“On the inheritance and mechanism of baculovirus resistance of the codling moth, *Cydia pomonella* (L.)”

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LIST OF ABBREVIATIONS

GENERAL

AD	activation domain
BD	binding domain
BC	backcross
BV	budded virus
bp	base pair
CD	cytochalasin D
cDNA	complemaentary deoxyribonucleic acid
CL	confidence limit
CM	codling moth
DNA	deoxyribonucleic acid
DNA-AD	DNA-activation domain
DNA-BD	DNA-binding domain
dscDNA	double stranded complementary deoxyribonucleic acid
EPNs	entomopathogenic nematodes
FB28	fluorescent brightener 28
ha	hectare
hpi	hours post infection
Ig	immunoglobulin
IPM	integrated pest management
IRAC	Insecticide resistance Action Committee
kbp	kilobase pair
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
LD-PCR	long distance polymerase chain reaction
LPS	lipopolysaccharides
MCS	multiple cloning site
MDT	mating disruption techniques

min	minute
MNPV	multiple nucleopolyhedrovirus
mRNA	messenger ribonucleic acid
OB	occlusion body
ODV	occlusion derived virus
ORF	open reading frame
ori	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PM	peritrophic membrane
PTU	phenylthiourea
qPCR	quantitative polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROS	reactive oxygen species
RTA	repressed transactivator
SMART	Switching Mechanism at 5' end of RNA Transcript
SNPV	single nucleoplyhedrovirus
Trx	thioredoxin
TtD	time to death
UAS	upstream activation sequence
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation
Y2H	yeast two-hybrid

VIRUSES

AcMNPV	*Autographa californica* MNPV
AgseGV	*Agrotis segetum* GV
AgMNPV	*Anticarsia gemmatalis* MNPV
ArGV	*Artogeia rapae* GV
BmDNV-2	*Bombyx mori* densovirus type 2

BmNPV	*Bombyx mori* NPV
CpGV	*Cydia pomonella* GV
HzSNPV	*Helicoverpa zea* SNPV
LaolGV	*Lacanobia oleracea* GV
LdMNPV	*Lymantria dispar* MNPV
PlxyGV	*Plutella xylostella* GV
SfMNPV	*Spodoptera frugiperda* MNPV
TnSNPV	*Trichoplusia ni* SNPV
XecnGV	*Xestia c-nigrum* GV

SUMMARY

The *Cydia pomonella* granulovirus (CpGV, *Baculoviridae*) is the most important biocontrol agent of the codling moth in apple production. CpGV has a very narrow host range, is harmless to humans and beneficial arthropods and is highly virulent against codling moth larvae. It is successfully used since the late 1980s, particularly in organinc apple production. All commercial CpGV products available in Europe contain the same CpGV isloate (CpGV-M), originally discovered in Northern Mexico in 1963. In Europe it is applied on about 150,000 ha per year. Since 2004, several codling moth populations with an up to thousand-fold decreased susceptibility to CpGV have been observed in Germany, France, the Netherlands, Italy, Switzerland and Austria. In order to understand this phenomenon of field resistance and to develop resistance management strategies, investigations on the biological and genetic background of CpGV resistance were initiated. This thesis is focused on the analysis of the inheritance and the mechanism of CpGV resistance.

Single pair crosses between a susceptible laboratory strain (CpS) and a homogeneous CpGV resistant strain (CpRR1) revealed that CpGV resistance is inherited by a single dominant gene, which is located on the Z-chromosome. This sex-linked mode of inheritance explains the rapid development of CpGV resistance in the field. Females (Z^RW) require only one copy of the resistance allele to survive virus exposure; this enables a faster initial selection response than autosomally inherited resistance. Resistance in the heterozygous males (Z^SZ^R) is dominant at low virus concentrations, but at high concentrations it manifests as recessive, accelerating the response to selection even more when applications are increased in numbers and intensity as a response to less effective pest control.

Mass crossing experiments between CpS and a heterogeneous resistant, field collected strain (CpR) initially suggested an autosomal incomplete dominant mode of inheritance. However, single pair crosses between CpS and CpR disclosed that CpR and CpRR1 share the same mode of inheritance of CpGV resistance. This thesis also discusses the advantages of single pair crossings compared to mass crossings in terms of elucidating the mode of resistance inheritance.

The efficacy of a new CpGV isolate (CpGV-I12) originating from Iran was tested against CpRR1 larvae in bioassays. CpGV-I12 is able to overcome resistance in all larval stages of CpRR1 and works almost as well as CpGV-M against CpS larvae.

CpGV-I12 is suitable for codling moth control in orchards where resistance against CpGV-M has occurred.

In order to investigate the mechanism underlying CpGV resistance, four different experimental approaches were followed: First, the peritrophic membrane was degraded by adding an optical brightener to the diet containing virus. However, the removal of this mechanical barrier in the midgut did not lead to enhanced CpGV infection in resistant larvae suggesting that the PM is not involved in resistance. Second, injection of CpGV budded virus directly into the insect's haemocoel did not overcome resistance. Accordingly, CpGV resistance is not restricted to the midgut but also present in secondary infection. Third, CpGV replication was traced by quantitative PCR in three different tissues of susceptible (CpS) and resistant (CpRR1) insects. After both oral and intrahaemocoelar infection, the amount of CpGV copies detected in all three tissue types of CpS increased with time elapsed. Virus replication could not be detected in any of the isolated tissue types of CpRR1. This indicates that CpGV resistance is present in each of the cell types. Fourth, CpGV caused mortality was analysed after transfusion of haemolymph between resistant (CpRR1) and susceptible (CpS) codling moth larvae to determine the possible action of a humoral factor involved in CpGV resistance. Thus, no immune response was observed and no factor in the haemolymph wich induces resistance could be identified. Injection of haemolyph from inoculated CpRR1 larvae into CpS larvae did not cause viral infection. Hence, the injected haemoplymph of inoculated CpRR1 larvae did not contain infectious budded virus. Based on these results it is proposed that virus replication is affected in all cell types, suggesting a virus-cell incompatibility in resistant codling moths. Because no DNA replication could be observed it is proposed that CpGV resistance is caused by an early block of virus replication.

The early transcribed gene *pe38* encodes for a protein which is putatively responsible for the resistance overcoming capacity of CpGV-I12. Interactions between the protein PE38 and proteins of CpRR1 were investigated using the yeast two-hybrid (Y2H) system. The interactions found are not confirmed by other methods yet. Nevertheless, the Y2H screening revealed two putative proteins located on the Z-chromosome and one located on chromosome number 15, which may be involved in resistance to CpGV.

In this thesis the phenomenon of host resistance against CpGV was investigated from two sides: the mode iheritance and the mechanism of resistance. Hereby, diverse methods were applied ranging from bioassays and classical crossing experiments to molecular methods like quantitative PCR and Y2H system. The results of the different approaches interlock and give important information about CpGV resistance that can be used for the development of adequate resistance management strategies.

1 GENERAL INTRODUCTION

The codling moth *Cydia pomonella* L. (Lepidoptera: *Tortricidae*), is one of the most important insects pests in apple, pear and walnut production worldwide. Applications of chemical insecticides have been the main tools for its control. However, this pest has developed resistance to many registered insecticides and only a few compounds are still effective against the codling moth in areas where insecticide resistance has emerged (Reyes, 2007). The *Cydia pomonella* Granulovirus (CpGV) is a biological control agent against codling moths and has been used successfully by integrated and organic farmers for decades. However, recently some codling moth populations developed resistance against CpGV products. Several resistant populations in Germany, France, the Netherlands, Italy, Austria and Switzerland have been detected so far (Fritsch et al., 2006; Sauphanor et al., 2006, Asser-Kaiser et al., 2007; Jehle unpublished). CpGV resistance is the first case of field resistance against a baculovirus. A spread of CpGV resistance is a severe threat to the efficient control of the codling moth, particularly in organic farming. To understand this phenomenon and to develop new control strategies or to restore high susceptibility towards CpGV, investigations on the biological and genetic background of CpGV resistance are essential. This thesis addresses the analysis of inheritance and the mechanism of CpGV resistance to gain crucial information, which shall help to develop a resistance management strategy.

1.1 *CYDIA POMONELLA* AS A PEST IN APPLE PRODUTION

1.1.1 LIFE CYCLE OF CODLING MOTH

The codling moth is a major pest in pome fruit production throughout most of the temperate fruit growing regions worldwide. The larvae of this species cause damage on various cultivated plants, mainly apple, pear, walnut, quince and apricot by feeding on the fruits (Barnes, 1991).

The insect hibernates as diapausing larva in a white, firm cocoon (hibernaculum), mostly behind bark scales or other hidden places in the orchard. Pupation takes place in spring and adult moths of the first generation emerge from May to June (Börner, 1997, Lacey & Shapiro-Ilan, 2008). The time between pupation and eclosion depends solely on temperature. The crepuscular moths fly at temperatures between 14°C and 16°C. The flight of the first generation spans over a period of approximately 10 weeks (Harzer, 2006). After copulation the females lay 60 to 100 eggs mostly on leaves (Börner, 1997).

The duration of egg development again depends on temperature and takes 80 to 85 degree days (T> 10°C). Ranging from a few hours to a few days after hatching, the first instar larvae bore into the young fruits and build a gallery to the core (Figure 1.1) (Harzer, 2006). The larvae of the codling moth are cryptic living insects which remain inside the fruit throughout their feeding stages. Fifth instar larvae leave the fruit and locate overwintering places behind bark scales for hibernation. In warmer regions like southern Germany, France, or Italy, one part of the larvae does not directly enter the diapause phase, but pupate to build up a second generation in the same season. Depending on climatic conditions there are one to four generations per year. In general, the second and later generations cause severer damage than the first generation, because the moths lay their eggs on the already ripening fruits. The first generation infests the unripe fruits of which some are discarded by the farmer due to reduction of the harvest for quality management or the trees themselves naturally drop off redundant fruits. Additionally, when the first generation was not controlled efficiently by chemical or biological insecticides, the population size highly increases from one to the next generation (Börner, 1997).

Figure 1.1 Adult codling moth (A) and apples infested by codling moth larvae: Fourth instar larva in the gallery inside the apple (B) and the typical damage caused by the *Cydia pomonella*: Holes with bore meal and frass caused by a codling moth larva (C).

1.1.2 CONTROL OF CODLING MOTH

1.1.2.1 THE NECCESSITY OF CONTROL

Fruits that are infested by the codling moth are not marketable as dessert fruits and can only be used for the production of fruit juice. Even small injuries on the surface of apples or pears are not tolerated by the consumer. The need of control measures against a pest is given when the expected damage exceeds the costs for the treatment (Steiner,

1968). There are different methods to predict infestation of codling moth in the apple orchard: First, pheromone traps are used to monitor adult moths in the orchards ready for mating and producing larvae that infest the fruit. Second, 1000 apples are harvested in June and infestation is scored in order to predict the second generation, and third, infestation rate of the last season harvest is used to forecast the infestation pressure of the current season. In case of apples, the economic threshold for control is 5-7 moths/ week in one pheromone trap, 0.5-2% infestation by the first generation in 1000 apples or 1% infestation in the last year respectively (Höhn et al., 2008).

1.1.2.2 CONTROL METHODS APPLIED IN ORCHARDS

Arthropod pests in temperate tree fruits and nuts are mainly controlled by broad-spectrum insecticides. Currently there are several chemical pesticides registered for codling moth control in Europe, including insect growth regulators (tebufenozid and tethoxifenozide), oxadiazine insecticides (indoxacarb) and neonicotinids (thiacloprid and acetamiprid). However, the frequent use of chemical insecticides over decades caused a series of problems: health risks for farmers and consumers, outbreaks of secondary pests that were normally controlled by natural enemies, environmental contamination, decrease in biodiversity and insecticide resistance (Lacey & Shapiro-Ilan, 2008). The reduction of the amount of chemicals applied in agriculture and the development of safe and environmentally benign methods are strongly demanded by the general public, scientists and governments. Integrated pest management strategies (IPM) combine preventive, biological and biotechnical methods, in order to minimize the application of chemical pesticides and to prevent development of resistance. Moreover, in organic farming the use of chemicals is completely banned.

The application of mating disruption techniques (MDT), entomopathogenic nematodes (EPNs), parasitoids (*Trichogramma*) and CpGV and formulations of *Bacillus thuringiensis* are environmentally benign alternatives to the usage of chemicals in codling moth control. They are used to control a multitude of insect species, but have a low efficacy against codling moth (Blommers, 1994).

Synthesized pheromones of the female codling moths are used for MTD. The underlying theory is that male moths are confused by the extremely high concentration of the attractant and are not able to find female moths. Accordingly, mating is disrupted. However, there are several parameters that impact the success of this method: Population density should be low (<1% infestation in the last season), otherwise males are able to find females by chance. The orchards should be uniform and larger than 2 ha. Dispensers containing the pheromone should be affixed to the upper third of the crown just before the moths start to fly. 500 dispensers per hectare are needed, whereupon the boundary area should be included to prevent mating near to the orchard

(Trautmann & Sheer, 2006). This method is mostly used in combination with chemical or biological control measures.

The target stages of EPNs are the diapausing cocooned larvae in the orchard. Protected habitats where diapausing larvae of the codling moth use as overwintering sites, exhibit favourable conditions for EPNs. The two nematode species *Steinernema carpocapsae* and *S. feltiae* are well studied in terms of codling moth control (Lacey & Shapiro-Ilan, 2008). EPNs carry bacteria of the genera *Xenerohabdus* and *Photorhabdus* in their intestinal lumen, which actually kill the insect host (Cross et al., 1999). The success of EPNs in control of *C. pomonella* highly depends on temperature (> 15°C) and sufficient moisture in the orchard. Therefore, irrigation before and after treatment and the use of strip mulches around tree bases can be necessary to ensure the efficacy of the EPNs (Lacey & Shapiro-Ilan, 2008).

The release of mass reared egg parasitoids of the genus *Trichogramma* is successfully used in biological control of the European corn borer *Ostrinia nubilalis* (Hübner) and in storage protection. Following this method, eggs of the Angoumois grain moth *Sitotroga cerealella* (Oliver) on cards parasitized by *Trichogramma* species are released. However, in codling moth control it plays only a minor role. Because of its variable and inconsistent efficacy and its high costs it is not established in commercial pome fruit production. However, it is commercialised for the use in private gardens (Zimmermann, 2004). The efficacy of treatments with *Trichogramma cacoeciae* to control codling moth in field trials ranged from 81 - 88%, (Sakr et al., 2002).

CpGV products are one of the most important and most effective biological control agents used in codling moth control. The first CpGV product was registered by Andermatt-Biocontrol AG in 1987 and it was introduced into the marked in 1988 under the trade name Madex (Huber, 1998). Today, there are further CpGV products on the European market: Granupom (Probis GmbH), Carpovirusine (Arysta LifeScience) and Cyd-X (Certis). In Europe, CpGV is successfully applied on more than 100,000 ha per year in organic and integrated pome fruit production. CpGV products contain infectious virus particles (occlusion bodies, OBs) which have a long shelf life and can be sprayed like normal insecticides with usual sprayers (Kienzle et al., 2006). CpGV has a narrow host range and is harmless to humans and to other non-target organisms including beneficial insects.

1.2 BACULOVIRUSES

1.2.1 INTRODUCTION

CpGV belongs to the family of *Baculoviridae* (Theilmann, et al., 2005). Baculoviruses are a diverse group of viruses that exclusively infect organisms belonging to the class *Insecta* (Theilmann et al., 2005). In ancient Chinese literature a disease of the silkworm which caused serious economic problems in sericulture was described. The portrayed symptoms were similar to symptoms caused by baculovirus infection (Miller, 1997). In those days, baculoviruses probably were far from being beneficial to human. Today, we mostly benefit from these viruses. Baculoviruses are widely applied to control pest insects. They are used as vectors for the expression of heterologous proteins in insect and mammalian cells and for medical purposes like vaccine production and health diagnostics (Possee, 1997; Kost & Condreay, 1999; van Oers, 2006). In addition, they possibly can be used as gene therapy vectors in the future (Hofmann et al., 1995). Baculoviruses are environmentally safe and selective biopesticides. The most successful example for the use of baculoviruses in pest control is the nucleopolyhedrovirus of the velvet bean caterpillar, *Anticarsia gemmatalis* (Hübner), in Brazil, where it is applied on 1-2 million ha per year (Moscardi, 1999). Other examples for successful applications of baculoviruses in insect pest control are *Helicoverpa amigera* NPV against the cotton bollworm in China, *Lymantria dispar* NPV against the gypsy moth in North American forests and application of CpGV for codling moth control in many countries worldwide (Hunter-Fujita et al., 1998). Baculoviruses have a very narrow host range and preserve natural antagonists and other beneficial arthropods. However, this attribute is not only an advantage but can be adverse for commercialisation since thebaculovirus products are extremely specific and target only single pest species. Other limiting factors for the use of baculoviruses in agriculture are the slow speed of kill in some cases, technical and economical difficulties for commercial large scale production, variable efficacies in field application and dependence on climate conditions (Moscardi, 1999).

1.2.2 STRUCTURE AND CLASSIFICATION

The genome of baculoviruses is characterized by a double-stranded, circular, supercoiled DNA (Rohrmann, 2008). The DNA is packed into nucleocapsids, which are enveloped and embedded in a proteinaceous matrix and form occlusion bodies (OBs) (Granados, 1980). The protein matrix protects the virions from environmental impacts, like UV light, outside their hosts. Baculoviruses produce two phenotypes of virions: the occlusion-derived virus (ODV) originates from OBs and has an envelope assembled *de novo* within the nucleus. It is responsible for *per os* infection. The budded virus (BV) buds out of infected cells and spreads the infection within an insect. BVs get their

envelope from modified cell membranes (Rohrmann, 2008; Hilton, 2008). BVs and ODVs are morphologically different, as reflected in a distinct protein composition, but they are genetically identical (Federici, 1997).

According to the OB morphology, two groups can be recognized: Nucleopolyhedroviruses (NPV) form 0.15-15 µm large polyhedron-like OBs, called polyhedra, whereas granuloviruses (GV) form smaller granules of 120-300 nm in diameter and 300-500 n min length (Figure 1.2). Different from OBs of GVs that contain only one virion, the polyhedra of NPVs include several to many virions (Miller, 1997). Some NPVs have virions with a single nucleocapsid (SNPV), whereas other NPVs consist of ODVs with multiple nucleocapsids (MNPV) (Granados, 1980).

Until 2006, classification and nomenclature of baculoviruses was primarily based on these morphological traits. Based on the complete genome sequences of 29 baculoviruses Jehle et al. (2006) proposed that phylogeny of baculoviruses followed the classification of the host rather than morphological parameters. Consequently, the family of *Baculoviridae* includes four genera according to the insect order their hosts belong to. *Alphabaculoviruses* are lepidopteran-specific NPVs, whereas *Betabaculoviruses* are lepidopteran-specific GVs. *Gammabaculoviruses* are hymenopteran-specific NPVs and *Deltabaculoviruses* are dipteran-specific NPVs. Nomenclature of baculovirus species was not affected by this re-classification of the genera. Species are named after the insect from which they were first isolated, followed by the indications GV or NPV (van Oers & Vlak, 2007).

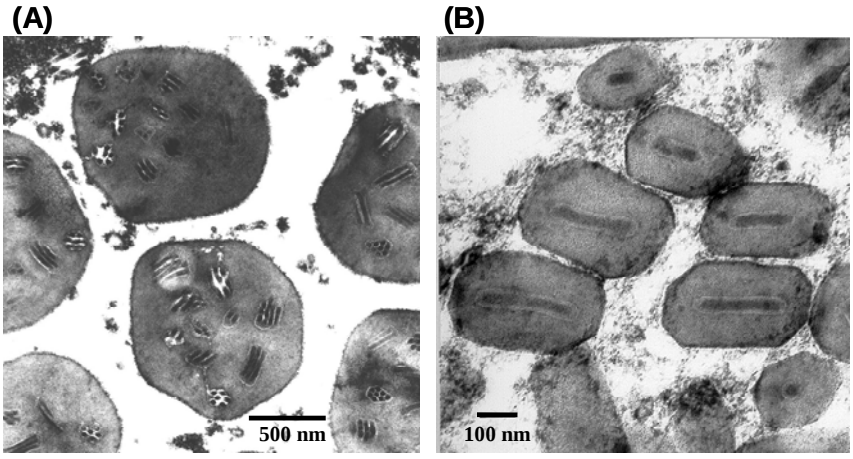

Figure 1.2 Electron micrographs of polyhedra (A) of a nucleopolyhedrovirus and (B) granules of the *Cydia pomonella* granulovirus. Dr. A. Huger (Julius-Kühn-Institut).

1.2.3 GENOME AND GENE EXPRESSION

Baculovirus genome sizes vary between 80 and 180 kilobase-pair (kbp) that encode between 90 and 180 genes. Viruses pathogenic to insects of the order *Hymenoptera* contain smaller genomes of ~90 kbp, whereas GV genomes have larger genome sizes between 101 up to 178 base-pair (bp) (Rohrmann, 2008). To date, genomes of more than 50 baculoviruses have been sequenced. Most of them are lepidopteran specific. The open reading frames (ORFs) of baculovirus genomes are tightly packed with minimal intergenic regions and equal distribution of the coding sequences over both strands. There are 30 conserved core genes, which are characteristic for baculoviruses. The majority of baculovirus genomes contain multiple homologous regions with repeated sequences (*hrs*) and often palindromic motifs (Van Oers & Vlak, 2007). Functional studies have demonstrated that *hrs* serve as enhancers of early gene transcription (Theilmann & Steward, 1992; Guarino & Summers, 1986) and most likely as origin of DNA replication (Kool et al., 1993; Pearson et al., 1992, 1995; Hilton, 2008). The genome of CpGV contains 13 imperfect palindromes of 74-76 bp that have been found to replicate in *C. pomonella* cells (Hilton, 2008). Field isolates of baculoviruses show considerable genomic heterogeneity, indicating a certain degree of plasticity. Variation evolves from the uptake of new genes due to recombination events and transposon insertions when a host is co-infected with more than one virus (Van Oers & Vlak, 2007). The horizontal transfer of transposable elements has been identified in the genomes of several baculoviruses including CpGV (Friesen, 1993; Jehle et al. 1995; Jehle et al., 1998).

Baculovirus gene expression is temporarily regulated in a cascade-like manner (Blissard & Rohrmann, 1990). According to their phase of expression, baculovirus genes are divided into four temporal classes of genes: immediate-early genes (IE), delayed-early genes (DE), late genes (L) and very late genes (VL) (Van Oers & Vlak, 2007). Early genes are expressed before DNA replication is started and are not affected by inhibitors of DNA synthesis. They are transcribed by the host RNA polymerase II. DE genes are activated by gene products of IE genes like the transactivator IE-1. Proteins encoded by early genes are needed for viral DNA replication and reprogramming the host cell towards virus replication. Structural proteins for BVs and ODVs are encoded by late genes (e.g. *F protein, odv-e18*), which are transcribed by a viral encoded RNA polymerase. For the transcription of the late genes virus encoded proteins, called late essential factors (LEFs), are needed (Lu & Miller, 1997). Proteins involved in OB formation and release (*polyhedrin/granulin, p10*) are encoded by very late and hyper-expressed genes (Vlak & Rohrmann, 1985).

1.2.4 *CYDIA POMONELLA* GRANULOVIRUS (CPGV) AS A MEMBER OF THE GENUS BETABACULOVIRUS

The *Cydia pomonella* Granulovirus is the type species of genus of *Betabaculovirus* (Jehle et al., 2006). Viruses belonging to this genus have been reported to infect lepidopteran hosts only. About 100 species within this insect order are known to be attacked by GVs (Volkman et al., 1995). The granule-like OBs of GVs are about 130-250 nm in diameter and 300-500 nm in length, with genome sizes from 100-180 kbp (Jehle et al., 2006; Winstanley & O'Reilly, 1999). The genome of CpGV is composed of 123,500 bp and is completely sequenced. It contains 143 ORFs of which 118 are homologous to genes previously identified in other baculoviruses (Luque et al., 2001). 62 genes are present in all lepidopteran baculoviruses, e.g. genes involved in replication or transcription or structural and auxiliary genes. CpGV also contains 22 GV specific genes which are not present in NPVs. Eleven genes of the CpGV genome are CpGV-specific genes which may encode host-specific factors (Hilton, 2008).

Research on CpGV started after it was discovered in Chihuahua, Mexico in 1963 (Tanada, 1964). Because of its narrow host range and high virulence against codling moth larvae investigations towards the use of this virus in biological codling moth control was started in the 1960s. After first commercialisation in 1988 it has been successfully used in integrated and organic apple and pear production for about 20 years (Huber, 1998). In 2005, first CpGV resistant populations were reported in Germany (Fritsch et al., 2005). In order to understand how this insect could have become resistant to CpGV, it is important to know the pathology and route of infection of the virus.

1.2.5 PATHOLOGY

Members of the baculovirus family have in common that they infect only larval instars, and exhibit a bi-phasic infection process. Insect-to-insect transmission is carried out by OBs, whereas the spread from cell-to-cell is conducted by the non-occluded BVs (Federici, 1997). However, pathology of NPVs slightly differs from the pathology of GVs. Since this thesis focuses CpGV, the life cycle of GVs is described in the following (Figure 1.3).

According to the tissue tropism of the virus infection GVs can be divided into three groups (Winstanley & O'Reilly, 1999): After invasion of the midgut epithelium, GVs of group I (e.g. *Adoxophyes orana* GV) infect only the fat body. Tissue tropism of group II GVs, which CpGV belongs to, is similar to that of NPVs. These viruses invade most of the major body tissues: haemocytes, tracheal matrix, epidermis and fat body. Group III GVs do not invade other tissues than the midgut epithelium. They replicate only in the midgut (Federici, 1997). Furthermore, there are "slow" and "fast" acting GVs. "Slow"

GVs, like *Xestia c-nigrum* (Xecn) GV, *Agrotis segetum* (Agse)GV and *Lacanobia oleracea* (Laol) GV, have high median lethal doses (LD_{50} values) and duration from infection to death normally spans several larval stages. The "fast" GVs (e.g. *Artogeia rapae* (Ar)GV, *Plutella xylostella* (Plxy) GV and CpGV) have lower LD_{50} values and rapid speeds of kill as they typically kill their hosts in the same instar in which it was infected (Winstanley & O'Reilly, 1999).

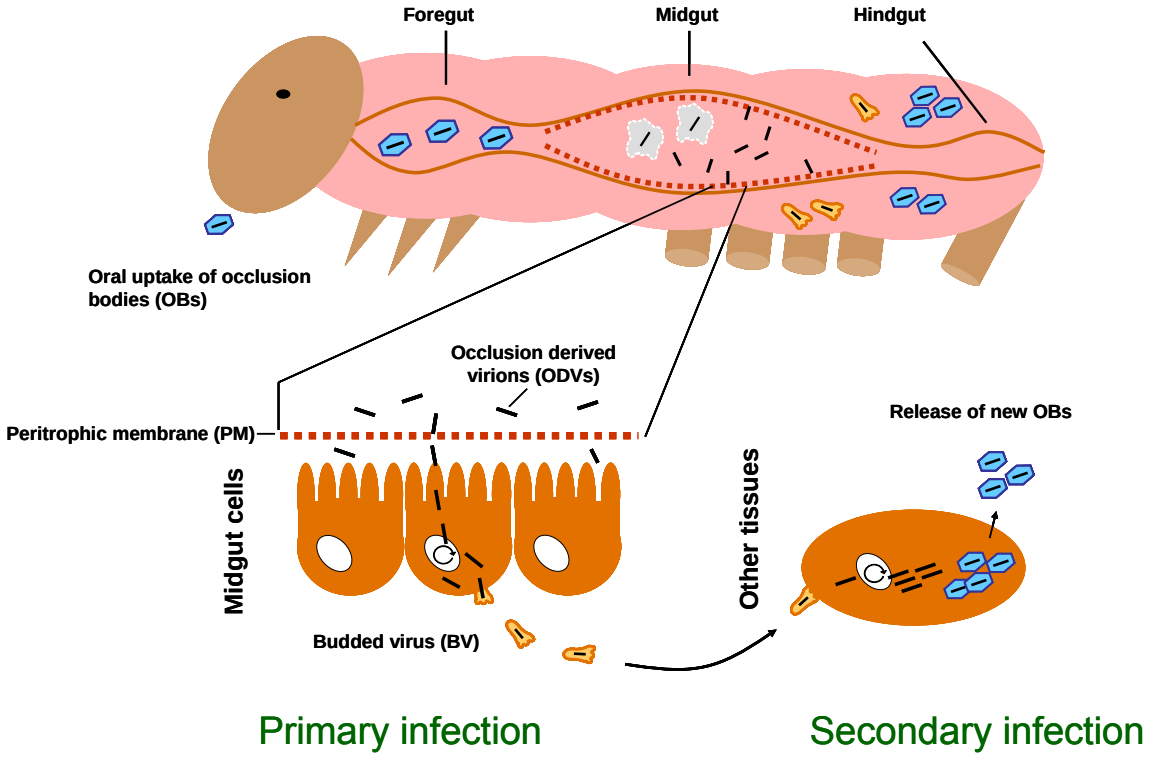

Figure 1.3 Schematic infection cycle of CpGV in a codling moth larva. Infection begins with ingestion *per os* and solubilization of the occlusion bodies in the larval midgut. The released (ODV) pass the PM and fuse with the membrane of the midgut epithelial cells. Nucleocapsids enter the nucleus where replication is started. New nucleocapsids are formed and leave the cells by budding through the membrane. Thereby BVs, which initiate secondary infection by infection of other tissues, are formed. BVs enter cells by membrane fusion where new nucleocapsids are produced and enveloped. Due to the formation of OBs cells greatly enlarge, eventually burst and release OBs.

Primary infection starts in the insect's midgut after the oral uptake of occlusion bodies. Due to the alkaline milieu (pH 8-11) and in presence of proteases in the midgut lumen, OBs quickly dissolve and release the occlusion derived virions (ODVs). ODVs pass through the peritrophic membrane (PM) lining the midgut, and bind to the microvillar membrane of columnar midgut epithelial cells. Via fusion of the virion envelope with the microvillar membrane the virions enter the cells. Different from nucleocapsids of NPVs, which pass through the nuclear pores, nucleocapsids of GVs are thought to inject the DNA into the nucleus at the nuclear pore (Summers, 1971). The initial phase of virus replication takes place in the nucleus and comes along with enlargement and margination of the nuclei, development of a virogenic stroma, hypertrophy of the nucleus and production of nucleocapsids (Walker et al., 1982). Eventually the nucleus membrane ruptures and nucleocapsids move to the cell membrane, where they bud through and form BVs along the basal membrane. BVs pass through the basal lamina and enter the haemolymph. With the formation of BVs the secondary infection is commenced. BVs spread the infection to other tissues by penetration through the basal lamina, or via tracheal junctions. CpGV infects tracheal matrix directly by penetrating from the midgut cells into tracheoblasts (Federici, 1997).

