots of intragenomic recombination (Van Oers and Vlak, 2007), contributing to the genomic plasticity described for baculoviruses. *Hr* regions were also shown to act as cis-acting transcriptional enhancers of early gene expression in *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) (Lu and Miller, 1997) and may function as origins of replication (*ori*) (Kool et al., 1994).

Comparison of the completely sequenced genomes of two genotypes of *Mamestra configurata* nucleopolyhedrovirus-A (MacoNPV-A) deriving from the same host population revealed 49 ORFs carrying point mutations, insertions and deletions as well as one additional ORF in one of the genotypes (Li et al., 2005). One of these isolates showed in bioassay a median lethal dose (LD50) value which was ten times higher compared to the other isolate. The genetic differences were for the most part found in the region of several *bro* genes, indicating that the *bro* genes are associated with baculovirus genome variation (Li et al., 2005). Comparison of four variants of AcMNPV showed that these field isolates contained an additional gene compared to the previously determined sequence (Yanase et al., 2000), indicating that genomic heterogeneity in baculoviruses can also be found between isolates of one species.

Genome analysis of two isolates of *Helicoverpa armigera* nucleocapsid nucleopolyhedrovirus (HearNPV) from the same host differing in their virulence also revealed variations in their *hrs* and *bro* genes (Zhang et al., 2005). Comparison of the HearNPV genome to the complete nucleotide sequence and genome organisation of *Helicoverpa zea* single nucleocapsid nucleopolyhedrovirus (HzSNPV) revealed that the major differences occurred in the organisation of the *hr* regions. Furthermore, these two virus variants contained different *bro* genes (Chen et al., 2002).

Several CpGV isolates are able to overcome resistance in codling moth (Chapter 3). As these resistance overcoming isolates differ in terms of RFLPs or genome types, the question arose which capacity they share for overcoming resistance to CpGV. In this Chapter 5, this question is addressed to by sequencing and comparing the genome sequences of two resistance overcoming isolates CpGV-I12 and -S and the re-sequenced isolate CpGV-M as an internal reference.

5.2 Methods

5.2.1 Whole genome sequencing

CpGV-I12 whole genome sequencing was performed using Sanger sequencing technology by Genterprise Genomics (Mainz). For creating a shotgun library, 60 µg DNA isolated from the virus stock TPCpGVI12_01 were used (see Chapter 2). DNA isolation was performed as described previously (see Chapter 3). As the CpGV-M1 sequence was already published (Luque et al., 2001) and the main genomic differences between CpGV-I12 and -M1 were already known from the REN profile, a genome coverage of three was regarded as sufficient. For closing the sequencing gaps, specific oligonucleotides were designed (Table 2.7) using the borders of the gaps as DNA template (primer walking strategy).

The virus genomes of CpGV-M and -S were sequenced using the 454 sequencing technology (University of Florida, Gainesville, USA). Between 15-20 µg DNA were used for each genome sequencing. Re-sequencing of CpGV-M became necessary as first differences between the isolates were found to be annotation mistakes in CpGV-M1 (Luque et al., 2001) when performing partial sequencing on putative differences between CpGV-M and resistance overcoming isolates. Therefore, DNA was isolated from the virus stock CpGV-M (EU).

5.2.2 Genome analysis using DNAStar

Trace files obtained from the sequencing facilities were checked by eye and minor mistakes were corrected. The files were assembled using SeqMan (DNAStar, Lasergene). The known CpGV-M1 sequence (Luque et al., 2001) was used as reference sequence. The alignment was adjusted manually when not done by the program. Predicted ORFs (methionine initiated, encoding more than 50 amino acids) were compared to public databases using the BLAST programs (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The ORFs were compared between the three sequenced genomes and to the previously published CpGV-M1 sequence (Luque et al., 2001) in order to find significant differences between the resistance overcoming isolates and CpGV-M.

5.3 Results: Genome comparison of CpGV-M, -I12, and -S

5.3.1 Whole genome sequence of CpGV-M

Sequence analysis and localisation of indel mutations were performed in comparison to CpGV-M1 (Luque et al., 2001). Therefore, the ORFs predicted in the re-sequenced CpGV-M were named according to the CpGV-M1 sequence annotation (Luque et al., 2001).

The largest contig contained 8319 sequences out of 9410 obtained ones. The average coverage was 17.23 reads per nucleotide. CpGV-M1 (Luque et al., 2001) was included into the assembly file as reference sequence. The CpGV-M sequence was 123,529 bp in length, 29 bp more than previously determined for CpGV-M1 (Luque et al., 2001). The G/C content was 45.27%. Instead of 143 ORFs (Luque et al., 2001), 142 ORFs were predicted for CpGV-M (Table 5.1). Thereof, 27 ORFs showed differences in their predicted amino acid sequence from CpGV-M1. These differences were due to single predicted additional or missing nucleotides, leading to frameshifts in the sequence, and to amino acid (aa) changes based on single nucleotide polymorphisms (SNPs). The nucleotide differences between CpGV-M1 and -M were mostly found in positions where one nucleotide was repeated several times in the sequence.

Differences in the prediction of ORFS were found in nine cases; cp28, cp29, cp36, cp50, cp51, cp52, cp82, cp129 and cp130 were either predicted splitted or fused compared to CpGV-M1. The ORFs cp28 and cp29 were predicted as a single ORF of 457 codons. This difference to the prediction in the CpGV-M1 annotation (Luque et al., 2001) was due to an additional T in nucleotide position 22,900 found in CpGV-M. Due to this frameshift, the predicted ORF cp28 did not end in nt 22,918 in the stop codon TGA, but continued to nt 24,049, the stop codon of cp29.

CpGV-M1 ORF cp36 was predicted as two ORFs 36a and 36b of 55 and 61 codons, respectively. The sequence differences underlying this changed prediction were two nucleotide positions. The G in nt position 30,984 was not present in CpGV-M; also the A in position 31,060 was missing. Due to these frameshifts, two overlapping ORFs were predicted.

An ORF corresponding to CpGV-M1 cp38 could not be found. One additional nt for CpGV-M led to a frameshift; therefore, there was only a very short ORF predicted from the same start codon as in CpGV-M1 (Fig. 5.1). ORF cp37 in CpGV-M was with 745 predicted aa longer than in CpGV-M1 (683 aa) (Table 5.1). Anyway, the putative cp38 region was covered by CpGV-M cp37.

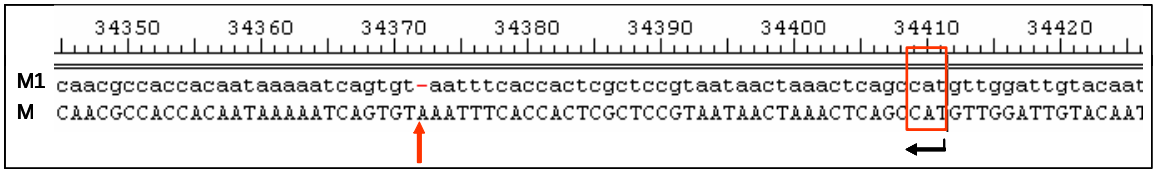


Fig. 5.1. An ORF corresponding to CpGV-M1 ORF cp38 was not found in CpGV-M. Due to an additional nucleotide (red arrow), no ORF was predicted from the start codon (highlighted in red) annotated for CpGV-M1.

The CpGV-M1 ORFs cp50 and cp51 were predicted as a single ORF cp50 of 1101 codons. This difference based on 20 single nucleotide differences between the CpGV-M1 and CpGV-M sequence in this region. In consequence of these differences, the stop codon TAA predicted for CpGV-M1 ORF cp51 was no longer inframe and the ORF ended 64 nucleotides later than predicted previously.

ORF cp52 was predicted as two ORFs cp52a and cp52b. These ORFs were with 121 and 247 codons longer than cp52 in CpGV-M1 (342 codons). ORF cp52a began 144 nucleotides upstream of the predicted start of CpGV-M1 cp52. Two additional nucleotides in CpGV-M led to the same reading frame as in CpGV-M1, so that the aa sequence did not differ downstream of aa position 35; the start codon of the previously predicted cp52. Due to one further frameshift, ORF cp52a ended after 247 codons in TAA at position 44,083. ORF 52b began 23 nt upstream, ending in the stop codon previously predicted for ORF cp52. Hereby, ORF cp52a was lying completely in the antecendent cp50/51. BLASTX analysis showed sequence similarity of cp52b to ORFs of further baculoviruses on amino acid level (Table 5.2). The homologues found for cp52b showed also a similar size as the predicted CpGV ORF. For ORF cp52a, no similarity could be found, indicating that this prediction should be discarded in the annotation as ORF as it is lying com-

pletely in a larger ORF and has no homologue in other baculoviruses, which were two criteria of ORF selection.

ORF cp82 was predicted as cp82a and cp82b, overlapping in 76 nt. The ORFs had a length of 86 and 231 codons, respectively. An additional C found in CpGV-M1 at nt 64,845 led to a shift in the reading frame, ending cp82b in position nt 64,747 in TGA.

CpGV-M1 ORF cp129 and cp130 were predicted as one ORF cp129. For CpGV-M1, 177 bp of non-coding sequence were predicted between the ORFs. In CpGV-M, an additional C in nt 112,703 led to a frameshift in cp130. Hence, cp130 did not end in the predicted stop codon but ran on until the stop codon of cp129.

Compared to the CpGV-M1 ORF predictions the cp6, cp31, cp35 and cp105 were found to be truncated (Table 5.1). Due to nt differences, they ran into earlier stop codons than predicted previously.

Beside the already mentioned fusions of ORFs, the following ORFs were larger than in CpGV-M1: cp26, cp27, cp37, cp55, cp76 and cp135.

Single nucleotide differences resulting in single amino acid changes or frameshifts due to missing or additional nucleotides were found in cp3, cp6, cp11, cp17, cp26, cp27, cp31, cp50/51, cp55, cp83, cp87, cp123, cp125, cp131 and cp135. In total, sequence identity between CpGV-M1 (Luque et al., 2001) and CpGV-M was 99%.

Table 5.1. Positions and orientations of 142 putative ORFs predicted for CpGV-M. The ORFs are numbered according to the previously determined 143 ORFs for CpGV-M1 (Luque et al., 2001). ORFs differing in their prediction from CpGV-M1 in terms of length or amino acid sequence are written in red. AA differences are specified as follows: fs = frameshift mutation, Δ = deletion, ▼ = insertion and by indicating aa changes. For details see text.

CpGV-M ORF	CpGV-M1 ORF	name	position in CpGV-M	length (nt)	length (aa)	position and type of aa differences
1	1	*granulin*	1>747	747	248	
2	2		749<1273	525	174	
3	3	*pk-1*	1254>2093	840	279	fs161-180
4	4		2173<2739	567	188	
5	5		2729>2971	243	80	
6	6		3122>3298	177	58	fs51-57, Δ59-72
7	7	*ie-1*	3391<4857	1467	488	
8	8		4963>5541	579	192	
9	9		5581<5886	306	101	
10	10	*chitinase*	6027<7811	1785	594	
11	11	*cathepsin*	7934>8935	1002	333	S231C
12	12		9015>9248	234	77	
13	13	*gp37*	9318<10073	756	251	
14	14	*odv-e18*	10,145<10,399	255	84	
15	15	*p49*	10,400<11,773	1374	457	
16	16		12,145<12,735	591	196	
17	17	*iap-3*	12,865>13,692	828	275	V166A
18	18	*odv-e56*	13,730<14,797	1068	355	
19	19	*orf15R*	15,171>15,398	228	75	
20	20	*orf16L*	15,458<16,165	708	235	
21	21	*orf17L*	16,434<16,613	180	59	
22	22	*orf17R*	16,836>17,879	1044	347	
23	23		17,970>18,428	459	152	
24	24	*pe38*	18,571<19,719	1149	382	
25	25		20,165<20,329	165	54	
26	26		20,328>21,314	987	329	C101L, ▼121-329
27	27		20,358<21,827	1470	490	fs191-197, ▼405-490
28	28+29		22,688>24,058	1371	457	fused
30	30		24,629>25,174	546	181	
31	31	*F-protein*	25,306>27,099	1794	598	H523Q, Δ599-601
32	32		27,325>28,665	1341	446	
33	33		28,737<29,621	885	294	
34	34		29,664<30,353	690	229	
35	35	*pif-3*	30,247>30,822	576	191	Δ192-199

CpGV-M ORF	CpGV-M1 ORF	name	position in CpGV-M	length (nt)	length (aa)	position and type of aa differences
36a	36		30,998<31,162	165	55	fs26, split
36b	36		30,901<31,083	183	61	split
37	37	*odv-e66*	31,205<33,439	2235	745	▼1-62
-	38					
39	39		33,481>33,798	318	105	
40	40		33,851<34,180	330	109	
41	41	*lef-2*	34,321>34,836	516	171	
42	42	*orf35RA*	34,928>35,176	249	82	
43	43		35,223<35,567	345	114	
44	44	*orf36L*	35,632<36,255	624	207	
45	45		36,314<36,778	465	154	
46	46	*mp-nase*	36,836<38,473	1638	545	
47	47	*p13*	38,480>39,289	810	269	
48	48	*pif-1*	39,333>40,451	1119	372	
49	49		40,448<40,837	390	129	
50	50+51		40,773>44,090	3318	1101	fused
52a	52		43,709<44,071	363	121	fs1-24, split
52b	52		44,099<44,839	741	247	split
53	53		44,849>44,995	147	48	
54	54	*ubiquitin*	45,071<45,355	285	94	
55	55		45,434>46,498	1065	355	fs266-281 fs309-321 ▼327-355
56	56		46,505>46,714	210	69	
57	57	*pp31/39K*	46,791<47,516	726	241	
58	58	*lef-11*	47,467<47,871	405	134	
59	59	*sod*	47,826<48,224	399	132	
60	60	*p74*	48,598<50,664	2067	688	
61	61		50,883<51,095	213	70	
62	62		51,067<51,636	570	189	
63	63	*bro*	51,774>51,941	168	55	
64	64		52,654>53,546	693	230	
65	65		53,444<53,683	240	79	
66	66	*ptp-2*	53,838>54,059	222	73	
67	67		54,134<54,397	264	87	
68	68	*p47*	54,369>55,751	1383	460	
69	69		55,790>56,452	663	220	
70	70		56,526<57,080	555	184	
71	71	*p24capsid*	57,150>57,761	612	203	
72	72		57,800<58,144	345	114	
73	73	*38.7kd*	58,338<58,934	597	198	
74	74	*lef-1*	58,915<59,622	708	235	
75	75	*pif-2*	59,748>61,364	1617	538	
76	76		61,523<62,203	681	227	▼196-227
77	77		62,271<62,582	312	103	
78	78		62,591<62,818	228	75	

CpGV-M ORF	CpGV-M1 ORF	name	position in CpGV-M	length (nt)	length (aa)	position and type of aa differences
79	79		62,842>63,312	471	156	
80	80	*lef-6*	63,309<63,614	306	101	
81	81	*dbp*	63,693<64,565	873	290	
82a	82		64,592<64,849	258	86	split
82b	82		64,773<65,465	693	231	split
83	83	*p45*	65,355>66,674	1320	439	fs53-55
84	84	*p12*	66,706>67,035	330	109	
85	85	*bv/odv-c42*	67,094>68,236	1143	380	
86	86	*p6.9*	68,268>68,417	150	49	
87	87	*lef-5*	68,518<69,246	729	242	fs88-91
88	88	*38K*	69,070>70,101	1032	343	
89	89		70,269<70,754	486	161	
90	90	*helicase*	70,738>74,133	3396	1131	
91	91	*odv-e25*	74,245<74,886	642	213	
92	92		74,976<75,461	486	161	
93	93		75,525>76,280	756	251	
94	94	*iap*	76,324<77,055	732	243	
95	95	*lef-4*	77,060<78,502	1443	480	
96	96	*vp39capsid*	78,574>79,431	858	285	
97	97	*odv-ec27*	79,573>80,439	867	288	
98	98	*ptp-2*	80753>81,238	486	161	
99	99		81,329<82,513	1185	394	
100	100		82,545>82,871	327	108	
101	101	*vp91capsid*	82,906<84,903	1998	665	
102	102	*tlp20*	84,884>85,534	651	216	
103	103		85,509>86,084	576	191	
104	104	*gp41*	86,110>86,979	870	289	
105	105		87,059>87,319	261	86	Δ1-25
106	106	*vlf-1*	87,276>88,412	1137	378	
107	107		88,508>88,762	255	84	
108	108		88,874>89,320	447	148	
109	109		89,415<89,990	576	191	
110	110		90,729<90,352	408	135	
111	111	*dnapol*	90,849<94,004	3156	1051	
112	112	*desmoplakin*	93,946>96,102	2157	718	
113	113	*lef-3*	96,313<97,374	1062	353	
114	114		97,343>97,723	381	126	
115	115		97,845>98,351	507	168	
116	116	*iap-5*	98,533>99,360	828	275	
117	117	*lef-9*	99,335>100,834	1500	499	
118	118	*fp25K*	100,870>101,355	486	161	
119	119		101,468>101,956	489	162	
120	120	*dna-ligase*	102,007<103,719	1713	570	
121	121		104,019>104,234	216	71	
122	122		104,328>104,528	201	66	
123	123	*fgf*	104,618<105,820	1203	400	S214P

CpGV-M ORF	CpGV-M1 ORF	name	position in CpGV-M	length (nt)	length (aa)	position and type of aa differences
124	124		105,973>106,272	300	99	
125	125	*alk-exo*	106,435>107,631	1197	398	H352N
126	126	*helicase-2*	107,555>108,928	1374	457	
127	127	*rr1*	109,017<110,891	1875	624	
128	128	*rr2a*	111,019>112,107	1089	362	
129	129+130		112,140<113,120	981	327	fused
131	131	*lef-8*	113,198<115,819	2622	873	Q738E
132	132		115,907>116,302	396	131	
133	133		116,364<116,552	189	62	
134	134		116,542>116,943	402	133	
135	135		117,012<118,142	1131	377	D281N ▼355-377
136	136		118,148<118,369	222	73	
137	137	*lef-10*	118,320>118,589	270	89	
138	138	*vp1054*	118,447>119,445	999	332	
139	139		119,335<119,655	321	106	
140	140		119,756>120,799	1044	347	
141	141	*egt*	120,882<122,336	1455	484	
142	142		122,354>122,557	204	67	
143	143	*me53*	122,530>123,441	912	303	

Table 5.2. BLASTX analysis of homologous genes found for ORF cp52b (247 codons). For cp52a, no similarities were found.

description/ GenBank accession n°	ORF length (aa)	score	expected value
ORF43 Pieris rapae GV/ ACZ63529.1	210	370	8.0E-101
hypothetical protein Cryptophlebia leucotreta GV/ NP_891897.1	206	324	5.0E-87
ORF43 Adoxophyes orana GV/ NP_872497.1	202	281	6.0E-74
ORF 40 Agrotis segetum GV/ YP_006298.1	219	259	1.0E-67
ORF50 Xestia c-nigrum granulovirus/ NP_059198.1	272	237	1.0E-60

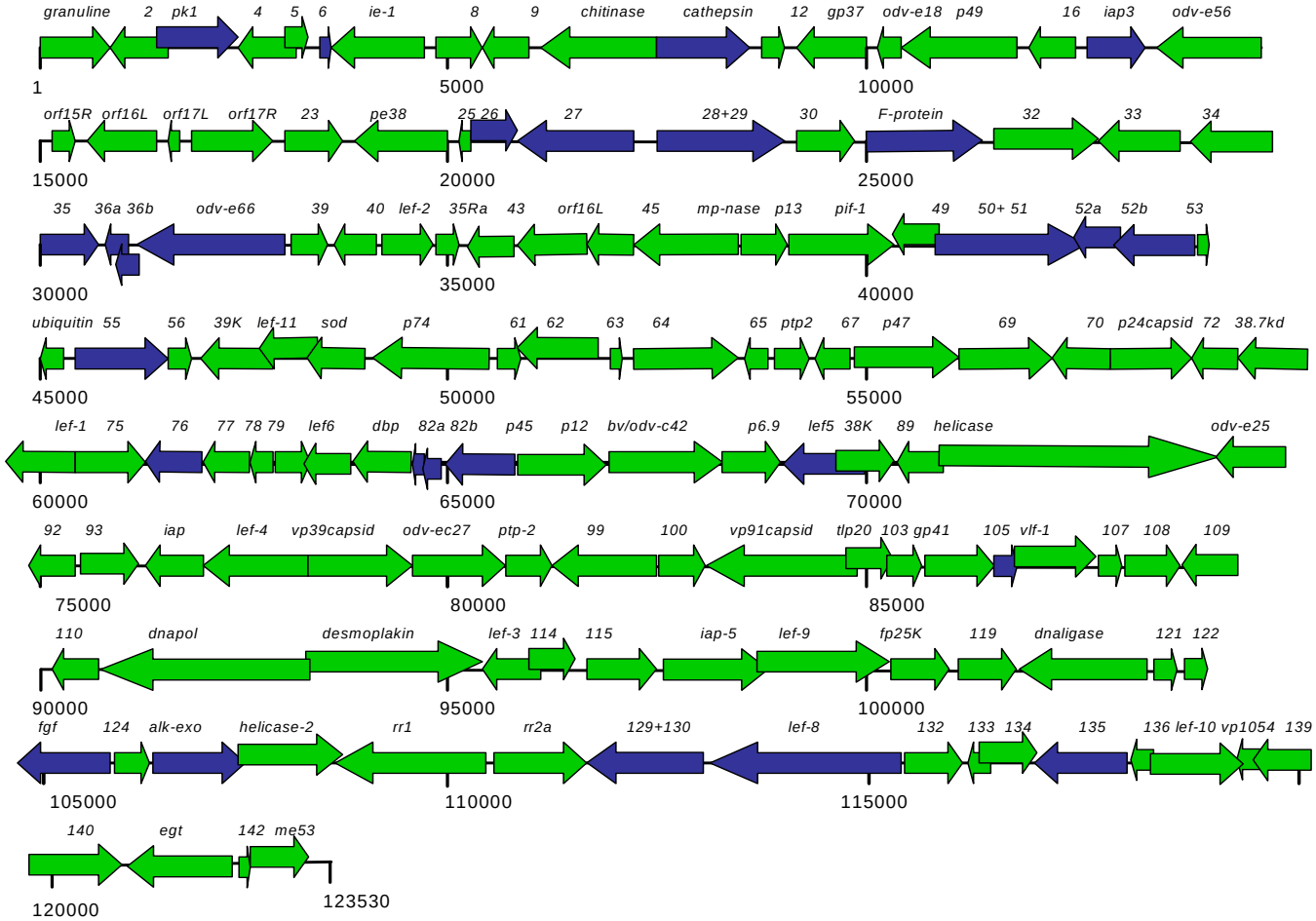


Fig. 5.2. Linear map of the CpGV-M genome (123,529 nt). Arrows symbolise CpGV-M ORFs and their transcriptional direction. CpGV-M ORFs which do not differ from CpGV-M1 in terms of the predicted aa sequence are coloured in green, ORFs showing aa differences to CpGV-M1 are coloured in blue.