CpGV replication first starts in the nucleus of all infected cells. Infected nuclei enlarge and finally the nuclear membrane ruptures. Nucleoplasm and cytoplasm become intermixed throughout the cell. Several virogenic stromata emerge as dense granular regions. These regions are connected to each other with nucleoprotein strands. Nucleocapsids begin to assemble, become enclosed with the envelope and accumulate outside the stroma. Late in infection the occlusion protein granulin is highly expressed and an OB embedding the virions is formed. In the end, the whole cell is filled with OBs. Eventually, the cells burst and release OBs (Federici, 1997). Many baculoviruses, including CpGV, express the enzymes chitinase and cathepsin (cystein protease), which are thought to facilitate the release of OBs from the insect cadaver. These enzymes degrade the chitin and protein in the insect cuticle late in infection (Cory & Meyers, 2003; Rohrmann, 2008).

1.2.6 SYMPTOMS OF CPGV INFECTION

CpGV is highly virulent against larvae of the codling moth. One single virus particle is able to kill a neonate larva. The median lethal dose (LD_{50}) for first instar larvae was determined by Steineke (2004) and ranged from 0.6 to 1.3 granules per larva. First signs of infection are paralysis and reduced activity. Healthy larvae show defence behaviour, like coiling up and giving off a defence secretion from the mouth parts, when they are touched, e. g. with forceps. In contrast, infected individuals react indolently and

lethargically upon touch. The bodies of infected larvae swell up and the surface becomes glossy (Figure 1.4 B). The slightly transparent cuticle of healthy larvae (Figure 1.4 A) changes to a milky appearance (Figure 1.4 B). Due to the degradation of the cuticle, the skin of killed larvae becomes fragile and easily bursts, releasing OBs to the environment ready to infect other codling moth larvae (Figure 1.4 C).

Figure 1.4 Healthy codling moth larva (L4) (A), larva infected with CpGV, showing typical symptoms (B) and larva killed by CpGV infection (C), where occlusion bodies are released from the liquefied body.

1.3 RESISTANCE

The development of resistance to pesticides is a great challenge to agriculture. The documented history of insecticide resistance began in 1914, when the first report of resistance was published. Melander (1914) described resistance of the San José scale (*Quadraspidiotus perniciosus* C.) to sulphur-lime. Today, there are more than 7,400 cases of resistance reported, involving 550 species (Onstad, 2008).

1.3.1 DEFINITION OF RESISTANCE

In 1957 resistance to insecticides was defined by the World Health Organisation (WHO): as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species". Furthermore, the WHO has set a standard of 10, which means, when a population requires 10times the amount of pesticide to kill 50% of a test population compared to a reference susceptible population, it is classified as resistant (Fleischer, 2004).

Since resistance to insecticides became more and more serious the Insecticide Resistance Action Committee (IRAC) was founded in 1984, and serves as a specialist technical group providing a coordinated industry response to prevent or delay the

development of resistance in insect and mite pests. IRAC defines resistance as follows: "A heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (www.irac-online.org/).

1.3.2 RESISTANCE TO BACULOVIRUSES

Insects have not only developed resistance to most chemical insecticide classes, but also to microbial control agents (Briese, 1986b). A pathogen and its insect host form a long-term co-evolving system with a high degree of variability at both sides. It is not surprising that insects have developed different methods to defend themselves against infection by a baculovirus over time. The application of baculoviruses in pest control, can lead to changes in the mean response of a population to it, similar to the selection of insects resistant to a chemical insecticide. But different to insecticide resistance, insects and viruses are in co-evolutionary relationship with each other. Therefore, virus genotypes overcoming host resistance are selected as well. Differences in susceptibility to baculoviruses have been identified in many host species (reviewed by Watanabe, 1987). Persistence of resistance is variable and some but not all mechanisms of resistance are costly (Rigby et al., 2002; Milks et al., 2002). There are quite a few cases of host resistance achieved by selection pressure under laboratory conditions. However, in many cases resistance decreased when exposure to the virus was discontinued (Briese, 1986a; Fuxa & Richter, 1989). Abot et al. (1996) reported the selection of resistance to *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) in strains of the velvet bean caterpillar, *A. gemmatalis,* originating from Brazil and the US. The Brazilian strain reached 1000-fold resistance after 13-15 generations, whereas the strain from the US did not exceed a resistance ratio of five. Without selection pressure resistance was quickly lost in the American strain because it was associated with reduced fertility (Fuxa & Richter, 1998). Milks (1998) also found a negative correlation between *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) resistance and fertility in *T. ni*.

Mechanisms of resistance to baculoviruses are diverse and can occur during the complete larval phase or restricted to certain larval instars. Kirkpatrick et al. (1998) observed that *H. virescens* larvae showed increasing resistance to fatal infections with age when they were infected orally with Autographa californica multiple nucleopolyhedrovirus (AcMNPV) OBs. First, it was assumed to be related to the increasing weight of the larvae. However, this effect could not be seen after intrahaemocoelic inoculation. Midgut cells undergo changes in composition metamorphosis to adult cell types. Thus, resistance could be caused by the different

physiology of the midgut cells. Another suggestion was that midgut cells are sloughed off at higher rates in later larval instars (Washburn et al. 1998).

The midgut seems to be the major barrier to baculovirus infection. The PM lines midgut lumen and serves as a physical fence protecting the midgut epithelial cells from the invasion of pathogens. Levy et al. (2007) observed reduced susceptibility of *A. gemmatalis* larvae to AgMNPV correlated with increasing thickness of the peritrophic membrane. After the virions have passed the peritrophic membrane primary infection starts with virions that infect midgut cells. Here, different resistance mechanisms can occur. In *Archips argyrospila*, the fruittree leafroller, the replication of GV is blocked in midgut epithelial cells (Pinnock & Hess, 1977). Haas-Stapleton et al. (2005) demonstrated that the oral infectivity of a virus could be influenced by the ability of the ODV to bind to the midgut of insects. They observed a reduced affinity of AcMNPV ODV to midgut cells of *Spodoptera frugiperda* compared to ODV of *Spodoptera frugiperda* (Sf) MNPV suggesting that SfMNPV might bind to a different receptor than AcMNPV.

The immune system also plays an important role in insect defence against virus infection. Although insects lack an adaptive immune system similar to vertebrates, insects have evolved innate defence reactions that are partially shared with higher organisms (Tzou et al., 2002; Hultmark, 2003; Hoffmann, 2003; Brennan & Anderson, 2004). Both, cellular and humoral mechanisms of immunity are involved in resistance to baculoviruses. Haemocytes are highly specialized cells circulating in the haemolymph. They combat virus invasion by phagocytosis, encapsulation and melanisation. *Helicoverpa zea* larvae are not susceptible to AcMNPV. Using a recombinant of AcMNPV containing the LacZ reporter gene it was observed that *H. zea* cells were susceptible to infection by AcMNPV but infected cells were encapsulated by haemocytes and further spread of infection was blocked (Washburn et al., 1996). The plasma enzyme phenoloxidase catalyzes oxidation of phenols like tyrosine into melanin and reactive oxygen intermediates that can directly be toxic to microbial pathogens. In *Heliothis virescens* the products of the enzyme phenoloxidase, melanin and superoxide, contribute to the virucidal activity against baculovirus infection. Elevated phenoloxidase activity is associated with melanisation not only in the haemolymph but also in the cuticle and the midgut (Shelby & Popham, 2006). Melanin is toxic to microorganisms and shows antiviral activity because it binds to many proteins (Montefiori & Zhou, 1991; Ourth & Renis, 1993; Sidibe et al., 1996). In many organisms activation of the Toll pathway, is needed to start innate immune reactions. Members of the Toll-like receptor family are described as sensors that recognize viral components like nucleic acids activating an immune response (Kawai & Akira, 2006).

The host range of baculovirus species differs in size: some baculoviruses replicate in only a very limited number of insect species others are known to replicate in numerous species. AcMNPV is not able to infect cells of the gypsy moth *Lymantria dispar*. The virus can enter the cell and viral DNA is synthesized, but late in infection protein synthesis is shut off. As a result, no viral progeny is produced (Guzo, 1992). Co-infection of a *L. dispar* cell line together with *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) resulted in successful replication of AcMNPV. The gene responsible for the promotion of AcMNPV replication, called host range factor 1 (*hrf-1*) was identified. A recombinant AcMNPV containing *hrf-1* of LdMNPV was able to infect the gypsy moth (Du & Thiem, 1997). Host range factors like *hrf-1* are essential for the infection of a host. A mutation of this gene could result in resistance of the host to the virus.

1.3.3 OCCURRENCE OF CPGV RESISTANT CODLING MOTH POPULATIONS IN EUROPE

The first reported case of codling moth resistant to a chemical was resistance to arsenates in the USA in 1928 (Hough, 1928). Since then cases of resistance to chemical insecticides in codling moth occurred in all apple growing regions worldwide and to several classes of insecticides used against this pest (Sauphanor et al., 1999). In contrast there are only a few cases of resistance to baculoviruses used in pest control (Moscardi, 1999; Briese, 1986a; Fuxa, 1993). Because of the biological characteristics of baculoviruses, development of resistance was assumed to be rather unlikely (Granados & Federici, 1986). Resistance of the codling moth against CpGV was the first case of documented field resistance against a baculovirus biocontrol product.

Beginning the 2000s some organic apple growers in south-west Germany first noticed that CpGV treatments failed to control the codling moth in their orchards. In 2003 diapausing codling moth larvae were collected in these orchards and reared to adulthood. Susceptibility of the offspring was tested in the following year. Surprisingly, two of the three populations were more than 1000fold less susceptible than a susceptible laboratory strain used as a reference (Fritsch et al., 2005). A second collection of diapausing larvae in autumn 2004 with adjacent susceptibility tests in 2005 confirmed these results and discovered two more resistant populations at different locations (Fritsch et al., 2006). In France collections of overwintering larvae were performed in autumn 2004 and tested in 2005. This study revealed that there were CpGV resistant populations existing also in France (Sauphanor et al., 2006). The emergence of resistance against CpGV products was a severe threat for the apple growers, especially in organic apple production where growers highly depend on CpGV. Because there was an urgent need to find solutions for this problem, investigations to understand the

phenomenon of CpGV resistance and to develop resistance monitoring methods and resistance management strategies were started.

1.4 AIMS OF THIS THESIS

The goals of resistance management are to avoid resistance, slow the rate of resistance development, and cause resistant populations to revert to more susceptible populations. But the success of a certain resistance management strategy depends on whether it fits to the kind of resistance that is present in the insect. For example the saturation strategy is used to prevent selection by making sure even resistant individuals are killed by very high doses, and sometimes adding synergists to block detoxification (Fleischer, 2004). But this implies that resistance is dose dependent and there is a dose of insecticide able to eradicate resistant insects. Otherwise, resistant individuals are selected and frequency of resistance alleles increases extremely fast. The multiple attack strategy takes aim of the use of products with different modes of action with rotations or tank mixtures (Fleischer, 2004; Onstad, 2008). Resistant individuals surviving the first pesticide application are killed with the second compound. This strategy requires that there is no cross resistance between these compounds. The principle of the refuge strategy is to maintain untreated areas where susceptible individuals are preserved (Fleischer, 2004). These individuals mate with resistant ones in order to maintain the susceptible allele in the population. However, this strategy does only work satisfactorily if the resistance gene is recessive and if there is a high rate of immigration of insects. Another strategy is to kill all heterozygotes. Here, the insecticide concentration or the efficacy of the treatment is increased in order to kill all heterozygous resistant insects of the population. This lowers the dominance of the resistant individuals relative to susceptibles. This strategy requires a low frequency of resistant heterozygotes in the population and a refuge for susceptibles to prevent evolution of resistance by promoting the mating of homozygous susceptibles with any rare homozygous resistant individuals (Onstad, 2008). In order to develop an adequate resistance management strategy and to predict the long-term effectiveness of a strategy, it is important to know the mode of inheritance and the mechanism of resistance.

Accordingly this work was focused on two main topics: First, how is CpGV resistance inherited? Is inheritance of resistance autosomal or linked to a sex chromosome? Is resistance inherited by a single gene (monogenic) or more genes (polygenic)? Is resistance recessive, dominant or intermediate inherited? And second, what is the mechanism of resistance? Is resistance restricted to the midgut or is it also present in secondary infection? Is resistance present in all five larval stages of the codling moth? Is resistance caused by an immune factor? What is the genetic basis of resistance? Does

cross resistance with *Bacillus thuringiensis* toxin exist? Are there other CpGV isolates that are able to overcome resistance?

Three years and eight moths of intensive work have passed since I have started my Ph.D. studies in order to explore the unknown territory of CpGV resistance and to find answers to all these questions. The experimental approach and the results of this research are described in this thesis.

Chapter 2 describes the elucidation of the mode of inheritance of CpGV resistance. Crossing experiments between a susceptible and a homogeneously resistant codling moth strain (CpRR1) revealed that resistance is inherited by a single, concentration dependent dominant gene located on the Z-chromosome.

Since in chapter 2 the mode of inheritance was identified using the homogeneous resistant codling moth strain CpRR1 that was generated by inbreeding, **chapter 3** addresses the question whether a field collected heterogeneous resistant codling moth strain (CpR) shares the same mode of inheritance. Chapter 3 also discusses the advantages of single pair crossings compared to mass crossings in terms of investigation of resistance inheritance.

In **Chapter 4** it is shown that resistance is not dependent to a specific larval instar and that a new virus isolate originating from Iran (CpGV-I12) is able to overcome resistance in all larval instars of the codling moth. CpGV-I12 is a promising alternative control agent of *C. pomonella* in orchards where conventional CpGV products fail. Furthermore it is demonstrated in bioassays with recombinantly expressed *Bacillus thuringiensis* toxin Cry1Ab that cross resistance to CpGV and *Bt* products is not likely.

Chapter 5 is focused on the analysis of the mechanism underlying CpGV resistance. Involvement of the PM was excluded using bioassays with a fluorescent brightener. Via intra-haemocoelar infection it is shown that resistance is not restricted to the midgut but also present in secondary infection. Virus replication was observed in different tissues via quantitative PCR. CpGV caused mortality was analysed after transfusion of haemolymph between resistant (CpRR1) and susceptible (CpS) codling moth larvae to determine the possible action of a humoral factor involved in CpGV resistance. However, haemolymph of CpRR1 did not convey resistance to CpS larvae. The results show that virus replication is blocked in the midgut, the haemolymph and the fat body at a very early stage of infection.

In **chapter 6** the findings of the previous chapters were combined and an approach to identify the resistance gene was started. Using the yeast two-hybrid technique, protein-protein interactions between the resistance overcoming virus isolate CpGV-I12 and the

resistant codling moth strain CpRR1 was investigated. This chapter should be considered as a first step towards the final identification of the resistance gene and a starting point for prospective research on this topic.

Chapter 7 is a general discussion of the research presented in this thesis and connects all results for general conclusions. Moreover, it provides an outlook on the use of CpGV products in the future preventing the spread of resistance and to control CM populations already resistant to CpGV-M.

2 RESISTANCE OF CODLING MOTH AGAINST *CYDIA POMONELLA* GRANULOVIRUS (CPGV) IS INHERITED BY A SINGLE, DOMINANT GENE LOCATED ON THE Z-CHROMOSOME

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ABSTRACT

Insect-specific baculoviruses are increasingly used as biological control agents of lepidopteran pests in agriculture and forestry, and they have been previously regarded as robust to resistance development by insects. However, in more than a dozen cases of field resistance of the codling moth *Cydia pomonella* to commercially applied *C. pomonella* granulovirus (CpGV) in German orchards, resistance ratios exceed 1000. The rapid emergence of resistance is facilitated by sex-linkage and concentration dependent dominance of the major resistance gene and genetic uniformity of the virus. When the gene is fixed, resistance levels approach 100,000 fold. Our findings highlight the need for development of resistance management strategies of baculoviruses.

2.1 INTRODUCTION

More than 2500 cases of insect resistance to chemical pesticides have been documented over the past 50 years (Mota-Sanchez et al., 2002; Mallet, 1989). In contrast, baculoviruses have been used to control lepidopteran pests on 2 to 3 million hectares

[1] **Author contributions:** J.K. managed and performed the collection of diapausing larvae from the orchards. E.F., K.U-S. and J.H. conducted bioassays with the field populations and determined the LC_{50} values. N.A.G., A.R., D.G.H., and C.P.W.Z. were responsible for W-Body visualisation and W-specific PCR. S.A.K. generated the homogeneous strain CpRR1 by inbreeding the field strain CpR, performed all crossing experiments, susceptibility tests with parental strains, F1 generations and backcross generations, morphological sex determination of surviving individuals and data analyses. S.A.K, J.A.J and D.G.H. wrote the manuscript.

per year worldwide, with high specificity and low environmental impact and with only sporadic and anecdotal reports of resistance (Moscardi, 1999; Briese, 1986a; Fuxa, 1993). Baculovirus virions are protected by a proteinaceous occlusion body (OB) that confers environmental stability and allows field application with conventional insecticide sprayers. Their narrow host range and high virulence make them one of the most selective pest control agents, widely used for the control of many agricultural and forestry pests including codling moth (*Cydia pomonella*, L., Lepidoptera: *Tortricidae*) (Hunter-Fujita et al., 1998). One of the most successful commercial baculovirus insecticides is the *C. pomonella* granulovirus (CpGV), now a cornerstone of codling moth control in both organic and integrated apple production. In many countries CpGV products are among the very few control measures for this pest allowed in organic apple production. Without control, codling moth infestations can result in severe damage and complete loss of marketable fruits. In the last three years, enigmatic field observations in organic apple orchards in Germany and France suggested that CpGV was failing to control apple damage (Fritsch et al., 2005; Sauphanor et al., 2006). A spread of this phenomenon is a severe threat to the efficient control of the codling moth, particularly in organic farming. In order to prevent this development, investigations on the population genetics and the mode of inheritance of resistance against CpGV of codling moth populations in Germany were initiated. Here we report resistance levels of several codling moth populations in Germany and the elucidation of the mode of inheritance using single pair crosses. These investigations will give information about velocity of spread of resistance and can help to develop new control strategies or to restore high susceptibility towards CpGV.

2.2 MATERIAL AND METHODS

2.2.1 VIRUS

The CpGV isolate used in the bioassays is a descendent from the CpGV collected in Northern Mexico (“Mexican strain”) (Tanada, 1964). It was propagated in host insects and purified by the method described by Huber (1981). The virus occlusion bodies (OBs) were enumerated with a Petroff-Hauser counting chamber (depth 0.02 mm) using dark field optics of a light microscope (Leica DMRBE).

2.2.2 TEST INSECTS

For resistance testing, wild codling moth larvae were collected in autumn using corrugated cardboard bands placed around the trunk of apple trees in the respective orchards and maintained in diapause in frost-free room during winter. In spring they

were kept on a sheltered balcony for pupation and adult emergence, which started at the beginning of May. The moths were transferred to rearing containers at room temperature and under natural light conditions for copulation and oviposition. Populations were collected in 2003-2005 and tested in 2004-2006.

Larvae of a susceptible strain ("CpS DA") of the codling moth served as a standard in the bioassays. This strain was established more than thirty years ago at the institute for Biological Control of the Julius Kühn-Institute (JKI) in Darmstadt. The rearing method has been described by Bathon (1981).

All insects used in crossing experiments were reared at the Agricultural Service Centre Palatinate (DLR Rheinpfalz), in Neustadt/Weinstraße. The CpGV susceptible codling moth strain (CpS) had been reared for nine years, whereas the resistant strain CpR was reared since 2004. It dates back to a population originally collected in 2003 in an organic orchard in South Baden, Germany. It was designated BW FI 03 and is identical to the resistant strain described by Fritsch et al. (2005), called "Suedbaden". Since the original collection, the CpR strain has been reared without selection by CpGV.

2.2.3 SINGLE PAIR CROSSES

Pupae of the different *C. pomonella* strains were sorted according to sex before eclosion. The sex of pupae was determined by their number of abdominal segments. The single pair crosses were performed in small plastic cylinders lined with foil for egg deposition. Round filter papers were placed into the cylinder and incubated at 26°C, 60% relative humidity and 16/8 hours light/dark photoperiod. As soon as the adult moths emerged, a piece of cotton wool soaked with water was added to the cylinder. After mating, females deposited eggs on the foil. The eggs were collected every two days by replacing the foil and were stored at 8-10°C in the refrigerator. From each pair 60-100 eggs could be collected. A family is defined as the offspring of such a single pair. After oviposition the moths were frozen at –80°C and the eggs were incubated in a climate chamber at 26°C, 60% relative humidity and 16/18 hours light/dark photoperiod. The susceptibility of freshly hatched neonates to CpGV was determined using a bioassay.

2.2.4 HOMOGENISATION OF CPR

Eleven single pair crosses between CpS males and CpR females were performed and the mortality of the progeny first instar larvae was determined in a 7 day bioassay using the discriminating virus concentration of 5.8×10^4 CpGV OB/ml. As mortality data among the families were very variable and reached up to 100% it was suggested that CpR

contained susceptible and resistant individuals. In order to eliminate the susceptible individuals from CpR, 20 single pair crossings of CpR females and CpR males were performed. Half of the progeny of each single pair family was subjected to susceptibility testing as noted above; the other half was used as a control. The families showed between 0-100% mortality in the bioassays. The control larvae of six families with 0% mortality (F1) were further used for single pair crossings within each family. After this second round of inbreeding half of the progeny of each family was tested for resistance and control insects of those 10 families with 0% mortality (F2) in the bioassay (251 individuals) were pooled to build up the homogeneous strain termed CpRR1.

2.2.5 BIOASSAYS

The bioassays for testing the offspring of field collected codling moth populations were conducted following the method described by Huber (1981). The virus was incorporated into an artificial diet by thorough mixing of CpGV suspensions of different concentrations (from 100 to 10^6 granules per ml of diet) with codling moth diet (Ivaldi-Sender, 1974). The mixtures were dispensed into special boxes (LICEFA, Bad-Salzuflen, Germany) with 50 separate cells (1.5 x 1.5 x 2 cm). One neonate larva was placed in each cell. The boxes were covered with a layer of tissue paper, a polyethylene sheet, and a hard plastic cover, and fixed with two rubber bands. The boxes were incubated at 26°C, 60-70% relative humidity with an 18 hours light/dark photoperiod. Larvae were examined for virus mortality 14 days after the start of the bioassay. Survivorship data were corrected for control mortality using Abbott's formula (Abbott, 1925). The virus concentration-mortality response was analysed using probit analysis based on a maximum likelihood procedure (MLP 3.08, NAG, Lawes Agricultural Trust, Rothamsted Experimental Station, 1987) in order to obtain LC_{50} values and other parameters of the dose mortality response.

Bioassays for analysing the susceptibility of CpS, CpRR1, F1a and the different backcrosses were performed according to the same method using a virus concentration of 5.8 x 10^4 CpGV OB/ml. These assays were scored at 7 days. The virus concentration –mortality response of CpS, CpRR1 and F1b was measured at 3 x1 0^2 – 1 x 10^6 OB/ml using the method described above. The surviving larvae at day 1 were considered as starting cohort. Mortality was scored at day 7 and day 14. After 14 days, the bioassay plates were placed in plastic boxes with corrugated cardboard for pupation. At day 21, the number of pupae was determined and the sex of the pupae and remaining larvae was determined. The bioassay data were corrected for control mortality using Abbott's formula (Abbott, 1925). The numbers of surviving larvae at day 7, day 14 and the

number of pupae at day 21 were subjected to the probit analysis procedure in ToxRat Standard Version 2.09 (ToxRat Solutions GmbH, 2005).

2.2.6 DETERMINATION OF THE SEX OF INDIVIDUALS

The method used depended on the life stage and condition of preservation of the individuals. The morphological methods are most reliable on living individuals. When larvae had already been completely homogenised for DNA extraction for linkage studies currently in progress, only the PCR method could be used. Both the W-body visualisation and W-specific PCR were performed on 97 frozen larvae as a cross-check of methods; the results agreed for all but 5 in which the PCR method clearly showed a W-specific product, but not enough cells could be examined microscopically to the W-body. These were classified as females.

2.2.6.1 MORPHOLOGICAL SEX DETERMINATION

The sex of pupae was determined under the binocular microscope according to the number of their abdominal segments; male larvae were identified by testes visible through the dorsal integument (Fuková et al, 2009).

2.2.6.2 W-BODY VISUALISATION

The posterior half of a frozen codling moth larva was excised and directly stained with 1.5% lactic acid orcein for 30-50 minutes. Highly polyploidy interphase nuclei in these cells were inspected under a light microscope at a magnification of 100x for the presence of sex chromatin. A female nucleus shows a deeply stained heterochromatic W-body whereas males have no W body because of the ZZ (male)/ZW (female) sex chromosome mechanism in the order *Lepidoptera* (Traut & Marec, 1996). Preparations were observed in a Zeiss Axiovert 200 microscope (Carl Zeiss Jena, Germany) at 100x. Black and white images were recorded with an Axio Cam MRc and captured with Axio Versin AC software.

2.2.6.3 W-SPECIFIC PCR

DNA was isolated from the anterior half of the frozen codling moth larva following a CTAB-based method (Reineke et al., 1998). PCR was conducted using primers CpW5-F1 (5'-GATTG GAATT TCTTT CTGGT G) and CpW5-R2 (5'-TCAGG AAGTA ACCGC AACC) designed to amplify the sex chromosome specific sequence CpW5 (Fuková et al., 2007). PCR reactions consisted of 20 ng template DNA, 10 pmol each forward and reverse primer, 0.2 mM each dNTP and MgCl, and 0.4 U Taq Polymerase (Metabion). Thermocycler conditions were 94°C for 4 min, followed by 30 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for

4 min. Products were electrophoresed on a 1% agarose gel. Female DNA produced an intense band of 92 bp and male DNA produced a very faint band of the same size.

2.3 RESULTS AND DISCUSSION

In 2003 to 2006, we undertook a systematic survey of 13 organic orchards in southern Germany where the efficacy of CpGV had been reported as unsatisfactory. Concentration-mortality relations were determined in 14-day bioassays to estimate the median lethal concentrations (LC_{50}) of the commonly used Mexican isolate of CpGV (Tanada, 1964; Huber, 1981).

Compared with susceptible laboratory and field populations with LC_{50} values of ~170 to ~970 OB/ml of artificial diet, most field populations showed resistance ratios of ~1000, and two even exceeded 10,000-fold resistance levels (Figure 2.1). One of the resistant populations collected in 2003 (BW FI 03) was used to establish the CpR strain, which was reared in the laboratory without selection by virus. It maintained a stable ~100-fold resistance level over many generations, compared with the susceptible strain CpS.

Mass crossing experiments between CpR and CpS had suggested an autosomal (i.e. not sex-linked), incompletely dominant inheritance of resistance (Eberle & Jehle, 2006). However, single pair crosses between CpR and CpS yielded heterogeneous bioassay results, indicating that CpR still contained some susceptible individuals. To produce a genetically homogeneous strain for inheritance studies, offspring of single pair CpR crossings (i.e. families) were screened with a discriminating CpGV concentration of 5.8×10^4 OB/ml. This concentration caused 95 to 98% mortality of CpS and <30% mortality (on average) of CpR in 7-day bioassays; however, single pair CpR families showed a wide range of mortalities. Progeny from only those families with 0% mortalities were selected and intercrossed in single pairs. After a second round of this screening process, progeny from the six single pair families with 0% mortality were used to start the homogenised CpRR1 strain.

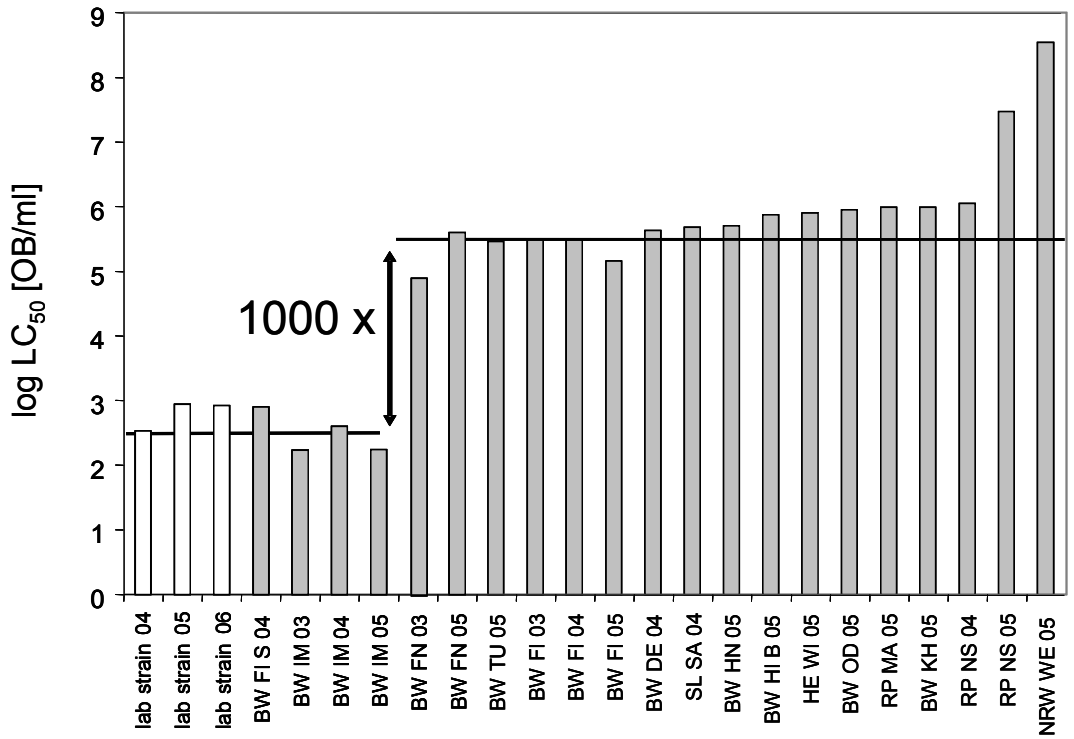

Figure 2.1 CpGV-susceptibility levels of codling moth populations from German orchards in 2003-05. The LC_{50}, in numbers of OBs per 1 ml of artificial diet, was estimated by probit analysis from 14-day mortality bioassays with neonate larvae. The resistance ratio is defined as the ratio of LC_{50} values of resistant and susceptible strains. Gray columns represent orchard-collected strains and white columns, the susceptible laboratory strain (CpS DA). The last two letters indicate the year of testing (CpS DA) or sampling (field populations). Laboratory reared progeny from the field populations were tested the year after sampling.

Inheritance of resistance in the CpRR1 strain was then investigated using single pair crosses with CpS. When F_1 offspring of 10 single pair crosses (F1a) between CpS males and CpRR1 females were tested at the discriminating concentration, 57% survived the 7-day bioassay (Table 2.1). These findings were consistent with the autosomal semidominant CpGV resistance previously reported for CpR (Eberle & Jehle, 2006), albeit at a higher resistance level. However, additional crosses combined with longer and more detailed bioassays led to a different conclusion. If resistance were determined by autosomal genes, then reciprocal single-pair backcrosses to the parental strains should yield similar results, independent of the sex of the F_1 parent. As shown in Table 2.1, this was not the case, and survivorship in the 7-day bioassay differed significantly from the hypothesis of autosomal inheritance (χ^2 = 343.7, df = 4; $P < 0.0001$). In *C. pomonella* as well as most Lepidoptera, the sex chromosomes are ZZ in males and ZW in females (Fukova et al., 2005). Figure 2.2 compares crossing schemes applied in this

study and the expected genotypes under the hypothesis of either Z-linked or autosomal inheritance of resistance. The bioassay data are consistent with a dominant resistance gene linked to the Z chromosome enabling 100% survivorship of either heterozygous Z^RZ^S or homozygous resistant Z^RZ^R males as well as Z^RW females ($\chi^2 = 1.6$, df = 4; $P > 0.5$). Z^SZ^S males and Z^SW females would experience nearly 100% mortality in this bioassay, consistent with the behaviour of CpS (Table 2.1).

Table 2.1 Survivorship at day 7 and pupation at day 21 in crosses with CpGV-susceptible (CpS) and resistant (CpRR1) strains of codling moth, exposed to a discriminating concentration of 5.8 10^4 OB/ml as neonates. BC, back cross; f, female; m, male. Progeny genotypes are shown for two hypotheses: (Z), a single Z-linked resistance gene, or (A), a single autosomal resistance gen. R and S denote the resistant and susceptible alleles, respectively. F/n indicates the total number of families (F) and neonates (n) tested. [Exp (Z)] is the expected fraction of survivors, under the hypothesis of a single Z-linked resistance gene, with Z^R fully dominant to Z^S. [Exp (A)] is the expected fraction of survivors, under the hypothesis of a single autosomal resistance gene, with the degree of dominance of A^R estimated from the response of F1a survivorship. At 21 days, "males" refers to the fraction of males among living pupae. The chi-square value applies to the goodness-of-fit test of the observed 7-day survivorships in the four backcrosses to the expected values und the Z-linkage (Z) or autosomal (A) hypothesis. *N.O., Sex ratio not observed because there were no living pupae at 21 days in this group.

Strain or cross	Progeny genotypes, by hypothesis		F/n	Fraction of survivors, day 7			Pupation, day 21	
	(Z)	(A)		Observed (SD)	Exp (Z)	Exp (A)	Pupated	Males
CpS	Z^SW, Z^SZ^S	A^SA^S	–/95	0.02 (N.O.)	0.00	0.02	0.00	(N.O.)*
CpRR1	Z^RW, Z^RZ^R	A^RA^R	10/251	1.00 (0.000)	1.00	1.00	0.97	0.56
F1a: CpSm × CpRR1f	Z^SW, Z^RZ^S	A^RA^S	10/394	0.57 (0.092)	0.50	0.57	0.09	1.00
BC1: F_1f × CpRR1m	Z^RW, Z^RZ^S	A^RA^S, A^RA^R	8/369	1.00 (0.008)	1.00	0.79	0.56	0.08
BC2: F_1m × CpRR1f	Z^SW, Z^RW, Z^RZ^S, Z^RZ^R	A^RA^S, A^RA^R	7/270	0.73 (0.103)	0.75	0.79	0.44	0.58
BC3: F_1f × CpSm	Z^SW, Z^SZ^S	A^SA^S, A^RA^S	10/458	0.00 (0.000)	0.00	0.30	0.00	(N.O.)*
BC4: F_1m × CpSf	Z^SW, Z^RW, Z^SZ^S, Z^RZ^S	A^SA^S, A^RA^S	7/308	0.47 (0.057)	0.50	0.30	0.25	0.09
χ^2, BC1-4					1.6	343.7		

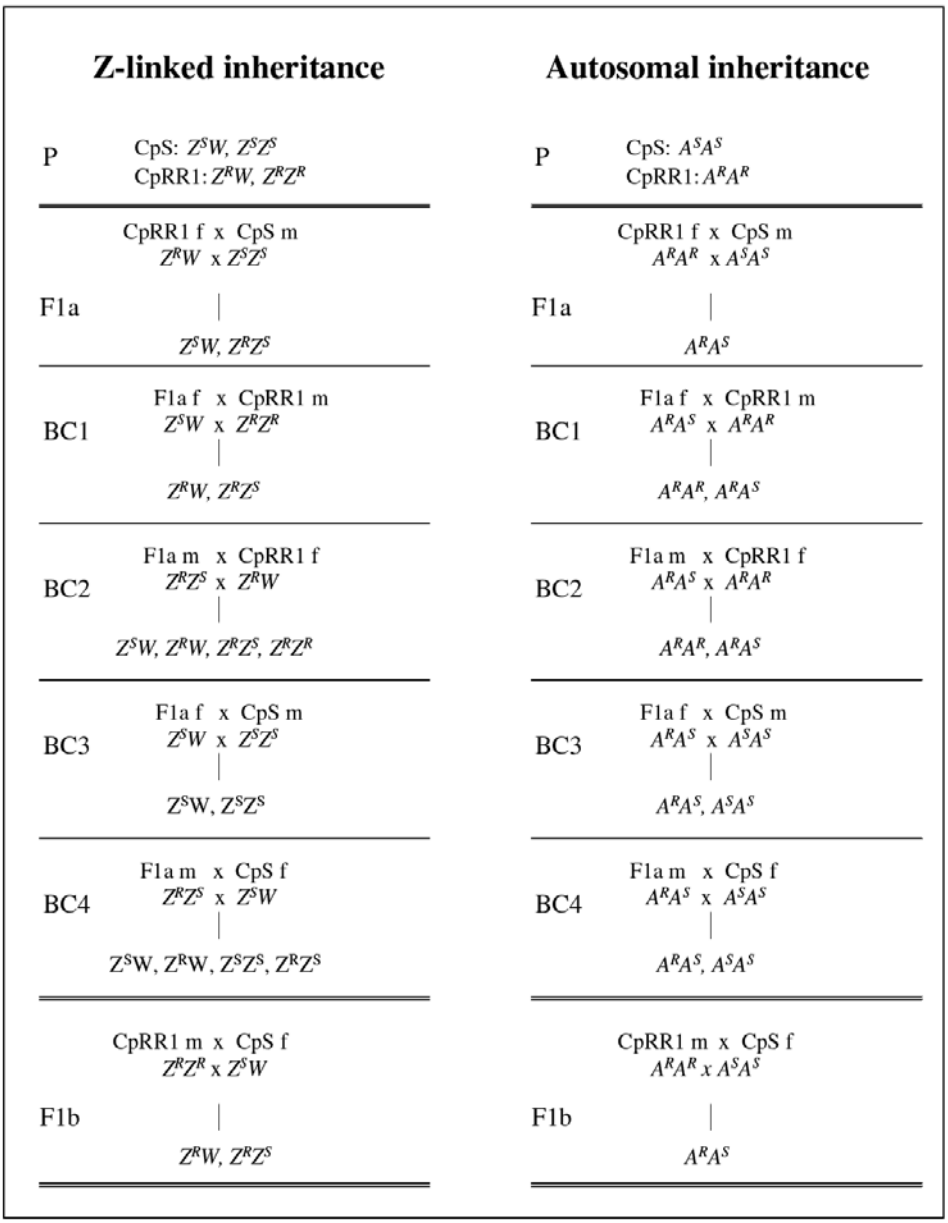

Figure 2.2 Crossing schemes of the F1a, back crosses BC1-BC4, and F1b crossings. Given are the crossings and expected genotypes under the hypothesis of Z-linked and autosomal inheritance. The suffixes f and m to the strains indicate female and male individuals used in the crossings.

The same bioassays were observed until day 21, when untreated control larvae and most of the treated CpRR1 larvae would have pupated. By this time, some crosses had many surviving larvae that had stopped feeding but not yet pupated; these died a few days later, still in the larval stage. By using morphological and molecular methods, most of these nonpupating larvae were determined to be males. The pupation success and sex ratio (Table 2.1) were also consistent with a Z-linked resistance gene, with the additional property that most of the Z^RW females and homozygous Z^RZ^R males successfully pupated, whereas heterozygous Z^RZ^S males surviving day 7 subsequently died or failed to pupate and never became adults. Thus, with respect to adult fitness at the discriminating concentration of 5.8×10^4 OB/ml, the Z^R allele is recessive, because males need two copies to survive to adulthood.

To explore the quantitative response of Z^RZ^S males to virus exposure, single pair crosses (F1b) between CpS females and CpRR1 males were made, and bioassays were conducted at eight different virus concentrations. F1b progeny were expected to consist of only Z^RZ^S males and Z^RW females in equal proportions at hatching, thus differing from CpRR1 only in the presence of Z^RZ^S instead of Z^RZ^R (Figure 2.2). Mortality was scored at days 7 and 14, and the fraction of treated individuals that had pupated was scored at day 21 (Figure 2.3).

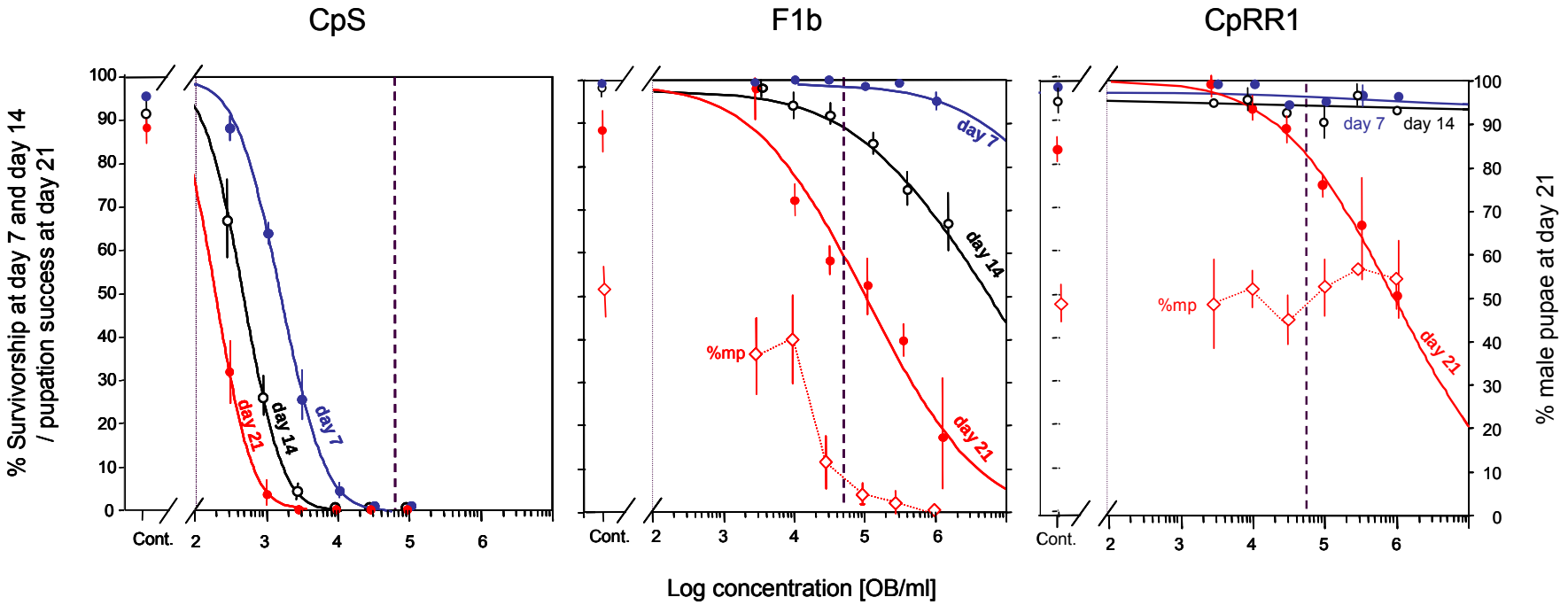

Figure 2.3 Concentration-mortality responses of neonate codling moth larvae of CpS, CpRR1 and F1b (CpS females x CpRR1 males) exposed to different concentrations of CpGV. The survivorship of larvae (day 7, day 14) and the effect on pupal development (day 21) were analysed using Probit analysis. The percentage of male pupae (%mp) was scored at day 21. On the left side of the graphs, the survivorships in the respective untreated controls (Cont.) are given. The dashed line marks the discriminating virus concentration applied to neonates in single pair crossings (see Table 2.1).

CpS survivorship showed a strong virus concentration-mortality response, with LC_{50} values of 1425 and 501 OB/ml at days 7 and 14, respectively. CpRR1 survivorship was nearly 100% at all concentrations up to day 14, so the LC_{50} values could not be estimated. The LC_{50} based resistance ratio of CpRR1 to CpS is likely to exceed 10^4 to 10^5. Further, a concentration-dependent decrease in successful pupation at day 21 was evident for CpRR1. The fraction of males among pupae fluctuated around 50%; thus, pupation success was dose-dependent but sex-independent in this strain. Mortality of F1b was very low by day 7, which confirmed the dominant action of Z^R in conferring early survivorship at virus concentrations that kill all Z^SZ^S males. The observed mortality on day 14 with increasing virus concentrations can be exclusively attributed to Z^RZ^S males, because Z^RW females survive all virus concentrations applied in the bioassays (compare CpRR1). Even more strikingly, the fraction of males among day 21 pupae decreased rapidly with increasing virus concentration as Z^RZ^S males died or remained living but did not pupate, rendering them effectively "genetically dead" as they did not develop to adulthood.

Thus, the surprisingly rapid emergence of CpGV in orchard populations of codling moth can be explained by the interaction of three factors: sex-linkage, concentration dependent dominance, and uniformity of the selective agent. First, the major resistance gene is Z-linked, and therefore, Z^RW females require only one copy of the resistance allele to survive virus exposure; this enables a faster initial selection response than the case of autosomally inherited resistance. Second, resistance measured as the probability of successful pupation after virus exposure in heterozygous Z^RZ^S males is dominant at low concentrations (further promoting a rapid initial selection response), but recessive at high virus concentrations. Hence, when Z^R occurs at al low frequency in an orchard population, we predict that it would be effectively selected for, even by low virus concentrations, both in Z^RW females and Z^RZ^S males as a dominant gene. When the resistance allele frequency increases and virus applications are also increased by growers to compensate for control failures, the Z^RZ^R males enjoy an advantage over Z^RZ^S males and Z^R would continue to increase in frequency, because it is selected for as a recessive in males. Third, all commercially available products in Europe contain the same CpGV isolate, which has high genetic homogeneity (Crook et al., 1997). Because organic apple growers rely heavily on CpGV and apply it repeatedly in each growing season, most of the organic orchards are continuously exposed to this virus isolate. Moreover, each OB of CpGV contains a single virion, in contrast to nucleopolyhedroviruses with up to several hundred virions per OB (Theilmann et al., 2005). The potential resistance delaying of a mixture of virus genotypes in a single infection would thus be much weaker for CpGV.

The aim of insecticide-resistance management is to prevent or delay the selection of resistance by controlling the factors affecting allele frequencies in field populations. Our results make clear that this area of applied evolutionary biology is also highly relevant to the application of baculoviruses as biological control agents. Implementation of resistance monitoring and resistance management will be needed in order to sustain the ecological and economic benefits of this environmentally friendly class of biological insecticides.

3 SEX LINKAGE OF CPGV RESISTANCE IN A HETEROGENEOUS FIELD STRAIN OF THE CODLING MOTH *CYDIA POMONELLA* (L.)

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ABSTRACT

The occurrence of codling moth populations in European apple orchards that were not controlled by *Cydia pomonella* granulovirus (CpGV) is the first reported case of field resistance against a baculovirus control agent. A monogenic, dominant sex-linked mode of inheritance was previously demonstrated in single pair crosses between a homogeneous resistant (CpRR1) and a susceptible (CpS) laboratory strain of codling moth. However, resistant field populations (CpR) are more heterogeneous in their levels of resistance, and the possibility that they could harbor different resistance genes to CpRR1 had not been directly addressed. Here we report single pair crossing experiments using a resistant codling moth strain collected from an apple orchard in the southwest of Germany. Single pair crosses within the field strain revealed a genetic basis to the heterogeneity of CpR concerning CpGV resistance. Hybrid crosses to a susceptible laboratory strain and backcrosses of the F1 generation to the resistant CpR strain confirmed that the homogenous strain CpRR1 and the heterogeneous field strain CpR share the same mode of inheritance. Thus the variable levels of CpGV resistance in field populations is likely due to frequency differences of the same resistance-conferring gene, rather than different genes, which will facilitate future efforts to monitor and manage resistance.

3.1 INTRODUCTION

In apple orchards, most methods of control are applied to control the codling moth *Cydia pomonella* L.. The larvae of this worldwide pest infest mostly apples, but also pears, apricots and walnuts. Without control it can cause very high yield losses of more than 95%. Along with chemical insecticides, pheromone based mating disruption and

Bacillus thuringiensis sprays, preparations of *Cydia pomonella* granulovirus (CpGV) [family *Baculoviridae*] are used to control the codling moth (Cross et al., 1999). In organic farming CpGV products are the most important agents for the control of codling moth. This highly specific and extremely virulent virus was originally discovered by Tanada (1964) in northern Mexico and has successfully been used in European fruit production since the late 1980s (Huber 1998; Fritsch et al., 2005). All commercially available CpGV products in Europe contain the same CpGV isolate (CpGV-M) that originated from Mexico (Eberle et al., 2008). Since 2003, in some organic orchards in the south west of Germany and France, CpGV products failed to control codling moth infestation (Fritsch et al., 2005; Sauphanor et al., 2006). In 2005 and 2006, susceptibility tests were performed on 21 field populations from orchards in southern Germany, where farmers reported high infestation rates despite intense CpGV treatment. 15 populations showed a resistance ratio of about 1000 and two populations even exceeded a value of 10,000 (Asser-Kaiser et al., 2007). Spread of this phenomenon is a severe threat to the efficient control of the codling moth, particularly in organic farming.

Knowledge of the mode of inheritance of CpGV resistance is extremely important for resistance management as it is needed for accurate prediction of the temporal and spatial spread of resistance. Mass-crossing experiments between a susceptible laboratory codling moth strain (CpS) and a field collected codling moth strain, termed CpR, which showed a 100-fold reduced susceptibility against CpGV suggested an autosomal, incomplete dominant inheritance (Eberle & Jehle, 2006). Conversely, single pair crosses between the susceptible laboratory strain and a genetically homogeneous strain originating from CpR, termed CpRR1, showed that resistance is based on a single, dominant gene that is located on the Z-chromosome (Asser-Kaiser et al., 2007). These conflicting results raised the question of whether the genetic basis of CpGV resistance was different in the two experiments, perhaps because an atypical type of resistance was selected for in the development of the CpRR1 strain. Alternatively, the genetic basis of resistance in CpR and CpRR1 strains is the same, but failure to account for heterogeneity in resistance allele frequency in the CpR strain could explain the results of the first experiment. Since strains collected from different orchards show different levels of resistance, it is important to determine whether the same genetic mechanism exists in all of them. The aim of this work was (i) to test whether sex linkage of the resistance gene shown in CpRR1 can be evidenced in the original field strain CpR by single pair crosses and (ii) to investigate whether the mode of inheritance had been altered during establishment of the homogeneous population CpRR1.

3.2 MATERIALS AND METHODS

3.2.1 INSECTS

Codling moths used in bioassays and crosses were derived from the codling moth rearing at the DLR Rheinpfalz (Agricultural Service Center Palatinate), Neustadt/Weinstr. The insects were kept at 26°C, 60% relative humidity and 16/8 hours light/dark photoperiod. The susceptible laboratory strain is designated as CpS. The CpGV resistant *C. pomonella* strain CpR has been reared at the DLR Rheinpfalz since 2005 (Eberle and Jehle, 2006). It was originally collected in 2003 from an organic orchard in south western Germany. This resistant strain is identical to the resistant strain described by Fritsch et al. (2005), called "Suedbaden", and Eberle et al. (2006), designated as "R". Larvae were kept in autoclavable 50-well plates on semi-artificial diet which was prepared according to Ivaldi-Sender (1974). Fifth-instar (L5) larvae were allowed to pupate in corrugated cardboard strips.

3.2.2 VIRUS

The CpGV isolate used for the bioassays was the so called "Mexican isolate" (CpGV-M), (Tanada, 1964). CpGV-M was propagated in fourth instar larvae of the susceptible *C. pomonella* strain (CpS). The occlusion bodies (OBs) were purified following the method described by Jehle et al. (1992). The number of OBs per µl of the stock solution was counted using a Petroff-Hauser counting chamber (depth 0.02 mm) in the dark field optic of a Leica light microscope (DMRBE).

3.2.3 BIOASSAYS

To distinguish between resistant and susceptible individuals, neonate larvae were exposed to a discriminating concentration of virus in the diet. For the discriminating concentration the ~95% lethal concentration (5.8×10^4 OB/ml diet) of the susceptible codling moth strain (CpS) was chosen (Eberle and Jehle, 2006). Bioassays were performed in autoclavable 50-well plates containing 45 ml of diet mixed with 5 ml aqueous virus suspension. For control plates the virus suspension was replaced by 5 ml H_2O. In order to prevent thermal inactivation of the virus, the amount of agar was halved, to assure that diet could cool down to 40°C without solidifying. Larvae that died from handling were scored 24 hours after they were put on the diet and excluded from the experiment. Mortality data were collected on the seventh day of the experiment.

3.2.4 SINGLE PAIR CROSSES

Pupae of the different *C. pomonella* strains were sorted according to sex before eclosion. The sex of pupae was determined by their number of abdominal segments (♀: 3, ♂:4) and the appearance of the genital area (Asser-Kaiser et al., 2007). The single pair crosses were performed in small plastic cylinders lined with plastic foil for egg deposition. Round filter papers were placed on the bottom of the cylinders to absorb moisture. A single male and female pupa were placed into the cylinder and incubated at 26°C, 60% relative humidity and 16/8 hours light/dark photoperiod. As soon as the adult moths emerged, a piece of cotton wool soaked with water was added to the cylinder to allow liquid intake. After mating, females deposited eggs on the foil. The eggs were collected every other day by replacing the foil and were stored at 8-10°C for at most six days in the refrigerator. From each pair up to 100 eggs could be collected. After oviposition was completed the eggs were incubated in a climate chamber at 26°C, 60% relative humidity and 16/8 hours light/dark photoperiod. The susceptibility of the freshly hatched neonates to CpGV was determined in a bioassay as described above. The offspring of every single pair was divided among one control plate without virus and one plate containing the discriminating concentration. Larvae on control plates were reared to adulthood and partially used for backcrosses. Mortality data were corrected for control mortality using Abbott's formula (Abbott, 1925).

3.2.4.1 SINGLE PAIR CROSSES WITHIN CPR

In order to test for homogeneity of the resistant field strain CpR, single pair crosses within the strain were performed. 21 single pairs of CpR (CpR♀ x CpR♂) were crossed according to the method described above. The offspring of the successful crosses was tested in a bioassay using the discriminating concentration of CpGV-M.

3.2.4.2 HYBRID CROSSES BETWEEN CPS AND CPR

Twenty single pairs consisting of a susceptible male (CpS male) and a female from the resistant strain CpR (CpR female) were crossed, and the susceptibility of the different F1 families to CpGV was tested in a bioassay using the discriminating concentration of CpGV-M. Larvae of the controls were reared until the pupal stage to be used in backcrosses.

3.2.4.3 BACKCROSSES OF THE F1 GENERATION TO CPR

The F1 generation of the hybrid cross as described in 2.4.2, was backcrossed to the resistant codling moth strain CpR. Two F1 families with the highest (#5, #11) and two F1 families with the lowest mortality (#8, #13) were selected and reciprocal single pair backcrosses were performed: BC1: F1♀ x CpR♂ and BC2: F1♂ x CpR♀. Five single

pairs for each variant were crossed and the progeny of the successful crosses was tested against the discriminative concentration of CpGV-M.

3.3 RESULTS

3.3.1 SINGLE PAIR CROSSES WITHIN CPR

Twenty one single pair crosses within the codling moth strain CpR (CpR♀ x CpR♂) were performed and the different F1 families were tested in a 7-day bioassay using the discriminating concentration of 5.8 x 10^4 OBs/ml. Seventeen out of the 21 crosses produced progeny that could be tested. The average mortality over all CpR♀ x CpR♂ crosses was 31%, which was consistent with the 28% mortality of the strain CpR tested at the same concentration (Table 3.1). There was a high variation between the different (CpR♀ x CpR♂) F1 families, ranging from 0% mortality up to 100% mortality (Figure 3.1). The families could be classified into 4 groups: Families that showed about 25%, 100%, and 0% mortality, and one family that responded with 60% mortality. The variable response of the different families suggested that the individuals selected for the crosses were not genetically homogeneous with respect to CpGV resistance.

Table 3.1 Average mortalities of the different codling moth strains CpS and CpR and the pooled progeny of single pair crosses within CpR (CpR♀ x CpR♂) and the hybrid cross between CpS and CpR (CpR♀ x CpS♂) at the discriminating concentration of 5.8 x 10^4 OB/ml, n= number of larvae tested, SD=standard deviation of the means.

	CpS	CpR	CpR ♀ x CpR ♂	CpR♀ x CpS♂
mean	99.3	28.1	31.0	65.8
n	201	93	522	361
SD	1.2	9.5	36.45	23.2

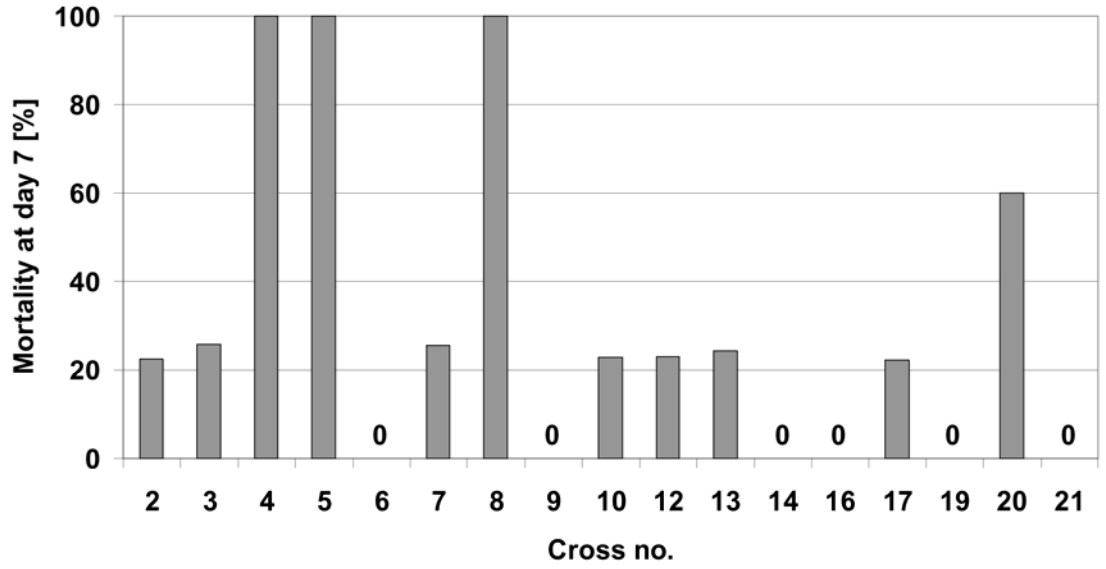

Figure 3.1 Mortalities of the different F1 families of single pair crosses within CpR after exposure to diet containing the discriminating virus concentration of 5.8 x 10^4 OB/ml for seven days. Mortalities are Abbott corrected.

Table 3.2 Average mortalities of the different F1 families (CpR♀ x CpS♂) after exposure to diet containing the discriminating virus concentration of 5.8 x 10^4 OB/ml for seven days. Mortalities are Abbott corrected. n= number of larvae tested, SD=standard deviation of the means.

Pair no.	n	Mortality [%]*	Mean (SD)
#5	24	100.0	
#11	29	100.0	99.0
#18	26	96.9	(+/-1.8)
# 3	25	61.0	
# 8	44	41.0	
# 9	41	64.9	
#10	31	42.1	53.3
#13	37	38.3	(+/- 10.9)
#15	30	61.7	
#17	49	57.5	
#20	25	60.0	

* Abbott corrected

3.3.2 HYBRID CROSSES BETWEEN CPS AND CPR

Twenty single pair crosses, between CpS males and CpR females were set up. Eleven out of the 20 crosses produced enough progeny to be tested in a bioassay with the discriminating concentration. The different F1 families resulting from these crosses showed a high variability in susceptibility to the virus. The mortalities varied from 38% up to 100% (Table 3.2). Three families (#5, #11, #18) displayed a mortality of about 100%, whereas the other eight families varied between 38% and 65% with a mean of 53.3%. The average mortality over all F1 families was 62%. Based on the mortality of 28.1% for CpR (Table 3.1) and assuming that resistance is inherited in CpR the same way as in CpRR1 the frequencies of the phenotypes of the F1 families can be predicted (Table 3.3). The expected frequency of the F1 phenotype "100% mortality" is 0.28. The observed frequency was 0.27 (3 out of 11). The phenotype "50% mortality" is expected to represent 72% (8 out of 11) of the F1 families. In 73% of the F1 families tested 50% of the larvae died in the bioassay (Table 3.3).

Table 3.3 Expected genotypes of the F1 families of single pair hybrid crosses CpR♀ x CpS♂ depending on the genotype of the CpR mother and expected and observed frequencies of the phenotypes "100% mortality" and "50% mortality" of the F1 generation.