5.3.2 Whole genome sequencing of CpGV-I12

The CpGV-I12 genome was 124,269 bp in size and coded for 142 putative ORFs. The largest contig contained 638 sequences, the average coverage was 3.9 reads per nucleotide. All differences found when comparing CpGV-M to the previously published CpGV-M1 sequence (Luque et al., 2001) could also be found in CpGV-I12. This indicated that the differences found between the CpGV-M and -M1 sequences were really based on sequencing errors in the CpGV-M1 annotation and not on real differences of the two isolates on genomic level. On amino acid level, only seven ORFs of CpGV-I12 differed in their predicted protein product from CpGV-M. As in CpGV-M, there was no CpGV-M1-like ORF cp38 found in CpGV-I12 (Table 5.3).

On nucleotide level, the alteration observed in cp23 was based on three nucleotide changes in positions nt 18,303 and 18,304, where two times C (CpGV-I12) instead of two times T (CpGV-M) occurred, whereas in position nt 18,329, CpGV-I12 revealed A instead of G. The predicted amino acid sequence of cp23 differed in two amino acids from that of CpGV-M, resulting in an ORF aa identity of 98.7% (Table 5.3). In aa position 111, an alanine residue was found instead of valine. In position 120, valine was predicted for CpGV-I12, for CpGV-M isoleucine. All of these amino acid changes concerned aliphatic residues, suggesting that these differences do not seriously change the proteins` 3-D-structure.

ORF cp24 encodes a *pe38* homologue (Luque et al., 2001). In CpGV-I12 this ORF (1125 nt, 374 aa) was shorter than in CpGV-M (1149 nt, 382 aa). From nucleotide position 18,771 on, an indel mutation of 24 bp was found. Hereby, a sequence motif of eight amino acids found in CpGV-M was not present in CpGV-I12: [Asp-Thr-Val-Asp-Asp-Thr-Val-Asp] from aa position 311 on. The motif [Asp-Thr-Val-Asp] was three times present in CpGV-M and only one time in CpGV-I12. The nt identity of CpGV-I12 cp24 to that of CpGV-M was 97.4%.

ORF cp62 of CpGV-I12 (606 nt, 202 aa) was 36 bp longer than predicted for CpGV-M (570 nt, 189 aa). A duplication of the motif [Glu-Glu-Pro-Arg-Val-Glu-Glu-Pro-His-Lys-

Val-Gln] was found in the aa positions 349-360. Compared to CpGV-M, the ORF identity based on aa was 94.0%.

The nucleotide sequence of CpGV-I12 cp83 differed in one nt from CpGV-M; due to a transversion from C to A, aa 252 was changed from leucine to methionine. ORF aa identity was 99.1% compared to CpGV-M.

Three amino acid differences were predicted for the protein product of cp128, resulting in an aa of 99.2% compared to CpGV-M. Transition C111,823T in cp128 resulted in a change of aa 278 from isoleucine to threonine. Based on a transversion C111,461A, amino acid 157 was leucine instead of isoleucine. Aspartic acid instead of tyrosine was found in aa 274 due to a change G111,809T. As aspartic acid carries an acidic group whereas tyrosine an aromatic one, this difference might cause changes in protein structure or function, depending on the function of this site.

As in CpGV-M, CpGV-I12 cp129 consisted of the CpGV-M1 ORFs cp129 and cp130 (Table 5.1). Additionally, aa 264 (serine) was deleted. Two further amino acid changes (G292C, L314I) were found in CpGV-I12 cp129.

In cp131, nt 113,703 revealed T (CpGV-I12) instead of G (CpGV-M). Compared to CpGV-M, ORF aa identity was 99.8%. Two differences were found in the predicted protein product. Methionine instead of threonine in aa position 506, isoleucine instead of serine in aa position 704. Thereby, nucleotide position 114,306 revealed a T (CpGV-I12) instead of C (CpGV-M). AA position 738 was glutamine like in CpGV-M1 and not glutamatic acid like in CpGV-M.

Table 5.3. Positions and orientations of 142 putative ORFs predicted for CpGV-I12. The ORFs are numbered according to the previously published sequence of CpGV-M1 (Luque et al., 2001). ORFs differing in their prediction from CpGV-M1 in terms of length or amino acid sequence are written in red. Real differences to CpGV-M are written in blue. AA differences are specified as follows: fs = frameshift mutation, Δ = deletion, ▼ = insertion and by indicating aa changes.

CpGV-I12 ORF	CpGV-M1 ORF	name	position in CpGV-I12	length (nt)	length (aa)	position and type of aa differences
1	1	*granulin*	1>747	747	248	
2	2		749<1273	525	174	
3	3	*pk-1*	1254>2093	840	279	fs161-180
4	4		2173<2739	567	188	
5	5		2729>2971	243	80	
6	6		3122>3298	177	58	fs51-57 Δ59-72
7	7	*ie-1*	3391<4857	1467	488	
8	8		4963>5541	579	192	
9	9		5581<5886	306	101	
10	10	*chitinase*	6027<7811	1785	594	
11	11	*cathepsin*	7934>8935	1002	333	S231C
12	12		9015>9248	234	77	
13	13	*gp37*	9318<10,073	756	251	
14	14	*odv-e18*	10,146<10,400	255	84	
15	15	*p49*	10,401<11,774	1374	475	
16	16		12,146<12,736	591	196	
17	17	*iap-3*	12,866>13,693	828	275	V166A
18	18	*odv-e56*	13,731<14,798	1068	355	
19	19	*orf15R*	15,172>15,399	228	75	
20	20	*orf16L*	15,459<16,166	708	235	
21	21	*orf17L*	16,435<16,614	180	59	
22	22	*orf17R*	16,837>17,880	1044	347	
23	23		17,971>18,429	459	152	V111A, I120A
24	24	*pe38*	19,314<20,438	1125	374	Δ311-319
25	25		20,884<21,048	165	54	
26	26		21,047>22,033	987	329	▼121-329
27	27		21,077<22,546	1470	490	fs191-197 ▼405-490
28	28+29		23,407<24,777	1371	457	fused
30	30		25,348>25,893	546	181	
31	31	*F-protein*	26,025>27,818	1794	598	H523Q Δ599-601
32	32		28,044>29,384	1341	446	
33	33		29,456<30,340	885	294	
34	34		30,383<31,072	690	229	
35	35	*pif-3*	30,966>31,541	576	191	Δ192-199

CpGV-I12 ORF	CpGV-M1 ORF	name	position in CpGV-I12	length (nt)	length (aa)	position and type of aa differences
36a	36		31,717<31,881	165	55	fs26, split
36b	36		31,620<31,802	183	61	split
37	37	*odv-e66*	31,924<34,158	2235	745	▼1-62
-	38					
39	39		34,200>34,517	318	105	
40	40		34,570<34,899	330	109	
41	41	*lef-2*	35,040>35,555	516	171	
42	42	*orf35Ra*	35,647>35,895	249	82	
43	43		35,942<36,286	345	114	
44	44	*orf36L*	36,351<36,974	624	207	
45	45		37,033<37,497	465	154	
46	46	*mp-nase*	37,555<39,192	1638	545	
47	47	*p13*	39,199>40,008	810	269	
48	48	*pif-1*	40,052>41,170	1119	372	
49	49		41,167<41,556	390	129	
50	50+51		41,492>44,794	3303	1101	fused
52a	52		44,413<44,775	363	121	fs1-24, split
52b	52		44,803<45,543	741	247	split
53	53		45,553>45,699	147	48	
54	54	*ubiquitin*	45,775<46,059	285	94	
55	55		46,138>47,202	1065	355	fs266-281 fs309-321 ▼327-355
56	56		47,209>47,418	210	69	
57	57	*pp31/39K*	47,495<48,220	726	241	
58	58	*lef-11*	48,171<48,575	405	134	
59	59	*sod*	48,530<48,928	399	132	
60	60	*p74*	49,302<51,368	2067	688	
61	61		51,587>51,799	213	70	
62	62		51,771<52,376	606	201	▼349-360
63	63	*bro*	52,514>52,681	168	55	
64	64		53,394>54,086	693	230	
65	65		54,184<54,423	240	79	
66	66	*ptp-2*	54,578>54,799	222	73	
67	67		54,874<55,137	264	87	
68	68	*p47*	55,109>56,491	1383	460	
69	69		56,530>57,192	663	220	
70	70		57,266<57,820	555	184	
71	71	*p24 capsid*	57,890>58,501	612	203	
72	72		58,540<58,884	345	114	
73	73	*38.7kd*	59,078<59,675	597	198	
74	74	*lef-1*	59,655<60,362	708	235	
75	75	*pif-2*	60,488>62,104	1617	538	
76	76		62,265<62,945	681	227	▼196-227
77	77		63,013<63,324	312	103	

CpGV-I12 ORF	CpGV-M1 ORF	name	position in CpGV-I12	length (nt)	length (aa)	position and type of aa differences
78	78		63,333<63,560	228	75	
79	79		63,584>64,054	471	156	
80	80	*lef-6*	64,051<64,356	306	101	
81	81	*dbp*	64,435<65,307	873	290	
82a	82		65,591<65,334	258	86	split
82b	82		65,515<66,207	693	231	split
83	83	*p45*	66,097>67,416	1320	439	fs53-55, L252M
84	84	*p12*	67,448>67,777	330	109	
85	85	*bv/odv-c42*	67,836>68,978	1143	380	
86	86	*p6.9*	69,010>69,159	150	49	
87	87	*lef-5*	69,260<69,988	729	242	fs88-91
88	88	*38K*	69,812>70,843	1032	343	
89	89		71,011<71,496	486	161	
90	90	*helicase*	71,480>74,875	3396	1131	
91	91	*odv-e25*	74,987<75,628	642	213	
92	92		75,719<76,204	486	161	
93	93		76,268>77,023	756	251	
94	94	*iap*	77,067<77,798	732	243	
95	95	*lef-4*	77,803<79,245	1443	480	
96	96	*vp39capsid*	79,317>80,174	858	285	
97	97	*odv-ec27*	80,316>81,182	867	288	
98	98	*ptp-2*	81,496>81,981	486	161	
99	99		82,072<83,256	1185	394	
100	100		83,288>83,614	327	108	
101	101	*vp91capsid*	83,649<85,646	1998	665	
102	102	*tlp20*	85,627>86,277	651	216	
103	103		86,252>86,827	576	191	
104	104	*gp41*	86,853>87,722	870	289	
105	105		87,802>88,062	261	86	Δ1-25
106	106	*vlf-1*	88,019>89,155	1137	378	
107	107		89,251>89,505	255	84	
108	108		89,617>90,063	447	148	
109	109		90,158<90,733	576	191	
110	110		91,064<91,471	408	135	
111	111	*dnapol*	91,591<94,746	3156	1051	
112	112	*desmoplakin*	94,688>96,844	2157	718	
113	113	*lef3*	97,055<98,116	1062	353	
114	114		98,085>98,465	381	126	
115	115		98,587>99,093	507	168	
116	116	*iap-5*	992,75>100,102	828	275	
117	117	*lef9*	100,077>101,576	1500	499	
118	118	*fp25K*	101,612>102,097	486	161	
119	119		102,210>102,698	489	162	
120	120	*dnaligase*	102,749<104,461	1713	570	
121	121		104,761>104,976	216	71	

CpGV-I12 ORF	CpGV-M1 ORF	name	position in CpGV-I12	length (nt)	length (aa)	position and type of aa differences
122	122		105,070>105,270	201	66	
123	123	*fgf*	105,360<106,562	1203	400	
124	124		106,716>107,015	300	99	
125	125	*alk-exo*	107,178>108,374	1197	398	H352N
126	126	*helicase2*	108,298>109,671	1374	457	
127	127	*rr1*	109,760<111,634	1875	624	
128	128	*rr2a*	111,762>112,850	1089	362	I157L, Y274D, I278T
129	129+130		112,883<113,860	978	326	fused Δ264,G292C,L314I
131	131	*lef-8*	113,938<116,559	2622	873	T506M, S704I
132	132		116,647>117,042	396	131	
133	133		117,104<117,292	189	62	
134	134		117,282>117,683	402	133	
135	135		117,752<118,882	1131	377	D281N ▼355-377
136	136		118,888<119,109	222	73	
137	137	*lef-10*	119,060>119,329	270	89	
138	138	*vp1054*	119,187>120,185	999	332	
139	139		120,075<120,395	321	106	
140	140		120,496>121,539	1044	347	
141	141	*egt*	121,622<123,076	1455	484	
142	142		123,094>123,297	204	67	
143	143	*me53*	123,270>124,181	912	303	

Beside these differences in coding regions, a few changes in non coding regions were observed. A deletion of 16 nt compared to CpGV-M1 (Luque et al., 2001) was found from nt 24,545 on. One deletion of 15 nt was found concerning the CpGV-M1 nt 43,601-43,616. A duplication of a 36 nt long motif was found from nt 51,240 on. Beside these indel mutations, only 45 SNPs compared to CpGV-M were found.

Overall, CpGV-I12 was found to be very similar to the isolate CpGV-M. G/C content in CpGV-I12 was with 45.2% identical to CpGV-M1 (45.2%) and to -M (45.2%). Beside the described differences and the insertion of 0.7 kb starting from nt 18,454 in the CpGV-I12 genome, the genome content and genome organisation of CpGV-I12 and -M were identical. Alignment with the CpGV-M sequence using BLAST (bl2seq under http://www.ncbi.nlm.nih.gov/) reaveled a sequence identity of 99% when not considering

the 0.7 kb insertion. Of the 13 CpGV *hr* regions (Luque et al., 2001), only *hr*8 showed a difference to CpGV-M. One additional T was found in CpGV-I12 at nt position 62,186. The other 12 *hrs* were identical to CpGV-M.

5.3.3 Whole genome sequencing of CpGV-S

The largest contig obtained for the CpGV-S sequence contained 12,127 sequences, resulting in an average coverage of 21.27 reads per nucleotide. With 123,193 bp, CpGV-S had the smallest of the three sequenced genomes. In contrast to CpGV-M and -I12, only 140 putative ORFs were detected. Fifty-nine of these ORFs showed differences to CpGV-M, when compared on amino acid level. Again all sequence differences between CpGV-M and CpGV-M1 (Luque et al., 2001) were also found in the CpGV-S sequence, corroborating the previous conclusion that these differences were due to errors in the sequence annotation of CpGV-M1. In contrast to CpGV-I12, where the sequence differences to CpGV-M were for the most part SNPs or insertions or deletions of single nucleotides, several indel mutations of different sizes were found throughout the CpGV-S genome (Table 5.4). Most of these indel mutations were flanked by several SNPs in the adjacent regions. Several differences were close to the previously described major repeat region between 20 and 22 kb (Luque et al., 2001) or to the 13 CpGV repeat sequences dispersed over the genome (Hilton and Winstanley, 2007).

Table 5.4. Insertions (+) and deletions (-) found in the CpGV-S genome when compared to the CpGV-M, -M1 (Luque et al., 2001) and -I12 genome sequences. Several indel mutations were found to be close to the 13 CpGV repeat sequences and the major repeat region described previously (Luque et al., 2001; Hilton and Winstanley, 2007).

indel mutation	nt position in CpGV-M1	sequence characteristics	closest CpGV-M1 repeat region (Hilton and Winstanley, 2007)
+ 27 bp	following nt 3242	cp6, duplication of the preceding motif	3042-3116 3260-3335
+ 56 bp	following nt 10,127	duplication of the preceding motif, AT-rich, non coding	11,981-11,906 12,144-12,071
+ 16 bp	following nt 12,065	corresponds to additional 16 bp stretch found in	

indel mutation	nt position in CpGV-M1	sequence characteristics	closest CpGV-M1 repeat region (Hilton and Winstanley, 2007)
		MCp5 close to the integration site of TCl4.7 (Jehle et al., 1995) (Fig. 5.3)	
- 9 bp	15,668-15,677	non coding sequence	
- 5 bp	16,823-16,828	deletion of the preceding motif, non coding	
- 38 bp	20,329- 20,366	cp25, cp26	major repeat region 20- 22 kb
+ 30 bp	following nt 20,450	CAT- rich sequence, cp26	
- 225 bp	20,820- 20,948	cp27	
- 32 bp	24,342- 24,347	repeated motifs, non codning	
- 16 bp	24,538- 24,553	AT-rich, non coding	
+ 12 bp	following nt 25,213	duplication TATA sequence, non coding	
+ 27 bp	following nt 27,700	cp32, duplication of the preceding motif	28,924-28,999
+ 18 bp	following nt 29,800	cp34, duplication of the preceding motif	
+ 12 bp	following nt 33,136	duplication of a repeated motif, cp37	
- 12 bp	33,159-33171	deletion of a repeated motif, cp37	
+ 8 bp	following nt 33,846	non coding, insertion TTCAATTA	
+ 18 bp	following nt 47,481	cp58 *lef-11*, duplication of the preceding motif	48,480-48,555
- 15 bp	51,133- 51,148	cp62, deletion of a repeated motif	50,946-50,871
- 45 bp	51,178- 51,222	deletion of a repeated motif, non coding	

indel mutation	nt position in CpGV-M1	sequence characteristics	closest CpGV-M1 repeat region (Hilton and Winstanley, 2007)
+ 211 bp	following nt 51,990	BLASTX similarity to *bro* genes	
+ 11 bp	following nt 52,156	non coding, duplication, AT-rich sequence	
- 18 bp	56,868- 56,885	deletion of a repeated sequence motif	
+ 63 bp -24 bp	following nt 56,924	cp70, duplication of a repeated motif	
- 9 bp	57,093-57,102	AT- rich motif	
- 148 bp	57,916- 58,063	cp72	
- 6 bp	62,502- 62,507	cp77, CCT repeats	61,463-61,338
- 176 bp	76,532- 76,707	cp94 *iap*	77,581-77,655
+ 12 bp	89,937- 89,949	cp109, duplication of the preceding motif	83,891-83,966
- 14 bp	90,056- 90,070	deletion of a repeated motif, non coding	
+ 19 bp	following nt 97,791	duplication of the preceding motif, non coding	96,269-96,194
- 19 bp	101,953- 101,972	repeated AT-rich sequence motif, non coding	103,721-103,795
- 27 bp	120,789- 120,815	AT-rich sequence, non coding	114,565-114,640

In CpGV-S, 59 ORFs showed differences on aa level compared to CpGV-M (Table 5.5). The ORFs cp25 and cp26 found in CpGV-M1, -M and -I12 could not be found in the genome of CpGV-S. The predicted start codons of the two anti-clockwise and clockwise orientated ORFs were lying in a 38 bp deletion area found in CpGV-S (Table 5.4). CpGV-M1 cp38 was not predicted as previously determined for CpGV-M and -I12 (see 5.3.2).