Single pair crosses between CpR females and CpS males									
Genotype parents		Genotypes F1					Phenotype	expected	observed
female	male						(F1 Mortality)	phenot. freq.*	phenotyp. Freq.
Z^SW	Z^SZ^S	Z^SZ^S	Z^SZ^S	Z^SZ^S	Z^SW	Z^SW	100%	0.28	0.27
Z^RW	Z^SZ^S	Z^RZ^S	Z^RZ^S	Z^SZ^S	Z^SW	Z^SW	50%	0.72	0.73

*Expected phenotype frequencies are based on the average mortality of CpR (28%, Tab. 1), suggesting that females in CpR consist of 28% susceptible (Z^SW) and 72% resistant (Z^RW) individuals.

3.3.3 BACKCROSSES OF THE F1 GENERATION TO CPR

The F1 generation of the hybrid cross was backcrossed to the resistant codling moth strain CpR. Two families with the highest (#5 and #11, Table 3.2) and two F1 families with the lowest mortalities (#8 and #13, Table 3.2) were selected and five reciprocal backcrosses of each family were performed: BC1: F1♀ x CpR♂ and BC2: F1♂ x CpR♀. The particular backcross families were tested with the discriminating concentration of CpGV-M. The different mortalities of the particular backcross families are shown in Table 3.4. The average mortality over all backcrosses was 29.1% in a 7-day bioassay with the discriminating concentration. The mean mortalities of the reciprocal backcrosses differed from each other: In BC1 the average mortality was 8.7% and was significantly lower (ANOVA, Tukey's Multiple Comparison Test, $P < 0.001$) than the average mortality of BC2 which was 50.8%. Within BC2, the mortalities were significantly higher (ANOVA Tukey's Multiple Comparison Test $P< 0.05$) when the F1 father descended from F1 families #5 and #11, which had the highest F1 mortalities (Table 3.4). In contrast, in BC1 there was no significant difference between backcrosses of families #5 and #11 compared to crosses where F1 animals descended from families #8 and #13 (ANOVA Tukey's Multiple Comparison Test).

Table 3.4 Mortalities of the different offspring from the reciprocal backcrosses of F1 families #5, #8, #11 and #13 of the backcross to CpR after exposure to diet containing the discriminating virus concentration of 5.8 x 10^4 OBs/ml for seven days. Mortalities are Abbott corrected. SD=standard deviation of the means, n= number of larvae tested.

	Pair no.	n	Mortality [%]*	Mean (SD)
BC1 (F1♀ x CpR♂)	#5.1	49	0.0	
	#5.2	33	17.7	12.7
	#5.3	21	0.0	
	#5.4	45	2.2	(+/- 18.9)
	#5.5	20	43.8	
	#11.1	49	32.7	
	#11.2	24	0.0	8.2
	#11.4	30	0.0	(+/- 16.4)
	#11.5	28	0.0	
	#8.1	41	29.3	
	#8.3	46	0.0	9.8
	#8.5	21	0.0	(+/- 16.9)
	#13.1	25	4.0	
	#13.2	22	9.8	4.6
	#13.3	17	5.9	(+/- 3.6)
	#13.4	17	0.0	
	#13.5	30	3.3	
BC2 (F1♂ x CpR♀)	#5.6	15	93.3	
	#5.7	44	54.5	76.2
	#5.8	48	61.5	(+/- 21.2)
	#5.9	44	95.5	
	#11.6	45	100.0	
	#11.7	47	87.2	75.6
	#11.8	46	32.6	(+/- 29.6)
	#11.9	46	82.6	
	#8.6	49	34.7	
	#8.8	39	7.7	22.75
	#8.9	45	31.1	(+/- 12.5)
	#8.10	12	17.5	
	#13.6	41	27.8	
	#13.7	45	20.0	28.5
	#13.8	48	37.5	(+/- 7.2)
	#13.10	47	28.7	

* Abbott corrected

3.4 DISCUSSION

Based on mass crossing experiments Eberle and Jehle (2006) proposed an autosomal incompletely dominant inheritance of CpGV resistance in CpR. For the homogeneous resistant codling moth strain CpRR1, on the other hand, it was clearly shown that inheritance of resistance to CpGV-M is governed by a single dominant gene that is located on the Z-chromosome (Asser-Kaiser et al., 2007). In codling moth, as in other Lepidoptera, males are homogametic (ZZ) and females are heterogametic (ZW). Heterozygous males, homozygous resistant males and homozygous resistant females survive the discriminating concentration. Strain CpRR1 was made genetically homogeneous by inbreeding the field strain CpR (Asser-Kaiser et al., 2007) (Chapter 2), thus the frequency of the resistant allele was fixed at 100% in CpRR1. Eberle and Jehle (2006) had assumed that CpR was also fixed for resistance, and hence homogeneous, because there was no decline in resistance for almost two years of rearing without selection pressure under laboratory conditions. It is commonly observed that resistance to biological and chemical insecticides imposes a fitness cost, and that resistance can decline in laboratory populations that are not fixed, in the absence of continual selection with insecticide. However, the results of the single pair crosses within the strain CpR shown here disproved the assumption of homogeneity. The F1 families resulting from single pair crosses within CpR responded differently to an exposure of the discriminating concentration of CpGV-M (Figure 3.1). The variability in mortality indicates that CpR consists of resistant and susceptible individuals. The average mortality over all crosses was 31% (Table 3.1). When strain CpR was tested against the discriminating concentration the average mortality of three replicates was 28% (Table 3.1), consequently CpR contains 28% susceptible and 72% resistant individuals.

Assuming that resistance to CpGV-M in CpR is inherited by a single, dominant gene, located on the Z-chromosome as in CpRR1, there are six different possible combinations of genotypes being crossed within CpR: A) A susceptible female is crossed to a susceptible male (Z^SW x Z^SZ^S). Here 100% of the progeny is susceptible. B): A susceptible female is mated with a homozygous resistant male (Z^SW x Z^RZ^R), then all of the offspring are resistant. C): A susceptible female is crossed to a heterozygous resistant male (Z^SW x Z^SZ^R). In this case 50% of the progeny die in the bioassay. In the other three cases the female is resistant and conveys her Z-chromosome carrying the resistance gene to her sons. In case D) this resistant female is crossed to a homozygous susceptible male (Z^RW x Z^SZ^S). 50% of the progeny of this cross is susceptible. When a resistant female is crossed to a homozygous resistant male (Z^RW x Z^RZ^R) all descendants are resistant (E). A resistant female mated with a heterozygous male (Z^RW x Z^RZ^S) produces offspring of which 25% are susceptible (F).

According to their mortalities in the discriminating bioassay, each of the different F1 families of the cross within CpR can be assigned to one of these six cases. Three F1 families showed a mortality of 100% and reflect case A). Six families responded with 0% mortality to the discriminating bioassay and can be related to the cases B) and E). Case F) is represented by seven families that show a mortality of about 25%. One family displayed a mortality of 60%. This family probably represents the phenotype "50% mortality" which corresponds to case C) or D). Thus, the different mortality rates of the F1 families of crosses within CpR are consistent with the mode of inheritance proposed by Asser-Kaiser et al. (2007) (Chapter 2) from studies on the CpRR1 strain.

In order to test whether inheritance of resistance was monogenic, single pair crosses between CpS and CpR, and backcrosses of the F1 generation to CpR were performed. Because of the limited amount of eggs deposited by one codling moth female it is not possible to implement a full range bioassay for the determination of LC values. One single female produces between 60 and 100 eggs (Börner, 1997). Thus, the standard test for monogenic or polygenic inheritance employing a range of different insecticide concentrations (Tabashnik, 1991) cannot be applied to individual families. The different F1 families of the crosses between CpS and CpR were therefore tested against the discriminating concentration in order to distinguish between susceptible and resistant phenotypes. Assuming that resistance to CpGV-M in CpR is due to the same mode of inheritance as in CpRR1 we can predict the frequency of susceptible and resistant genotypes in the F1 generation of a hybrid cross between CpR females and CpS males (Table 3.3). With genes located on a sex chromosome, the genotype frequency of the heterogametic sex is equal to the frequency of the allele in the population at Hardy-Weinberg equilibrium (Sperlich, 1988). The mortality of strain CpR (28%, Table 3.1) thus provided the basis for the frequency estimation of susceptible (Z^SW) females in the strain. The probability for a susceptible female to be chosen out of CpR for the hybrid cross is 28% and for a resistant female it is 72%. The progeny of a cross between a resistant female and a susceptible male consists of the genotypes given in Table 3.3. When a susceptible female of CpR is crossed to a CpS male neither the father nor the mother conveys resistance to the F1 generation, resulting in 100% mortality in the bioassay. This phenotype was present in three out of eleven crosses (#5, #11 and #18, Table 3.2). The observed frequency of the phenotype "100% mortality" in the experiment was 0.27 and is very close to the expected frequency of 0.28 (Table 3.3), since CpR contains 72% females that carry a Z-chromosome with the resistance gene. When a resistant female is chosen for the cross, all males in the F1 are heterozygous resistant and survive the discriminating concentration whereas all females are susceptible and die, resulting in a mortality of 50%. The heterozygous males survive the exposure to the virus. Nine out of eleven crosses, which are 73% of all crosses, displayed this phenotype. The actual experimental phenotype frequencies are very close

to the expected frequencies and heterozygous males survive the discriminating concentration as they do in CpRR1, confirming the monogenic, dominant inheritance in CpR.

In order to test for sex-linkage of the resistance gene, reciprocal backcrosses of the F1 generation to CpR followed by bioassays were performed (Table 3.4). In backcrosses where the father descended from CpR (BC1) mortalities were significantly lower than in backcrosses with a CpR mother (BC2). This clear difference can only be explained by a linkage of CpGV resistance to the sex chromosome Z of the CpR parent, as it was also found for CpRR1 (Asser-Kaiser et al., 2007) (Chapter 2). In backcrosses performed with F1, insects from families #5 and #11 have Z^SZ^S/ Z^SW genotypes and susceptible phenotypes (Table 3.3). In BC1 the mothers have the same genotype regardless of which F1 family they come from. Therefore it does not matter from which family the mother was chosen for the backcross. Variation between the BC1 families is caused by the genotype of the CpR father. When the resistance gene is located on the Z-chromosome, male genotypes follow the Hardy-Weinberg ratios at equilibrium $p^2 : 2pq : q^2$ (Sperlich, 1988). Here q represents the frequency of Z-chromosomes that do not carry the resistance gene Z^S and p is related to the frequency of the Z^R. Applying this formula males in CpR consist of 7.8% Z^SZ^S (q^2), 51.8% Z^RZ^R (p^2) and 40.3% Z^SZ^R (2pq). There are three different phenotypes expected for the BC1 families: 0%, 100% and 50% mortality. Nine out of 17 BC1 families show 0% mortality in the bioassay, which is a proportion of 52.9%. Assuming the same mode of inheritance as in CpRR1, the predicted frequency of this phenotype in BC1 is 51.8%. The remaining families (47.1%) can be assigned more or less to the phenotype “50%” mortality. This phenotype is predicted to be represented by 40.3% of the backcross families. 7.8% of the families are expected to show 100% mortality, but none of the families displayed this phenotype in the experiment. The predicted and the observed proportions of the phenotypes do not match exactly, but are very close. This is probably caused by the limited number of families and can be explained by the random choice of homozygous (Z^RZ^R) and heterozygous (Z^SZ^R) resistant males at the expense of susceptible males (Z^SZ^S) for the backcrosses.

Within backcross BC2 there is a significant difference between the progeny of different pairs depending on the origin of the F1 father (Table 3.4). Under the assumption of a monogenic sex-linked inheritance F1 males of the hybrid cross CpR♀ x CpS♂ are either homozygous susceptible (Z^SZ^S) or heterozygous resistant (Z^SZ^R) (Table 3.3). For the backcross BC2 with F1 families #5 and #11 which represent the phenotype “100% mortality”, males had a homozygous susceptible genotype. These males were crossed to CpR females Z^SW (28%) or Z^RW (72%). Thus the backcross progeny is expected to consist of families with “50% mortality” and families with “100% mortality”. The

predicted average mortality of these families is 64% and the observed mortality was 75.9%.

In BC2 backcrosses performed with F1 families #8 and #13 that represent the phenotype "50% mortality" F1 males were heterozygous resistant (Z^SZ^R). When these males are crossed to CpR females mortality of the progeny is predicted to be 25% regardless which genotype the female had. The predicted average mortality of the offspring of BC2 with F1 males from families #8 and #13 is 25%, which is consistent with the actual average mortality of 25.75%. Hence differences in the backcross families within BC2 can be explained by the sex-linked mode of inheritance stated for CpRR1.

Based on reciprocal mass crosses between CpS and CpR, Eberle and Jehle (2006) proposed an incompletely dominant, autosomal inheritance of resistance. Sex linkage and maternal effects were not suspected, because of the lack of differences between the LC_{50}s in the F1 generation of the reciprocal hybrid crosses. It is now evident that this comparison was confounded by the heterogeneity of CpR. The only indication of sex linkage of CpGV resistance in the experiments by Eberle and Jehle (2006) were the different slopes of the probit lines in the reciprocal crosses. The slope of the concentration-mortality relationship was significantly steeper when a CpR female was crossed to a CpS male, than the slope of the reciprocal cross. At higher CpGV-M concentrations, the difference between the reciprocal hybrid crosses was more pronounced. Comparing the LC_{95} values, as when a discriminating concentration is used, may have facilitated the detection of sex linkage in these mass crossing experiments (Roush & Miller, 1986); however the discrepancy in the mode of inheritance of the LC_{50} vs. the LC_{95} values would have required explanation.

Given the conclusion that the genetic basis of resistance was autosomal, the observation of an intermediate level of resistance in the F1 was used by Eberle and Jehle (2006) to further conclude that it was semi-dominant as well. The loss of information caused by mass crossing obscured the mortality differences of offspring of parents with different resistance genotypes, so that even if mortalities of sons and daughters had been separately recorded, they probably would not have differed significantly after averaging over all the different types of families.

From our experiments we conclude that the field strain CpR has the same mode of inheritance concerning CpGV-M resistance as the genetically homogeneous strain CpRR1. This was revealed by the use of single pair crosses. Single pair crosses are often inconvenient, and difficult to perform in some species. Compared to mass crosses, single pair crosses have one additional disadvantage: because of the limited number of progeny of one single pair, full range bioassays with different concentrations to

determine LC values cannot be applied. On the other hand, single pair crosses provide much more information about the variability within populations and between families of hybrid- and backcrosses. Another example where careful examination of single-pair crosses revealed a previously-unsuspected genetic heterogeneity was shown by Tabashnik et al. (1997). In studies of resistance to Cry1A toxins from *Bacillus thuringiensis* in the diamondback moth *Plutella xylostella*, it was found that a susceptible strain actually harbored low levels of a recessive resistance allele, that could be detected in single-pair but not in mass crosses to the resistant strain.

Our findings strongly suggest that the heterogeneous CpR strain collected from an apple orchard with intermediate levels of resistance, and the homogenous CpRR1 strain with extremely high levels of resistance that was selected from it, do not have different genetic mechanisms of resistance. Instead, they differ substantially only in the frequency of the same resistance-conferring allele. Although without fine-scale linkage mapping we cannot rule out the formal possibility that a second, distinct gene on the Z chromosome had mutated in the development of the CpRR1 strain, which also conferred high levels of CpGV resistance, we believe that this is very unlikely. Therefore additional laboratory studies on the mode of action and the cloning of the resistance-conferring allele will be highly relevant to detecting and managing CpGV resistance in the field.

A further conclusion relevant to resistance management can be drawn from our experiments: long-term maintenance of a stable level of virus resistance in a laboratory population of insects does not necessarily show that the strain is fixed for resistance. The single-pair crosses show that CpR was heterogeneous concerning resistance to CpGV, and had thus the potential to revert to full susceptibility if the frequency of the resistant allele had declined to zero. However, mortality rates of CpR were stable over three years of rearing without selection pressure, indicating stable ratios of susceptible and resistant animals in the strain. In contrast, other studies showed that resistance to baculovirus was lost when exposure to the virus was discontinued (Briese and Mende, 1983). Two populations of *A. gemmatalis* from Brazil and one from the United States were submitted to selection pressure by *A. gemmatalis* nucleopolyhedrovirus (AgMNPV) in the laboratory. Both strains developed resistance to the virus (Abot et al. 1996). However resistance decreased in both strains when they were released from selection pressure indicating that there are fitness costs related to resistance (Fuxa and Richter, 1998). Similar to the case of a cabbage looper strain resistant to *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) described by Milks et al. (2002), there were no fitness costs for resistance that would have selected for susceptible insects in CpR, at least under laboratory conditions. There is thus an urgent need to determine whether the CpGV resistance allele of codling moth suffers a fitness cost in the field in the absence

of virus selection pressure. Such a fitness cost has been shown to be very useful in managing insecticide resistance by a strategy that employs sequential rotation of insecticides with different modes of action (Roush & Miller, 1986). With the identification of novel CpGV isolates, which overcome CpGV resistance, a first step towards a diversification of CpGV application is done (Eberle et al., 2008; Berling et al., 2009; Eberle et al., 2009). Determining whether a strategy of applying different CpGV isolates will be useful in managing CpGV resistance in apple orchards could be a key step in prolonging the efficacy of this means of biological control.

4 RESISTANCE OVERCOMING CHARACTER OF THE IRANIAN ISOLATE CPGV-I12 AND TESTING ON CROSS RESISTANCE TO CRY1AB

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ABSTRACT

Recently, codling moth (*Cydia pomonella* L., CM) populations with a significantly reduced susceptibility to C. pomonella granulovirus (CpGV) have been observed in Germany. A novel CpGV isolate, designated CpGV-I12, is able to overcome CpGV resistance. CpGV-I12 originated from Iran and showed superior efficacy in laboratory bioassays against CM strain CpR, which has a 100-fold reduced susceptibility to commercially used CpGV-M. Determination of the median lethal concentration (LC_{50}) indicated that CpGV-I12 is nearly as efficient in a homogeneous resistant strain (CpRR1) as CpGV-M in a susceptible CM strain (CpS). Beyond, CpGV-I12 caused superior mortality in CpS. Infection experiments showed that the resistance breaking effect can be observed in all instars of CpR. CpGV-I12 is a promising alternative control agent of CM in orchards were conventional CpGV products fail. In addition, we demonstrate in bioassays with recombinant expressed Cry1Ab that cross-resistance to CpGV and *Bacillus thuringiensis* products is not likely.

4.1 INTRODUCTION

The codling moth (*Cydia pomonella* L., CM) is one of the major insect pests of apples, pears and walnuts in all fruit growing regions of the world. Without control it can cause severe damage and unmarketable fruits. Chemical insecticides, pheromone based mating disruption, *Bacillus thuringiensis* sprays and application of *C. pomonella* granulovirus products (CpGV) are the most commonly used control measures of CM. CpGV is an extremely specific and highly virulent pathogen of CM and has been developed to one of the most efficient and environmentally safe insecticides (Huber,

1998). The active substance of nearly all registered CpGV products is the isolate CpGV-M, which was first discovered in Mexico in 1963 (Tanada, 1964). A detailed physical map and the complete genome sequence of CpGV-M have been established (Crook et al., 1985, 1997; Luque et al., 2001). Two other CpGV isolates originating from England (CpGV-E) and Russia (CpGV-R) were described later (Harvey & Volkman, 1983; Crook et al., 1985). Recently, several new field isolates from Georgia and Iran, including the isolate CpGV-I12, were characterized by DNA restriction fragment length polymorphism, partial genome sequence data and single nucleotide polymorphisms (SNPs) in selected genes and revealed a considerable heterogeneity in their genetic content (Rezapanah et al., 2008; Jehle, unpublished).

Since 2004, several local codling moth populations with a reduced susceptibility to CpGV have been reported from Germany and from France (Fritsch et al., 2005; Sauphanor et al., 2006; Asser-Kaiser et al., 2007). These populations were detected in organic apple plantations, where CM control failed despite intensive CpGV application. Bioassays in the laboratory further demonstrated that the median lethal concentrations (LC_{50}) of these populations increased up to 1000- to 10,000-fold indicating a significant resistance to CpGV. Mass crossing experiments between a susceptible laboratory codling moth strain (CpS) and a resistant field population (CpR) initially suggested that the resistance is autosomally and incompletely dominant inherited (Eberle and Jehle, 2006). The incomplete dominance of CpGV-resistance was recently supported by single pair crossing experiments of a genetically homogenized resistant codling moth strain CpRR1. However, these experiments clearly indicated that the CpGV resistance is monogenic and sex-linked to the Z-chromosome of *C. pomonella* (Asser-Kaiser et al., 2007) (Chapter 2).

In order to develop a better insight into resistance and to find possible measures to control the CpGV resistant codling moth populations, the ability of CpGV-I12 to overcome the CpGV resistance was tested. As a result, CpGV-I12 is able to kill CpRR1 and CpS in a similar efficient way. Further, the potential of cross resistance between CpGV and *B. thuringiensis* products was investigated by testing the susceptibility of CpS and CpR to Cry1Ab, which is the main lepidopteran specific toxin of *B. thuringiensis* products.

4.2 MATERIAL AND METHODS

4.2.1 TEST INSECTS

All codling moth larvae were derived from the insect rearing at the DLR Rheinpfalz (Agricultural Service Centre Palatinate), Neustadt/Weinstr., where the insects are kept at 26°C, 60% relative humidity and a 16/8 hours light/dark photoperiod. Larvae were reared on semi-artificial diet (Appendix I) (Ivaldi-Sender, 1974). The CpGV-susceptible CM strain (CpS), has been reared free of virus for nine years at the DLR. The CM strain resistant to CpGV-M (CpR) was reared since 2004 without selection pressure. It was originally collected in 2003 in an organic orchard in South Baden, Germany and is identical to the resistant strain described by Fritsch et al. (2005), called "Suedbaden" (BW FI 03). Concerning resistance to CpGV-M, the CpR strain is heterogeneous as it contains about 30% susceptible individuals. CpR was used for the instar specific infection experiments and the test on cross resistance with Cry1Ab. Later, a homogeneous resistant *C. pomonella* strain (CpRR1), which was generated by inbreeding of CpR (see 2.2.4), was available. Bioassays to estimate concentration-mortality relationships were performed with CpS and CpRR1.

4.2.2 VIRUS

Two different virus isolates of the *C. pomonella* granulovirus (CpGV) were used in the bioassays. The so-called "Mexican isolate" (CpGV-M) (Tanada, 1964) is identical to the virus used in the commercial products registered in Europe (Huber, 1998). The isolate CpGV-I12 originated from Iran (Rezapanah, 2008). It is not commercially used yet. Both isolates were propagated in fourth instar larvae of the susceptible *C. pomonella* strain (CpS). The occlusion bodies (OBs) were purified following the method described by Jehle et al. (1992). The number of OBs per ml in this stock solution was scored using a Petroff-Hauser counting chamber (depth 0.02 mm) in the dark field optic of a Leica light microscope (DMRBE).

4.2.3 BIOASSAYS WITH CPGV

Bioassays were performed in autoclavable 50-well plates containing 45 ml of diet (Ivaldi-Sender, 1974) mixed with 5 ml virus suspension of different concentrations per plate. For control plates the diet was mixed with 5 ml H_2O instead of virus 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 to prevent thermal inactivation of the virus. 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. The time when the bioassays were evaluated depended on the different experiments that are specified below.

For instar-specific infection experiments larvae were kept on virus-free diet until they reached the particular age. Then they were transferred onto diet containing $2x10^5$ OB/ml of CpGV-M and CpGV-I12, respectively. This concentration was chosen to reach a >99% in all instars of CpS. Mortality was daily determined, up to 14 days after the larvae were transferred onto the virus containing diet. For each assay about 25-30 larvae were used. Each bioassay was independently repeated five times with CpGV-M and three times with CpGV-I12. The means of the results were calculated. Mortality data were corrected for control mortality (Abbott, 1925).

In order to estimate the median lethal concentrations (LC_{50}) of the resistant strain CpRR1, 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) of CpGV-M and CpGV-I12. Thirty-five neonate larvae (L1) were used for each concentration; and 70 L1 larvae for the untreated control. Mortality was determined after one, seven, and 14 days. Control mortality was general below 9% (7 days) and below 21% (14 days). Three independent replicates were applied. Mortality data were corrected for control mortality (Abbott, 1925).

4.2.4 BIOASSAYS WITH CRY1AB PROTEIN

Bioassays with Cry1Ab were performed with neonate codling moth larvae. Artificial diet (1 ml) was dispersed in every well (surface area of 1.77 ± 0.08 cm^2/well) of the bioassay tray (Bio-ba-128, Color-Dec Italy). Trypsinised Cry1Ab protein was obtained from Dr. Hang T. Nguyen (Nguyen et al., 2004). The plasmid pMP encoding the open reading frame of Cry1Ab protein of *B. thuringiensis* HD-1 was cloned in Escherichia coli HB101 as host bacteria (Masson et al., 1989). After purification, the trypsin resistant 62 kDa fragment of Cry1Ab was used in bioassays with codling moth. Cry1Ab protein was diluted to seven different concentrations ranging from 0.03 – 30 µg/cm^2 diet. The dilutions were stored at 4°C and were pipetted on the surface of the diet. After the diet had dried, one larva was placed on each well. Thirty-two larvae were used per dilution, 64 larvae were used as control. The assays were conducted at 25°C and after 7 days the mortality was assessed. Three independent replicates were performed.

4.2.5 STATISTICAL ANALYSIS

Estimation of median lethal concentrations (LC_{50}) and slopes of the concentration-mortality lines were done using probit analysis procedure in ToxRat Standard version 2.09 (ToxRat Solutions GmbH, 2005).

4.3 RESULTS

4.3.1 BIOASSAYS WITH CPGV-M AND CPGV–I12

The response of CpS and CpRR1 to the virus isolates CpGV-M and –I12 was tested at 7 and 14 days post incubation. After 7 days, CpGV-M caused high mortality of CpS with a LC_{50} of 1.4 x 10^3 OB/ml (95% confidence limit (CL) = 1.2-1.7 x 10^3 OB/ml, n = 613, slope (s) = 2.0, χ^2 = 1.2) (Figure 4.1a). In contrast CpRR1 showed a highly reduced susceptibility to CpGV-M, which did not reach 50% mortality, even at the highest concentration of 1 x 10^5 OB/ml (CL not determined, n = 651, slope (s) = 0.5, χ^2 = 1.4). The isolate CpGV-I12 showed an LC_{50} = 8.97 x 10^2 OB/ml (95% CL = 0.7-1.1 x 10^3 OB/ml, n = 854, s = 1.53, χ^2 = 6.94) for CpS. In CpRR1, CpGV-I12 showed an LC_{50} of 8.03 x 10^4 (95% CL = 4.2-19.8 x 104, n = 621, s = 1.27, χ^2 = 24.05) indicating an increased virulence compared to CpGV-M.

After 14 days of exposure, mortality for CpRR1 incubated with CpGV-M did not reach 50%, accordingly an LC_{50} could not be determined (CL not determined, n = 651, slope (s) = 0.78, χ^2 = 0.18). In contrast, CpGV-I12 showed an LC_{50} of 1.8 x 10^3 on CpRR1 (95% CL = 1.4-2.2 x 10^3 OB/ml, n = 621, s = 1.55, χ^2 = 2.08), which was slightly higher compared to that of CpGV-M against CpS (LC_{50} = 5.01 x 10^2 OB/ml) (95%CL not determined, n = 613, s = 2.14, χ^2 = 186.7). Moreover, its efficacy on CpS (LC_{50} = 1.89 x 10^2 OB/ml, 95% CL not determined, n = 854, s = 1.83, χ^2 = 275.0) was similar to that of CpGV-M (Figure 4.1b).

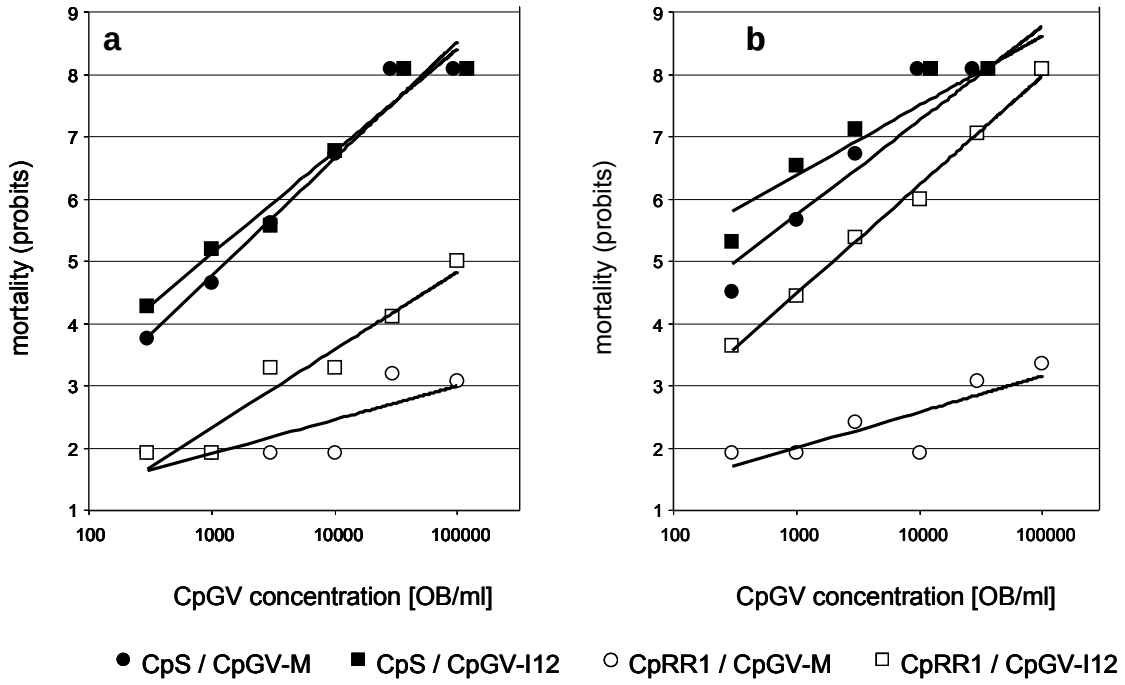

Figure 4.1 Concentration–mortality response (probits) of codling moth strains CpS and CpRR1 after (a) 7 days and (b) 14 days of incubation with CpGV-M or CpGV-I12.