Single amino acid changes between CpGV-M and -S were found in 47 ORFs (Table 5.5). Eighteen ORFs differed in their length from the ORFs predicted for CpGV-M due to insertion or deletions (Table 5.5). In ORF cp2, the codon for aa 114 (Gly) was duplicated. In cp6, a motif coding for nine amino acids was duplicated (see also Table 5.4). Cp16 started 13 aa earlier than predicted for CpGV-M. The amino acids 66-67 were not found in cp20. ORF cp24 (*pe38*) showed, corresponding to CpGV-I12, eight amino acids less than CpGV-M (311-319). ORF cp27 lacked the amino acids 366-440 and 476-479, attended by an insertion of five aa (Table 5.5). Cp31 lacked the aa 599-601. A motif of nine amino acids was duplicated in cp32. In cp34, aa positions 194-199 were duplicated and the ORF endet earlier than predicted for CpGV-M due to an additional T. Cp46 showed a duplication of aa 471, in cp52b, one additional aa in position 27 was found. In cp58 (*lef-11*), a motif of six amino acids was duplicated. Cp59 started 24 aa earlier than predicted for CpGV-M. In cp62, nineteen amino acids were missing due to a deletion. One additional codon was found in cp70. Two amino acids were not found in cp77 due to a deletion site. Cp94 started 16 aa later as predicted for CpGV-M and was missing the last 133 aa due to a deletion site in this area. Cp109 harboured a duplication of four aa (see also Table 5.4). Position 129 in cp112 was deleted.

In the 13 CpGV *hr* regions (Luque et al., 2001), four nt differences to CpGV-M were found. In *hr*2, CpGV-S nt 3316 was A instead of G (CpGV-M), nt 3321 A instead of G. In *hr*4, CpGV-S nt 12,218 was T instead of A, and in *hr*12, nt 103,488 was C instead of T. The other *hrs* were identical to CpGV-M.

An insertion site of 211 bp between the ORFs cp63 and cp64 showed similarity to baculovirus *bro* genes when analysed with BLASTX (Fig. 5.4). Translation was in frame + 1. The sequence itself did not show any repeat structure nor were target site duplica-

tions found, indicating that this structure did not derive from a transposition event. There was also no similarity to the 0.7 kb insertion found in CpGV-I12 or -E2. The sequences bordering the insertion revealed no BLASTX hits. Also, no in frame start or stop codon was found, and there was no aa sequence similarity to the cp63 *bro* gene.

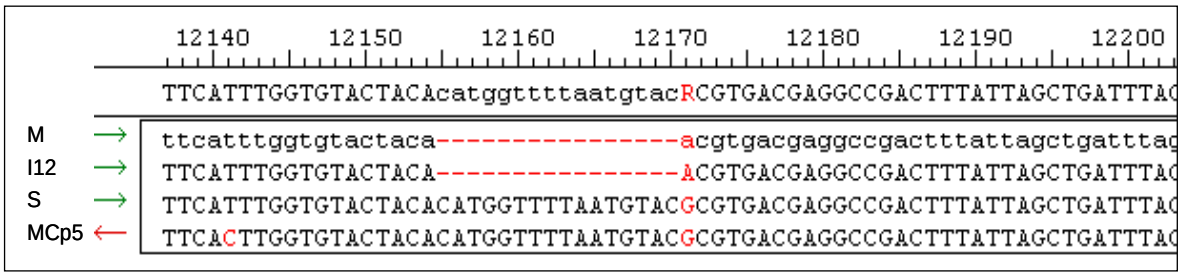


Fig. 5.3. Alignment of CpGV-M, -I12 and -S with the reverse complement of the MCp5 insertion region of TCl4.7 (gi|727219|). Sixteen additional bp found close to the integration site of transposon TCl4.7 in MCp5 (Jehle et al., 1995) were also present in CpGV-S, as well as one nt change from A (M, I12) to G (S, MCp5). These differences were not found in CpGV-M or -I12.

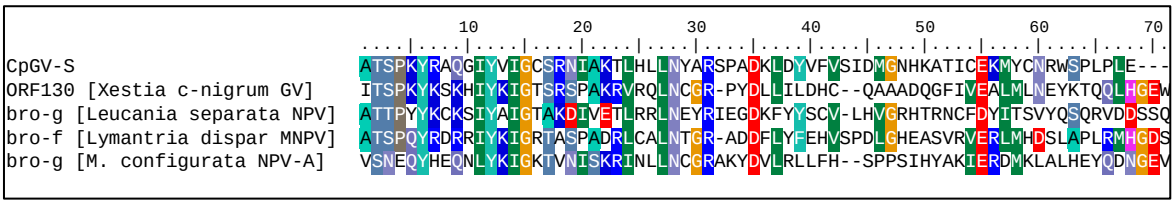


Fig. 5.4. BLASTX analysis followed by ClustalW multiple alignment of the 211 nt insertion in CpGV-S reveald similarities on amino acid level to the baculovirus *bro* genes of Xestia c-nigrum GV (gi|9635380), Leucania separata NPV (gi| 114679974), Lymantria dispar MNPV (gi|9631041) and Mamestra configurata NPV-A (gi|33331834). Colours: identity and similarity shading (BioEdit 7.0.5.3)

Table 5.5. Position and orientation of 141 putative ORFs predicted for CpGV-S. ORFs differing in their prediction from CpGV-M1 in terms of length or amino acid sequence are written in red. Real differences to CpGV-M are written in blue. AA sequence differences are described as follows: fs = frameshift mutation, Δ = deletion, ▼ = insertion and by indicating single amino acid changes.

CpGV-S ORF	CpGV-M1 ORF	name	position	length (nt)	length (aa)	positin and type of aa differences
1	1	*granulin*	1>747	747	248	
2	2		749<1276	528	175	▼114
3	3	*pk-1*	1257>2096	840	279	fs161-180
4	4		2741<2175	567	188	
5	5		2731>2973	243	80	
6	6		3124>3327	204	67	▼29-37, fs51-57, Δ59-72
7	7	*ie-1*	3420<4886	1467	488	E368D
8	8		4992>5570	579	192	
9	9		5610<5915	306	101	
10	10	*chitinase*	6055<7839	1785	594	M555L, D473E
11	11	*cathepsin*	7962>8963	1002	333	S231C
12	12		9043>9276	234	77	
13	13	*gp37*	9348<10,103	756	251	
14	14	*odv-e18*	10,232<10,486	255	84	
15	15	*p49*	10,487<11,860	1374	457	D30V
16	16		12,248<12,877	630	209	▼1-13
17	17	*iap-3*	12,969>13,796	828	275	V61A, V166A
18	18	*odv-e56*	13,834<14,901	1068	355	
19	19	*orf15R*	15,275>15,502	228	75	
20	20	*orf16L*	15,562<16,260	699	232	A10V, Δ66-67
21	21	*orf17L*	16,529<16,708	180	59	
22	22	*orf17R*	16,925>17,968	1044	347	
23	23		18,059>18,517	459	152	
24	24	*pe38*	18,661<19,785	1125	374	Δ311-319
-	25					
-	26					
27	27		20,370<21,644	1275	425	fs191-197 Δ366-440, Δ476-479,▼459-464.
28	28+29		22,505>23,875	1371	457	fused
30	30		24,399>24,944	546	181	
31	31	*F-protein*	25,088>26,881	1794	598	Δ599-601, H523Q L505I, G323E
32	32		27,107>28,474	1368	455	L71M, V250A, V264A, M438T, ▼364-372
33	33		28,546<29,430	885	294	
34	34		29,567<30,181	612	204	G160A, ▼194-199, fs 203-205,Δ206-229

CpGV-S ORF	CpGV-M1 ORF	name	position	length (nt)	length (aa)	positin and type of aa differences
35	35	*pif-3*	30,075>30,650	576	191	Δ192-199
36a	36		30,827<30,991	165	55	split
36b	36		30,730<30,912	183	61	fs26, split
37	37	*odv-e66*	31,033<33,267	2235	745	▼1-62, fs94-101, M48V, P68S
-	38					
39	39		33,309>33,626	318	105	R71K, N77G
40	40		33,687<34,016	330	109	
41	41	*lef-2*	34,157>34,672	516	171	V47A, F96L
42	42	*orf35RA*	34,764>35,012	249	82	
43	43		35,059<35,403	345	114	
44	44	*orf36L*	35,468<36,091	624	207	I8L
45	45		36,150<36,614	465	154	
46	46	*mp-nase*	36,672<38,312	1641	546	V87L, D178E, ▼G471
47	47	*p13*	38,319>39,128	810	269	V211I
48	48	*pif-1*	39,172>40,290	1119	372	
49	49		40,287<40,676	390	129	
50	50+51		40,612>43,929	3318	1106	fused
52a	52		43,910<43,545	366	122	▼L27, split
52b	52		43,938<44,678	741	247	split
53	53		44,688>44,834	147	48	
54	54	*ubiquitin*	44,910<45,194	285	94	
55	55		45,273>46,337	1065	326	fs266-281, fs309-312, ▼327-355
56	56		46,344>46,553	210	69	
57	57	*pp31/39K*	46,630<47,373	744	248	Q2K, A130V, N136D
58	58	*lef-11*	47,306<47,728	423	140	▼122-124
59	59	*sod*	47,683<48,156	474	157	▼1-24
60	60	*p74*	48,458<50,524	2067	688	
61	61		50,742<50,954	213	70	M24T
62	62		50,926<51,435	510	173	Δ139-158, fs159-162
63	63	*bro*	51,573>51,740	168	55	
64	64		52,675>53,367	693	230	
65	65		53,465<53,704	240	79	
66	66	*ptp-2*	53,859>54,098	240	80	
67	67		54,150<54,413	264	87	
68	68	*p47*	54,385>55,767	1383	460	V355M
69	69		55,806>56,468	663	220	
70	70		56,541<57,116	576	192	▼27
71	71	*p24capsid*	57,178>57,789	612	203	
72	72		57,828<58,103	276	92	fs1-47, Δ48-71
73	73	*38.7kd*	58,218<58,814	597	198	C19W
74	74	*lef-1*	58,795<59,502	708	235	N28K
75	75	*pif-2*	59,628>61,244	1617	538	V319M

CpGV-S ORF	CpGV-M1 ORF	name	position	length (nt)	length (aa)	positin and type of aa differences
76	76		61,403<62,083	681	227	D85E, ▼196-227
77	77		62,151<62,456	306	101	Δ24-25
78	78		62,465<62,692	228	75	
79	79		62,716>63,186	471	156	D63E
80	80	*lef-6*	63,183<63,488	306	101	
81	81	*dbp*	63,567<64,439	873	290	split
82a	82		64,466<64,723	258	86	split
82b	82		64,647<65,339	693	231	I34T
83	83	*p45*	65,229>66,548	1320	439	Y4C, G20C,I283M, fs53-55
84	84	*p12*	66,580>66,909	330	109	
85	85	*bv/odv-c42*	66,968>68,110	1143	380	
86	86	*p6.9*	68,142>68,291	150	49	
87	87	*lef-5*	68,392<69,120	729	242	fs88-91
88	88	*38K*	68,944<69,975	1032	343	
89	89		70,143<70,628	486	161	S137P
90	90	*helicase*	70,612>74,007	3396	1131	
91	91	*ODV-e25*	74,119<74,760	642	213	
92	92		74,850<75,335	486	161	I110M
93	93		75,399>76,154	756	251	V145M
94	94	*iap*	76,424<76,705	282	91	D9G, Δ1-16, Δ110-243
95	95	*lef-4*	76,758<78,200	1443	480	N271S
96	96	*vp39capsid*	78,272>79,129	858	285	D90S
97	97	*odv-ec27*	79,271>80,137	867	288	
98	98	*ptp-2*	80,451>80,936	486	161	
99	99		81,027<82,211	1185	394	A116V
100	100		82,243>82,569	327	108	
101	101	*vp91capsid*	82,604<84,601	1998	665	S517N
102	102	*tlp20*	84,582>85,232	651	216	
103	103		85,207>85,782	576	191	
104	104	*gp41*	85,808>86,677	870	289	
105	105		86,757>87,017	261	86	Δ1-25
106	106	*vlf-1*	86,974>88,110	1137	378	D159E
107	107		88,205>88,459	255	84	
108	108		88,579>89,017	447	148	V100A
109	109		89,112<89,699	588	195	▼12-15
110	110		90,432<90,016	408	135	
111	111	*dnapol*	93,698<90,543	3156	1051	A823T
112	112	*desmoplakin*	93,640>95,793	2154	718	N19D,Δ129
113	113	*lef-3*	97,065<96,004	1062	353	
114	114		97,034>97,414	381	126	
115	115		97,555>98,061	507	168	
116	116	*iap-5*	98,243>99,070	828	275	
117	117	*lef-9*	99,045>100,544	1500	499	

CpGV-S ORF	CpGV-M1 ORF	name	position	length (nt)	length (aa)	positin and type of aa differences
118	118	*fp25K*	100,580>101,065	486	161	
119	119		101,178>101,666	489	162	A77V
120	120	*dnaligase*	101,698<103,410	1713	570	
121	121		103,710>103,925	216	71	
122	122		104,019>104,219	201	66	V45I
123	123	*fgf*	104,309<105,511	1203	400	
124	124		105,664>105,963	300	99	
125	125	*alk-exo*	106,126>107,322	1197	398	H352N, K90R
126	126	*helicase*	107,246>108,619	1374	457	
127	127	*rr1*	110,582<108,708	1875	624	Y542F
128	128	*rr2a*	110,710>117,798	1089	362	E349K
129	129+130		112,811<111,831	981	327	fused
131	131	*lef-8*	115,510<112,889	2622	873	Q738E
132	132		115,597>115,992	396	131	T25N
133	133		116,242<116,054	189	62	
134	134		116,232>116,633	402	133	
135	135		117,832<116,702	1131	377	D281N, ▼355-377
136	136		118,059<117,838	222	73	
137	137	*lef-10*	118,010>118,279	270	89	
138	138	*vp1054*	118,137>119,135	999	332	V232I
139	139		119,345<119,025	321	106	D44G
140	140		119,446>120,489	1044	347	D263E
141	141	*egt*	122,000<120,546	1455	484	A280G
142 143	142 143	*me53*	122,018>122,221 122,194>123,105	204 912	67 303	 143

Summarizing, the CpGV-S sequence revealed several differences to CpGV-M and -I12, most of them due to insertions and deletions between three to more than 200 bp (Table 5.4, Table 5.5). No indication for the 0.7 kb insertion present in CpGV-I12 was found in CpGV-S.

Comparing the CpGV-S and -I12 sequences, there were several ORFs which differed from CpGV-M1 or -M. But for the most part, the mutations found differed also between CpGV-I12 and -S.

Only one difference was shared by the two isolates and distinguished them from CpGV-M and CpGV-M1: the nucleotide stretch of 24 bp in ORF24 (*pe38*) was present in CpGV-M1 and -M, but not in the CpGV-I12 and -S. This nucleotide stretch consisted of

the motif TGTGTCATCCAC, coding for [Asp-Thr-Val-Asp-Asp-Thr-Val-Asp]. This motif was three times present in CpGV-M, but only one time in CpGV-I12 and -S.

As this was the only common difference to CpGV-M, the *pe38* region of further isolates was amplified by PCR using the oligonucleotides designed for analysis of the 18-20 kb region (Table 2.5) . It was found that all CpGV-M-like isolates, such as CpGV-G03 and -AZI, carried 24 nt more in *pe38* than other isolates (Fig. 5.4) which were previously found to differ from CpGV-M in terms of RFLPs and SNPs (see Chapter 3, Chapter 4).

Fig. 5.5. Alignment of the partial *pe38* sequences of eight different CpGV isolates. Twenty-four nt found in CpGV-M, -AZ1 and -G03 were not present in further genome types.

```
CpGV-M    AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACAGTGGATG ACACAGTGGA TGACACAATT ATGCGTGATG ATTC
CpGV-AZ1  AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACAGTGGATG ACACAGTGGA TGACACAATT ATGCGTGATG ATTC
CpGV-G03  AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACAGTGGATG ACACAGTGGA TGACACAATT ATGCGTGATG ATTC
CpGV-I01  AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACA------- ---------- -------ATT ATGCGTGATG ATTC
CpGV-E2   AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACA------- ---------- -------ATT ATGCGTGATG ATTC
CpGV-I07  AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACA------- ---------- -------ATT ATGCGTGATG ATTC
CpGV-I12  AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACA------- ---------- -------ATT ATGCGTGATG ATTC
CpGV-S    AAACCGAAGA TGATATCACA AAGTCGGTAG CAAATGACAC AGTGGATGAC ACA------- ---------- -------ATT ATGCGTGATG ATTC
```

To summarize, the *pe38* sequence differed between A type genomes and all other so far described genome types. Genomes grouped to type A (Chapter 4) were not able to overcome resistance in CM (Chapter 3). In contrast, isolates differing from type A genomes showed an improved efficacy against resistant CM strains and were able to overcome resistance when tested in bioassay (Chapter 3). Despite the RFLP differences among them, they did not differ in their *pe38* sequence referring to the 24 nt strech found in CpGV-M. This insertion was found only in A type genomes, which were not able to overcome resistance, suggesting that the difference in virulence of the isolates is based on this difference of the *pe38* sequence.

5.4 Discussion

With CpGV-I12 and -S, two genomes of resistance overcoming isolates have been sequenced. CpGV-M was re-sequenced as internal reference. This was necessary, as putative frameshifts and SNP mutations between CpGV-I12 and the published sequence of CpGV-M1 were identified to be errors in the annotation of CpGV-M1 and not due to real nucleotide changes. This was corroborated by the finding that the same differences were also found in the genomes of CpGV-I12 and -S.

Twenty-six ORFs of CpGV-M differed in their predicted protein product from CpGV-M1 (Fig. 5.2). With 123,529 bp, the CpGV-M sequence was 29 bp longer than predicted previously (Luque et al., 2001). In total, 142 ORFs were predicted for CpGV-M. CpGV-M1 cp38 was not found. Also cp52a should not be considered as ORF: it was lying completely in a larger ORF cp52b and did not show homology to further baculovirus ORFs, as it was the case for the cp52b (Table 5.2).

With 124,269 bp, CpGV-I12 had the largest genome of the three virus isolates. Most of these additional bp derived from the 0.7 kb insertion, which was described previously (Fig. 4.4) (Eberle et al., 2009). Also CpGV-I12 did not reveal an ORF corresponding to CpGV-M1 cp38. Therefore, in total 142 ORFs were predicted. Only seven ORFs differed from CpGV-M in their predicted aa sequences, limiting the number of putative genes responsible for overcoming CpGV resistance.

CpGV-S was the smallest of the three genomes with 123,193 bp in size. Compared to CpGV-I12, it reveald more differences on nt level compared to CpGV-M, but nucleotide identity was still more than 98% (blast2seq). 141 ORFs were found in total, beside CpGV-M1 cp38, the ORFs cp25 and cp26 were not predicted as their start codons were lying in a deletion area. This indicates that these ORFs are possibly not transcribed, as they have no known homologues in other granuloviruses and are lying in the repeat region between 20 and 21 kb (Luque et al., 2001). Several insertions and deletions were found throughout the CpGV-S genome (Table 5.4). Most of them occurred in regions located near the previously described 13 repeated sequences (Luque et al., 2001; Hilton

and Winstanley, 2007) and were due to the duplication or deletion of a repeated sequence motif. Beyond this mechanisms, the integration of transposons also plays a role in the plasticity of baculovirus genomes (Jehle et al., 1998). Apparently, this seems to be a factor in the variety of CpGV-S to the other two isolates. A stretch of additional 16 bp was found in the CpGV-S genome (Table 5.4, Fig. 5.3) which was identical to a 16 bp sequence described close to the integration site of the transposon-like element TCl4.7 into the genome of the CpGV mutant MCp5 (Jehle et al., 1995).

The aim of sequencing the genomes of CpGV-I12 and -S was to identify putative genes responsible for overcoming resistance to CpGV. The assumption that overcoming resistance is due to an effect on protein level is due to the fact that numerous CpGV isolates are able to overcome the 100,000 fold resistance observed in CM populations (Asser-Kaiser et al., 2007; Eberle et al., 2008). Beyond that, resistance to CpGV is not an unspecific mechanism such as immune response, as it is apparently specific to A type genomes but not to other genome types. A mechanism based on transcriptional level only is very unlikely, as it would be hardly possible to reach this high level of signal amplification. The fact that CpGV-I12 and -M were very similar made it easier to limit the number of possible genes involved in overcoming CpGV resistance. Only one difference was found to be shared by the two resistance overcoming isolates CpGV-I12 and -S: a 24 nt insertion, which was present in CpGV-M1 and -M, but not in CpGV-I12 and -S. This stretch consists of a motif of 12 bp, that was three times present in CpGV-M1 and -M, but only one time in CpGV-I12 and -S. Sequencing of the *pe38* region in further isolates of different genome types revealed that only type A CpGV genomes encode a PE38 protein with an insertion of 8 aa, like CpGV-M, -AZ01 or -G03. Type B genomes (CpGV-E2), type C (CpGV-I07), type D (CpGV-I12, -I01) or type E (CpGV-S) genomes did not carry this insertion (Fig. 5.5, Fig. 5.6).

Regarding the site of this indel mutation, it is striking that the genome region between 18-22 kb revealed a multiplicity of insertions, deletions and duplication events when comparing the differences of the three genomes CpGV-M, -I12 and -S (Fig. 5.7). The predominant part of the CpGV-S indel mutations is located in the area between 18-35 kb. Three out of five indel mutations of CpGV-I12 are also located in this area. A major repeat region is described for the CpGV-M1 region of 20-21 kb (Luque et al., 2001),

consisting of AT-rich sequences. An imperfect palindrome of 74-76 bp shows 13 copies dispersed over the CpGV genome (Luque et al., 2001; Hilton and Winstanley, 2007; Van Oers and Vlak, 2007) with possibly similar function as the *hr* regions found in further baculoviruses (Van Oers and Vlak, 2007; Hilton and Winstanley, 2007). *Hrs* are known as target sites for intragenomic recombination and rearrangements (Van Oers and Vlak, 2007; Hilton and Winstanley, 2007).

The 4.7 kb transposon TCl4.7 originating from *Cryptophlebia leucotreta* was also found to be integrated between the palindromes 3 and 4 (Jehle et al., 1995). When comparing the sites of CpGV-S indel mutations, it was found that they are located for the most part with the previously described 13 CpGV repeat sequences (Luque et al., 2001). Another region harbouring several indel mutations is the area between 50,000 and 60,000 kb (Fig. 5.7). This is the genomic area, where the insertion of four ORFs cp63-cp66 was found for several CpGV genotypes (Eberle et al., 2009). Cp63 showed homology to baculovirus *bro* genes. In CpGV-S, an insertion of 211 bp between the ORFs 63 and 64 showed homology to baculovirus *bro* genes. The *bro* genes are known to be target sites for recombination events and genome heterogeneity (López-Ferber et al., 2003; Van Oers and Vlak, 2007). Comparing the CpGV-S indel mutations to CpGV-M, the region around ORF63 (51,774-51,941) is also subject to several insertions and deletions (Fig. 5.7).

The fact that the differences between CpGV-S and -M are for the most part deletions or duplications of repeated motifs lying in or close to repeated regions indicates that a molecular mechanism like recombination could be involved rather than random mutations. When comparing two genotypes of *M. configurata* nucleopolyhedrovirus-A (MacoNPV-A), which differed in REN profiles and in their virulence against the host, one genotype was found to carry a *bro* gene that could not be found in the other isolate (Li et al., 2005). It was suggested that the *bro* genes could be involved in the virulence difference. Also the comparison of two isolates of *H. armigera* nucleocapsid nucleopolyhedrovirus (HearNPV) showing differences in virulence suggested that *hrs* or *bro* genes could be a factor for strain virulence, as rearrangement in these regions might be responsible for strain difference in replication (Zhang et al., 2005). As no start or stop codon was found for the *bro* like inserted sequence in CpGV-S and as this sequence is

not present in CpGV-I12, it seems not to play the major role in the virulence differences of CpGV-S, -I12 and -M against resistant codling moth populations. This insertion might be the trace of a former *bro* gene deleted in phylogeneticaly younger CpGV isolates. Nevertheless, the *bro* genes seem to be an important factor in generating baculovirus diversity, not only when comparing different virus genomes, but also between isolates of one virus. Taking into account the phylogenetic tree based on the partial *polh/gran* and *lef-8* sequences (Chapter 4), it can be seen that the 24 nt insertion only appears in the phylogenetic youngest type A isolates. This finding suggests that the 24 nt are not a deletion in some isolates, but a recent insertion into type A genomes. Thus, all genome types phylogenetically older than type A genomes do not carry this stretch, and also all resistance overcoming isolates do not carry it (Fig. 5.6).

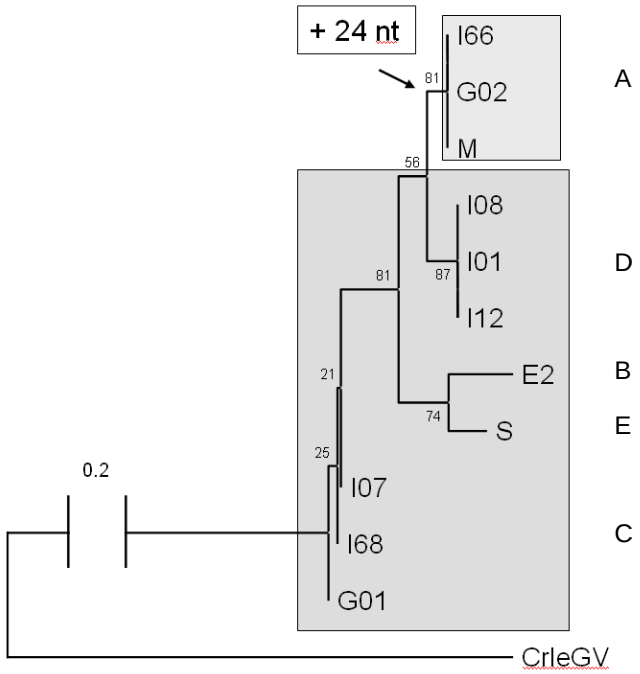


Fig. 5.6. Minimum evolution tree based on the *polh/gran* and *lef-8* sequences. Genome types A to E of the CpGV isolates are given on the right. The insertion of 24 nt into *pe38* was only found in the phylogenetically youngest A type genomes.

In AcMNPV, PE38 was found to be an important factor in DNA synthesis and budded virus production. The knock out of *pe38* resulted in a reduced infection of insects when applying the virus orally (Milks et al., 2002). The fact that *pe38* is not essential for infection could explain why CpGV-M still is infective to most codling moth populations. Tracing of virus replication by qPCR in midgut, fatbody cells and heamocytes of virus infected CM larvae revealed that CpGV was not replicating in any of these cell types (Asser-Kaiser, 2010). This finding showed that resistance to CpGV is located during an early event in the replication process, which corresponds to the putative role of PE38 in transactivation of early gene expression (Kovacs et al., 1991; Krappa and Knebel-Mörsdorf, 1991). Assuming that this mutation is the reason why different genome types are able to overcome resistance, it can be concluded that the observed resistance is not a resistance to CpGV in general, but a resistance to CpGV-M or to specifiy it, to CpGVs with A type genomes.

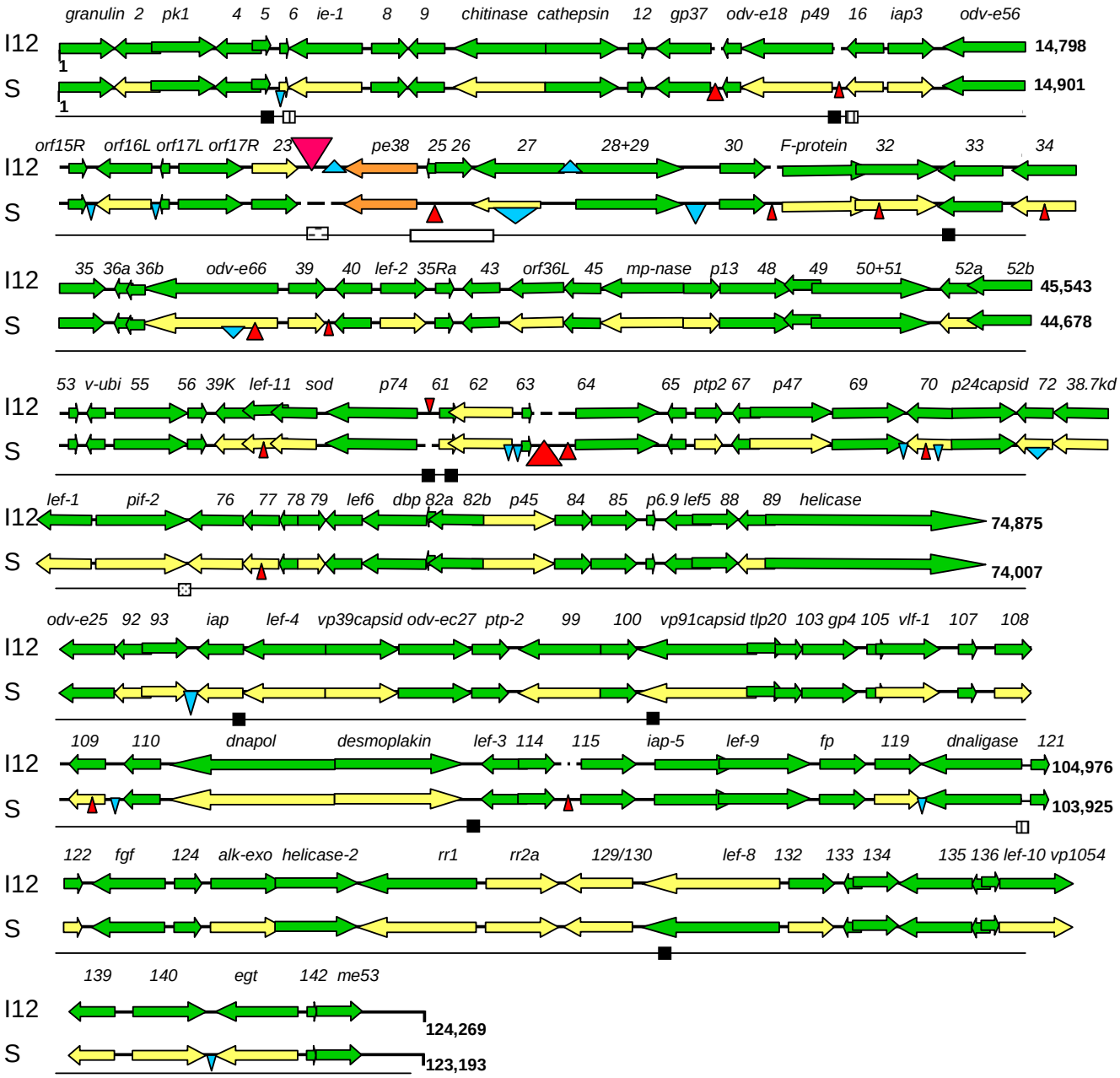


Fig. 5.7. Linear maps of the CpGV-I12 and -S genomes. Green, ORFs corresponding in their predicted protein product to CpGV-M. Yellow, ORF differences between CpGV-I12 or -S to -M in terms of aa sequence. Orange: common ORF difference of CpGV-I12 and -S compared to -M. Numbers in bold give the nt position in the particular genome. ▽ = insertions and △ = deletions found in the CpGV-I12 and -S genomes relative to CpGV-M (see Table 5.1, 5.3 and 5.5). Black boxes indicate the positions of the 13 CpGV *hr* regions. Filled boxes: *hr* region identical to CpGV-M, striped boxes: *hr* difference to CpGV-M in CpGV-S, dotted box: *hr* difference to CpGV-M in CpGV-I12. White box: major repeat region between 20-22 kb (Luque et al., 2001). Dashed box: putative hr repeat within the 0.7 kb insertion site in CpGV-I12 (compare Fig. 4.5).

6 Investigations on the role of *pe38* in overcoming codling moth resistance using CpGV bacmids

6.1 Introduction

Until 2008, more than 35 codling moth populations resistant to CpGV-M have been reported in Europe (Jehle, 2008). The fact that all registered CpGV products in Europe are based on the same isolate CpGV-M deriving from Mexico (Tanada, 1964) motivated a search for other CpGV isolates. Field isolates originating from Iran, Georgia and England have been characterised by RFLPs and phylogenetic analysis (Eberle et al., 2009). Several isolates, such as CpGV-I12, -E2, -S and others were shown to overcome CpGV resistance in laboratory bioassays (Eberle et al., 2008, Chapter 3). Beyond that, the isolate CpGV-I12 has been successfully tested in the field (Zingg, 2008; Berling et al., 2009). Beside the search for resistance overcoming isolates, it is inevitable to determine the factor for this improved virulence in order to evaluate future strategies of CpGV application as control agent.

Resistance to virus infection may occur at different steps during the infection process. In contrast to vertebrates, invertebrates lack an acquired or adaptive immune system (Narayan, 2004). Insects posses innate immunity characterised by non-specific reactions. Insect immunity is based on cellular and humoral processes, including structural barriers like cuticule, chemical properties in the gut and the peritrophic membrane as well as cellular reactions or the activation of immune proteins (Narayan, 2004). For baculoviruses, the peritrophic membrane is a barrier to infection of the midgut cells before starting the primary infection (Volkman, 1997). Beyond that, some resistance mechanisms are based on the midgut, for example by sloughing of infected midgut cells as observed for fourth instar larvae of *Trichoplusia ni* infected with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Engelhard et al., 1994). The physiological mechanism of resistance of *Heliothis zea* to AcMNPV infection was shown to be based on encapsulation of virus-infected cells (Washburn et al., 1996). For codling moth resistance, a mechanism based on the midgut or on the immune system could be excluded (Asser-Kaiser, 2010).

The genome comparison of three CpGV isolates, which differed in their virulence against resistant CM larvae, revealed that isolates overcoming CpGV resistance shared one ORF alteration when compared to the CpGV-M sequence (see Chapter 5). In contrast to CpGV-M, which was not able to infect resistant CM, CpGV isolates overcoming resistance missed eight predicted aa in their ORF cp24, which codes for PE38 (Luque et al., 2001). The amino acid motif TVDD was only one time present in PE38 of resistance overcoming isolates, but three times in type A genomes like CpGV-M. Phylogenetic analysis indicated that the phylogenetically youngest type A genomes of CpGV, which are not able to infect resistant CM populations, contain an 24 bp insertion in their *pe38* gene (Chapter 5). As this is the only ORF difference shared by resistance overcoming isolates, it is supposed that this mutation in *pe38* is the factor for different virulence against resistant CM larvae. Codling moth larvae resistant to CpGV developed a resistance mechanism to CpGVs with type A genomes, such as CpGV-M (see Chapter 5). It could be located to an early stage of infection by blocking of virus DNA replication in the insect (Asser-Kaiser and Jehle, unpublished). This finding supports the assumptation that *pe38* could be involved in overcoming resistance in CM.

Homologous genes for the immediate-early gene *pe38* are not described for all baculoviruses. Genome analyses indicated that *pe38* is predominantly present in group I NPVs (Milks et al., 2002). By genome sequencing, a gene homologue of *pe38* could be identified in CpGV (Luque et al., 2001; Herniou et al., 2001). *Pe38* was described to encode a 38-kDa nuclear protein PE38 (Krappa and Knebel-Mörsdorf, 1991). PE38 shows two potential DNA and protein binding motifs: one N-terminal RING (really interesting new gene) finger motif and one C-terminal leucine zipper (Krappa and Knebel-Mörsdorf, 1990) (Fig. 6.1 A, B). The basic leucine zipper is a motif involved in the dimerisation of DNA binding proteins. Every seventh amino acid position in the protein α-helix consists of leucine. Due to this configuration, all hydrophobic leucine residues of the protein domain are on the same side of the helix. The residues of two α-helices can then interact like a zipper due to hydrophobic bindings. A RING finger motif is a specialised type of zinc finger. The zinc finger is another frequent DNA binding motif: Zn^{2+} is coordinated by four cysteine or histidine residues. Zn^{2+} fixes the α-helix against a β-sheet structure; recognition of a DNA sequence is then mediated by the α-helix (Krauss, 1997).

A

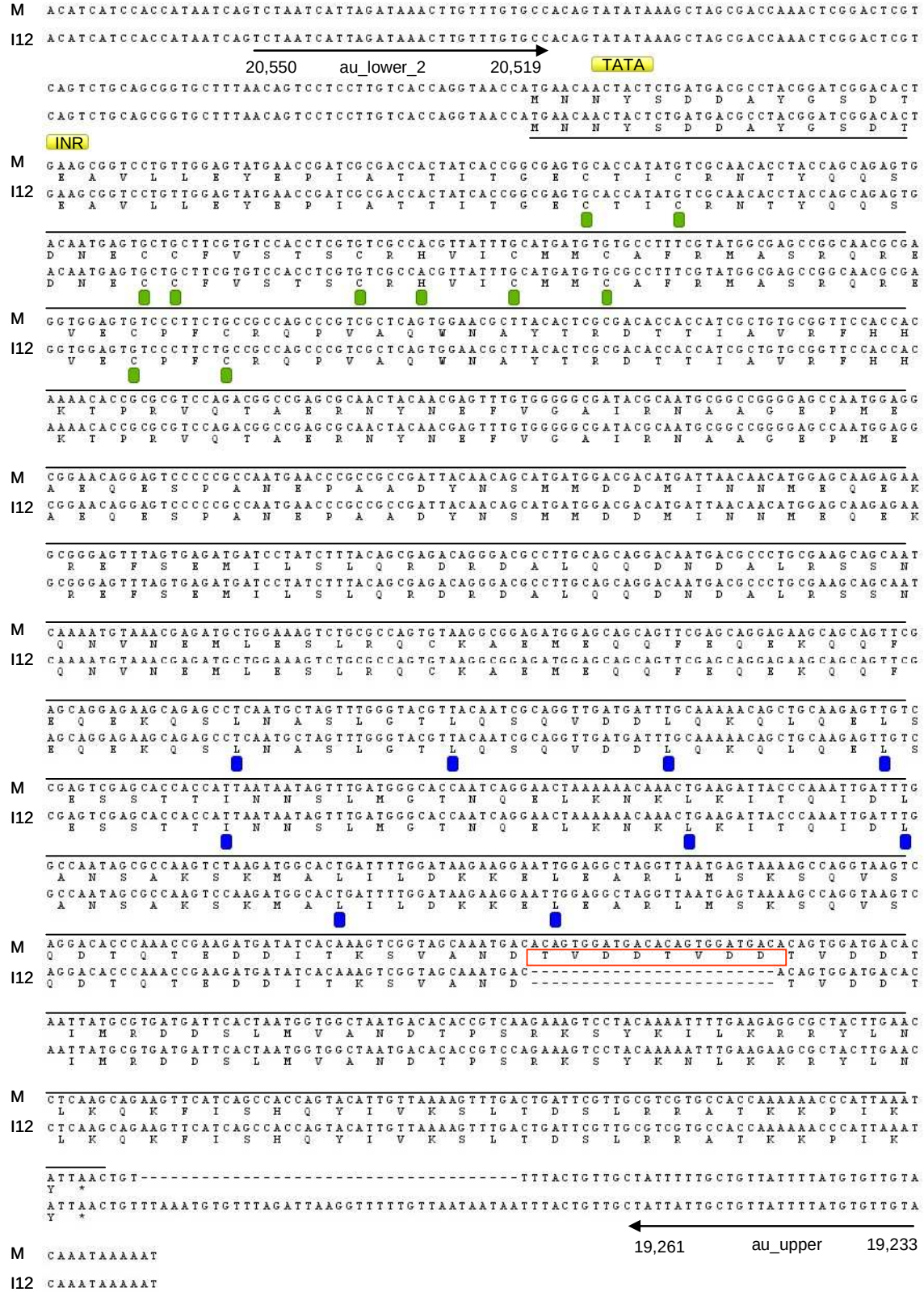


Fig. 6.1. (A) For description see next page.