At 14 days, CpGV-M was also not able to cause 50% mortality of CpRR1, not even at the highest concentration of 1 x 10^5 OB/ml. When the concentration of CpGV-M was increased up to 3 x 10^9 OB/ml a mortality of 100% was reached after 14 days of incubation with CpGV-M (data not shown). Based on these data LC_{50} after 7 days of exposure was LC_{50} = 6.92 x 10^8 (95% CL = 2.9 x 10^8 – 2.6 x 10^9, n = 752, s = 1.0, χ^2 = 29.11) and in the 14 days bioassay the LC_{50} was 8.53 x 10^6 OB/ml (95% CL = 5.3 x 10^6 – 1.4 x 10^9, n = 752, s = 0.67, χ^2 = 5.76).

4.3.2 INSTAR-SPECIFIC MORTALITY OF CPS AND CPR

To investigate if virus resistance is related to a particular larval stage, larvae were infected at different instars (L1-L5) using a single virus concentration of 2 x 10^5 OB/ml. The virus induced mortality was scored every day and the 25%, 50% and 75% percentiles of time to death (TtD) were determined (Figure 4.2a-d). Within 14 days, a 100% mortality of CpS was observed with CpGV-M in all larval stages. The median TtDs ranged from 4 days (L1) to 7 days (L5) (Figure 4.2a). In CpR, the reduced susceptibility to CpGV-M is distinct in all larval stages (Figure 4.2b). A mortality of 50% could only be induced in L1 after an incubation time of 13 days. For the instars L2-L5, mortality was lower than 50%, with 34.2% in L5 larvae as the lowest mortality value.

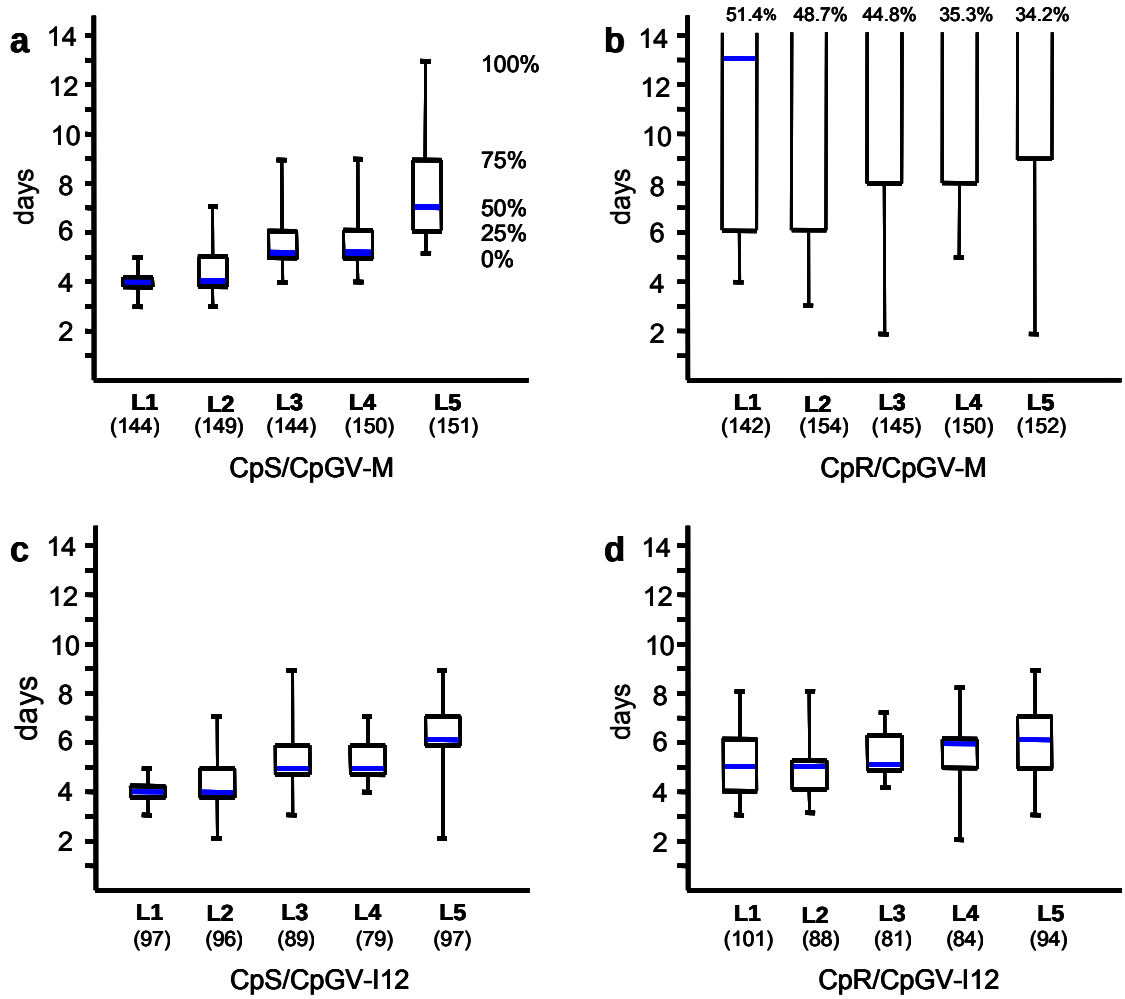

Figure 4.2 Box-plot analysis of instar-specific mortality of CpS and CpR larvae after incubation at 2 x 10^5 OB/ml CpGV-M or CpGV-I12 during 14 days incubation (a-d). Given are the mean values from five replicates (CpGV-M) and three replicates (CpGV-I12), respectively. Mortality was scored daily. The open box indicates 25-75% percentile of time to death, where as the horizontal line in the box gives the day when 50% mortality was reached. The vertical line indicates the days when >0% (lower end) and 100% mortality was observed. Numbers in brackets indicate the number of tested insects.

CpGV-I12 showed similar efficacy in CpS as CpGV-M in all larval instars, with a median time to death ranging from 4 days (L1) to 6 days (L5) (Figure 4.2c). Furthermore, CpGV-I12 caused similar mortalities in CpR as in CpS (Figure 4.2d). However, the 50% mortality of L1 and L2 larvae of CpR caused by CpGV-I12 was retarded by 1 day compared to CpS. With extended incubation time no difference in the reaction of CpS and CpR could be observed.

4.3.3 BIOASSAYS WITH BACILLUS THURINGIENSIS CRY1AB PROTEIN

In addition to the comparison of virulence of CpGV-M and CpGV-I12 in larvae of CpS and CpRR1 it was determined whether CpGV resistance of codling moth is correlated with a decreased susceptibility to Cry1Ab protein. As shown in Figure 4.3 the Cry1Ab concentration–mortality response of CpS (n = 1128, slope (s) = 0.54, χ^2 = 2.54) and CpR (n = 1108, slope (s) = 0.64, χ^2 = 7.43) showed that CpR is similarly susceptible to Cry1Ab as CpS. The control mortality after 7 days was 4.3% (CpR) and 2.2% (CpS), respectively.

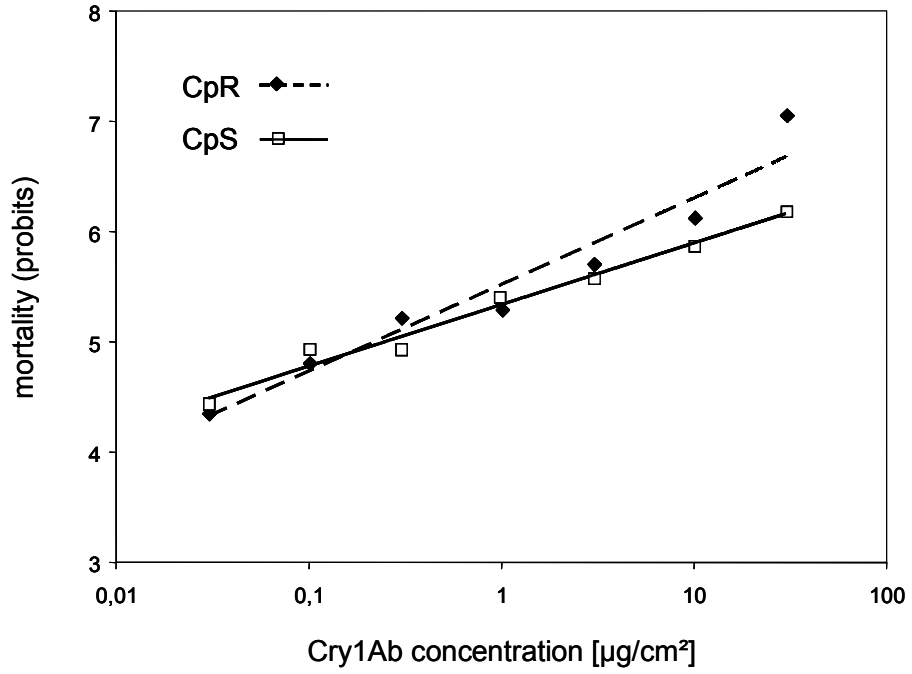

Figure 4.3 Concentration-mortality response (given in probits) of codling moth strains CpS and CpR to recombinantly expressed Cry1Ab after 7 days of incubation.

4.4 DISCUSSION

As some local codling moth field populations in Germany had lost their susceptibility to the widely used CpGV-M, we were looking for alternatives which may overcome this resistance phenomenon (Eberle and Jehle, 2006; Asser-Kaiser et al., 2007)(Chapter 2). A promising alternative for the control of codling moth populations resistant to CpGV-M is the application of CpGV-I12. Determination of the median lethal concentration (LC_{50}) indicated that CpGV-I12 is similarly efficient in resistant CpRR1 as CpGV-M in the susceptible CpS codling moth strain. When CpRR1 was infected with CpGV-I12 in

7-day bioassays it showed a resistance ratio of only 57.1, whereas with CpGV-M the resistance ratio was 4.9 x 10^6. In 14-day bioassays with CpGV-I12 the resistance ratio of CpRR1 was only 3.6. Thus, the new isolate CpGV-I12 showed its potential to overcome resistance already after 7 days, but the full efficacy was obtained after an incubation time of 14 days. A slight delay of the efficacy of CpGV-I12 in CpR compared to CpGV-I12 or CpGV-M in CpS was also visible in the instar-specific experiments performed with CpR L1 and L2 larvae. 50% mortality was reached at day 5 after exposure to the virus, and 100% mortality at day 8. For CpS, 50% mortality was reached with CpGV-M and CpGV-I12 at day 4, and a complete mortality between 5 (L1) and 7 (L2) days. Hence in resistant first and second instar larvae, the time to death of CpGV-I12 was delayed by about 1-2 days, this effect was not visible in experiments with CpS. Although the efficacy of CpGV-I12 slightly higher in CpS than in CpRR1, indicated by the lower LC_{50}s in CpS, it was shown that CpGV-I12 is able to overcome resistance, and to cause 100% mortality in CpRR1.

Resistance of the field derived codling moth strain CpR to CpGV-M could be observed in all five instars (Figure 4.2). With increased larval development, age dependent susceptibility decreased as expected (Briese, 1986a) and the mortality declined continuously for L5 larvae. With CpGV-I12, it was possible to overcome resistance in all instars and to obtain complete mortality in all larval stages after an incubation time of at most 9 days for L5 larvae.

Considering the 100-fold resistance level of CpR and the fact that CpGV-M still induces a mortality ranging between 30 to 50% in all larval stages suggest that CpR strain still may contain susceptible individuals. Indeed, recent studies demonstrated that CpR is a mixture of susceptible and resistant individuals (Asser-Kaiser et al., 2007)(Chapter 2). Based on single pair crossing experiments it was estimated that up to 30-40% of all individuals of CpR were still susceptible to CpGV-M (Asser-Kaiser and Jehle, unpublished). Hence, the observed CpGV-M caused mortality in CpR of about 30-40%, which did not further increase from day 7 to day 14, may be attributed to the mortality of susceptible individuals within the CpR strain. On the other hand, for the other 60-70% of the CpR individuals a 1000-100,000 fold resistance to CpGV-M is expected (Asser-Kaiser et al., 2007) (Chapter 2). This resistance can be overcome by CpGV-I12.

Cross-resistance of CpR to CpGV-M and Cry1Ab protein of *B. thuringiensis* could not be substantiated. When Cry1Ab protein was fed to L1 larvae of CpR and CpS, similar susceptibilities were observed for both strains in bioassays. Apparently, there is no common mechanism in the development of resistance against CpGV-M and Cry1Ab (Heckel et al., 2007). Hence CpGV resistant codling moth populations may be also successfully targeted using *B. thuringiensis* products. Bt products are generally

considered to be not efficient enough to compete with other codling moth measures (Lacey & Unruh, 2007). Their application, however, may be worth considering in orchards where CpGV resistance is present but resistance overcoming isolates are not available.

Based on restriction analysis the resistance overcoming Isolate CpGV-I12 was found to be a genetically rather homogeneous isolate, which shows a very similar DNA endonuclease restriction profile to CpGV-M (Eberle et al., 2008). For a better understanding of the resistance mechanism as well as the molecular factor(s) involved in overcoming CpGV resistance a detailed knowledge of the genome sequence of CpGV-I12 will be crucial. Knowing the molecular mechanism involved in overcoming CpGV resistance is also of utmost importance for the design of robust and sustainable application strategies of improved CpGV products. A broadened genetic basis of CpGV products by using different isolates will be one step forward to lower the risk of resistance development.

Whether CpGV-I12 is 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 by on a resistant codling moth population (Kienzle et al., 2007; Zingg, 2008). Thus these isolates provide well-founded optimism that the emergence of CpGV resistant codling moth populations can be pushed back.

5 BACULOVIRUS RESISTANCE IN CODLING MOTH (*CYDIA POMONELLA* L.) CAUSED BY EARLY BLOCK OF VIRUS REPLICATION

A modified version of this chapter will be submitted for publication soon.

ABSTRACT

After the successful use of CpGV in codling moth control in organic and integrated farming for more than 20 years, some populations of *Cydia pomonella* in Europe developed resistance against CpGV products. In order to investigate the mechanism underlying CpGV resistance, four different experimental approaches were followed: First, degradation of the peritrophic membrane (PM) using an optical brightener (FB28) did not lead to enhanced CpGV infection in resistant animals suggesting that the PM is not involved in resistance. Second, injection of budded virus directly into the insect's haemocoel did not overcome resistance. Accordingly, CpGV resistance is not caused by an altered midgut receptor. Third, virus replication was traced by quantitative PCR in three different tissues of susceptible and resistant insects after oral and intra-haemocoelar infection. In contrast to the tissues taken from susceptible larvae in none of the tissues from resistant insects virus replication could be detected suggesting a systemic block of virus infection. Fourth, humoral and cellular immune responses as a reason for resistance were investigated by transfusion of haemolymph from resistant into susceptible insects. Transfusion of haemolymph from resistant insects into susceptible ones could not convey resistance. In conclusion, the different lines of evidence suggest that CpGV resistance in codling moth is caused by an early block of virus replication.

5.1 INTRODUCTION

The *Cydia pomonella* granulovirus (CpGV) is worldwide used for biological control of codling moth (*Cydia pomonella* L.), the major insect pest of apples, pears and walnuts (Huber, 1998). CpGV belongs to the genus *Betabaculovirus* of the family *Baculoviridae* (Jehle et al., 2006). Until recently baculoviruses were considered to be robust against development of insect resistance (Moscardi, 1999; Cory & Meyers, 2003). Early laboratory selection experiments using different baculovirus host systems suggested that

insects may develop an up-to 10-fold resistance but that resistance is not stable without selection pressure (Huber, 1974; Fuxa, 1993). However, in 2005 first codling moth populations in Germany and France with up to 1000-fold reduced susceptibility to CpGV were reported (Fritsch et al., 2005; Sauphanor et al., 2006). Until now, about 35 codling moth populations resistant to CpGV products have been observed in several European countries (Fritsch et al., 2005; Asser-Kaiser et al., 2007; Jehle, unpublished) (Chapter 2). Due to the dominant, monogenic and sex-linked mode of inheritance resistant individuals are selected rapidly against susceptible ones when CpGV is used in codling moth control (Asser-Kaiser et al., 2007) (Chapter 2). Beside the knowledge of heredity, it is mandatory to know the mechanism of resistance for the development of successful resistance management strategies.

Insects have developed many different ways to defend themselves against pathogens like fungi, bacteria, nematodes and viruses. In both, laboratory and field populations of insects, differences in viral susceptibilities have been observed (Fuxa & Richter, 1993; Briese, 1980; 1982). Insects resist virus infection through morphological barriers, behavioral resistance, developmental, physiological, nutritional, biochemical and molecular mechanisms (reviewed by Narayanan, 2004). Many insects show developmental resistance to baculovirus infection with decreasing susceptibilities at older larval stages (Briese, 1986a; Kirkpatrik et al., 1998; Teakle et al., 1986). However, resistance to CpGV in *C. pomonella* is prevalent in all five larval instars (Eberle et al., 2008) (Chapter 4). Resistance may occur at different steps during the infection process. Baculoviruses enter the insect as viral occlusion bodies (OBs) by oral uptake through the larvae. After the OB is dissolved due to the alkaline milieu in the midgut occlusion derived virions (ODVs) are released (Federici, 1997). Primary infection is started due to infection of the insect's midgut epithelial cells but before, virions have to pass through the PM lining the midgut epithelium and being a morphological barrier to baculovirus invasion of the midgut cells (Brandt et al., 1978; Granados et al., 1980). Optical brighteners, such as Calcoflour white M2R or Tinopal LPW, are used in baculovirus formulations to protect viral occlusion bodies from inactivation by UV light in field application (Shapiro, 1992). Furthermore, optical brighteners enhance the susceptibility of insects to baculoviruses by degeneration the peritrophic membrane and preventing sloughing of infected midgut epithelial cells (Washburn et al., 1998; El-Salamouny et. al., 2005). Here we report bioassays to test the influence of fluorescent brightener 28 on CpGV infection in resistant insects.

Once the ODVs have passed the PM primary infection starts with the infection of the midgut epithelia cells (Federici, 1997). There are different mechanisms of virus resistance described that are based on the midgut: For example, Engelhard and Volkman (1994) showed that fourth instar larvae of *Trichoplusia ni* can completely clear

infection with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) by sloughing of infected midgut cells. Resistance of *Bombyx mori* to *Bombyx mori* densovirus type 2 (BmDNV-2) is caused by the 6-kb deletion in a gene that encodes a transmembrane protein which is a functional receptor of the virus in the midgut (Ito et al., 2008). Larvae of the fall armyworm *Spodoptera frugiperda* are highly resistant to oral infection by AcMNPV, but susceptible to infection BVs within the haemocoel (Haas-Stapleton et al. 2003). This resistance is due to an aberrant binding of ODVs to the midgut cells (Haas-Stpleton et al., 2005). Furthermore, baculovirus replication can be blocked or inhibited in the midgut epithelial cells. As a result no BVs are produced to cause fatal infection (Pinnock & Hess, 1977).

After the initial infection of the midgut, baculoviruses form a second viral phenotype, the budded virus (BV). BVs spread the infection from cell to cell, often transported through trachea and/or haemolymph within the host (Engelhard et al., 1994; Flipsen et al., 1995). Eventually, most of the insect's tissues are infected and new OBs are produced (Federici, 1997).

Vertebrates have both innate and acquired immunity with an "immunological memory" to defend themselves against microbial infections. In contrast, insects only possess an innate immune system, which is characterized by non-specific immune reactions. Insects do not produce immunoglobulin for the recognition of foreign antigens and they do not produce alpha/beta interferon to respond to viral infections. Though, invertebrates lack the acquired immune system, they are capable of cellular and humoral immune reactions (reviewed by Cory & Meyers, 2003; Narayanan, 2004). Phagocytosis, aggregation of haemocytes and encapsulation are cellular defence mechanisms, whereas melanisation and induction of immune proteins (lysozymes, lectins and anti-bacterial and anti-fungal proteins) belong to humoral reactions (Narayanan, 2004). Increased concentrations of phenoloxidase in the insect's plasma affect baculovirus infection (Wilson et al., 2001). Popham et al. (2004) showed that the plasma of *Heliothis virescens* exhibit virucidal activity against BVs of *Helicoverpa zea* single nucleopolyhedrosis virus (HzSNPV) which is caused by phenoloxidase activity (Shelby et al., 2006). In *Lepiodptera* the haemolymph protein hemolin is induced by bacterial and baculovirus infection (Faye et al., 1975; Hirai et al., 2004). It binds to bacteria, lipopolysaccharides (LPS), lipid A and haemocytes (Sun et al., 1990; Dafre & Faye, 1997; Bettencourt et al., 1999). Hemolin is thought to function as an opsonin or as a pattern recognition molecule and thus to be involved in antiviral immune response (Faye & Kanost, 1998; Hirai et al., 2004).

Lepidopteran larvae resist baculovirus infection by programmed cell death, called selective apoptosis (reviewed by Clem, 2001). To overcome this defense strategy

baculoviruses have evolved apoptosis inhibitors, which block the cell death (Clem et al., 1991). Infection of *S. frugiperda* cells with a mutant of AcMNPV lacking a functional anti-apoptitic gene *p35*, lead to apoptosis and inhibition of OB formation (Clem et al. 1991).

Codling moth larvae that are resistant to CpGV have developed a mechanism that prevents lethal virus infection. In order to explore at which part of the infection cycle this mechanism becomes effective and to gain information about the mechanism's mode of action four different experimental approaches were followed: First, the PM was degenerated in susceptible as well as in resistant codling moth larvae to see whether a modification of the PM is involved in resistance. Second, by-passing the midgut, budded virus was injected directly into the insect's haemocoel in order to investigate whether resistance is midgut based only. Third, susceptible and resistant fifth instar larvae were infected either orally or intra-haemocoelarly and spread of virus infection was traced in midgut, haemolymph and fat body using quantitative PCR. Fourth, CpGV caused mortality was analysed after transfusion of haemolymph between resistant (CpRR1) and susceptible (CpS) codling moth larvae to determine the possible action of a humoral factor involved in CpGV resistance.

5.2 MATERIALS AND METHODS

5.2.1 INSECTS

Codling moth larvae used for the experiments were reared at the DLR Rheinpfalz (Agricultural Service Center Palatinate), Neustadt/Weinstraße, where three different strains of *C. pomonella* are kept. Strain CpS is a CpGV susceptible strain, which has been reared in Neustadt for ten years. The strain CpR is reared in Neustadt since 2005 and was obtained from Andermatt BIOCONTROL AG (Switzerland). It was originally collected in 2003 in an organic orchard in South Baden, Germany where CpGV application failed to control infestation with codling moth. This resistant strain is identical to the resistant strain described by Fritsch et al. (2005), called "Suedbaden" or CpR (BW FI 03) (Asser-Kaiser et al., 2007). CpR is about 100 times less susceptible to CpGV-M than CpS (Eberle & Jehle, 2006). This strain is genetically heterogeneous concerning CpGV-resistance (Asser-Kaiser et al., 2009) (Chapter 3). The homogeneous resistant *C. pomonella* strain CpRR1 was selected by single pair crosses within CpR (Asser-Kaiser et al., 2007) (Chapter 2).

The insects were reared at 26°C, 60% relative humidity and 16/8 hours light/dark photoperiod. The same conditions were used for the experiments. Larvae were kept in autoclavable 50-well plates on semi-artificial diet. According to Ivaldi-Sender (1974) the diet was composed of water and agar-agar, which were autoclaved for 20 min at 120°C and then mixed with maize meal, wheat germ, brewer's yeast, ascorbic acid and methyl-4-hydroxybenzonate dissolved in ethanol. After accomplishing the last larval stage the insects were allowed to pupate in corrugated cardboard stripes.

5.2.2 VIRUS

The CpGV isolate used for the bioassays was the so called "Mexican isolate" (CpGV-M), which was originally collected in Northern Mexico and described by Tanada (1964). It is identical to the virus used in the commercial products Madex (Andermatt BIOCONTROL AG, Switzerland), Granupom (Hoechst A.G., Frankfurt) and Carpovirusine (Calliope SA, Beziers) (Huber, 1998). This virus was propagated in fourth instar larvae of the susceptible strain CpS. The OBs were purified following the method described by Jehle et al. (1992). The number of OBs per µl of the stock solution was scored using a Petroff-Hauser counting chamber (depth 0.02 mm) in the dark field optic of a light microscope (Leica DMRBE).

Budded virus (BV) was produced in fifth instar larvae of the susceptible strain CpS. After starvation for eight hours the larvae were fed with small pieces of diet (2 mm^3) containing 1000 OBs. Larvae that did not ingest the diet during a period of 12 hours were excluded from the experiment. Three days later haemolymph was extracted: After disinfection with 70% ethanol larvae were anesthetized with diethyl ether vapour for 2-3 min. The second proleg was cut of using microscissors. Haemolymph droplets were collected using a 0.5-10 µl Eppendorf pipette. Haemolymph was transferred into IZD04 cell culture medium containing 50 µg/ml streptomycin, 100 units/ml penicillin and a small crystal of phenylthiourea (Winstanley, 1993). After centrifugation at 1000 x g and 4°C for 5 min, the supernatant was stored at -70°C in aliquots of 250 µl. Concentration of budded virus was estimated by quantitative PCR described below.

5.2.3 BIOASSAYS WITH FLUORESCENT BRIGHTENER 28

In order to investigate the effects of fluorescent brightener 28 (FB28) ($C_{40}H_{44}N_{12}O_{10}S_2$) on CpGV induced mortality diet incorporation bioassays were conducted with the strains CpS and CpR. Bioassays were performed in autoclavable 50-well plates containing 50 ml diet in total. Artificial diet was prepared as described above but using 10% less water and half of the amount of agar in order to assure that diet could cool down to 40°C without solidifying to prevent thermal inactivation of the virus. 40 ml diet

was mixed with 5 ml of the appropriate virus suspension and 5 ml of FB28 diluted in distilled water. For the susceptible strain CpS a constant CpGV concentration of 2 x 10^3 OB/ml diet was used. Due to the resistance factor of 100 a CpGV concentration of 2 x 10^5 OB/ml diet was chosen for strain CpR. For both strains four different concentrations of FB28 were used: 0.0%, 0.05%, 0.075%, 0.1 % and 0.3%. For each FB28 concentration one control, where the virus suspension was replaced by 5 ml distilled water, was performed. Fifty larvae of the fourth instar were used for each concentration, and four replicates were performed. Mortality due to virus infection was scored 10 days after the larvae were exposed to the virus and FB28 containing diet. Mortality data were corrected for control mortality according to Abbott (1925). Data were statistically analyzed using ANOVA Kruskal-Wallis- and Dunn's Multiple Comparison test.

5.2.4 INTRA-HAEMOCOELAR INFECTION

Freshly molt fourth instar CpS and CpRR1 larvae were anesthetized with diethyl ether vapour for 2-3 minutes. Prior to injection each larva was ventrally disinfected with a 0.4% hyamine solution using a sterile cotton bud. 2 µl of haemolymph containing 2 x 10^5 BVs or haemolymph extracted from not infected larvae without BVs for the controls were injected into the second proleg using a gas-proof 25 µl Hamilton syringe (0.21 mm in diameter). 25 larvae of each strain were injected with BV and the same number was injected with control haemolymph free of virus. After the larvae have recovered they were transferred onto virus free artificial diet. Larvae that died the next day were regarded as dead by handling and were excluded from the test. Mortality data were collected seven days post injection. Three independent replicates of each experiment were conducted.

5.2.5 TISSUE SPECIFIC VIRUS REPLICATION

Newly emerged fifth instar larvae of CpS and CpRR1 were infected either orally or intra-haemocoelarly. For oral infection larvae were starved for twelve hours before they were fed with small pieces of diet (2 mm^3) containing 1000 OBs. Intra-haemocoelar infection was performed as described above, with a dose of 2 x 10^5 BVs. Twenty-four, 48, 72 and 96 hours post infection, larvae were dissected and the midgut, 7 µl haemolymph and fat body were transferred into single 1.5 ml micro centrifuge tubes containing 180 µl PBS buffer (Appendix I), and stored at -20°C before DNA was extracted. Tubes for the collection of fat body were weighed before and after the fat bodies were added in order to determine the weight of the collected tissue.

5.2.6 DNA ISOLATION AND QUANTITATIVE PCR

Prior to DNA isolation midgut and fat body samples were grinded with sterile plastic microtube pestles. In order to generate a standard curve for quantitative PCR (qPCR) the following dilutions of CpGV occlusion bodies in sterile deionised water were performed: 1.25×10^6, 3.75×10^5, 1.25×10^5, 3.75×10^4, 1.25×10^4, 3.75×10^3, 1.25×10^3, 3.75×10^2 and 1.25×10^2 OB/5µl. For solubilisation of OBs 10 µl of 1 M Na_2CO_3 was added to the homogenized tissue samples as well as to the haemolymph samples and 100 µl of the serial dilutions. After incubation at room temperature for 10 min the pH was adjusted to 8.0 using 1 M HCl. DNA was isolated using DNeasy® Blood and Tissue Kit (Qiagen). DNA was eluted in 400µl elution buffer (AE) provided with the kit. For the quantification of CpGV genomes in the tissue samples oligonucleotides located in the granulin gene ware used: nested_PRCP1 upper (5`GGC CCG GCA AGA ATG TAA GAA TCA 3`) and nested_PRCP1 lower (5`GTA GGG CCA CAG CAC ATC GTC AAA 3`). The PCR reaction generated a 422 bp fragment. PCR reaction and fluorescence detection were performed using DNA engine Opticon™ System (MJ Reserarch, Biozym Diagnostic GmbH). For detection of the accumulation of PCR products as a function of amplification cycles QuantiTect SYBRGreen I PCR Kit (Qiagen) containing the double-strand DNA-specific fluorescent dye, SYBRGreen I, was used. The amount of PCR product copies was calculated with Opticon Monitor™ Software.

5.2.7 HAEMOLYMPH TRANSFUSION

Haemolymph of orally infected CpRR1 and not infected CpS and CpRR1 larvae was prepared following the method described above. Larvae of the susceptible strain CpS were reared on virus free diet until they reached the fourth instar. Larvae were injected intra-haemocoelarly as described above with 2 µl of Sf900 cell culture medium, CpS haemolymph, and infected and non infected haemolymph of CpRR1. After recovering the injection, the larvae were fed with a piece of diet either contaminated with 1000 OBs or free of virus. Mortality data was collected nine days later. Each variant consisted of 20-25 larvae. Each experiment was four times replicated.