B

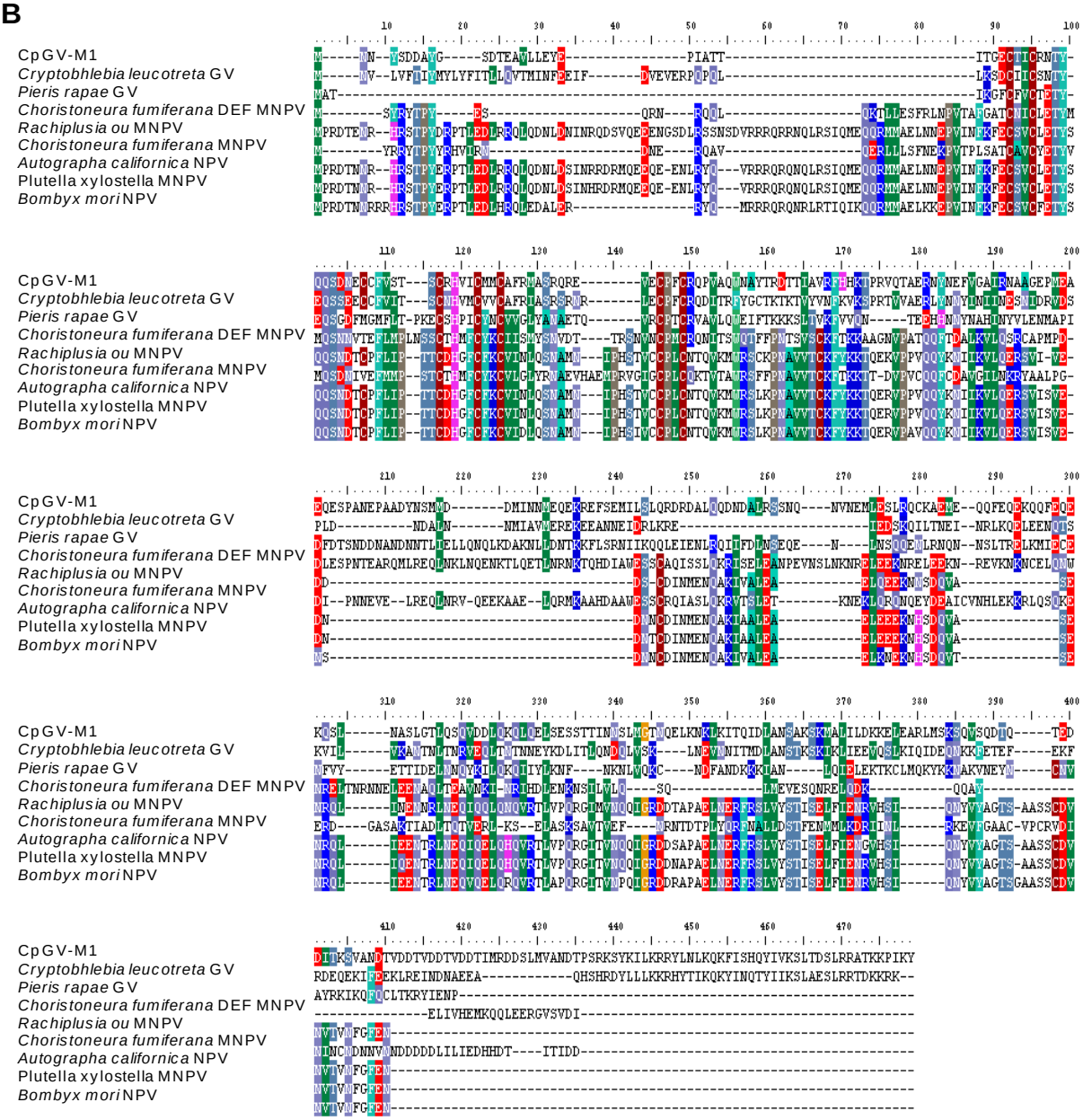


Fig. 6.1. (A) Alignment of CpGV-M and -I12 *pe38* and their corresponding translated aa sequences (underlined). Cysteine and histidine residues of the predicted RING finger motif are highlighted in green, leucine residues probably involved in the leucine zipper are highlighted in blue. The eight additional amino acids in CpGV-M are marked by a red box. Yellow boxes indicate early promotor elements: TATA = TATA box, INR = initiator region. Oligonucleotides for *pe38* amplification and their position in CpGV-I12 are given by arrows. (B) Alignment of CpGV-M1 and -I12 PE38 and its homologues found by BlastP search (http://www.ncbi.nlm.nih.gov): *Crypthophlebia leucotreta* GV (gi|33622331), *Pieris rapae* GV (gi|288804662), *Choristoneura fumiferana* DEF MNPV (gi|37651373), *Rachiplusia ou* MNPV (gi|23577864), *Choristoneura fumiferana* MNPV (gi|30387376), *Autographa californica* NPV (gi|9627896), *Plutella xylostella* MNPV (gi|114680204) and *Bombyx mori* NPV (gi|9630952). Color code: BioEdit 7.0.5.3, identity and similarity shading. The leucine zipper and RING finger motifs are conserved in the different baculoviruses.

Along with the products of other immediate early genes (*ie0*, *ie1*, *ie2*), PE38 transactivates early gene expression (Kovacs et al., 1991; Krappa and Knebel-Mörsdorf, 1991). Concerning the phase of virus infection, PE38 was found as a nuclear 38-kDa protein during the early phase with a peak between six and 12 hours past infection (Milks et al., 2002), but as a cytoplasmatic 20-kDa protein in the late phase of infection (Krappa et al., 1995).

So far, the function of PE38 is not completely elucidated. Deletion of *pe38* in AcMNPV resulted in a significantly reduced budded virus (BV) production and DNA replication compared to the wild-type (Milks et al., 2002). Hereby, the beginning of BV production was delayed in SF-9 cells and the production reduced by more than 90%, whereas initiation of DNA synthesis was not affected. Size and morphology of occlusion bodies (OBs) showed no difference between wild-type and *pe38* knock out mutant (Milks et al., 2002).

During oral infection in bioassay, deletion of *pe38* resulted in a seven fold reduction of AcMNPV virulence against *Heliothis virescens*. When bypassing the midgut by injecting BV directly into hemocoel, there was no difference in efficacy of wild-type and *pe38* knock out mutant (Milks et al., 2002). It was found that *pe38* was not an essential viral gene, but that knock out resulted in a dramatic reduction of virulence during the natural, oral way of infection. In contrast, CpGV BV injection into the hemocoel of susceptible and resistant CM larvae revealed that resistant CM larvae were not infected when bypassing the midgut. If *pe38* is involved in overcoming resistance, its function or mode of action might therefore be different as described for AcMNPV in *H. virescens*. CM resistance to CpGV was found to be 100,000 fold compared to susceptible larvae. Due to this high level of resistance, it is likely that resistance is based on protein interactions of viral and host proteins (maybe involved in a signal transduction cascade), and not on DNA transcription level.

The aim of this study was the investigation of the potential role of *pe38* in overcoming CM resistance. Susceptible codling moth larvae CpS are infected by CpGV-M and -I12, resistant CM larvae are resistant to CpGV-M, but susceptible to CpGV-I12 (Eberle et al., 2008). By knocking out *pe38* of a CpGV-M based bacmid, the effect of *pe38* during the infection process of CM can be evaluated. Amplification of *pe38* from the resistance overcoming isolate CpGV-I12 and swapping into the knock out bacmid was done to receive a CpGV-M based bacmid which differs only in its *pe38* sequence from the CpGV-M bacmid. Infection experi-

ments were attempted to evaluate the role of *pe38* in infecting susceptible and resistant codling moth larvae.

6.2 Methods

6.2.1 Transformation of *E.coli* cells

Electro-competent *E.coli* cells (DH5α, Epi300™) were stored in aliqouts of 50 µl at -75°C. The cells were thawed on ice for electroporation. One to three µl maximum of DNA were applied for transformation. Electroporation was performed in cooled cuvettes (2 mm) at 2.5 kV for 4.5 ms. Directly after electroporation, one ml SOC medium was added. Incubation was done at 37°C and 250 rpm for one to three hours, depending on the number of antibiotic resistance genes in the construct. The SOC medium was centrifuged for 30 sec at 14,000 rpm and the cell pellet resolved in 100 µl SOC medium. From this solution, 10, 20 and 70 µl were pipetted on selective agar plates and incubated for 24 h at 37°C.

6.2.2 BAC DNA Isolation of *E.coli* cells

The CpGV bacmid (CpBac) used for the construction of a *pe38* knock out and swapping bacmid was kindly provided by Dr. Wael Elmenofy (Elmenofy, 2008). CpBac was provided in Epi300™ cells already containing the expression plasmid pRed/ET necessary for homologous recombination. For isolation of bacmid DNA from *E. coli* clones, colonies were transferred from a selective agar plate into 100 ml selective LB medium and incubated over night at 37°C and 250 rpm. To harvest the cells, the overnight culture was centrifuged at 4500 × g and 4°C. Bacmid DNA isolation was performed using the Qiagen Plasmid Midi Kit. The DNA pellet was resolved in maximum 100 µl TE buffer (pH 8.0). DNA concentration was estimated by agarose gel electrophoresis and comparison to a DNA marker.

6.2.3 Deletion of *pe38* using Red/ET- Recombination

By homologous recombination using the Red/ET technology (Quick and Easy BAC Modification Kit, GeneBridges), it is possible to modify bacmids *in vivo*.

Red/ET recombination is based on the expresion plasmid pRed/ET, which is brought into *E. coli* cells containing the bacmid of interest. pRed/ET codes for the recombination system of the phage λ. This system contains the protein Redγ protecting linear constructs in the cell from degradation, the 5`-3` exonuclease Redα and the DNA binding protein Redβ necessary for recombination with the target molecule. The expression of these genes is under control of the promoter pBAD, which is induced by L-Arabinose. Unspecific recombination events are minimised by increasing the incubation temperature from 30°C to 37°C after adding the inductor. The expression plasmid containing the temperature sensitive *ori* pSC101 is not further replicated, the recombination activity is limited to a short time period.

The 50 bp homology arms necessary for recombination are added by PCR on a linear DNA fragment. To construct a *pe38* knock out mutant of CpBac, the oligonucleotides “left arm pe38 k.o.” and “right arm pe38 k.o.” (Table 2.8) were designed. The nucleotides one to 50 of the left arm primer corresponded to the CpGV-M1 (Luque et al., 2001) genome position 18,624-18,673 (left homology arm). The primer “right arm pe38 k.o.” corresponded in its nucleotides one to 50 to the genome position 19,671-19,618 (right homology arm). These two homologous arms served as target sequences for homologous recombination of CpBac and knock out cassette. The adjacent 25 and 24 nt, respectively, were specific for the amplification of the Tn5 neomycin knock out cassette, coding for resistance to kanamycin and used as selection marker for a successful knock out (Fig. 6.2).

Amplification was done by PCR from the template Tn5-neo provided by the supplier. The size of the Tn5-neo cassette was 1082 bp. By homologous recombination, 946 bp of *pe38* were replaced, including the 24 bp from nt 18,747 on, which differ in CpGV-M from further isolates.

TransforMaxEpi300™ cells containing CpBac and the expression plasmid pRedET were plated on LB plates (12.5 µg/ml chloramphenicol, 3 µg/ml tetracycline) and incubated over night at 30°C. For providing electro-competent cells, colonies were picked and incubated in selective LB medium (12.5 µg/ml chloramphenicol, 3 µg/ml tetracycline) overnight at 30°C and 270 rpm.

To 30 µl of the overnight culture 1.4 ml fresh selective LB media (12.5 µg/ml chloramphenicol, 3 µg/ml tetracycline) was added the next day and incubated for 2 h at 500 rpm and 30°C

until OD_{600} reached 0.3. To induce the expression of Red/ET protein, 50 µl of a 10% L-Arabinose solution were added. Tubes without the inductor were used as negative control. Control reactions as positive control were done according to the kit`s manual using the cells (BAC-control-neo) and reagents provided with the kit.

The *E. coli* cells were shaked for 1 h at 37°C to assure expression of the recombination proteins. The cells were harvested by centrifugation at 11,000 rpm for 30 sec and 2°C and resuspended in 1 ml cold ddH_2O. This washing step was repeated three times. The electrocompetent cells were transformed directly (2.5 kV, 4.5 ms) using 1 µl (0.1-0.2 µg) linear PCR fragment Tn5-neo. The transformed cells were resuspended in 1 ml LB medium and shaked at 37°C for 70 min. 100 µl cells were plated on LB plates containing 12.5 µg/ml chloramphenicol (marker gene for CpBac) and 15 µg/ml kanamycin (marker gene for the knock out cassette). Incubation was done over night at 37°C. Colonies were picked and recombination verified by PCR using the oligonucleotides ”k.o. check upper” and “-lower” (Table 2.8). The lower primer was located in the CpGV genome on nt position 18,124-18,143, the upper primer in the Tn5-neo cassette. Beyond this, recombination to CpBacΔpe38 was verified by DNA restriction analysis with *Bgl*II.

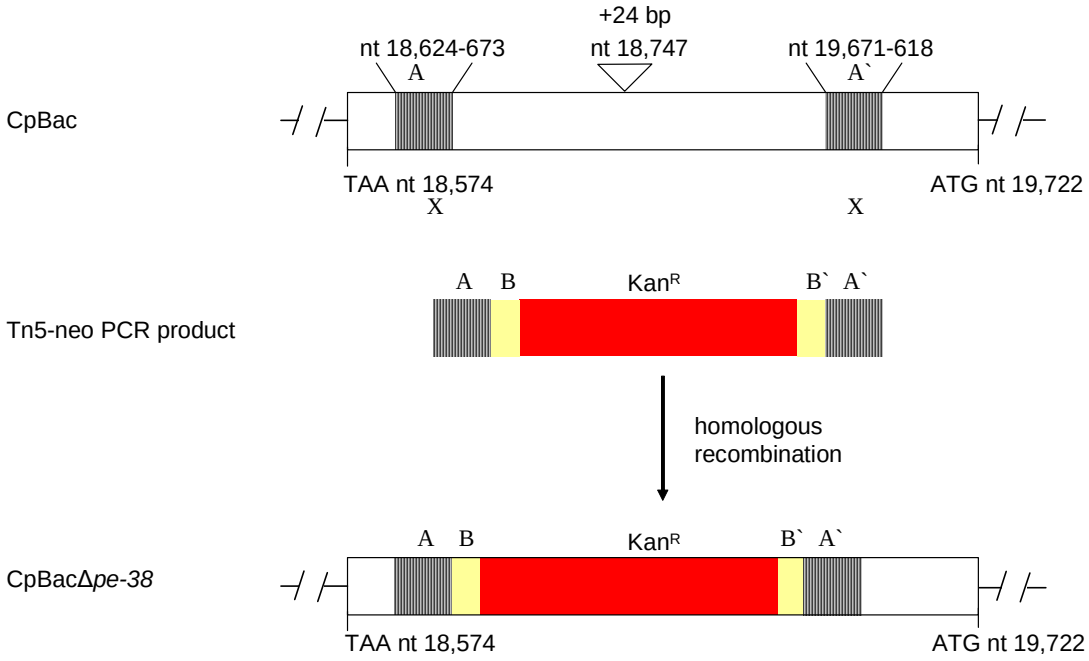


Fig. 6.2. Deletion of *pe38* (nt 18,574-19,722) of CpBac by homologous recombination using the Red/ET system. Homology arms were added to the Tn5-neo cassette (red) using PCR. The oligonucleotides used for amplification contained two regions (A and A`, striped) which were homologous to two regions inside the *pe38* gene. The regions B and B`(yellow) served as primer for amplification of Tn5-neo from its template. 946 bp of *pe38* were replaced by the Tn5-neo cassette.

6.2.4 Insertion of CpGV-I12 *pe38* into CpBacΔpe38 using Red/ET-Recombination

Amplification of *pe38* of CpGV-I12 was done by PCR using the oligonucleotides "au_upper" and "au_lower-2" (Table 2.9). The oligonucleotides were designed to amplify a 1317 bp product containing the *pe38* ORF and the region of 100 bp upstream the start codon, to include its early promoter region. A restriction site for *Eco*RI and *Bam*HI, respectively, were included into the 5`ends of the primers to allow directed cloning of the PCR product into a vector. Two PCR samples of 50 µl each were purified (GFX PCR DNA and Gel Band Purification Kit, Amersham, Freiburg) and eluted in two steps of 25 µl elution buffer. Restriction with *Eco*RI and *Bam*HI was done as double digestion (buffer: Tango) for three hours at 37°C. Subsequently another column purification (GFX PCR DNA and Gel Band Purification Kit, Amersham, Freiburg) was performed, the sample was eluted in two rounds using 10 µl elution buffer.

PUC19 was chosen as cloning vector as it contains an ampicillin resistance gene which was amplified together with the cloned *pe38* as resistance marker gene. Five µg pUC19 were digested using one µl *Eco*RI and *Bam*HI (3 h, 37°C, buffer Tango), purified over a column (GFX PCR DNA and Gel Band Purification Kit, Amersham, Freiburg) and eluted in two rounds using 10 µl elution buffer.

Concentration of vector and PCR product was estimated by agarose gel electrophoresis. Ligation of vector and product was done using 200 ng vector and 100 ng PCR product (one µl T4 DNA ligase, one h, 22°C). Electrocompetent DH5α cells were transformed with one µl ligation reaction and plated on selective LB plates (100 µg/ml ampicillin, 100 µg/ml X-Gal, 40 µg/ml IPTG). White colonies containing the *pe38* insert in the *lacZα* of their multiple cloning site (MCS) were picked and amplified in three ml overnight culture. pUC19-*pe38* DNA was isolated of *E. coli* cells with illustra Plasmid MiniPrep Kit (GE Healthcare).

Amplification of *pe38* together with the ampicillin resistance gene of pUC19 was done using the primer pair "BacAmpCp15-rep_F" and "BacAmpCp15-rep_R" (Table 2.9) designed by Dr. Wael Elmenofy (Elmenofy, 2008). The homology arms added to the primer pair were designed

to allow recombination of the amplified *pe38*-Amp^R product with the inactive GFP gene of CpBacΔpe38. “BacAmpCp15-rep_F” contained on its 3` end 24 nt specific for pUC19 (nt 1587- 1602 in the empty pUC19). The following 50 nucleotides corresponded to the sequence upstream the *hr5* region of the GFP cassette. “BacAmpCp15-rep_R” was in 22 nt at its 3` end specific for pUC19 (nt 502-523 in the empty pUC19). The following 50 nt corresponded to the region downstream of the GFP ORF of CpBac. Prior to PCR amplification, pUC19-*pe38* was digested with *Afl*III to avoid a background due to plasmid. pUC19 contains only one restriction site for *Afl*III in position 897. This restriction site was chosen because it did not interfere with the amplified region but was between the binding sites of the primers. To assure a correct amplification of the 3040 bp fragment, PCR was performed using iProof™ High-Fidelity DNA polymerase:

reagent	**volume**	**program**	**temperature [°C]**	**time**
5 X iProof HF buffer	10 µl	1. denaturation	98°C	30 sec
10 mM dNTP Mix	1 µl	2. denaturation	98°C	10 sec
10 mM primer for	1 µl	3. annealing	71°C	30 sec
10 mM primer rev	1 µl	4. extension	72°C	1 min, 30 sec
50 mM $MgCL_2$	2 µl	5. repetition of step 2-4, 29 X		
DNA pUC19-pe38	1 µl	6. final extension	72°C	5 min
iProof polymerase	0.5 µl			
ddH_2O	33.5 µl			

Purification of the PCR product Amp^R-*pe38* was done with GFX PCR DNA and Gel Band Purification Kit (Amersham, Freiburg). DNA was eluted in 10 µl elution buffer, concentration was estimated by agarose gel electrophoresis. To digest putative traces of plasmid not visible on the gel, DNA was digested with *Dpn*I and purified again. *Dpn*I only cuts methylated DNA strands and was therefore used for removing traces of DNA template.

To allow homologous recombination of CpBacΔpe38 with the PCR product Amp^R-*pe38*, the *E.coli* cells containing the knock-out bacmid had to be transformed with the expression plasmid pRed/ET. To transform pRed/ET into Epi300™ cells containing CpBacΔpe38, the cells were grown overnight in one ml LB medium (15 µg/ml chloramphenicol, 3 µg/ml kanamycin). To 30 µl of this culture 1.4 ml fresh LB medium (15 µg/ml chloramphenicol, 3 µg/ml kanamycin) were added the following day and shaked for two to three h at 37°C and 270 rpm. To prepare electrocompetent cells, the cells were pelleted at 11,000 rpm, 2°C and resuspended in one ml cooled ddH_2O. This washing step was repeated two times. One µl pRed/ET was added to the cell pellet, the cells were transformed by electroporation. One ml LB without antibiotics was added and the cells were shaked for 70 min at 30°C, as the plasmid pRed/ET is not stable at 37°C. 100 µl were plated on selective LB plates (15 µg/ml kanamycin, 3 µg/ml tetracyclin) and incubated over night at 30°C.

Homologous recombination was performed as described in 6.2.3. Screening for succesful recombination was performed using the primer pair “swap_upper” and “swap_lower” (Table 2.11). Primer “swap_upper” was located in the chloramphenicol resistance gene of the bacmid cassette. Primer “swap_lower” was located on the ampicillin resistance gene amplified together with *pe38* of pUC19. Clones with succesful recombination of PCR product and bacmid showed a PCR product of 1734 bp.