5.3 RESULTS

5.3.1 BIOASSAYS WITH FLUORESCENT BRIGHTENER 28

In a bioassay using CpGV concentrations of 2×10^3 OB/ml for CpS and 2×10^5 OB/ml for CpR, fluorescent brightener 28 (FB28) was added to the diet at different

concentrations (Figure 5.1). The average mortalities of CpS larvae increased due to the addition of fluorescent brightener from 67% without FB28 up to 91% at a FB28 concentration of 0.075% (Figure 5.1). At higher FB28 concentrations mortality decreased to 67% at 0.3% FB28. The average mortalities of the resistant strain CpR were much lower compared to the susceptible strain CpS and did not exceed a value of 27%. Differences between the different treatments within CpS and CpR respectively were statistically not significant when Kruskal-Wallis test and Dunn's Multiple Comparison test were applied.

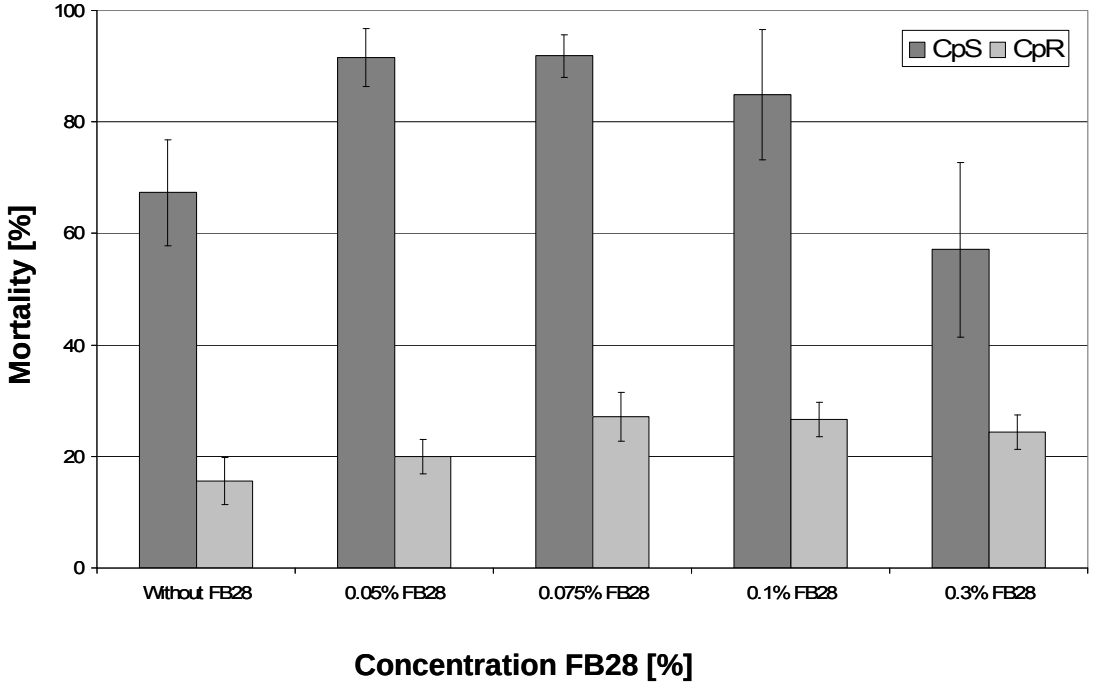

Figure 5.1 Mortalities of susceptible (CpS) and resistant (CpR) larvae in a ten days bioassay with Fluorescent brightener (FB28) added to virus concentrations of 2 x 10^3 OB/ml for CpS and 2 x 10^5 OB/ml for CpR. FB28 was added in concentrations of 0.0%, 0.05%, 0.075%, 0.1% and 0.3%.

5.3.2 INTRA-HAEMOCOELAR INFECTION

A dose of 2 x 10^5 BV particles was injected into the haemocoel of susceptible CpS and resistant CpRR1 fourth instar larvae and mortality data were collected every day. The mortality of CpS individuals caused by CpGV infection reached 100% seven days post

injection (Table 5.1). Larvae of CpRR1 did not show any signs of CpGV infection and all survived the BV injection. The surviving CpRR1 individuals were further observed until pupation but mortality did not further increase (data not shown). Mortality in the untreated controls in both strains was negligible.

Table 5.1 Average mortality of susceptible (CpS) and resistant (CpRR1) larvae at day 7 after intra-haemocoelar injection of Sf900 cell culture medium (untreated) or medium containing 2×10^5 budded virus (BV). Data are the mean of three replicates of 24-27 larvae per treatment.

	Control		BV injection	
	CpS	CpRR1	CpS	CpRR1
Mean mortality [%]	2.7	0	100	0
Standard deviation	4.6	0	0	0

5.3.3 REPLICATION OF CPGV IN DIFFERENT TISSUES

Fifth instar larvae of CpS and CpRR1 were infected either orally with 1000 OBs or intra-haemocoelarly with a dose of 2×10^5 BVs. Virus replication in different tissues was determined 24, 48, 72 and 96 hours post infection (hpi) using qPCR. After oral infection of CpS larvae virus replication was detected first in the fat body after 48 hours (Figure 5.2). At 72 hpi, virus replication was observed also in the haemolymph and the midgut and increased until 96 hpi. In contrast, virus replication was not detected in tissues of resistant CpRR1 larvae after oral inoculation, independently of the time point. When budded virus was injected into CpS larvae, CpGV copies were first detected in the haemolymph at 24 hpi, and after 48 hours also in the fat body (Figure 5.3). In the haemolymph of CpRR1 larvae, there was no detection of the PCR product until 72 hours post injection. Some weaker CpGV signals were detected in CpS and CpRR1 controls as well as in CpGV-M infected CpRR1 at 96 hpi (Figure 5.3). These signals, however, did not derive from the CpGV-M inoculum but from a contamination with CpGV-I12 in the reared insects as substantiated by PCR using CpGV-I12 specific primers (data not shown).

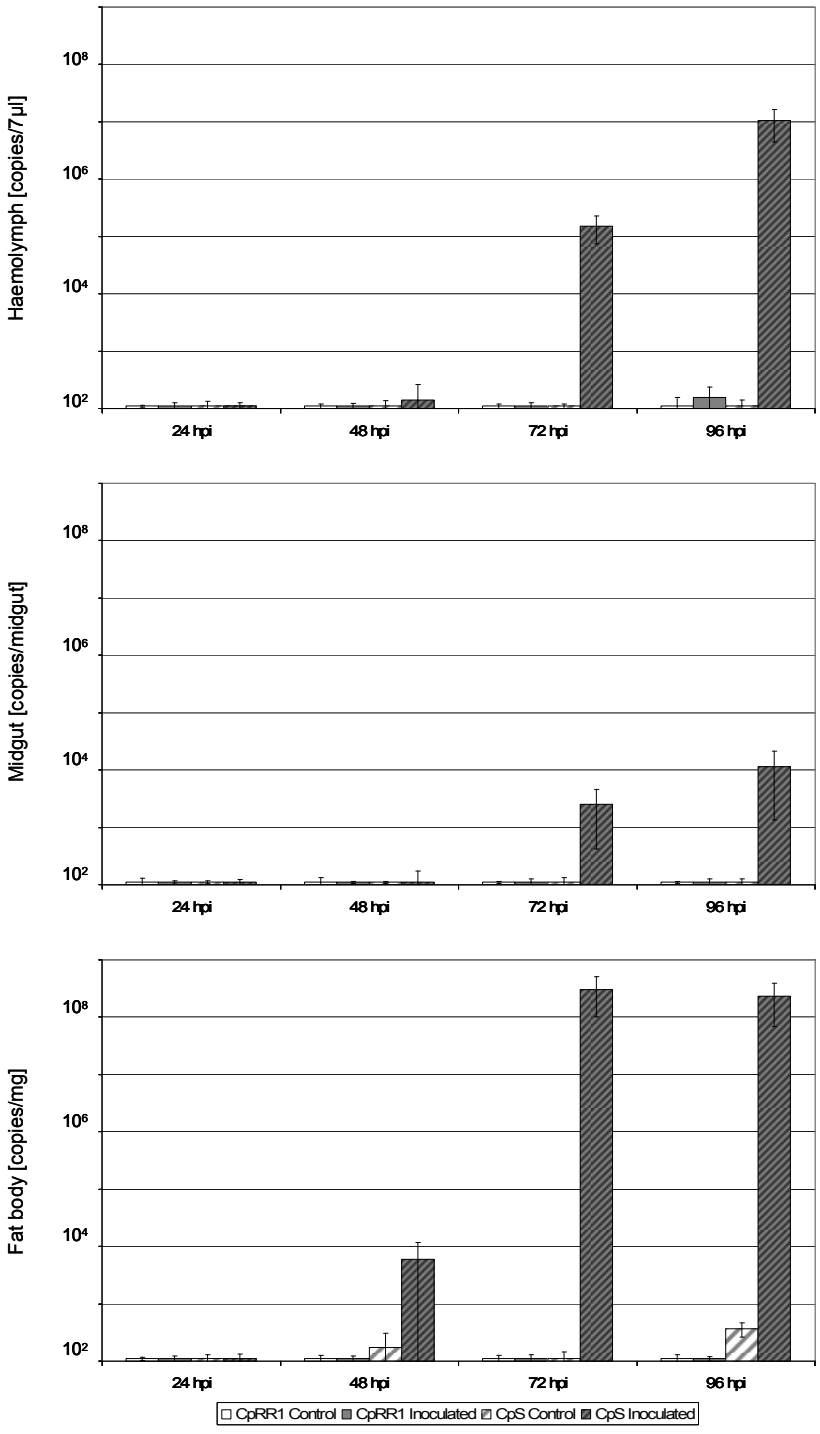

Figure 5.2 Tissue specific replication of CpGV in susceptible (CpS) and resistant (CpRR1) fifth instar larvae at 24, 48 72 and 96 hours post oral infection using 1000 OB/larva.

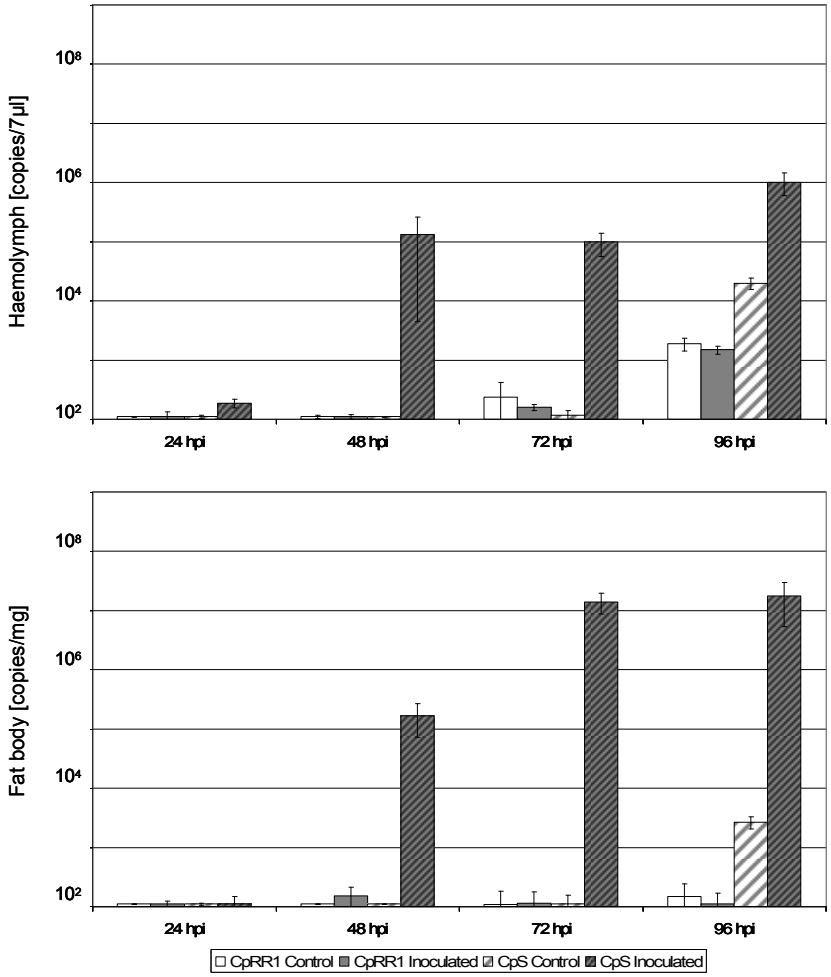

Figure 5.3 Tissue specific replication of CpGV in susceptible (CpS) and resistant (CpRR1) fifth instar larvae at 24, 48 72 and 96 hours post intra-haemocoelar injection of 2 x 10^5 BV/larva.

5.3.4 HAEMOLYMPH TRANSFUSION

In order to investigate whether a humoral factor in the haemolymph of CpRR1 larvae may convey resistance against CpGV, haemolymph from resistant larvae was injected into susceptible larvae before they were orally infected with CpGV (Table 5.1). Haemolymph from CpS larvae was injected into CpS larvae in order to explore whether injection of haemolymph from just another individual affects CpGV infection (treatment A). To see if resistance in CpRR1 larvae is consistently present or can be induced by CpGV infection, haemolymph from uninfected CpRR1 (treatment B) and from CpGV infected CpRR1 individuals (treatments C and D) was injected to CpS larvae. As a control cell culture medium Sf900 was injected into CpS larvae which were

afterwards either fed with virus free diet (treatment E) or a piece of diet containing 1000 OBs (treatment F). The results of the different treatments A to F and are shown Table 5.2. Mortality of CpS larvae nine days post injection with Sf900 medium and oral infection was 76.1% (treatment F). The variant of CpS larvae that were injected with Sf900 medium and not infected with occlusion bodies afterwards displayed 1.2% mortality (treatment E). The treatments B and C, where CpS larvae were injected with CpRR1 haemolymph prior to oral inoculation, showed slightly lower mortalities compared to the treatments F and A where CpS larvae were injected with Sf900 medium or CpS haemolymph, respectively. However, these differences were not statistically significant indicated by the high standard deviation (SD). The mortality of treatment D was 2.5 % which is similar to the control (treatment E).

Table 5.2 Mortality data of CpS larvae in different treatments (A-F) nine days post inoculation. In treatment F, CpS larvae were injected with Sf900 medium before they were orally infected with 1000 OBs. Treatment E represents injection of CpS larvae with Sf900 medium without subsequent oral inoculation. In treatment A, CpS larvae were injected with haemolymph taken from CpS larvae and were orally infected with 1000 OBs afterwards. Larvae which were injected with haemolymph taken from CpRR1 larvae and orally infected with OBs belong to treatment B. Treatments C and D represent injection of CpS larvae with haemolymph from CpRR1 larvae which were inoculated with 1000 OBs before haemolymph was taken. In treatment C CpS larvae were orally infected post injection whereas CpS larvae in treatment D were not orally infected.

Treatment	Oral infection of CpS	Injection of haemolymph (HL) or Sf900 medium prior to oral infection	Mortality [%] of CpS	SD
A	yes	HL of CpS, uninfected	79.4	15.8
B	yes	HL of CpRR1, uninfected	63.1	17.4
C	yes	HL of CpRR1, infected	61.6	16.6
D	no	HL of CpRR1, infected	2.5	5.0
E	no	Sf900 medium (control)	1.2	2.4
F	yes	Sf900 medium (control)	76.1	16.2

5.4 DISCUSSION

Optical brighteners are described to have an enhancing effect on the virulence of baculoviruses by disintegration of the PM (El-Salamouny et al., 2005). The loss of this mechanical barrier results in more efficient passage of virus to the midgut cells (Corsaro et al., 1993). Addition of FB28 to *Agrotis segetum* nucleopolyhedrovirus and *A. segetum* granulovirus increased the susceptibility of *A. segetum* (Lepidoptera: *Noctuidae*) to both viruses (El-Salamouny et al., 2005). Furthermore, optical brighteners affect sloughing of infected midgut epithelial cells which is also a defence mechanism

of insects to viral infection (Washburn et al., 1998). In the experiments shown here, a significant affect of FB28 on the efficacy of CpGV against CpS and CpR larvae was not observed (Figure 5.1). In *C. pomonella* larvae the PM is very thin, leaving doubts that it functions as a physiological barrier of CpGV (R. Kleespies, personal communication). For CpR it can be at least stated that there is no evidence that the PM is modified in that way that addition of FB28 would increase the susceptibility to CpGV. When FB28 was added to the diet at a concentration of 0.3% the mortality decreased. This may be explained by the acidic effect of the compound which lowers the pH value in the midgut. Sheppard et al. (1994) observed a significant reduction in midgut luminal pH due to the fluorescent brightener Tinopal. Solubilisation of OBs in the midgut could be affected by the lower pH value, resulting in a passage through the midgut without a release of infectious virions from the OBs. The mortality rates observed for CpR in this experiments correspond to the average mortality generally observed for strain CpR (Chapter 3) (Eberle et al., 2006; Asser-Kaiser et al., 2009). This mortality can be ascribed to the fraction of susceptible individuals present in the heterogeneous strain CpR which die from virus infection, whereas the resistant individuals survive the CpGV concentration of 2 x 10^5 OBs/ml (Chapters 2 and 3) (Asser-Kaiser et al., 2007; Asser-Kaiser et al., 2009).

To differentiate between midgut based and systemic resistance to a baculovirus injection of BVs into the haemocoel is an appropriate experiment. Fuxa and Richter (1990) investigated the resistance of *Spodoptera frugiperda* larvae against *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV). There was no significant difference in mortality when SfMNPV was injected into the haemocoel. Thus, they concluded that resistance to the virus was associated with the gut of the insect. There are several examples for midgut-based resistance to a virus described in literature (Watanabe, 1971; Fraser & Stairs, 1982; Engelhard & Volkman, 1994; Hoover et al., 2000; Haas-Stapleton et al., 2005). However, in the present study injection of BVs of CpGV killed all susceptible larvae within seven days, whereas all larvae of the resistant strain CpRR1 survived (Table 5.1). Consequently, CpRR1 larvae are resistant to both, infection of the midgut epithelium by ODVs and secondary infection induced by BVs. Resistance solely due to an altered midgut receptor, as described by Ito et al. (2008) or an unspecific binding of ODVs to midgut receptors as reported by Haas-Stapleton et al. (2005) can be excluded. This was confirmed by the investigation of tissue specific virus replication, where replication was blocked after both oral and intra-haemocoelar infection (Figure 5.2, Figure 5.3).

In order to get more detailed information about the location of resistance in the infection route, replication of the virus in susceptible and resistant larvae was traced after oral and intra-haemocoelar infection with CpGV using qPCR. The results clearly show that

replication of the virus was inhibited in all three tissues of CpRR1 (Figure 5.2, Figure 5.3). Replication is apparently blocked in midgut and fat body cells and also in haemocytes, indicating that CpGV is unable to replicate in any cell type of.

We further investigated whether an immune factor present in the haemolymph of CpRR1 triggers resistance to CpGV. Haemolymph of CpRR1 was transfused into CpS larvae in order to see whether resistance can be carried over with the haemolymph (Table 5.2). Using this method there was no evidence that transfusion of haemolymph from CpRR1 larvae could reduce susceptibility of CpS larvae to oral infection with CpGV. Phenylthiourea (PTU) was added to the haemolymph during its preparation in order to prevent melanisation. However, PTU is an inhibitor of the enzyme phenoloxidase and eliminates its virucidal activity (Popham et al., 2004). If an increased level of phenoloxidase in the plasma of CpRR1 was related to resistance, similar to the virucidal effect of plasma of *Heliothis virescens* larvae described by Popham et al. (2004), we would not have seen it in experiments using PTU. Immune reactions like an increased activity of the enzyme phenoloxidase in the plasma can reduce susceptibility of a host to its virus (Popham et al., 2004; Shelby et al., 2006). However, it is unlikely that a virus infection is completely blocked by this mechanism, as it is the case in CpRR1. Furthermore, the virucidal effect of phenoloxidase is unspecific and would be expected to affect both CpGV-M and CpGV-I12. But, CpGV-I12 is able to overcome resistance in CpRR1 very effectively (Eberle et al., 2008) (Chapter 4). Therefore and with regard to the results of the tissue specific virus replication experiment we propose that resistance is caused by inhibition of virus replication in the cells rather than an humoral immune response.

Beside the information about a possible immune response there is another conclusion that can be drawn from the transfusion experiment. Mortality of CpS larvae injected with haemolymph from CpRR1 larvae that were inoculated with 1000 OBs prior to haemolymph extraction (Table 5.2, F), did not differ significantly from the mortality of CpS larvae injected with Sf900 medium (Table 5.2, B). Hence, haemolymph derived from infected CpRR1 larvae did not contain active BVs that would infect susceptible insects. This is consistent with the observation that there is no virus replication in the haemocytes or other cell types of CpRR1.

In summary, the mechanism of resistance to CpGV in CpRR1 is prevalent in the whole insect. Furthermore, the mechanism must be a very specific as it inhibits infection by CpGV-M but not by CpGV-I12 (Eberle et al., 2008) (Chapter 4). All unspecific mechanisms like a modified PM, sloughing of infected cells, inactivation of BVs by the enzyme phenoloxidase and other unspecific immune responses can be excluded. As no virus DNA replication could be detected in the resistant insects, it is obvious that the

mode of resistance is located during an early event in the replication process. Resistance occurred with BVs and ODVs with the same efficiency. The envelopes of BVs and ODVs comprise different attachment proteins binding to cellular receptors (Rohrmann, 2008). Therefore, a modification of the attachment protein as a reason for resistance is unlikely. Apoptosis occurs early in the infection process (Clem, 2001). CpGV exhibits an inhibitor of apoptosis (*iap*) that prevents apoptosis in *C. pomonella* leading to a successful infection (Crook et al., 1993). A mutation in the insect and/or the virus could abolish inhibition of apoptosis by CpGV-M in CpRR1 larvae, resulting in a block of virus infection and replication. The different experiments undertaken to dissect the infection process of CpGV-M in susceptible and resistant *C. pomonella* larvae provided clear evidence that CpGV resistance is caused by a blockage of CpGV replication at an early stage of infection and that it is specific to CpGV-M.

6 INVESTIGATIONS ON PROTEIN-PROTEIN INTERACTIONS BETWEEN PE38 OF CPGV-I12 AND HOST PROTEINS OF A CPGV RESISTANT CODLING MOTH STRAIN

6.1 INTRODUCTION

6.1.1 BACKGROUND AND AIM OF THE STUDY

In Europe, several codling moth populations have developed resistance to the *Cydia pomonella* granulovirus (CpGV) (Fritsch et al., 2005; Sauphanor et al., 2006; Asser-Kaiser et al., 2007) (Chapter 2). All CpGV products used for codling moth control in European apple and pear production are based on the same virus isolate CpGV-M, which was originally discovered in Northern Mexico in 1963 (Tanada, 1964, Huber, 1998). Inheritance of resistance to CpGV-M is described to be due to a single gene which is located on the Z-chromosome of CpRR1 (Asser-Kaiser et al., 2007) (Chapter 2). When resistant codling moth populations were detected, an intensive search for CpGV isolates capable to overcome resistance has been launched. The genomes of eight new field isolates of CpGV originating from Iran and Georgia and one isolate from England have recently been analyzed and compared to the isolate CpGV-M (Eberle et al., 2009). These isolates were not only characterized by restriction fragment length polymorphism (RFLP) and partial genome sequencing. They were also tested for their virulence to codling moth larvae by comparing their efficacy against a susceptible laboratory strain CpS and a strain that is resistant to the isolate CpGV-M, called CpRR1 (Eberle & Jehle, personal communication, Eberle et al., 2008) (Chapter 4). Several of these new isolates were able to overcome resistance in CpRR1 (Eberle & Jehle, unpublished). The sequences of the isolates showed several differences to the sequence of CpGV-M on nucleotide and amino acid basis, but there was only one difference in which CpGV-M differed from all resistance overcoming isolates: CpGV-M showed an insertion of 24 bp in the open reading frame (ORF) 24, which is coding for the gene *pe38* (Eberle & Jehle, personal communication). These findings highly suggest that *pe38* could be the gene responsible for the ability of these isolates to overcome resistance against CpGV-M. In *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *pe38* is described to be an immediate-early gene, which is first detectable 1 h post inoculation. The predicted amino acid sequence of PE38 contains two putative DNA binding and dimerisation domains: a zinc-finger motif near the N-terminus and a

leucine zipper motif located at the C-terminus of the protein (Krappa & Knebel-Mörsdorf, 1991) and it appears to activate DNA replication (Kool et al., 1994). Furthermore, deletion of *pe38* in AcMNPV results in a delay of infection, a 99% reduction of budded virus production, reduced levels of DNA synthesis and a reduction in oral infectivity in larvae (Milks et al., 2003).

Investigations on the mechanism of CpGV resistance revealed that in resistant CpRR1 larvae CpGV-M replication is blocked at an early stage of infection (Chapter 5). This finding supports the assumption that the early transcribed gene *pe38* may be involved in the resistance overcoming mechanism of CpGV-I12.

In summary, CpS larvae are susceptible to both CpGV-M and CpGV-I12, whereas CpRR1 larvae are resistant to infection by CpGV-M but not to infection by CpGV-I12. The putative gene that enables CpGV-I12 to infect CpRR1 is *pe38*. The insertional mutation of the gene *pe38* in CpGV-M is the only difference between CpGV-M and all known resistance overcoming isolates. It can be hypothesized that this mutation possibly disables the interaction of PE38 of CpGV-M with its protein partner in CpRR1, and as consequence CpGV-M replication may be blocked. The aim of this study was to investigate protein-protein interactions between the early expressed viral protein PE38 and proteins of *Cydia pomonella*. For this purpose the not mutated *pe38* of CpGV-I12 ($pe38^{I12}$) was chosen to find interacting host proteins. There are several methods available to investigate protein-protein interactions. Classical biochemical approaches are co-purification, affinity purification or co-immunoprecipitation of protein complexes, which require *in vitro* handling of protein extracts. Furthermore, these techniques exhibit limitations like restricted sensitivity and bias towards high affinity interactions. The yeast two-hybrid (Y2H) system allows detection of interacting proteins in a more natural environment *in vivo* (Brückner et al., 2009). As a first step towards elucidation of the functional role of PE38, the Y2H system was chosen to identify possible interaction partners in CpRR1.

6.1.2 YEAST TWO-HYBRID SYSTEM

The Y2H system, developed by Fields and Song (1989), is used to identify protein interaction partners for a protein of interest. It is based on a yeast genetic assay in which the interaction of two proteins is measured by the reconstitution of functional transcription activator in yeast. Transcription factors in yeast consist of two physically separable domains: a DNA-binding domain and a transcription activation domain. The DNA-binding domain recognizes a specific promotor sequence and binds to it (designated as upstream activation sequence UAS) whereas the activation domain initiates transcription of a specific gene. When these two domains are expressed as

separate proteins, the DNA-binding domain (BD) will still bind to the UAS, but the activation domain (AD) is not in the right place to activate transcription. To test interaction of two proteins, they are expressed in fusion with the BD and the AD, respectively. The known protein is expressed in fusion with the BD and serves as a bait hybrid. The AD is fused with unknown possible protein partners and forms the prey hybrid. If the bait protein binds to a prey protein, the transcription factor is reconstituted, and gene expression is activated (Fields & Song, 1989). A schematic illustration is shown in Figure 6.1. To monitor transcriptional activation, reporter proteins such as b-galactosidase are expressed under the control of the GAL4-upstream activating sequence. In order to select transformants, where interaction between the bait hybrid and the prey hybrid has occurred, yeast cells harbouring a prey cDNA fragment coding for a protein that binds to the bait are isolated. Analysis of positive clones is performed by sequencing of the library insert. Reliable interactions are selected by simple statistic criteria, such as frequency of the occurrence with the specific bait is used as information about the reproducibility of the interaction. Hereby the frequency of occurrence with other baits, the so called “stickyness” of the prey has to be considered as well. Sticky preys are excluded (Albers et al., 2005).

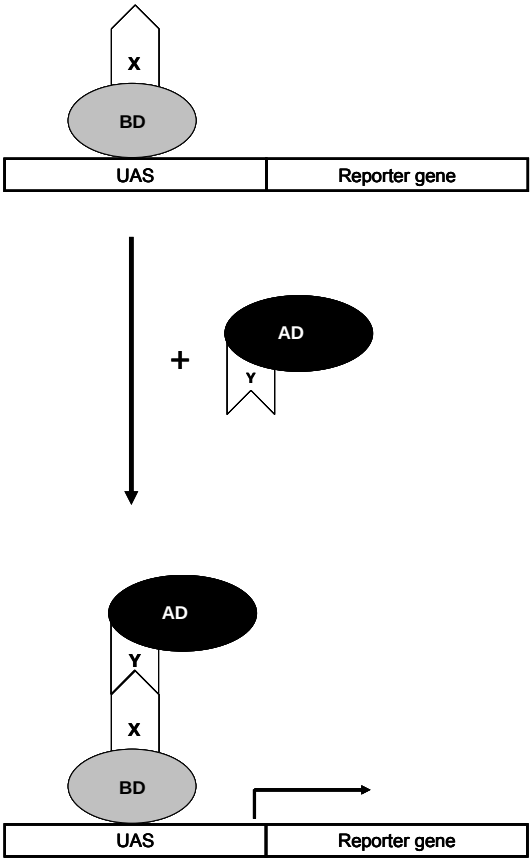

Figure 6.1 A schematic representation of the yeast two-hybrid system. The bait hybrid consists of protein X fused to a DNA-binding domain (BD) while the prey hybrid consists of protein Y fused to an activation domain (AD). Neither of these alone is able to activate transcription of the reporter gene. However, if interaction of Protein X and Y occurs, a functional transcription activator is generated and results in the transcription of the reporter gene.

The aim of this study was to detect protein-protein interactions of the viral protein PE38 and a protein partner in *C. pomonella* using the yeast two-hybrid system. Protein PE38 of the resistance overcoming virus isolate CpGV-I12 was used as bait protein and was expressed in fusion with DNA-binding domain of the vector pGBKT7. A cDNA library of the CpGV resistant strain CpRR1 was generated and used for the construction of an AD fusion library in pGADT7-Rec. Interactions between PE38 and proteins of the cDNA library were screened at the German Cancer Research Centre in Heidelberg and potential interaction partners are discussed in this chapter.

6.2 MATERIAL AND METHODS

6.2.1 TEST INSECTS

Codling moths larvae used for RNA isolation were reared at the DLR Rheinpfalz (Agricultural Service Center Palatinate), Neustadt/Weinstraße. Strain CpS is a strain which is susceptible to both virus isolates, CpGV-M and CpGV-I12, and has been reared in Neustadt for ten years. Strain CpRR1 is resistant to CpGV-M but susceptible to CpGV-I12 (Chapter 4) (Eberle et al., 2008; Asser-Kaiser et al. 2007, 2009). The insects were reared at 26°C, 60% relative humidity and 16/8 hours light/dark photoperiod. Larvae were reared on semi-artificial diet containing water, agar-agar and maize meal, wheat germ, brewers yeast, ascorbic acid and methyl-4-hydroxybenzoate (Appendix I) (Ivaldi-Sender, 1974).