6.2.5 Biological activity of the different constructs

The biological activity of the different bacmid constructs was tested by DNA injection into susceptible (CpS) and resistant (CpRR1) codling moth larvae. L1 larvae were reared on virus free diet until larval stage L4 (10 days). Freshly molted L4 larvae were anaesthetised for two min with ether vapor. Using a sterile tip, larvae were sterilised with 0.4% hyamin solution. Bacmid DNA was mixed with lipofection; one to two µl of the bacmid-lipofection complex was injected through a proleg into the hemocoel of 20 larvae per treatment. When injecting budded virus (BV), two µl BV suspension were applied as well. Larvae were then transferred on fresh virus free diet and incubated at 26°C until death or pupation.

6.2.6 Transfection of Cp14 cells

The day before transfection, Cp14 cells were thawed into 6-well plates for sedimentation overnight. For every bacmid construct, one 6-well plate was prepared. For every transfection, one to two µg of bacmid DNA (5 to 10 µl) were diluted using serum free cell culture media (IZDO4). Six µl lipofection reagent (Invitrogen) were diluted using 100 µl serum free media (IZDO4). Both solutions were incubated for 45 min at room temperature. After mixing the solution was incubated for 10 min at room temperature. IZDO4 media was removed from Cp14 cells and the cells were washed with two ml serum-free media. 0.8 ml of serum free media was added to the DNA-lipofection mixture, mixed and pipetted onto the cells. The cells with transfection mix were incubated for 5 h at 26°C. The transfection mix was then replaced by two ml IZDO4 containing 10% SDS. Time points were taken on day three, six and 10 for estimation of the DNA concentration.

6.2.7 Estimation of DNA concentration by qPCR

DNA concentration in BV suspension from the supernatant of infected Cp14 cells was estimated using real time PCR (qPCR). DNA isolation was done using 500 µl supernatant (Ron`s Tissue Kit, Bioron) from infected cells. DNA amplification was performed using the oligonucleotides nested PRCP1U (5`-GGCCCGGCAAGAATGTAAGAATCA-3`) and nested PRCP1L (5`-GTAGGGCCACAGCACATCGTCAAA-3`) (Steineke, 2004). The expected product size was 422 bp. PCR products were detected by fluorescence (SYBRGreen) in every PCR cycle. SYBRGreen binds to double stranded DNA with a maximum of absorbance at

~497 nm and a maximum of emission of ~520 nm. An increase of SYBRGreen fluorescence is directly proportional to the increase of amplified PCR products. Identification and testing for the homogeneity of the PCR products was done by melting curve analysis.

For every sample, 35 µl master mix were mixed with five µl DNA. The master mix was prepared as follows:

reagent	volume
SYBRGreen	20 µl
nested PRCP1U	2 µl
nested PRCP1L	2 µl
ddH_2O	11 µl

As a reference for estimation of concentration, a dilution series of CpGV DNA of known concentrations was used. CpGV DNA isolated of occlusion bodies (OBs) were used as positive control, ddH_2O as negative control.

6.3 Results

6.3.1 Knocking out *pe38* from CpBac

The Tn5-neo knock out cassette for the deletion of *pe38* of CpBac was amplified using the oligonucleotides “left arm pe38 k.o.“ and “right arm pe38 k.o.“ from its template. The PCR product obtained corresponded to the expected size of about 1 kb (Fig. 6.3).

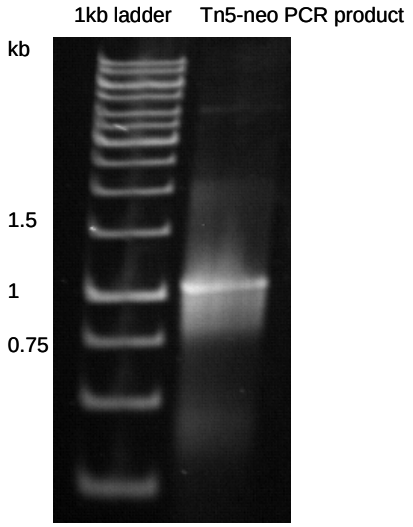


Fig. 6.3. Amplification of Tn5-neo knock out cassette. The expected product size was 1082 bp. One µl DNA were loaded onto a 0.8% (w/v) agarose gel and run at 90 V for 45 min.

After transformation of Epi300™ cells containing CpBac with two µl of Tn5-neo DNA, selection for recombinant bacmids was done by screening for kanamycin (knock-out cassette) and chloramphenicol (bacmid) resistance. Thirty-five colonies of the sample were picked. PCR screening for successful homologous recombination was done using the oligonucleotides “k.o.check_upper” and “-lower”. Five clones were taken together in one PCR reaction. Two of these samples showed the expected PCR product size of 750 bp. For each of the clones single PCR reactions were performed. The clones #1 and #27 showed the expected PCR product (data not shown) and were therefore picked for verification of recombination by REN analysis.

Verification of the insertion of the Tn5-neo cassette into clone #27 (CpBacΔpe38) was done by restriction analysis with *Bgl*II. The CpGV-M1 fragment A (Crook et al., 1997) of 67.7 kb was cut in CpBac due to three additional REN sites in the GFP gene into four fragments A1 to A4. By insertion of the knock-out cassette, another *Bgl*II site was brought into fragment A: fragment A1 (54.5 kb) was cut revealing a truncated fragment A1* of about 42.5 kb and an additional fragment A5 of about 12 kb (Fig. 6.4, 6.5). Fragment A5 could be identified as expected in the REN profile of the clones. As all other bands corresponded to the CpBac profile, a putative second, incorrect recombination at a different genome position could be excluded (Fig. 6.5).

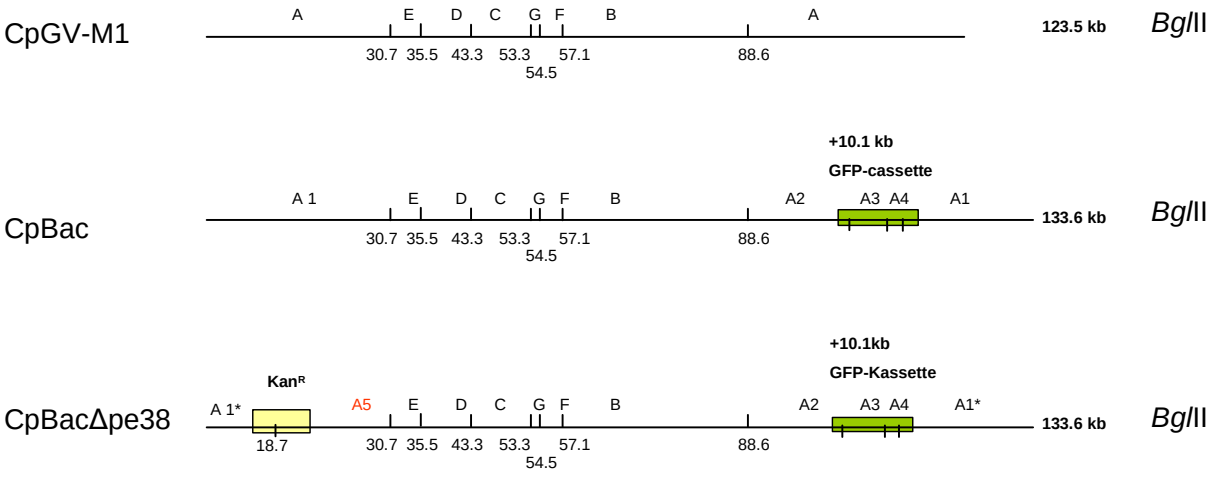


Fig. 6.4. Restriction mapping of CpGV-M1 (Crook et al., 1997), CpBac and CpBacΔpe38 using *Bgl*II. Insertion of the GFP cassette (green) into CpGV-M revealed three additional *Bgl*II sites into the genome of CpBac, revealing the fragments A1 to A4. Due to insertion of the Tn5-neo cassette (yellow), another *Bgl*II site was brought into the genome. After successful homologous recombination, an additional fragment A5 (red) of about 12 kb is to be expected.

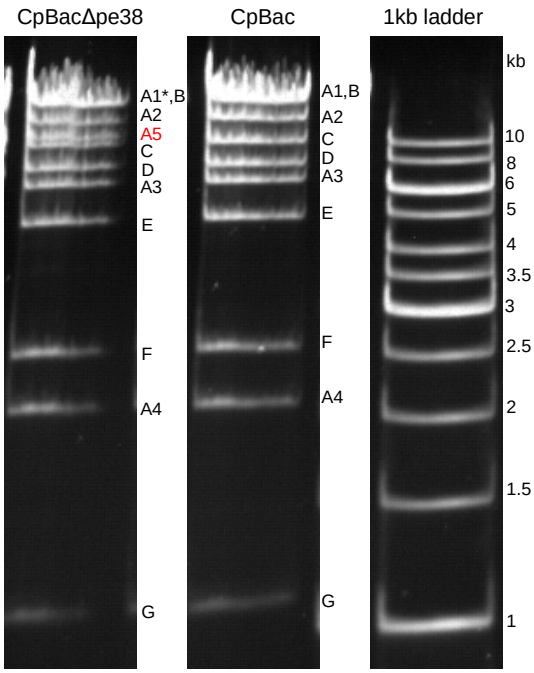


Fig. 6.5. *Bgl*II restriction analysis of CpBac and CpBacΔpe38 (clone #27). Above fragment C (10 kb), CpBacΔpe38 revealed the expected additional fragment A5 (red) deriving from the insertion of the knock out cassette. CpBac fragment A1 (54.5 kb) is about 12 kb larger than CpBacΔpe38 (A1*), due to the additional *Bgl*II REN site in A1*. Electrophoresis was done in 0.8 % agarose, 1 x TAE at 80 V for 1h.

6.3.2 Swapping of I12-*pe38* into CpBacΔpe38

Amplification of *pe38* of CpGV-I12 was performed using the oligonucleotides "außen_upper" and "außen_lower2" (Table 2.9). The obtained PCR product corresponded to the expected product size of 1.3 kb (Fig. 6.6).

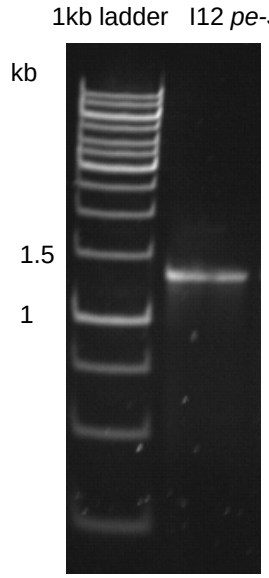


Fig. 6.6. PCR amplification of *pe38* and its promotor region of CpGV-I12. One µl PCR sample was electrophoresed in a 0.8% (w/v) agarose gel (1 x TAE, 90 V, 30 min).

The PCR fragment was cloned into pUC19 after restriction with *Eco*RI and *Bam*HI. Amplification of *pe38* together with the Amp^R gene of pUC19 was done using the primer pair "BacAmpCp15-rep_F" and "BacAmpCp15-rep_R2" (Elmenofy, 2008). The expected product size was 3040 bp (Fig. 6.7 A).

Homologous recombination took part in two regions of 50 bp each (C and C` in Fig. 6.7 B). The non-functional GFP gene, *hr5* region and *ie-1* promotor of CpBacΔpe38 were replaced. In total, 1848 bp of the GFP cassette were replaced by 2915 bp of the Amp^R-*pe38* cassette. The total size of the bacmid cassette was after recombination therefore 11,249 instead of 10,182 bp.

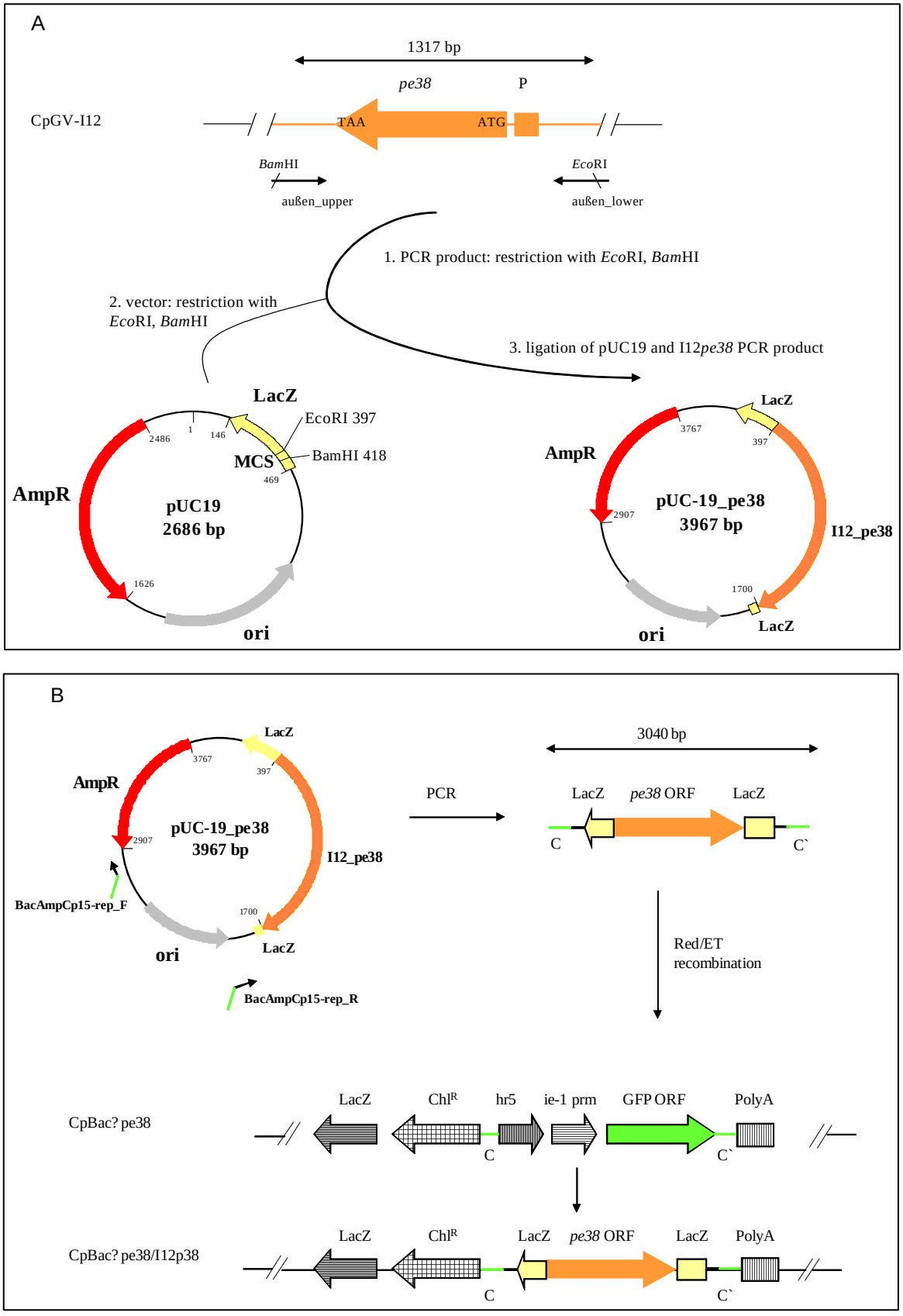


Fig. 6.7. For description see next page.

C

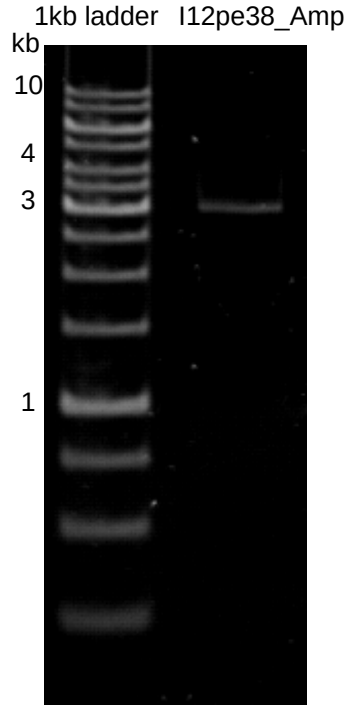


Fig. 6.7. Cloning and recombination of the Amp^R-*pe38* cassette. (A) Schematic drawing of the PCR amplification of *pe38* of CpGV-I12. Directed cloning into the pUC19 MCS was performed by including an *Eco*RI- and *Bam*HI REN site into the PCR primer. P = early promotor region of *pe38*. (B) Amplification of the Amp^R-*pe38* cassette of pUC19-*pe38*. To the oligonucleotides "BacAmpCP15-rep_F" and" _R", the 50 bp homology arms C and C` were added (green). They corresponded to two regions between Chl^R and PolyA signal of the GFP cassette. (C) PCR product Amp^R-*pe38* of pUC19-*pe38*. Product size corresponded to the expected product size of 3040 bp (1 µl DNA, 0.8 % Agarose (w/v), 1 x TAE).

Screening for successful homologous recombination was performed using the oligonucleotides "swap_screen_upper" and "swap_screen_lower" (Table 2.11). Clones with insertion of the recombination cassette into the GFP locus showed a PCR product of 1.7 kb, clones without recombination did not show any PCR reaction. Primer "swap_screen_lower" was located in the Amp^R gene of the *pe38* cassette. A putative bacmid background would also not have produced a PCR signal, as the primer "swap_screen_upper" was located in the Chl^R gene of the bacmids GFP cassette. Only for successful recombination a PCR product could be generated.

Following DNA isolation of positive clones, a verification of the insertion was done by PCR and REN analysis. Using the oligonucleotides "Pacsite_upper" and "Pacsite_lower" (Table 2.11) located in the CpGV-M genome around the insertion site of the GFP cassette, the whole cassette was amplified. The bacmid cassettes of CpBac and CpBacΔpe38 had a size of 10.1 kb, the cassette of CpBacΔpe38/I12pe38 a size of 11.2 kb. PCR products of the clones revealed the expected product sizes (Fig. 6.8).

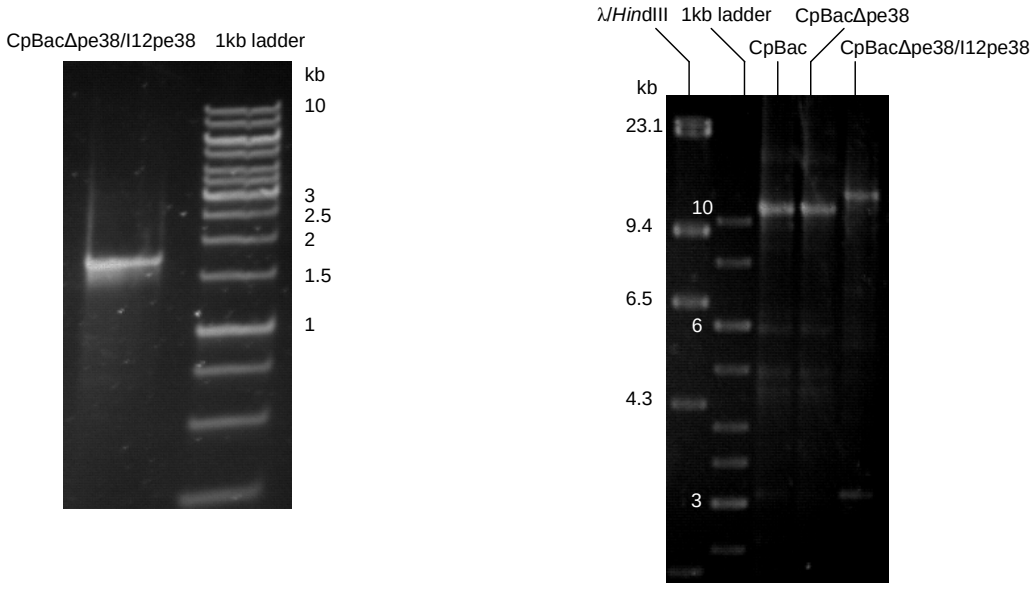


Fig. 6.8. PCR screening (0.8% agarose, 1 x TAE, 80V, 1h) for successful homologous recombination and creation of the construct CpBacΔpe38/I12pe38. (A) Recombinant clones showed a PCR product of 1.7 kb. (B) PCR for the GFP cassettes of different constructs. For CpBac and CpBacΔpe38, a band size of about 10.1 kb was expected, for CpBacΔpe38/I12pe38 a size of 11.2 kb.

Verification of recombination by REN analysis was performed using *Bam*HI. *Bam*HI was chosen as only one difference between the constructs CpBacΔpe38 and CpBacΔpe38/I12pe38 was expected, making it easier to identify the constructs. Beyond, the expected fragments were not running together with other fragments, which would make an allocation more difficult.