6.2.2 VIRUS

The resistance overcoming isolate CpGV-I12 (Eberle et al., 2008) was used for the construction of the as DNA-BD fusion vector. The isolate was propagated in about 250 fourth instar larvae of the susceptible *C. pomonella* strain CpS. The occlusion bodies were purified following the method described by Jehle et al. (1992). DNA isolation was performed according to the method described by Arends and Jehle (2002).

6.2.3 AGAROSE GEL ELECTROPHORESIS OF DNA

PCR products, cDNA and DNA fragments resulting from digestion with DNA restriction endonucleases were separated and visualized on horizontal 0.8% agarose gels using 1 x TAE buffer (Appendix I). Gels were prepared by melting 0.8 g agarose per 100 ml 1 x TAE buffer in a microwave and pouring the solution into an appropriate frame to solidify. DNA samples were mixed with 6 x loading dye (Appendix I), before they were loaded on the gel. To determine the size of the polymerase chain reaction (PCR) products or the restriction fragments GeneRuler^{TM} 1 kb DNA ladder (Fermentas) was used as a marker and loaded on the gel next to the samples. In case of visualization of cDNA O'GeneRuler^{TM} DNA Ladder Mix (Fermentas) was used. Gels were electrophoresed at 80 V for 60 minutes and then stained in an ethidium bromide bath (0.5 µg/ml) for 15-30 minutes followed by destaining in water for 5-10 minutes. DNA fragments were visualized using a UV-transilluminator (wavelength 321 nm) and photographs were taken using INTAS documentation system.

6.2.4 CONSTRUCTION OF THE DNA-BD FUSION VECTOR

6.2.4.1 AMPLIFICATION OF THE FUSION GENES

Three different DNA-BD fusion vectors were constructed: the complete gene *pe*38 as well as the 5' leucine zipper coding motif and the 3' zinc-finger coding motif were cloned into the DNA-BD vector pGBKT7 (Clontech) (Appendix II) to be expressed in fusion with the GAL4 DNA binding domain. For the amplification of the three different fusion genes by PCR, oligonucleotides containing the restriction sites *Eco*RI (GAATTC) and *Bam*HI (GGATCC) at their 5' ends were designed in order to ligate the PCR products into the multiple cloning site (MCS) of the DNA-BD vector (Figure 6.2).

Oligonucleotides to amplify the complete gene *pe*38:

pe38_au_upper 5'-GCC CAG GAT CCA AAA TAA CAG CAA TAA TA-3'

pe38_au_lower 5'-CTA GGA ATT CAA CTC GGA CTC GTC AGT-3'

Oligonucleotides to amplify the leucine zipper motif of *pe*38:

pe38_mi_upper 5'-ACT GGA TCC CCA TCT CCG CCT TAC A-3'

pe38_au_lower 5'-CTA GGA ATT CAA CTC GGA CTC GTC AGT-3'

Primers to amplify the zinc-finger motif of *pe*38:

pe38_au_upper 5'-GCC CAG GAT CCA AAA TAA CAG CAA TAA TA-3'

pe38_mi_lower 5'TGT GAA TTC GAG ATG GAG CAG CAG TT-3'

Figure 6.2 (Next page) Location of the primers for amplification of the three different bait genes of the virus isolate CpGV-I12 (a). Nucleotide sequence of $PE38^{I12}$ (highlighted in grey) and flanking regions separated by start and stop codons (highlighted in red) of the CpGV-I12 genome (position 20563-19214) (b). The predicted amino acid sequence is shown underneath. Periodic repetition of leucine residues are highlighted in pink and cluster of cysteine and histidine residues are highlighted in green. The location of oligonucleotides used for the amplification of the full-length pe38, the leucine zipper and the zinc finger domain are highlighted in yellow and direction of amplification marked with arrows.

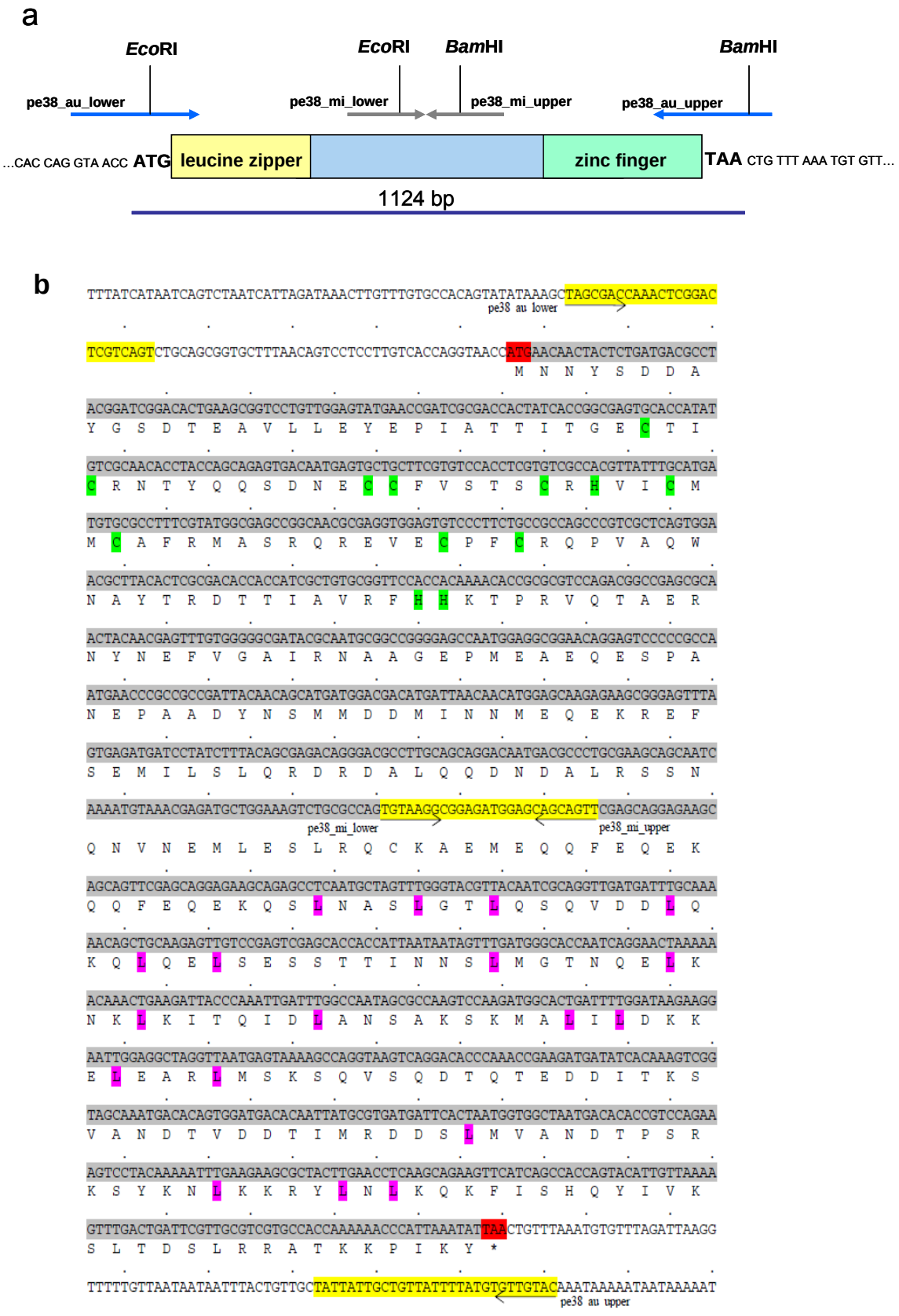

Figure 6.2 (Legend see page before)

The PCRs were performed (Mastercycler, Eppendorf) with Taq DNA polymerase (Axon Labortechnik, Kaiserslautern). The following reactions were performed in 0.2 ml PCR reaction tubes:

PCR reaction:

5 µl	10 x PCR buffer minus $MgCl_2$
1.5 µl	50 mM $MgCl_2$
1.5 µl	dNTP mixture (10 mM)
1 µl	upper Primer (10 pmol/µl)
1µl	lower Primer (10 pmol/µl)
1 µl	Taq polymerase (5 U/µl)
1-3 µl	Template DNA (CpGV-I12)
Ad 50 µl	autoclaved bidestilled water

PCR program:

Initial denaturation	94°C for 3 minutes
then 30 cycles:	
Denaturation	94°C for 45 seconds
Annealing	56°C (complete *pe38*) / 59°C (leucine zipper) / 54°C (zinc finger) for 45 seconds
Extension	72°C for 60 seconds
Final extension:	72°C for 5 minutes

PCR products were identified by agarose-gel electrophoresis (0.8 % agarose TAE).

6.2.4.2 DNA RESTRICTION AND LIGATION OF THE PCR PRODUCTS INTO PGBKT7

The three PCR products were purified using Illustra GFX DNA and Gel Band purification Kit (GE Healthcare). Each of the three PCR products and the vector pGBKT7 were digested with the restriction endonucleases *Eco*RI and *Bam*HI (FastDigest, New England Biolabs).

Reaction:

4 µl	10 x NE buffer *Eco*RI
2 µl	*Bam*HI
2 µl	*Eco*RI
0.5 µl	bovine serum albumin (100 mg/µl) (BSA)
2 µl	DNA (600 ng/µl)
29.5 µl	autoclaved bidestilled water

The reaction components were mixed by gently pipetting up and down before the reaction tubes were incubated at 37°C for 5 minutes. Afterwards the tubes were placed on ice and DNA was purified with illustra™ GFX DNA and Gel Band purification Kit (GE Healthcare). Concentration of the DNA was estimated by agarose gel electrophoresis (0.8% agarose TAE) by comparing with defined DNA amounts. The PCR fragments were ligated into the linearized vector at a ratio of 3:1 (vector to insert) using T4 DNA ligase (Fermentas).

Reaction:

2 µl	10x ligation buffer
2 µl	T4 DNA ligase
0.5-1 µl	insert (pe38, leucine zipper or zinc-finger)
1 µl	pGBKT7 vector

Ad 20 µl autoclaved bidestilled water

Ligation was performed in 0.5 ml reaction tubes (Eppendorf) at 22°C for 1 h. Ligase was inactivated by incubating the reaction mixture at 65°C for 10 minutes.

6.2.4.3 TRANSFORMATION OF *ESCHERICHIA COLI* CELLS

The three different ligation reactions were transformed into competent DH5α *Escherichia coli* cells by electroporation. Frozen 50 µl aliquots of electro-competent *E. coli* were thawed on ice and transferred into pre-chilled electroporation cuvettes (MicroPulser™ cuvettes, 0.2 cm gap, BioRad) before 1 µl of the ligation reaction was

added. For the negative control, 1 µl autoclaved bidestilled water was added instead of ligation reaction. Transformation was performed in MicroPulser™ electroporation apparatus (BioRad) at 2.5 kV for ~4.5 ms. 950 µl SOC medium (Appendix I) was added to the cells immediately after electroporation. The suspension was transferred to sterile 1.5 ml Eppendorf reaction tubes and incubated for 60 min at 37°C with shaking at 225 rpm. After that, the bacterial culture was diluted 1:10 and 1:100 and 100 µl of the each dilution were plated on LB agar plates (Appendix I) containing 50 µg/ml kanamycin A monosulfate (Duchefa Biochemie). The plates were incubated at 37°C for 24 h.

6.2.4.4 PLASMID PREPARATION

Five single clones of each transformation were picked from the plate using sterile pipette tips and incubated in 2 ml LB medium containing 50 µg/ml kanamycin A monosulfate (Duchefa Biochemie) in 15-ml Falcon tubes (Greiner Bio-One GmbH) at 37°C with shaking at 225 rpm for 16 h. Plasmid DNA was isolated from the bacterial culture using the Illustra™ PlasmidPrep Mini Spin Kit (GE Healthcare).

6.2.4.5 IDENTIFICATION OF THE INSERT-CONTAINING PLASMIDS BY RESTRICTION

Plasmid DNA of the three different constructs and the original vector pGBKT7 was digested using the restriction endonuclease *Hin*dIII (Invitrogen).

Reaction:

2 µl	10 x buffer ReAct2
1 µl	*Hin*dIII
1 µl	DNA (600 ng/µl)
16 µl	autoclaved bidestilled water

The reaction components were mixed by gently pipetting up and down before the reaction tubes were incubated at 37°C for 2.5 h. After heat inactivation for 15 min at 65°C the fragment sizes were determined by agarose gel electrophoresis (0.8% agarose TAE).

6.2.4.6 PREPARATION OF COMPETENT YEAST CELLS WITH THE LIAC METHOD

A small portion of frozen yeast stock (AH109 and Y187, Clontech Laboratories, Inc., USA) was streaked on an YPDA agar plate (Appendix I). The plates were sealed with Parafilm® "M" (Pechiney Plastic Packaging, Chicago, USA) and incubated upside down at 30°C for three days. One colony of each strain was inoculated into 3 ml of YPDA medium in a sterile 15-ml Falcon tube (Greiner Bio-One GmbH). In order to suspend

yeast cells they were vigorously mixed by vortexing, before they were incubated for 8 h at 30°C with shaking at 250 rpm. 5 µl of the culture were transferred to 250-ml flask containing 50 ml of YPDA medium (Appendix I) and incubated at 30°C with shaking at 230-250 rpm for 16-20 hours. At an OD_{600} of 0.15-0.3 cells were centrifuged at 700 x g for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 100 ml of YPDA medium. After incubation at 30°C for 3-5 h (OD_{600} = 0.4-0.5) cells were centrifuged again at 700 x g for 5 min. The cell pellet was suspended in 60 ml of sterile, deionised water before they were centrifuged for a third time at 700 x g for 5 min at room temperature. The supernatant was discarded again and cells were suspended in 3 ml of 1.1 x TE/LiAc solution (Appendix I). Subsequently the cells were split between two sterile 1.5-ml Eppendorf tubes and centrifuged at high speed for 15 seconds. Each pellet was resuspended in 600 µl of 1.1 x TE/LiAc solution. Competent cells were used for transformation immediately following the preparation.

6.2.4.7 TEST OF THE DNA-BD FUSION FOR TRANSCRIPTIONAL ACTIVATION

The yeast strains AH109 and Y187 were transformed with the hybrid construct using a small scale protocol. Selective SD agar plates (100 mm in diameter) were prepared (Appendix I):

SD/-Trp/x-α-GAL

SD/-His/-Trp/ X-α-GAL

SD/-Ade/-Trp/ X-α-GAL

For the transformation of yeast strains AH109 and Y187 the following components were added together in a sterile 1.5 ml reaction tube:

50 µl	AH109 or Y187 competent yeast cells
5 µl	Herring Testes Carrier DNA (10mg/ml), denatured
500 µl	PEG/LiAc Solution
1 µl	Plasmid DNA (fusion genes cloned into pGBKT7 and the original vector pGBKT7 without insert)

The components were mixed by gently vortexing and incubated at 30°C for 30 min, and gently vortexed every 10 min. Then, 20 µl DMSO were added to each tube, placed into a 42°C water bath for 15 min and vortexed every 5 min. The tubes were centrifuged at high speed in a microcentrifuge for 15 sec. The supernatant was removed and the pellet was resuspended in 1 ml of YPD Plus Liquid Medium (provided in the Mathcmaker™

Library Construction & Screening Kit, Clontech Laboratories, Inc., USA) by gently pipetting up and down. After incubation at 30°C for 90 min the tubes were centrifuged at high speed for 15 sec. The supernatant was discarded and the pellet was resuspended in 1 ml sterile NaCl (9%, w/v) solution by gently pipetting up and down. Three dilutions of each variant were prepared: 1:10, 1:100, and 1:1000 and 100 µl of each dilution were spread onto selective SD-agar plates. The plates were incubated for 3 days at 30°C.

6.2.4.8 VERIFICATION OF THE CORRECT INSERTION OF THE FUSION GENES BY SEQUENCING

In order to verify the correct insertion and to look for mutations in the insert, purified DNA of the three DNA-BD fusion vectors was sent for sequencing to GENterprise GmbH in Mainz, Germany.

6.2.5 GENERATION OF CDNA LIBRARIES OF CPS AND CPRR1

6.2.5.1 TOTAL RNA ISOLATION FROM MIDGUT TISSUE USING TRIZOL REAGENT (GIBCO/BRL)

Midguts of 20 fourth instar CpS and CpRR1 larvae were homogenised in 1 ml of TRIzol® Reagent (Invitrogen) in a sterile 1.5 ml Eppendorf tube using a micro pistil, and incubated at room temperature for 5 min. After centrifugation at 12,000 x g at 4°C for 10 min the top layer containing fat was removed. The RNA containing supernatant was transferred to sterile 1.5 ml reaction tube. After adding 0.2 ml of chloroform, the tubes were shaken vigorously for 15 sec and incubated for 2-3 min at room temperature. Then, the samples were centrifuged at 7,500 x g at 4°C for 5 min. The supernatant was discarded and the pellet containing the RNA was washed with 1 ml of ethanol (75%, v/v) by vortexing. After a second centrifugation step at 7,500 x g at 4°C for 5 min the supernatant was removed and RNA was allowed to air dry before it was dissolved in RNase-free water. RNA was stored at -80°C prior to use for cDNA synthesis.

6.2.5.2 SYNTHESIS OF CDNA

For cDNA synthesis the SMART (Switching Mechanism at 5' end of RNA Transcript) technology was used. Messenger RNA (mRNA) is efficiently copied into double stranded complementary DNA (dscDNA) and this technique and is particularly well suited for two-hybrid library construction because it consistently delivers high yields of cDNA while maintaining sequence representation (Zhu et al., 2001). Reagents used for the synthesis were provided by the Mathcmaker™ Library Construction & Screening Kit (Clontech Laboratories, Inc., USA).

For first-strand cDNA synthesis the following reagents were combined in a sterile 0.2 ml Eppendorf tube:

1.5 µl	RNA (CpS or CpRR1) (~ 2.5 µg RNA)
1 µl	CDSIII primer
1.5 µl	nuclease free water

The components were mixed by pipetting up and down and incubated at 72°C for 2 minutes. After cooling the tubes on ice for 2 min, the following reagents were added to each tube:

2 µl	5 x First strand buffer
1µl	DTT (20 mM)
1µl	dNTP (10 mM)
1µl	MMLV Reverse Transcriptase

After gently mixing the components the tubes were incubated at 42°C for 10 min. Then 1 µl SMART III oligonucleotide was added, before the tubes were incubated at 42°C for 60 min, followed by incubation at 50°C for 45 min and 75°C for 10 min. The samples were cooled down at room temperature before 1 µl RNase H was added. Lastly, the tubes were incubated at 37°C for 20 min.

Using Long distance PCR (LD-PCR) dscDNA was amplified.

Reaction:

1.5 µl	first-strand cDNA
35 µl	nuclease free water
5 µl	10 x Advantage® 2 PCR buffer (Clontech Laboratories, Inc., USA)
1 µl	dNTP Mix
1µl	5’ PCR primer (TTC CAC CCA AGC AGT GGTATC AA CGC AGA GTG G)
1 µl	3’ PCR primer (GTA TCG ATG CCC ACC CTC TAG AGG CCG AGG CGG CCG ACA)
5 µl	GC-melt solution
1 µl	50 x Advantage® 2 Polymerase mix (Clontech Laboratories, Inc., USA)

The components were gently mixed by pipetting. The thermocycler was preheated to 95°C before the tubes were placed in.

Program:

95°C	30 seconds
Then 22 cycles:	
95°C	10 seconds
68°C	6 minutes
68°C	5 minutes

The amplified dscDNA of CpS and cpRR1 was visualized by agarose gel electrophoresis and purified from the PCR reaction with CHROMA SPIN™ TE-400 Column provided by the Mathcmaker™ Library Construction & Screening Kit (Clontech Laboratories, Inc., USA). Concentration was determined with a NanoDrop™ 2000 spectrometer (Thermo Scientific).

6.2.6 CONSTRUCTION OF A GAL4-AD FUSION LIBRARY

In order to construct an AD fusion library yeast was transformed with cDNA of CpRR1 and the fusion vector pGADT7-rec. The protocol of the Mathcmaker™ Library Construction & Screening Kit (Clontech Laboratories, Inc., USA) was followed but instead of the yeast strain AH109 the strain Y187 was used. About 200 SD agar plates (150-mm) lacking the amino acid leucine and competent Y187 yeast cells were prepared according to the protocol of the kit and described above. The following ingredients were combined in a sterile, pre-chilled 15-ml Falcon tube (Greiner Bio-One GmbH):

20 µl	cDNA of CpRR1
6 µl	pGADT7-rec (0.5 µg/µl)
20 µl	Herring Testes Carrier DNA (10mg/ml) denatured
600 µl	competent Y187 cells

After gently mixing by using the vortexer, 2.5 ml PEG/LiAC solution was added. The tube was gently vortexed and incubated for 45 min at 30°C. Every 15 min the cells were mixed. After addition of 160 µl DMSO, the cells were mixed and placed in a 42°C water bath for 20 min with mixing every 10 min. After centrifugation at 700 x g for 5 minutes the supernatant was discarded and cells were resuspended in 3 ml of YPD Plus Liquid Medium. The cells were incubated for 90 min with shaking (225 rpm) for

90 min at 30°C and subsequently centrifuged at 700 x g for 5 min. After removal of the supernatant the cell pellet was resuspended in 30 ml of sterile NaCl solution (0.9%, w/v). On each of the 200 SD/-Leu plates 150 µl of the cells were spread. The plates were sealed with Parafilm® "M" (Pechiney Plastic Packaging, Chicago, USA) and incubated upside down at 30°C until colonies appeared. In order to estimate the transformation efficiency 100 µl of each dilution of a series of 1:10 up to 1:10,000 were plated on 100-mm SD/-Leu plates.

After colonies have grown, the plates were chilled at 4°C over night. Five ml of pre-chilled YPD medium containing 25% (v/v) glycerol together with sterile glass beads (ColiRollers™ Plating Beads, Novagen) were added to each plate. The cells were dislodged into the liquid by gently swirling the glass beads. The YPD medium containing the cells was transferred into a sterile 50-ml Falcon tube (Greiner Bio-One GmbH). The cells of all plates were combined and density was adjusted to an OD_{600} of 100. Before the library was used for screening it was stored at -80°C.

6.2.7 SCREENING

The GAL4-AD fusion library and the DNA of the three bait vectors were sent to the Y2H protein interaction screening service of the German Cancer Research Center (DKFZ) in Heidelberg, where protein interaction screens were performed in an automated high-throughput Y2H screening facility. The CpRR1 library was screened on interactions with the baits. The library inserts of positive clones were amplified by PCR and PCR products were sent for sequencing to GATC Biotech AG, Konstanz. The achieved sequences were aligned to the database of known sequences using NCBI's basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/). The "blastx" program, which compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) to a protein sequence database, was applied.

6.3 RESULTS

6.3.1 CONSTRUCTION OF THE DNA-BD FUSION VECTOR

Three different DNA-BD fusion vectors were constructed in order to investigate interactions of the viral protein PE38 of CpGV-I12 ($PE38^{I12}$) with a protein of its host *C. pomonella*. PCR primers were constructed which specifically amplified the complete gene *pe38*, one fragment that coded the leucine zipper coding motif and one fragment containing the zinc finger coding motif of $PE38^{I12}$. The primers also contained the restriction sites *Bam*HI and *Eco*R1 at their 5' ends to allow positional cloning of the

fragments into the multiple cloning sites (MCS) of the DNA BD vector in the right direction. The expected fragment sizes were 1275 bp for the complete gene *pe38*, 697 bp for the leucine zipper and 621 for the zinc-finger.

Prior to ligation of the PCR fragments with the DNA BD vector pGBKT7, the fragments as well as the vector were double digested with the restriction enzymes *Bam*HI and *Eco*RI (Figure 6.3). After ligation, DH5α *E. coli* cells were transformed with the ligation mixtures and positive bacterial clones were selected on LB medium containing kanamycin.

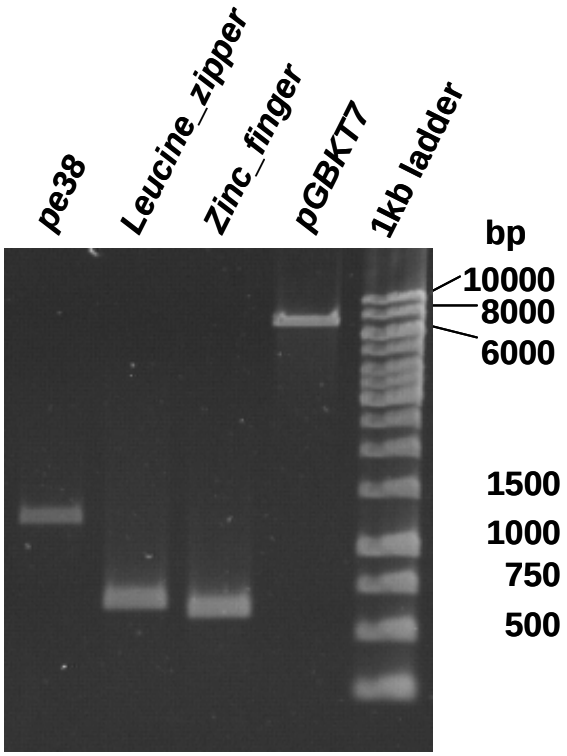

Figure 6.3 Agarose gel (0.8%, w/v) of the restriction digestion of the DNA BD vector pGBKT7 and the PCR fragments pe38, leucine zipper and zinc finger usingthe endonucleases *Bam*HI and *Eco*RI. Fragment sizes of the 1 kb ladder are given to the right (bp).

DNA of positive clones was isolated and analyzed by restriction endonuclease digestion using *Hin*dIII (Figure 6.4). The vector pGBKT7 has three *Hin*dIII restriction sites at the positions 738, 1606 and 6544, whereas *Hin*dIII does not cut in any of the three fusion genes. Therefore restriction digestion will result in three fragments in case of all three

different DNA-BD fusion vectors as well as the empty DNA-BD vector. The restriction fragment sizes were analyzed by agarose-gel electrophoresis (Figure 6.4) They corresponded to the expected fragment sizes given in Table 6.1. The empty DNA-BD vector pGBKT7 and the three DNA-BD fusion vectors pGBKT7_pe38, pGBKT7_Leucine_zipper and pGBKT7_Zinc_finger showed one big fragment of 4938 bp and one fragment of 1498 bp after digestion with *Hin*dIII (Figure 6.4). The PCR fragments were integrated between the *Hin*dIII sites at position 738 and 1606. The fragment between these sites differed in size depending on the inserted PCR fragment: The empty DNA-BD vector shows a fragment of 868 bp, whereas pGBKT7_Zinc_finger showed a second 1498 bp fragment. pGBKT7_pe38, containing the complete gene *pe*38 showed a fragment of 2143 bp and pGBKT7_Leucine zipper showed a fragment of 1547 bp (Figure 6.4).

Table 6.1 Expected fragment sizes of the empty DNA-BD vector pGBKT7 and the three DNA-BD fusion vectors pGBKT7_pe38, pGBKT7_Leucine_zipper and pGBKT7_Zinc_finger after digestion with *Hin*dIII endonuclease.

pGBKT7_pe38	pGBKT7_leucine_zipper	pGBKT7_zinc_finger	pGBKT7
4938	**4938**	**4938**	**4938**
2143			
	1547		
1498	**1498**	**2x1498**	**1498**
			868

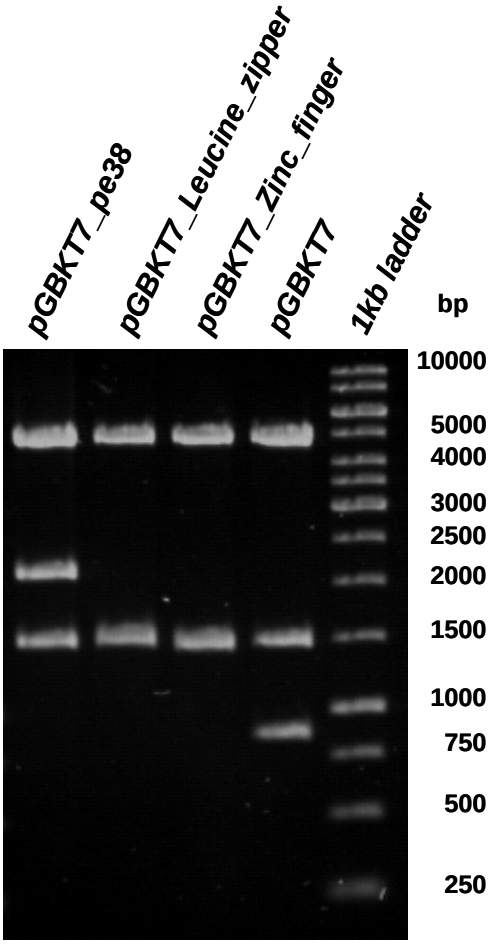

Figure 6.4 Agarose gel (0.8%, w/v) of restriction digestion analysis of the DNA-BD fusion vectors pGBKT7_pe38, pGBKT7_Leucine_zipper and pGBKT7_Zinc_finger and the empty DNA-BD vector pGBKT7 using *Hin*dIII endonuclease. Fragment sizes of the 1 kb ladder are given to the right (bp).

6.3.2 TEST OF THE DNA-BD FUSION VECTOR FOR TRANSCRIPTIONAL ACTIVATION

In order to assure that the bait protein does not activate transcription of the repoter genes after cloning it into the pGBKT7 vector, yeast strains AH109 and Y187 were transformed with the different the DNA-BD fusion vectors and screened on three different selective media containing X-α-Gal. On SD agar plates lacking tryptophane and histidine or tryptophane and adenine there was no yeast growth. White colonies were growing on SD agar plates lacking only tryptophane indicating that the bait protein did not transactivates transcription of the reporter genes and expression of tryptophane by the vector is working.

6.3.3 GENERATION OF CDNA LIBRARIES OF CPS AND CPRR1 AND CONSTRUCTION OF A GAL4-AD FUSION LIBRARY

After RNA was extracted from the midguts of 20 CpS and CpRR1 larvae, cDNA libraries were synthesized by SMART™ technology and visualized by agarose gel electrophoresis (Figure 6.5).