There was no *Bam*HI restriction site in the area of the *pe38* gene, meaning that the constructs CpBac and CpBacΔpe38 did not differ in their *Bam*HI profiles (Fig. 6.9). The GFP cassette of CpBac and CpBacΔpe38 contained three additional *Bam*HI sites compared to CpGV-M1 (Crook et al., 1997; Luque et al., 2001). CpGV-M1 fragment A was cut in these constructs in four fragments A1 to A4. After recombination with the Amp^{R}-*pe38* cassette, two of these *Bam*HI sites were replaced (Fig. 6.11). As *pe38* was cloned using *Bam*HI into pUC19, one additional *Bam*HI site was located in the recombinant GFP cassette. This difference was visible in fragment A3: a fragment of 2 kb present in CpBac and CpBacΔpe38 (nt region 102.3-104.3 kb) disappeared. In contrast, a fragment A3 of 4.8 kb (nt region 102.3-107.1 kb) appeared (Fig. 6.10).

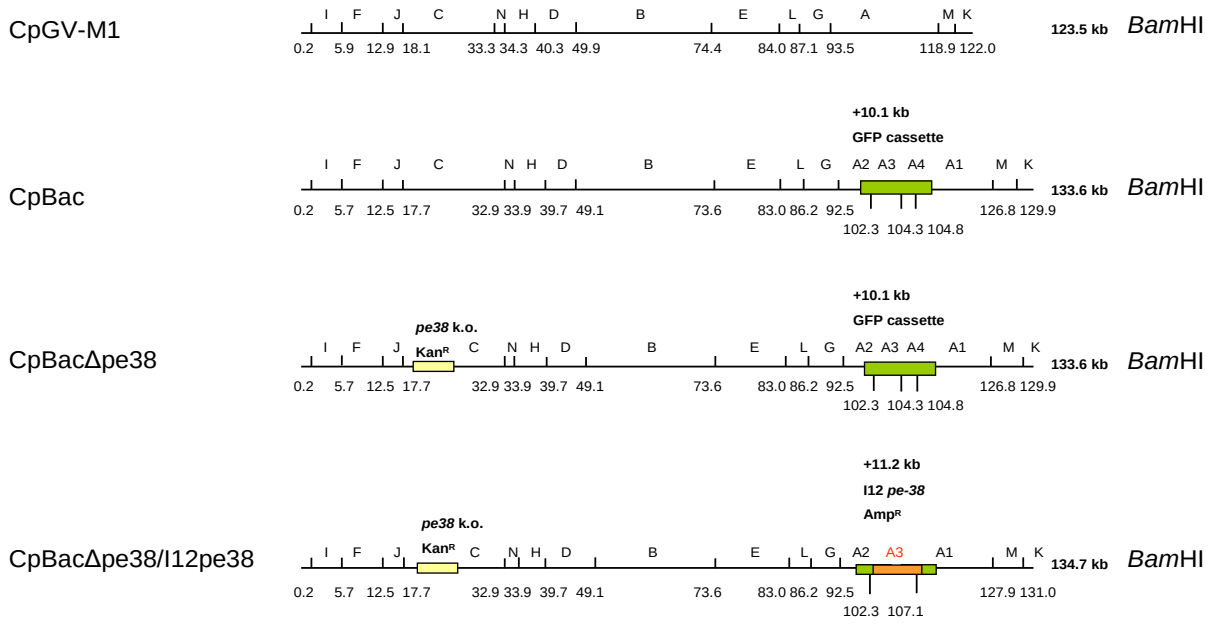


Fig. 6.9. *Bam*HI restriction profile differences between CpBac, CpBacΔpe38 and CpBacΔpe38/I12pe38. (A) Restriction maps of CpGV-M1 (Crook et al., 1997) and the three bacmid variants. CpBac and CpBacΔpe38 do not differ from each other in their *Bam*HI restriction sites, but from CpGV-M1 due to three additional REN sites. In CpBacΔpe38/I12pe38, fragment A4 was missing. Fragment A3 was with 4.8 kb larger compared to CpBac-A3 and CpBacΔpe38-A3 (2 kb).

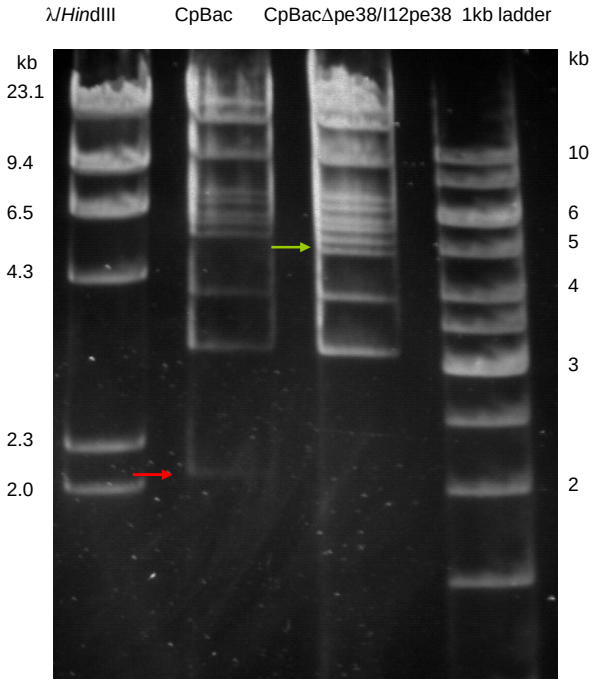


Fig. 6.10. *Bam*HI restriction profile of CpBac and CpBacΔpe38/I12pe38. As expected, the fragment of 2 kb is not present in CpBacΔpe38/I12pe38 like in CpBac (red arrow), in exchange a band of about 4.8 kb (green arrow) appeared.

6.3.3 Biological activity of the different constructs

6.3.3.1 Hemocoelar injection of DNA into CpS and CpRR1

As a first attempt for testing a succesful knock out of *pe38* of CpBac, 15 µl DNA of the constructs CpBac and CpBacΔpe38 (~1 µg) were mixed with 7.5 µl lipofection and 7.5 µl cell culture media SF900 and 1 µl injected into each of 25 susceptible (CpS) larvae (30 ng/larva). As negative control, lipofection was mixed with serum free cell culture media and with the QF elution buffer used for DNA preparation provided with the Qiagen Plasmid Midi Kit. Larvae were scored daily for infection. Larvae which died the first day after injection because of handling (black larvae showing symptoms of bacterial infection) were excluded from the evaluation.

Table 6.1. Testing the biological activity of CpBac and CpBacΔpe38 by DNA injection into L4 larvae of CPS. V_{inj} = volume injected per larva. n°CpS= number of tre ated CpS larvae.

treatment	n° CpS (L4)	DNA [ng/µl]	lipofectin [µl]	V_{inj}	infected after 14 d
CpBac DNA	25	~30	7.5	1 µl	0
CpBacΔpe38 DNA	25	~30	7.5	1 µl	0
SF900 + lipofectin + QF buffer (negative control)	25	~30	7.5	1 µl	0

After 10 days of incubation, evaluation became difficult because of strong growth of fungi. No viral infection or mortality of the virus treated larvae was observed during the experiment. This was expected for the negative control and the knock-out bacmid CpBacΔpe38. However, for the CpBac injection, a virus infection of CpS should have been induced (Elmenofy, 2008). A mistake in handling the syringe could be excluded, as the experiment was repeated and

carried out by two persons with the same result. A possible reason could be that the amount of DNA used for injection was to low to initiate infection of L4 larvae.

After the succesful construction of the swapped bacmid CpBacΔpe38/I12pe38 (see 6.4), the three constructs CpBac, CpBacΔpe38 and CpBacΔpe38/I12pe38 were tested for their infectivity by DNA injection into CpRR1 larvae. In these injection experiments, higher DNA concentrations as in the previous experiment should be used to increase the feasibility of a virus infection. As expected for CpRR1, no virus mortality was found in the negative control, in the CpBac DNA treatment and for the injection of CpBacΔpe38 DNA (Table 6.2). However, no mortality was observed for CpBacΔpe38/I12pe38 DNA which would be expected if *pe38* was active and the factor for overcoming resistance.

For evaluating and improving the conditions of infecting CM larvae by DNA injection, the importance of DNA and lipofectin concentration was concurrently tested, to find a possible effect why DNA injection had so far not led to virus infection. DNA of CpGV-I12 was chosen as a positive control for injection, as it should infect both susceptible and resistant CM larvae. CpGV-I12 DNA was mixed in different concentrations and relations (Table 6.2) with lipofectin and SF900 media. CpGV-I12 DNA was concentrated using a speed vac centrifuge (Maxi dry lyo, Thermo Scientific) and three variants of forming the DNA-lipofection complex were tested. The treated larvae were scored daily until day 14. As no virus infection was found in any of the treatments, though CpGV-I12 should have infected the larvae, it was intended to increase the DNA concentration again. CpS larvae were treated, as they should be infected by every construct except the knock out bacmid. The DNA injection experiments using different amounts of DNA and lipofectin are summarised in Table 6.2.

Table 6.2. Testing the biological activity of the three bacmid constructs CpBac, CpBacΔpe38 and CpBacΔpe38/I12pe38 by DNA injection into susceptible (CpS) and resistant (CpRR1) larvae using different DNA dilutions and volumes. Different concentrations of DNA and lipofectin were applied to improve the conditions of DNA injection. CpGV-I12 DNA was included as positive control. V_{inj}= volume injected into CM L4 larvae.

treatment	n° CpRR1 (L4)	n° CpS (L4)	DNA [ng/µl]	SF900 [µl]	lipofectin [µl]	V_{inj} [µl]	DNA/larva [ng]	infected after 14 d
CpBac DNA	25	-	~130	7.5	7.5	1	67	0
CpBacΔpe38 DNA	25	-	~130	7.5	7.5	1	67	0
CpBacΔpe38/ I12pe38 DNA	25	-	~130	7.5	7.5	1	67	0
SF900 + lipofectin + QF buffer (negative control)	25	-	-	7.5	7.5	1	67	0
CpGV-I12 DNA	10	-	274	200	2	1	18.9	0
	10	-	274	200	2	2	18.9	0
CpGV-I12 DNA	10	-	274	200	10	1	18.2	0
	10	-	274	200	10	2	18.2	0
CpGV-I12 DNA	10	-	274	5	5	2	137	0
CpBac DNA	-	10	~500	-	6.5	2	> 750	2
CpBacΔpe38 DNA	-	10	~300	-	6.5	2	> 450	2
CpBacΔpe38/ I12pe38 DNA	-	10	~500	-	6.5	2	> 750	1
CpGV-I12 DNA	-	10	274	-	6.5		> 400	0

When injecting high DNA concentrations of CpBac, CpBacΔpe38 and CpBacΔpe38/I12pe38 into CpS, in total five virus dead larvae were observed, which had turned black (Table 6.2).

The virus dead larvae were collected in Eppendorf reaction tubes and ten µl of the OB suspension obtained from each treatment were directly taken from the larvae. The OB suspensions were then diluted 1:10 with ddH_2O to increase the volume. By feeding the OB suspension to 40 CpS larvae each, the OBs were propagated to obtain enough material for DNA isolation and REN analysis. The fifth day after infection, all treated larvae had turned black. Larvae were collected and DNA prepared from the OB suspension to estimate the constructs present in the larvae.

The different DNA isolations were tested by PCR to identify if the CpBac, CpBacΔpe38 DNA and CpBacΔpe38/I12pe38 DNA had replicated in the larvae (Table 6.3). The CpBacΔpe38 DNA and CpBacΔpe38/I12pe38 DNA should be detected by the oligonucleotides designed for checking the knock out of *pe38*, k.o._check_upper and k.o._check_lower (product size 748 bp), because they do not contain a wild-type *pe38* anymore. To distinguish between CpBacΔpe38 DNA and CpBacΔpe38/I12pe38 DNA, the oligonucleotides specific to swapped constructs were used (swap_screen_upper and swap_screen_lower, expected product size 1734 bp). As positive control for the PCR, DNA of the different constructs isolated from transfected *E. coli* was used. For the detection of CpBac DNA, a PCR for *pe38* was performed using the oligonucleotids pe38-au_upper and pe38-mi_lower (product size 621 bp). Only CpBac DNA or CpGV DNA were supposed to generate a PCR band, because the *pe38* gene was knocked out in the other two constructs (Table 6.3).

Table 6.3. Testing of the DNA isolated from virus dead CM larvae for the replication of the three bacmid constructs by PCR. Oligonucleotides specific for the different features were used, bacmid DNA propagated in *E. coli* was included as control. + = expected PCR fragment, - = no PCR fragment observed.

construct	primer	target	expected fragment	observed
CpBacΔpe38/I12pe38	k.o._check_upper k.o._check_lower	knock-out cassette	748 bp	-
CpBacΔpe38/I12pe38 (*E.coli*) (positive control)	k.o._check_upper k.o._check_lower	knock-out cassette	748 bp	+
CpGV-I12 (negative control)	k.o._check_upper k.o._check_lower	knock-out cassette	-	-
CpBacΔpe38	swap_screen-upper swap_screen-lower	I12-*pe38* in CpBacΔpe38/I12pe38	-	-
CpBacΔpe38/I12pe38	swap_screen-upper swap_screen-lower	I12-*pe38* in CpBacΔpe38/I12pe38	1734 bp	-
CpBacΔpe38/I12pe38 (*E.coli*) (positive control)	swap_screen-upper swap_screen-lower	12-*pe38* in CpBacΔpe38/I12pe38	1734 bp	+
CpGV-I12 (negative control)	swap_screen-upper swap_screen-lower	I12-*pe38* in CpBacΔpe38/I12pe38	-	-
CpBac	pe38-au_upper pe38-Mi_lower	*pe38*	621 bp	-
CpBacΔpe38/I12pe38 (negative control)	pe38-au_upper pe38-Mi_lower	*pe38*	-	-
CpGV-I12 (positive control)	pe38-au_upper pe38-Mi_lower	*pe38*	621 bp	+

In every PCR reaction, only the positive controls (DNA isolated of *E. coli* or CpGV, respectively) revealed PCR products (Table 6.3). With the DNA deriving from infected CM larvae, no fragments were obtained with any of the specific oligonucleotides. Therefore, it was not possible to decide, if the correct bacmids were present in the DNA isolations (and therewith in the treated larvae), of if the larvae were contaminated, e.g. with CpGV DNA.

The DNA of CpBac and CpBacΔpe38/I12pe38 isolated from larvae was therefore subjected to *Bam*HI restriction analysis, to identify if the observed virus infection was due to CpGV contamination of the larvae used or really to an infection with a bacmid construct. DNA concentration of CpBacΔpe38 was too low for REN analysis. Parallely, DNA was isolated from the CpBac occlusion bodies which were characterised and tested in bioassay previously (Elmenofy, 2008). The CpBac DNA was digested using *Bam*HI and *Pac*I to check if the previous bioassay results were really due to an infection with CpBac and not due to a CpGV contamination of the larvae.

Restriction analysis revealed that the construct CpBac (DNA isolated from OBs) showed the expected restriction profiles in the *Bam*HI and *Pac*I digests, verifying that the previously performed injection and bioassay experiments were successfully performed with the construct CpBac (Fig. 6.11). The construct CpBac used as basis for the knock out and swapping constructs and used in the injection experiments here also showed the correct restriction profile: the additional *Bam*HI-fragment was present in the profile. DNA isolated from larvae infected with CpBacΔpe38 DNA and CpBac-I12pe38 DNA did not show restriction profiles corresponding to the bacmid constructs, but corresponding to CpGV-M. Therefore, injection of DNA into CM larvae only led to an amplification of CpBac DNA when using very high DNA concentrations. DNA isolated of larvae infected with CpBac-I12pe38 showed a REN profile similar to CpGV, but not to bacmid constructs.

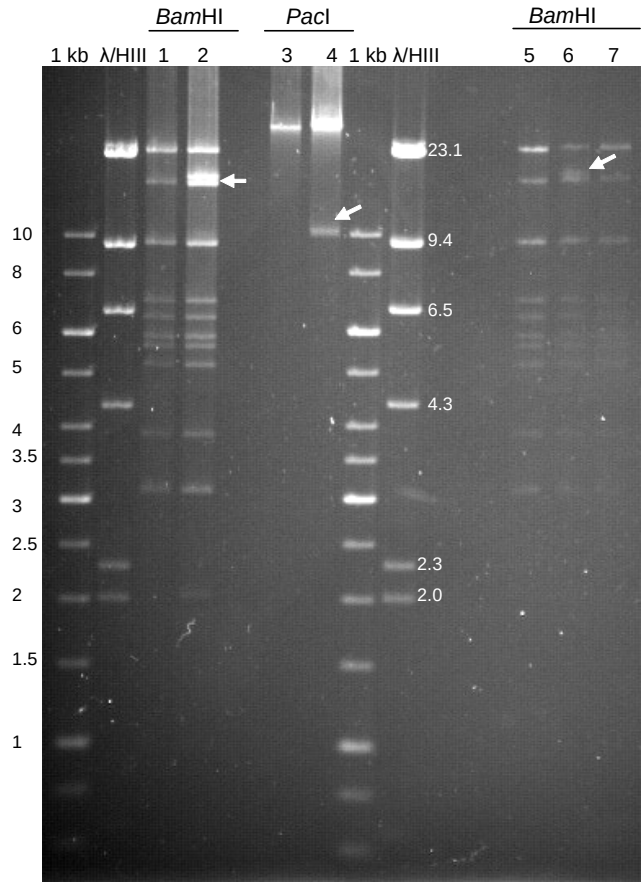


Fig. 6.11. Agarose gel (0.8 % (w/v), 25 V, 16 h) to identify the different constructs isolated from infected larvae. 1 = CpGV-M, 2 = CpBac from OBs, 3 = CpGV-M, 4 = CpBac from OBs, 5 = CpGV-M, 6 = CpBac from infected larvae, 7 = CpBacΔpe38/I12pe38 from infected larvae. One additional band in the CpBac *Bam*HI profile is due to the bacmid cassette. Restriction with *Pac*I reveals the bacmid cassette as a band of 10.1 kb.

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6.3.3.2 Hemocoelar injection of BV into CpS and CpRR1

Concurrently, Cp14 cells were transfected using the constructs CpBac DNA and CpBacΔpe38/I12pe38 DNA to prepare BV for intrahemocoelar injection, in order to test an alternative to DNA injection. As a positive control, cells were also transfected with CpGV-I12 DNA to obtain BV, as CpGV-I12 should infect both susceptible and resistant CM larvae. For every construct, a six well-plate was transfected, containing one virus-free, untreated control which was prepared the same way as the transfected wells. DNA was isolated from the supernatant (which should contain BV) and the concentration was estimated for the different time points taken (see 6.2.7). The time point with the highest DNA concentration was chosen for BV preparation for injection.

For the first time point (0-3 d) there was only little difference in viral DNA concentration between the treatments and their controls (Fig. 6.12). After six days, DNA concentration in the supernatant of Cp14 cells infected with CpBac DNA was about 10 times higher as for CpGV-I12 and 2.6 times higher than for CpBacΔpe38/I12pe38 DNA. The determined CpGV-I12 DNA concentration was lower as in its negative (virus free) control. After 10 days, the DNA concentration in the CpGV-I12 transfected supernatant increased 100 fold. Concurrently, the value for the CpGV-I12 uninfected control also increased. CpBac DNA concentration was 4-5 times higher compared to the other two treatments. Its negative control signal increased slightly. The DNA concentration estimated for CpBacΔpe38/I12pe38 was similar to the value obtained for CpGV-I12. Also for this construct, the signal of the untreated control still increased. After 14 days, DNA concentration of CpBac in its negative control decreased. For the CpGV-I12 and CpBacΔpe38/I12pe38 treatment as well as their controls, no value was determined, because the number of estimated DNA copies was under the detection limit of qPCR (Fig. 6.12).

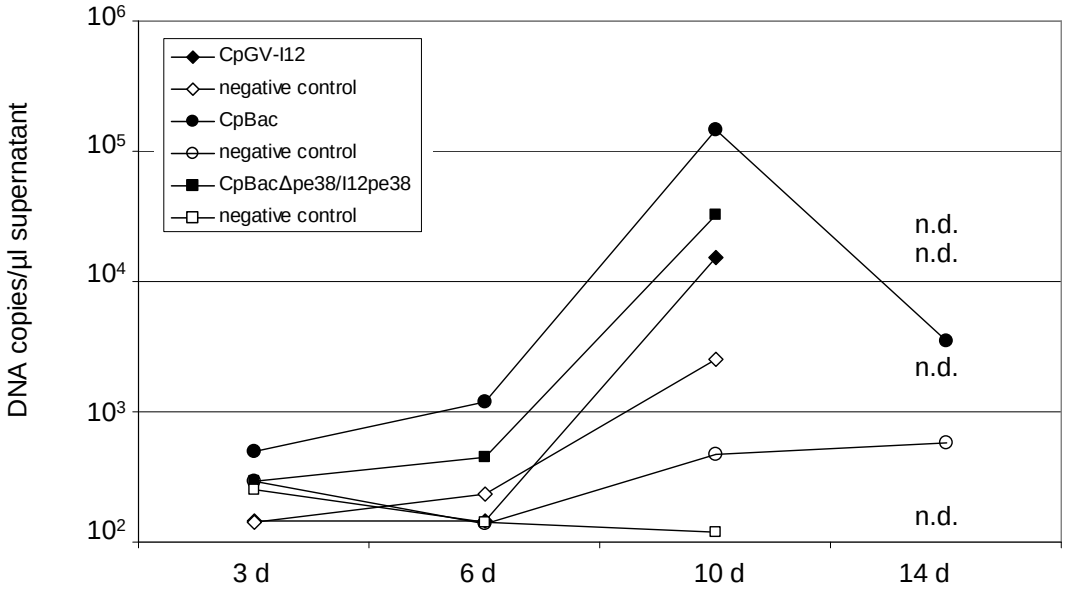

Fig. 6.12. DNA copies estimated for four time points past transfection of Cp14 cells with CpGV-I12 DNA, CpBac DNA and CpBacΔpe38/I12pe38 DNA. n.a.= not available, value was below the detection limit of qPCR (125 copies).

Time point #3 (7-10 d) was chosen for BV preparation. The supernatant of transfected cells containing BV was sterile filtered and kept at -80°C until injection, as BV are not stable when frozen at -20°C (Doreen Winstanley, personal communication). To infect CpS and CpRR1 larvae with an approxmimately similar BV concentration of each construct, the CpBac supernatant was diluted 1:10 and only one µl was used for CpBacΔpe38/I12pe38. Twenty larvae per treatment were injected with BV suspension (Table 6.4).

Table 6.4. Testing the biological activity of CpGV-I12, CpBac and CpBacΔpe38/I12pe38 against susceptible (CpS) and resistant (CpRR1) larvae by budded virus injection. Cell culture media was injected as negative control. No virus infected larvae were observed in any of the treatments 11 days past infection (dpi).

treatment	n° CpS/-RR1 (L4)	V_{inj}	n° DNA copies	virus infected 11 dpi
CpGV-I12 BV	25	2 µl	3.07×10^4	0
CpBac BV	25	2 µl	2.93×10^4	0
CpBacΔpe38/I12pe38 BV	25	1 µl	3.22×10^4	0
cell culture media	25	2 µl	0	0

In none of the treatments, either with CpRR1 or CpS, virus infection could be observed after supernatant injection. From day 10 on, larvae started to pupate and the growth of fungi complicated the evaluation.

6.3.3.3 Oral infection of CM larvae

As injection of DNA or BV did not result in virus infection, oral infection of the larvae using OBs produced in cell culture was attempted. Cp14 cells were transfected with CpBac DNA, CpBacΔpe38 DNA and CpBacΔpe38/I12pe38 DNA in order to feed the pellet of transfected cells to CpS larvae. Cells were transfected as described previously, the transfected cells were incubated for 10 days, harvested and centrifuged for two min at 13,000 rpm. The pellet was taken up in 100 µl H_2O and five µl inoculated onto small plugs of diet (Hilton et al., 2008). Twenty larvae were infected per construct. Larvae were kept single in Petri dishes instead of 50 well plates, in order to minimise the risk of fungi growth.

After four days, CpS larvae infected orally with CpBac transfected cell pellet started to show symptoms of virus infection; five days past infection, nine larvae infected with CpBac died because of virus infection. After 14 days, all larvae infected with CpBac were dead due to virus

infection, indicating that infectious CpBac virus was produced in the transfected Cp cells. None of the larvae infected with CpBacΔpe38 and CpBacΔpe38/I12pe38 transfected cells showed symptoms of virus infection and the treated larvae pupated, suggesting that both CpBacΔpe38 and CpBacΔpe38/I12pe38 did not render viable OBs after transfection of Cp14 cells.

Summarising, the injection experiments using DNA and BV failed. Infection of larvae was only obtained using transfected Cp14R cells and subsequent oral administration of cell pellets to larvae. However, only feeding of CpBac transfected cell pellets leaded to viral infection. CpBacΔpe38 and CpBacΔpe38/I12pe38 seemed not to be active in Cp14R cells, and most likely not active in larvae, too.

6.4 Discussion

Based on genome comparison of CpGV-M, -I12 and -S (Chapter 5), the early expressed *pe38* gene is suggested to be the putative factor which is responsible for the resistance overcoming effect of CpGV isolates different from CpGV-M. The mode of action of the *pe38* protein product is so far not determined. As described previously, PE38 contains two putative DNA and protein binding and dimerisation domains: a C-terminal leucine-zipper and a N-terminal zinc finger motif (Krappa and Knebel-Mörsdorf, 1991). A GATA motiv located 50 nt upstream of the PE38 transcriptional start site has been shown to be recognised by an insect GATA-binding protein detectable in uninfected and early-phase infected *S. frugiperda* cells (Krappa et al., 1992). If PE38 interacts directly with another viral protein or is part of a signal transduction cascade is not yet determined. The interaction of PE38 with an insect protein is another possibility, which is currently investigated (Asser-Kaiser and Jehle, unpublished). Knock out of *pe38* and swapping of it *pe38* from the resistance overcoming isolate CpGV-I12 into a CpGV-M bacmid were used to investigate the role of *pe38* for infection of susceptible and resistant CM larvae.

Pe38 was knocked out of the bacmid CpBac, which is based on CpGV-M (Elmenofy, 2008). This bacmid was previously propagated in CpS larvae after hemocoelic DNA injection and tested in bioassay (Elmenofy, 2008). The correct knock-out bacmid CpBacΔpe38 could be identified by its restriction profile and by PCR analysis. Amplification of *pe38* of CpGV-I12 and its swapping together with a resistance marker into the GFP locus of CpBacΔpe38 could be verified at every step by restriction and PCR analysis. The restriction profiles corresponded to the profiles expected when simulating the profiles *in silico*. Thus, it could be concluded that cloning and construction of the different bacmids was successful.

Infection of L4 larvae with different bacmids was intended by hemocoelic DNA or BV injection. As the resistance mechanism in the insect is not based on the midgut or peritrophic membrane but on an early block of replication (Asser-Kaiser, 2010), it should be possible to distinguish between resistance and resistance overcoming effects by hemocoelic DNA or BV injection. Infection of L4 larvae by injection of BV was successfully performed previously,

using BV deriving from haemolymph extracted from CpGV infected larvae. In these experiments, the CpGV DNA concentration in the haemolymph was used for correlation with BV concentration (Asser-Kaiser, 2010). With BV obtained from cell culture, no infection of susceptible CM larvae was visible in the experiments performed. The detection of viral DNA by qPCR as a measure for BV concentration in the supernatant also revealed relatively high DNA concentrations for the untreated controls. Infection of CP cells using a GFP expressing bacmid led to narrow bordered sources of infection, indicating that infection is spread somehow directly from cell to cell (Pit Radtke, personal communication). For a spread of infection due to BV, a spread of infection over the whole monolayer would be expected. The 10 day time point for harvesting the supernatant with the meassured BVs might have been to late and the DNA concentration estimated not due to BV, but already due to the formation of OB or lysed cells releasing virus DNA. During the occlusion phase, the production of infectious BV of AcMNPV in SF cells is greatly reduced or terminated (Lee and Miller, 1979). Infection of Cp cells with CpGV is difficult to identify by eye, a bacmid construct containing an active GFP gene would be a better attempt to facilitate the observation of the infection process.

For unknown reason, no virus infection could be induced by DNA injection, neither in CpS nor in CpRR1. Infection of L4 larvae by injection of bacmid DNA was succeeded previously using high DNA concentrations of 500 ng/µl in the transfection mix (Elmenofy, 2008). Only when using very high DNA concentrations (>400 ng bacmid DNA/larva) in the injection experiments, virus dead larvae could be observed. After propagation of the virus suspension collected from these larvae, it was not possible to prove the DNA identity by PCR analysis. For the samples (DNA isolated from larvae) no PCR product could be observed with any of the oligonucleotides specific for the constructs. Optimisation of the PCR protocol might be necessary, but the positive controls (the corresponding bacmid DNA isolated from *E. coli*) revealed the expected bands. DNA restriction analysis revealed that only dead larvae from the CpBac treatment were infected with the original bacmid construct CpBac, whereas the larvae from the CpBacΔpe38 and CpBacΔpe38/I12pe38 treatments were infected most likely with CpGV, as the REN profile did not correspond to the bacmids, but to the CpGV-M profile. Thus, virus dead larvae in the CpBacΔpe38 and CpBacΔpe38/I12pe38 treatments were most likely due to a CpGV-M contamination.

Hilton et al. (2008) propagated a CpGV bacmid containing the bacterial cassette deriving from the AcMNPV Bac-to-Bac system® in larvae by feeding them the pellet of transfected cells, because infection by DNA injection did not work very efficiently (Hilton, personal communication). As heamocoelic injection did not leed to success in the experiments described here, this method was applied to infect the larvae by the normal, the oral way of infection. Feeding of the pellet of transfected cells revealed a clear and complete virus infection of CM larvae when using CpBac. For CpBacΔpe38, no infection was visible, as expected for this construct if *pe38* is an essential factor for infection. Feeding the pellet of cells transfected with CpBacΔpe38/I12pe38 also did not lead to infection. This is a strong evidence that the rescue of CpGV-I12 *pe38* into CpBac functioned on DNA level, but modified somehow the biological activity. With an active *pe38* in CpBacΔpe38/I12pe38, infection of susceptible CM larvae would have been expected in the same way as observed with CpBac. Technically, the rescue of *pe38* was succesful; that the biological activity of the CpBacΔpe38 construct could not be restored by swapping *pe38* of CpGV-I12 in its GFP locus could be due to several reasons. *Pe38* is described as immediate early gene (Kovacs et al., 1991; Krappa and Knebel-Mörsdorf, 1990). Early genes exploit host RNA polymerase II and associated factors. Therefore, early promotors contain core transcription elements like TATA box and a ATCA(G/T)T(C/T) motif cooperating with auxiliary *cis*-acting elements. Changing the position of *pe38* into the GFP-locus of the bacmid cassette might have interrupted interactions with *cis*-acting factors necessary for gene transcription. PE38 transcription is described to be stimulated by IE-1 (Pullen and Friesen, 1995). Also the homologous repeat sequences function as transcriptional enhancers (Lu and Miller, 1997). In AcMNPV, *pe38* is located close to the homologous region 1 (Krappa and Knebel-Mörsdorf, 1990). In CpGV, *pe38* is also located close to the major repeat region between 20 and 22 kb (Luque et al., 2001). The proximity of *pe38* to a repeat region is also described for CfMNPV (Carstens et al., 2002), suggesting that these repeats function as origins of transient DNA replication or transcription enhancers.

In summary, the knock-out of *pe38* was succesful, revealing a bacmid unable to induce virus infection in susceptible CM larvae. L4 larvae could be infected by CpBac orally and by DNA injection using high DNA concentrations, but not by CpBacΔpe38. Intending further DNA injection experiments, it is necessary to use high DNA concentrations. A disadvantage is that

solutions with high DNA concentrations are jelly and more difficult to handle for injection, as the DNA should not break. A better method is the oral way of infection, using cells transfected with bacmid DNA.

The rescue of *pe38* into the inactive GFP locus of CpBacΔpe38 could not reveal its function. This might be due to the lack of some unknown factors influencing transcription or activation of *pe38*. A method to test if a position effect plays a role in the activity of *pe38* would be the direct mutation of the 24 nt stretch in the knock-out bacmid, without changing the original genomic position of the *pe38* gene. Another possibility to retain the original position of *pe38* is to swap CpGV-I12 *pe38* into the knock-out cassette of CpBacΔpe38, in combination with a marker gene to allow screening for recombinants.

7 Final Discussion

The granulovirus of codling moth (*Cydia pomonella,* L.), CpGV, is a highly selective and efficient biological control agent in apple production. During the last decades, it gained high importance in ecological and integrated pest management. The recent occurrence of field resistance to CpGV led to an intense research concerning inheritance and mechanism of codling moth (CM) resistance to CpGV as well as overcoming this resistance in CM. The mode of inheritance was published in 2007 (Asser-Kaiser et al.), and its elucidation revealed that alternatives to the conventionally applied virus isolate CpGV-M are inevitable.

Alternatives to CpGV-M were provided in this thesis: in Chapter 3, five out of 16 tested novel CpGV isolates were able to overcome CpGV resistance in CM. These isolates derived from distinct parts of the world and showed differences in their genetic content when investigated by restriction fragment length polymorphism (RFLP) analysis and partial sequencing (Chapter 3, Chapter 4). For future CpGV application, the exploitation of this baculovirus diversity is crucial for resistance management, in order to prevent a further selection for resistance. However, one question will immediately arise when proposing the application of novel CpGV isolates: is CM able to develop again resistance against these novel isolates?

To give a statement about the possibility of resistance development, it is important to understand the mechanism of resistance in detail. The application of CpGV-M worked efficiently for more than 20 years, and the occurrence of resistance was always regarded as rather unlikely. Apparently, this is not the case, and the development of resistance to novel CpGV isolates cannot be excluded as well. With the experiences and approaches so far made by investigating CM resistance to CpGV and its overcoming by novel isolates, it is possible to avoid repeating the same problem in the future. The finding that resistance to CpGV is a resistance to type A CpGV genomes, as elucidated in this thesis, implies that numerous further isolates exist worldwide which are able to overcome this resistance. The application of mixtures of isolates will prevent the selection for resistance to one isolate, as it was the case with application based on CpGV-M only.

This approach is actually a return to the natural situation in the field. Continued application of a single CpGV isolate against a CM population is an artificial host-pathogen system; naturally occurring CpGV isolates are often mixtures of different genotypes. As shown in Chapter 3,

the isolate CpGV-E2 showed the highest virulence against resistant CM populations among the tested isolates. The REN profile of -E2 revealed that this isolate was a mixture of a type A and a type B genome. In contrast to the other resistant overcoming isolates, which were found to be rather clear isolates, CpGV-E2 worked already efficiently after seven days, whereas CpGV-I12, -I01, -I08 and -S showed a slight delay in their efficacy. Interactions between virus genotypes have been described for co-infections of defective and complete virus genotypes, whereas the complete genotype acts as a helper for faciliating the transmission of the deletion genotype (López-Ferber et al., 2003). Due to its smaller size, the deletion mutant has a replicative advantage. In presence of a complete genotype, the deletion mutant can can benefit from its gene products to replicate itself (Barrett and Dimmock 1986). Genetic heterogenity of natural occuring CpGV isolates (not only regarding defective, but also co-existing complete genomes) might be a possibility to adapt quickly to the host`s ecology. Natural mixtures of genotypes contribute to the genetic diversity of baculovirus genomes, as intra- and intergenomic recombinations play an important role in maintaining their genomic plasticity. The genomic plasticity of CpGV isolates was also mirrored by the isolates investigated in this thesis: the uptake of ORFs (type C genomes), several duplication events (type E genome) or the integration of large insertions (type D and B genomes) were observed when comparing several isolates (Chapter 4, Chapter 5). As there is a high diversity between CpGV isolates (but concurrently also a high conservation of several viral genes and the *hr* regions), the question arises about the evolutionary “direction” of CpGV isolates.

Based on the phylogenetic analysis provided in Chpater 4, there is for example no trend to smaller or larger genomes: the phylogenetically eldest C type isolates lack four open reading frames (ORF), which are inserted into all younger isolates. Type B and D isolates reveal an additional 0.7 kb non-coding insertion. The phylogenetically youngest type A isolates lack this insertion, but are still larger in size than the phylogenetically elder isolate CpGV-S (type E genome). Intuitively, one would say that CpGV genomes evolve to more virulent genomes, but different genotypes operate better under different ecological conditions. This differential selection maintains, among other factors (interspecific competition, multiple infections), the baculovirus variability (reviewed in Cory and Myers, 2003). The uptake of the four ORFs cp63-cp66 and their establishment in the phylogenetically younger CpGV genomes indicate that these ORFs are somehow benefitial to the propagation or efficacy of the virus. The role of these four ORFs is so far not yet determined. Comparison of the genomes of CpGV-I12

and -S (Chapter 5) revealed that the area of cp62-cp66 harbours several insertions and deletions in coding and non-conding regions. In contrast to other regions, this area is prone to genomic rearrangements. Also the benefit of the 0.7 kb insertion present in CpGV-I01, -I12, -I08 and -E2 is unclear. Only few nucleotides differed in this inserted sequence when comparing CpGV-I01, -E2 and -I12. As supposed for the *hr* regions, the similarly structured 0.7 kb insertion could have *cis*-acting influence on gene transcription or act as origin of DNA replication. However, this insertion is not present in type A genomes.

Regarding the virus-insect interaction, selection pressure on viral genotypes is complex and even conflictive. Selection should not only favour the genome type which induces the shortest time to death of the host, but also optimise the time to death to maximise the delivery of newly synthesised occlusion bodies (OB). To evaluate the benefit of genomic differences between CpGV genome types, it might be necessary to consider a system of mixtures of genome types instead of an isolated, pure genome type.

Until the occurrence of resistant CM populations, CpGV-M worked efficiently against CM for more than twenty years. Whole genome sequencing of CpGV-M and two resistance overcoming isolates (Chapter 5) revealed that CpGV-M differed on ORF level only in a 24 bp insertion in the ORF cp24, which codes for the immediate early gene *pe38*. This raises the question about the function of *pe38*. What is its role, regarding that the insertion of these additional nucleotides was for a long time apparently not a critical disadvantage for CpGV-M? CpGV-M still shows high virulence against susceptible CM populations. Generally, a mutation neutral under specific conditions might by advantageous or deleterious when conditions change. This was the case with the appearance of CM populations resistant to CpGV, assumed that *pe38* is responsible for the resistance overcoming character of the isolates described in this thesis. PE38 of the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was found to play an important role in viral replication, budded virus production and virulence (Milks et al., 2003). Investigations on the resistance mechanism revealed that CpGV-M replication is affected at an early stage of infection in resistant CM larvae (Asser-Kaiser, 2010). PE38 comprises two putative DNA and protein binding domains: a C-terminal leucin zipper and a N-terminal zinc finger motif (Krappa and Knebel-Mörsdorf, 1991). Both motifs are typical for transcription factors. It is likely that the insertion of the 24 additional nt (eight additional amino acids) has an effect on the proteins 3-D structure. Assuming that PE38

is an activator of transcripton, a possible mechanism could be that CpGV-M PE38 can bind to its DNA or protein target sequence, but that its interactions with further proteins or ligands are not ideal. Nevertheless, there is still enough enhancement of transcriptional activity of its target gene to maintain virulence in susceptible CM. This would be consistent with the results in Chapter 6, where a complete knock out of *pe38* resulted in a loss of virulence against susceptible CM larvae. This mechanism would presume an interaction of PE38 with a viral target, but PE38 could also directly interact with a DNA or protein target of the host. Preliminary studies on protein-protein interactions of PE38 with host proteins using the Yeast Two-Hybrid System revealed several host proteins interacting with either one of the binding domains or with the full-lenght PE38 and provide a starting point for the search of the PE38 interaction partner (Asser-Kaiser, 2010).

To summarise, to prevent a further selection of resistance to CpGV-M, a broad genetic base of the applied CpGV products is important. Beyond that, the application of CpGV-M should also be continued. Cases of field resistance to CpGV appeared in several European countries, but compared to the total number of orchards where CpGV is successfully applied, resistance is still the exceptional case. However, CM populations with homozygous or heterozygous resistant individuals are able to establish quickly a high resistance level under CpGV selection pressure. Application of novel isolates, ideally as mixtures of different genotypes, could prevent a further selection of resistance. Several of the novel isolates were already tested successfully in the field (Kienzle et al., 2007; Zing, 2008). As already observed in bioassay, the full efficacy of the tested resistance overcoming isolates was visible with a delay of 1-2 days. Therefore, CM control should not only be based on CpGV application, but include further approaches for minimising the populations: the removal of infested apples, the cleaning and desinfection of apple boxes to avoid spreading of CM individuals between orchards and the application of mating disruption are approaches to keep population densitiy at a lower level. For the future, the development of resistance monitoring based on molecular markers will help to screen for resistant alleles in the populations and to develop the best CpGV application strategies for the particular orchard based on different CpGV isolates. Screening of the susceptibility of CM populations to novel CpGV isolates will also be essential in future resistance management. To develop adequate CpGV application strategies for different orchards, it is crucial to registrate and apply the novel developed products as soon as possible. The meth-

ods and results described in this thesis for the identification and characterisation of resistance overcoming isolates contribute to this aim.

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