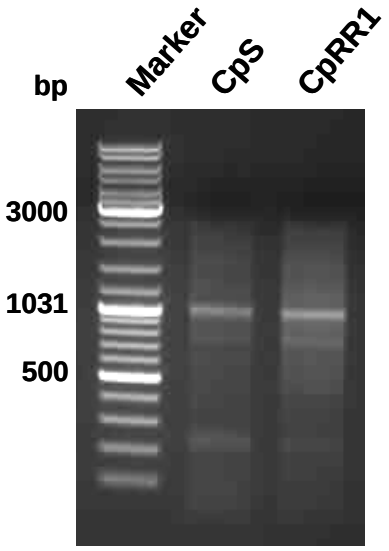

Figure 6.5 Agarose gel (0.8%, w/v) electrophoresis of cDNA of CpS and CpRR1. Fragment sized of the DNA ladder mix are given to the right (bp).

The yeast strain Y187 was transformed with cDNA of CpRR1 together with the vector pGADT7-rec (Appendix II). Hereby, cDNA fragments were integrated into the pGADT7-rec vector by homologous recombination *in vivo*. Positive transformants were selected on SD agar plates lacking leucine. After harvesting the colonies the GAL4-AD fusion library was sent together with DNA of the three different DNA-BD fusion vectors to the German Cancer Research Centre (DKFZ) in Heidelberg where the screening for interactions was performed. In total 151 positive yeast clones were detected and the cDNA inserts were sequenced.

6.3.4 IDENTIFICATION OF POTENTIAL HOT PROTEON INTERACTION PARTNERS OF PE38

The sequences were aligned to the database of known sequences using NCBI's basic local alignment search tool (BLAST) algorism (http://www.ncbi.nlm.nih.gov/). The "blastx" program, which compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) to a protein sequence database, was applied. Since the genome of *C. pomonella* is not completely sequenced the database of *Bombyx mori* was applied instead. Hits with high similarity, indicated by a high score and a low E-value, were selected and their location on *B. mori* chromosomes was mapped using the silkworm genome database (www.silkdb.org) (Biology analysis group et al., 2004). The "score" gives information about the number of corresponding positions (the more, the better) and the "E-value" indicates the probability that the hit is by random (the smaller, the better). Hits with high similarity to proteins in the *B. mori* genome, are specified in Table 6.2. Twelve proteins were found to interact with the full-length protein PE38, 20 proteins interact with the leucine zipper domain of PE38 and 7 proteins showed interaction with the zinc-finger domain of PE38. The number of interactions that were detected for a specific protein ranged from 1 to 12 interactions.

Table 6.2 Results of sequence analysis using the basic local alignment search tool (BLAST) of NCBI. Given are names of the proteins of the best hits, the score, E-value, the chromosome on which the proteins encoded in *B.mori,* the percentage of identities between the sequence query and the sequence in *B. mori* and the number of interactions detected between the protein and PE38 (NID).

Bait	Protein	Score	E-value	Identities [%]	Chromosome	NID
Full-length pe38	seronin 1	42	2-3 x 10^{-5}	40-41	5	2
	thioredoxin	133	1 x 10^{-5}	84	**15**	**3**
	electron-transfer-flavoprotein beta polypeptide	346	7 x 10^{-97}	90	25	6
	ribosomal protein L31	127	1 x 10^{-31}	94	13	2
	Hsp40 protein	218	2 x 10^{-55}	88	5	1
	ribosomal protein L10	122	1 x 10^{-26}	84	10	1
	muscle LIM protein isoform 1	43	1 x 10-^{5}	30	13	1
	myosin heavy chain variant	54	5 x 10^{-9}	86	8	3
	ribosomal protein L30	223	6 x 10^{-60}	98	15	1
	dynein molecular motor protein light chain 1	68	3 x 10^{-13}	93	7	1
	ubiquitin conjugating enzyme 4	27	0.89	44	**1 (Z)**	**1**
	cellular retinoic acid binding protein	36	1 x 10^{-4}	25	13	12
leucine zipper domain	Bm kettin	49	3 x 10^{-7}	23	**1 (Z)**	**2**
	muscle LIM protein isoform 1	48	4 x 10^{-7}	23	13	1
	glyceraldehyde-3-phosphate dehydrogenase	268	1 x 10^{-73}	95	3	1
	odorant-binding protein 4	35	3 x 10^{-6}	32	13	1
	acyl-CoA binding protein	49	2 x 10^{-7}	66	n.d.	1
	egg-specific protein	90	2 x 10^{-20}	44	19	2
	electron-transfer-flavoprotein beta polypeptide	343	3 x 10^{-96}	89	25	2
	Hemolin	73	2 x 10^{-14}	45	24	1
	Ribosomal protein S13	278	2 x 10^{-76}	97	23	1
	1-Cys peroxiredoxin	155	2 x 10^{-39}	71	**1 (Z)**	**1**
	mitochondrial cytochrome c	187	3 x 10^{-49}	97	3	1
	electron-transfer-flavoprotein beta polypeptide	330	3 x 10^{-92}	89	25	2
	TPA: putative cuticle protein	185	5 x 10^{-48}	75	10	1
	Elongation factor-1 alpha	219	7 x 10^{-59}	97	5	2
	Promoting protein	104	3 x 10^{-24}	40	18	1
	odorant binding protein	82	2 x 10^{-17}	33	18	1
	prophenoloxidase activating enzyme	36.6	1 x 10^{-4}	29	28	1
	Ribosomal protein S20	178	1 x 10^{-46}	100	20	1
	ubiquitin conjugating enzyme 4	25	1.9	42	**1 (Z)**	**1**
	cellular retinoic acid binding protein	31	4 x 10^{-3}	25	13	2
zinc-finger domain	myosin heavy chain variant	65	3 x 10^{-12}	95	8	4
	transaldolase	246	3 x 10^{-86}	85	13	2
	ribosomal protein L31	218	1 x 10^{-58}	95	13	1
	ribosomal protein S20	178	1 x 10^{-46}	100	20	1
	odorant binding protein	82	2 x 10^{-17}	33	18	1
	hypothetical protein LOC692536	49	3 x 10^{-7}	33	13	1
	ribosomal protein L21	87	5 x 10^{-33}	97	13	1

6.4 DISCUSSION

Protein-protein interactions induce many important cellular events including ligand-receptor interaction, cell adhesion, antigen recognition, immune response, and viral infection (Young, 1998). The early expressed viral protein PE38 seems to play an important role in baculovirus infection and it is the putative protein that enables CpGV-I12 to overcome CpGV resistance in CpRR1 larvae. Information about its interactions with proteins in the insect could help understanding the mechanism of CpGV resistance. The Y2H system was used to investigate interactions between $PE38^{I12}$ and proteins of the CpGV-M resistant codling mot strain CpRR1. After the two haploid yeast strains containing either one of the three DNA-BD fusion vectors or the GAL4-AD vector were mated, 151 positive yeast clones with interactions between $PE38^{I12}$ and proteins of the CpRR1 cDNA library were detected. The cDNA inserts of the GAL4-AD vector were sequenced and aligned with the genome database of *B. mori* using the basic local alignment search tool (BLAST) of NCBI. Hits with high similarity indicated by a high score and a low E-value are shown in Table 6.2. Four of these proteins found in the *B. mori* genome are encoded by genes located on the Z-chromosome: ubiquitin conjugating enzyme 4, which showed interaction with the DNA-BD vector containing the complete gene pe38 and the DNA-BD vector containing only the leucine zipper motif of pe38, Bm kettin, and 1-Cys-peroxiredoxin interacting with the leucine zipper domain of PE38. The protein thioredoxin (Trx) and the ribosomal protein L30 showed interaction with the full-length $PE38^{I12}$. In the genome of *B. mori* the genes coding for Trx and ribosomal protein L30 are located on chromosome number 15. There is some speculation that chromosome 15 may be fused to the Z-chromsome in *Tortricidae* (Heckel, personal communication). Therefore, genes located on chromosome 15 of *B. mori* may be interesting as well. These proteins are putative interaction partners of PE38 that could be involved in CpGV-resistance. However, the interactions found between PE38 and CpRR1 proteins have to be confirmed by other methods e.g. co-immunoprecipitation, pull-down-assay, or *in situ* hybridisation (Brückner et al., 2009). Due to the limited time, this was not performed in this study but could be a starting point for further investigations. Furthermore, the cDNA sequences resulting from positive interactions detected in the Y2H system were aligned to the genome of *B. mori*. It has to be considered that it is not proven that the Z-chomosome and chromosome 15 of *B. mori* are identical with the Z-chromosome of *C. pomonella*. In the following, preliminary results will be discussed with regard to the method's limitations and how these potential interaction proteins could be involved in CpGV resistance.

The Y2H system and other protein interaction assays normally screen for interactions between full-length proteins. However, most proteins are composed of multiple distinct domains and motifs and many protein-protein interactions are mediated through independently folding modular domains (Bornberg-Bauer et al., 2005; Pawsen & Nash, 2003, Boxem et al., 2008). Boxem et al. (2008) published interactome mapping of early embryogenesis for *Caenorhabditis elegans* using Y2H based on protein domains. As a result, the sensitivity of Y2H screens was increased compared to classical Y2H system. The amino acid sequence of PE38 comprises a leucine zipper domain at the carboxy terminus and zinc finger domain at the amino terminus (Krappa et al., 1995) (Figure 6.2). In order to increase the sensitivity of the Y2H system and the probability to find the interaction of interest these two domains and the full-length protein were fused separately to the DNA- binding domain of the bait vector (see 6.2.4). The results show that there are interactions that are seen for both, the full-length PE38 and one of the two domains. But there are also interactions that were found for only one of the three different baits (Table 6.2). If only the full-length protein was used for the screening the interactions between the leucine zipper domain and two proteins located on the Z-chromosome (*Bm kettin and 1-Cys peroxiredoxin*) (Table 6.2) would probably not have been detected.

An important limitation of the Y2H is the occurrence of false positive and false negative interactions. False negatives are protein-protein interactions which cannot be detected due to limitations of the screening method. Protein interactions involving membrane proteins are mostly undetectable using classical Y2H (Brückner et al., 2009). If PE38 interacts with transmembrane protein this interaction would not have been detected using this method. Another reason of false negative interactions is due to different or lacking post-translational protein modifications in yeast cells, when interactions of proteins of higher eukaryotes are tested (Brückner et al., 2009). In many cell signalling systems post-translational modification of proteins initiate a signalling cascade based on the interactions that occur subsequently (Pawson, 1995). Since there is no information about post-translational modifications of PE38 this point was ignored and it has to be considered as a source of false negative interactions in the Y2H screening.

False positives are physical interactions detected in the Y2H screening, which are not reproducible in an independent system (Brückner et al., 2009). Therefore, unspecific interactions found between PE38 and proteins of CpRR1, using Y2H, have to be excluded. False positive interactions can have diverse reasons. A high expression level of bait and prey and their localisation in a compartment which does not correspond to their natural cellular environment can cause false positive interactions. Another reason is interaction of prey with reporter proteins or with membrane anchors fused to the bait. Some proteins are able to overcome nutritional selection and when they are

overexpressed they might be detected as positives by mistake. So called "sticky preys" are preys, which bind to many different baits and show unspecific (false positive) interactions. There is a list available at the DKFZ homepage (http://www.dkfz.de/gpcf/251.html) and another list published by E. Golemis (http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html), where sticky preys are summarised. According to these lists some of the interactions listed in Table 6.2 are probably false positives: Hsp 40 protein, the different ribosomal proteins, which were found quite frequently in this screening, electron-transfer-flavoprotein beta polypeptide, the elongation factor-1 alpha, and the ubiquitin conjugating enzyme 4.

The classical Y2H system cannot be applied with baits that can directly activate transcription because they would trigger transcription of the reporter gene in absence of any interaction with a prey. In such a case modified Y2H systems have to be used: the repressed transactivator (RTA) system or split-ubiquitin systems (reviewed by Brückner et al., 2009). PE38 is described to transactivate transcription of early genes (Krappa and Knebel-Mörsdorf, 1991; Kool et al. 1994; Jiang et al., 2006). However, transcriptional activation of the DNA-BD fusion vectors containing the complete gene PE38, the leucine zipper domain or the zinc finger domain has been excluded by test on transcriptional activation (6.3.2).

Deducting the sticky preys from the list of possible interactions (Table 6.2) there are only two genes left that are located on the Z-chromosome: *Bm kettin* and *1-Cys peroxiredoxin* and one gene located on chromosome 15: *Trx*. Bm kettin and 1-Cys peroxiredoxin showed interaction with the leucine zipper domain of $PE38^{I12}$, whereas Trx interacted with the full-length $PE38^{I12}$. Although these interactions are not confirmed yet, information about the three proteins was collected in order to explore how they could be involved in the infection processes of CpGV in *C. pomonella*. The interaction between the leucine zipper domain of $PE38^{I12}$ and *1-Cys peroxiredoxin* was detected only one time, whereas interaction with *kettin* was found two times and interaction between Trx and the full-length $PE38^{I12}$ was detected three times. Regarding the number of interactions found, Trx is the most promising candidate as resistance gene in CpRR1. However, its location on the Z-chromosome is highly hypothetical.

The *B. mori kettin* (*BmKettin*) is a homolog of the *kettin* gene of *Drosophila*, which encodes a large modular protein present in the Z-disc of muscles in *Drosophila* (Koike et al., 2003; Suzuki et al., 1999; Lakey et al., 1993). But how can a muscle protein be involved in CpGV infection? *Kettin* is a chain of immunoglobulin (Ig) domains separated by linker sequences and it binds to F-actin with high affinity (van Straaten et al., 1999). Many viruses interact with the actin cytoskeleton and use it to their own advantage at various stages of their infection cycles (reviewed by Cudmore et al., 1997).

F-actin partially colocalizes with the major capsid protein (p39) in the nucleus during nucleocapsid morphogenesis (Volkman et al., 1992). Cells infected with *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) fail to produce infectious BVs in the presence of cytochalasin D (CD), a fungal toxin that binds to actin. Electron microscopic surveys revealed that BVs are still produced but they lack nucleocapsids (Cooper, 1987; Volkman et al., 1987). Ohkawa and Volkman (1999) demonstrated that F-actin is required for AcMNPV nucleocapsid morphogenesis by using AcMNPV recombinants engineered to express either wild-type- or CD-resistant actin. CpRR1 larvae do not produce infectious BVs when they are inoculated with CpGV-M (Chapter 5). Disability of CpGV-M to interact with F-actin in the resistant strain CpRR1 in order to form nucleocapsids might be a reason for CpGV resistance. In susceptible larvae, PE38 possibly interferes with nucleocapsid morphogenesis by interaction with Kettin. This could be a hypothetical explanation how Kettin could be involved in CpGV infection cycle and thus in CpGV resistance. However, in CpRR1 larve replication of CpGV-M, which precedes nucleocapsid morphogenesis, is blocked in midgut cells, haemocytes and cells of the fat body (Chapter 5). Therfore, it is rather unlikely that an interference with nucleocapsid formation is the reason for resistance against CpGV-M in CpRR1.

The second Z-linked protein found to interact with the leucine zipper domain of PE38 is 1-Cys peroxiredoxin. Peroxiredoxins are believed to play an important role in insects for protection against toxicity of reactive oxygen species (ROS). Peroxiredoxins represent a large group of antioxidant proteins present in both prokaryotic and eukaryotic species and share the same basic catalytic mechanism: an active site cysteine is oxidized to a sulfenic acid by the peroxide substrate (superoxide anion, hydrogen peroxide, nitric oxide and hydroxy radical) (reviewed by Rhee et al., 2005). In *B. mori* 1-Cys peroxiredoxin is expressed in all tissues and developmental stages. Its expression is upregulated by external temperature stimulus (Wang, et al., 2008) and by baculovirus infection (Lee, et al., 2005). However, there is no information available whether peroxiredoxins play a role in insect defense against baculoviruses.

In the genome of *B. mori* thioredoxin (BmTrx) is encoded by a gene located on chromosome number 15. Assuming that chromosome 15 and the Z-chromosome are fused in the family of *Tortricidae* this gene might be linked to CpGV resistance in *C. pomonella* (Heckel, personal communication). The results of the Y2H screening revealed three interactions of *Trx* with the full-lenth protein PE38 (Table 6.2). Trx is ubiquitous in many different procaryotes and eucaryotes and is described to act as substrate for reductive enzymes, to function as a protein disulfide oxido-reductase, to be a regulatory factor for enzymes and receptors and to serve as a subunit of a virus DNA polymerase or as an essential component for assembly of small viruses. Trx is known as

a hydrogen donor for ribonucleotide reductase, which is the essential enzyme providing deoxyribonucleotides for DNA replication (Holmgren, 1985, 1989). Phage T7 uses the Trx of its host *E. coli* as an essential subunit of its phage-encoded DNA polymerase (Holmgren, 1998). The cDNA sequence, expression, and functional characterization of BmTrx were described by Kim et al. (2005). The cDNA encoding BmTrx was expressed as a 12-kDa polypeptide in baculovirus-infected insect Sf9 cells. Furthermore, the expression of *BmTrx* mRNA was upregulated in the fat body when *B. mori* larvae were exposed to low or high temperatures, or injected with *B. mori* nucleopolyhedrovirus (BmNPV) similar to the upregulated expression of 1-Cys peroxiredoxin described by Lee et al. (2005). These results suggest that BmTrx is possibly involved in protection against oxidative stress and virus infection (Kim et al., 2007). Since Trx is directly involved in viral replication, it seems to be the most promising candidate for the resestance conferring gene in CpRR1.

Assuming that PE38 binds to kettin, 1-Cys peroxiredoxin or Trx, what kind of interaction could that be? PE38 of the BmNPV has been described to have ubiquitin ligase activity (Imai et al., 2003). Modification of proteins by ubiquitin is essential for numerous cellular processes and also plays a key role during viral infection. For example herpesvirus-encoded ubiquitin ligase promotes ubiquitination and subsequent degradation of immune recognition molecules to evade the immune system of the host (Coscoy et al., 2001; Imai et al., 2008). Most of the lepidopteran baculoviruses, including CpGV encode for ubiquitin-like genes (van Oers and Vlack, 2007; Rohrmann, 2008). PE38 is possibly involved in the ubiquitin pathway, acting as ubiquitin ligase and attaches ubiquitin to the target protein. Kettin, 1Cys peroxiredoxin or Trx could be possible target proteins that might be modified this way leading to fatal infection.

As already described above, PE38 comprises two putative DNA binding and dimerisation domains: an N-terminal zinc finger motif and a C-terminal leucine zipper motif (Krappa & Knebel-Mörsdorf, 1991). PE38 has been described to transactivate transcription of early genes. The possibility that PE38 of the resistance overcoming isolate CpGV-I12 first interacts with a viral protein or transactivates the expression of a viral protein which subsequently leads to fatal infection of the CpGV resistant codling moth larvae cannot be ruled out. In this case none of the interactions detected here is relevant for the resistance mechanism.

The very early expressed viral protein PE38 has various functions in baculovirus life cycle. Its role in the resistance mechanism of CpRR1 larvae is still unknown. However, the preliminary results of the Y2H screening offer some important information and a starting point for the ongoing research to identify the gene responsible for CpGV resistance.

7 GENERAL DISCUSSION

Baculoviruses show great promise for an environmentally friendly control of insect pests and are increasingly used as biological control agents in agriculture and forestry. However, the recently observed case of field resistance of the codling moth (*Cydia pomonella* L.) to products based on *Cydia pomonella* Granulovirus (CpGV) shows that implementation of resistance monitoring and resistance management strategies are needed to sustain the ecological and economic benefits of this class of biological insecticides. Here, the phenomenon of resistance of the codling moth against CpGV was investigated from two sides: the inheritance and the mechanism of resistance. This chapter intends to bring all the results together and to discuss them against the background of possible resistance management strategies.

Resistance of the codling moth against CpGV was not only the first case of resistance evolved by application of a baculovirus in the field; it also shows a very special and uncommon mode of inheritance. Crossing experiments between a susceptible (CpS) and a homogeneously resistant laboratory strain (CpRR1) disclosed the linkage of the resistance gene to the Z-chromosome (Asser-Kaiser et al., 2007) (Chapter 2). In Lepidoptera the female is the heterogametic sex (ZW) and thus requires only one copy of the resistance allele to become resistant. Compared to autosomally inherited resistance, sex linked inheritance enables a faster initial selection response (Asser-Kaiser et al., 2007) (Chapter 2). At lower concentrations heterozygous males Z^RZ^S survive virus exposure, are selected against susceptible males (Z^SZ^S) and convey their resistance allele to the next generation. Homozygous resistant males (Z^RZ^R) and females carrying a Z chromosome with the resistance allele are completely resistant even to very high virus concentrations.

One possible resistance management strategy could be to reduce selection pressure by lowering the intensity of each treatment (lower concentrations), in combination with untreated refuges (Onstad, 2008). This approach would be successful if the resistance gene was recessive (Fleischer, 2004). The aim of this strategy is to kill fewer susceptibles. Susceptible individuals can then mate with resistant individuals, lowering the proportion of homozygous resistant genotypes in the population (Onstad, 2008). However, due to the concentration dependent dominance, heterozygous males (Z^RZ^S) carrying the resistance gene would not be killed at low concentration and the frequency of the resistance gene would further increase. Moreover, the success of this strategy also depends on the association of resistance with fitness costs, allowing surviving susceptible insects to reproduce more successfully than the resistant ones (Onstad,

2008). However, this does not seem to be the case for CpGV resistance in codling moth. The resistance level of the heterogeneous field strain CpR did not decrease during several years or rearing without selection pressure, indicating that the allele frequency of the resistance gene remained stable (Asser-Kaiser et al., 2009) (Chapter 3). At least under laboratory conditions, resistance to CpGV is not connected with fitness costs.

The opposite of this "low-concentration strategy" would be to purge all heterozygotes of a population by using a concentration which is high enough to kill them. But this has to be done when the frequency of heterocygotes in the population is very low (Onstad, 2008). The German populations tested from 2004 to 2006 showed resistance ratios of ~1000-fold compared to the susceptible reference strain indicating that the frequency of the resistance gene is already highly increased (Asser-Kaiser et al., 2007) (Chapter 2). The allele frequency and the frequency of the different genotypes in a certain population can be estimated by using crossing experiments between susceptible laboratory strain the field population. This was shown in chapter 3, for the field collected codling moth strain CpR, which was 100 times less susceptible than the susceptible reference strain (CpS) (Asser-Kaiser et al., 2009). This method could be used to monitor CpGV resistance, and to estimate resistance levels of different populations. However, sex-linkage and dominance of resistance inheritance lead to a very fast emergence of CpGV resistance in field populations. When farmers observe a decreased efficacy of CpGV treatments, a dramatic increase of the frequency of the resistance alleles has already occurred. The strategy to use high concentrations in order to kill all heterozygotes could be used to prevent or to delay evolution of resistance in populations where farmers did not observe any control failures yet. In populations where the presence of resistance to CpGV has already become obvious this strategy is counterproductive. So, what can farmers do when resistance to CpGV has occurred in their orchards?

In this case the "multiple attac" strategy seems to be promising. Here, mixtures or rotations of insecticides with different modes of action are used in order to delay the evolution resistance (Onstad, 2008; Fleischer, 2004). The most important precondition for this strategy is that there is no cross resistance between the different control agents. Different methods to control the codling moth with different modes of action, e.g. chemical insecticides, entomopathogenic nematodes or *Bacillus thuringiensis* are described in chapter 1 and represent possible compounds to be used in this strategy. Since organic farmers disapprove the application of chemical insecticides they have to cope with a limited number of options. CpGV resistance mainly occurred in organic orchards, whereas orchards with integrated pest management are less affected (Kienzle, personal communication). This can be explained by the use of different measures to control insects in integrated farming compared to organic farming where CpGV is predominantly used. Furthermore, in Europe all commercial CpGV products contain the

same CpGV isolate originally found in Mexico, CpGV-M (Tanada, 1964). The uniformity of the selective agent also accelerates the development of resistance to CpGV. Chapter 4 describes the resistance overcoming character of a CpGV isolate originating from Iran and some more resistance overcoming isolates were recently found in Iran and elsewhere (Eberle et al., 2008; Eberle & Jehle, unpublished). Selection of CpGV-M in resistant codling moths also generated virus genotypes which are able to overcome resistance (Kienzle et al., 2007; Zingg, 2008; Berling et al., 2009). The mixture of different virus genotypes seems to be promising in terms of resistance management, especially in organic farming. However, rotations of different virus genotypes could be problematic because codling moth has a limited number of generations per year. The control of the first generation has to be highly efficient. When the first isolate applied fails to control the first generation the second generation is very hard to control by any isolate, increasing the infestation pressure for the next season. Therefore, tank mixes are more reasonable. In a 14 days bioassay the isolate CpGV-I12 showed similar efficacy in homogeneous resistant larvae (CpRR1) as CpGV-M in the susceptible larvae (CpS) (Chapter 4). However, this was not the case when mortality was scored after 7 days of exposure, CpGV-I12 was about 50 times less effective to CpRR1 than CpGV-M to CpS. The complete overcoming of resistance is delayed; larvae die later and thus cause more damage to the fruits. This in turn, could be negative for the acceptance of this new isolate by the farmers. Field trials in order to test the efficacy of new isolates under field conditions are necessary before they are implemented into practice. But, what if codling moth develops resistance to the new isolates? This question should not be neglected. The best resistance management is prevention of resistance. Therefore research on the resistance to other isolates should be accomplished before the use of new isolates is established. Cross resistance of resistant codling moths to CpGV-M and Cry1Ab protein of *B. thuringiensis* could not be substantiated (Eberle et al., 2008) (Chapter 4). Although *Bt* products are considered to be not efficient enough to compete with other measures to control the codling moth, their application could be rational to control populations with high levels of CpGV resistance.

Investigation on the mechanism underlying CpGV resistance revealed that resistance is not restricted to a certain developmental stage (Chapter 4). Therefore, it is not possible to choose the most susceptible larval stage as a time point for CpGV application. Field trials have shown that CpGV application is also effective against older larvae which have already bored into the apple (Steineke, 2004). There are several reports about midgut based baculovirus resistance (Watanabe, 1971; Fraser & Stairs, 1982; Engelhard & Volkman, 1994; Hoover et al., 2000; Haas-Stapleton et al., 2005). Different to these cases CpGV resistance is systemic. Injection of BVs into the insect's haemocoel did not overcome resistance (Chapter 5). Furthermore, CpGV replication in the midgut,

haemocytes and the fat body was traced after oral and intra-haemocoelarly by quantitative PCR. In none of the three tissues of CpRR1 larvae an accumulation of viral DNA could be detected (Chapter 5). Thus, an early block of virus replication as a reason for CpGV resistance is proposed. Transfusion of haemolymph from resistant to susceptible larvae could not convey resistance indicating that CpGV resistance is not caused by immune response (Chapter 5). Compared to vertebrates, immune response of insects is rather unspecific. Insect immunity is characterized by non-specific immune reactions against foreign material (Narayanan, 2004). The fact that the isolate CpGV-I12 is able to overcome resistance also argues against an immune response as a reason for CpGV resistance.

The genomes of several CpGV isolates have been sequenced recently (Eberle et al., 2009, Eberle & Jehle, personal communication). These studies revealed that CpGV-M differs from resistance overcoming CpGV isolates (e.g. CpGV-I12) in a small insertion in the open reading frame (ORF) 24, which codes for the immediate early gene *pe38* (Eberle, personal communication). Therefore, the gene *pe38* is hypothesized to be responsible for the resistance overcoming character of CpGV-I12. *Pe38* of the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) plays an important role in viral replication, budded virus production and virulence (Milks et al., 2003). Investigations on the resistance mechanism (Chapter 5) revealed that virus replication is affected at an early stage of infection in resistant codling moth larvae, indicating a blocked interaction between CpGV-M and CpRR1 cells. This is consistent with the assumption that PE38 is involved in the resistance mechanism. In order to identify interaction partners of the protein PE38 in CpRR1 the yeast two-hybrid system was applied (Chapter 6). Two putative proteins were detected that are encoded by genes on the Z-chromosome and one on chromosome number 15. These interactions have not been confirmed yet by other methods exploring protein-protein interactions. However, these first results on putative host protein interaction partners provide first hints for the future research on the identification of the CpGV resistance gene in codling moth.

CpGV products are a cornerstone in the sustainable control of the codling moth. To maintain the continuing ability to use CpGV successfully in the future the development of anti resistance strategies is crucial. The results of this thesis provide essential information for the achievement of this objective. The concentration dependent dominant, sex-linked mode of inheritance argues for the “multiple attac” strategy. Investigations on the mechanism of resistance showed that resistance is systemic but specific to a certain virus isolate. Resistance overcoming CpGV isolates (e.g. CpGV-I12) as well as Bt products can be used in this strategy to maintain or restore susceptibility to CpGV.

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APPENDIX I (MEDIA AND BUFFERS)

A.I.1 INSECT SEMI-ARTIFICIAL DIET (500 ML)

10 g	Agar-Agar
32 g	maize meal
33 g	wheat germ

Components were filled up to 500 ml with tap water and autoclaved at 120°C for 20 minutes. The following components were added to the autoclaved maize meal agar mixture and mixed well.

33 g	wheat germ
15 g	brewer's yeast
2.85 g	Ascorbic acid dissolved in 25 ml deionized, autoclaved water
1.15 g	Nipagin (hydroxybenzoic acid methyl ester) dissolved in 10 ml of Ethanol (96%)

A.I.2 LB MEDIUM (LURIA-BERTANI MEDIUM) (FOR 1 LITRE)

10 g	Tryptone
5 g	Yeast extract
10 g	NaCl
(15 g	Agar-Agar for solid medium)

Components were filled up with deionized water up to 1 litre and pH was adjusted to 7.2-7.5 using 5 N NaOH. Medium was sterilized by autoclaving for 20 minutes at 120°C.

A.I.3 SOC MEDIUM

20 g	Tryptone
5 g	Yeast extract
0.5 g	NaCl
10 ml	0.25 M KCL
5 ml	2 M MgCl2
20 ml	1 M Glucose

Components were filled up with deionized water up to 1 litre and pH was adjusted to 7.0 using 5 N NaOH. Medium was sterilized by autoclaving for 20 minutes at 120°C.

A.I.4 1X PBS BUFFER (FOR 1 LITRE)

8 g	NaCl
0.2 g	KCl
1.44 g	Na2HPO4
0.24 g	KH2PO4

Components were dissolve in 800 ml bdH2O. pH value was adjusted to 7.2 using HCl before the volume was filled up to 1 liter with deionized water. Afterwards, the buffer was sterilized by autoclaving for 20 min at 121°C.

A.I.5 10 X TAE BUFFER (FOR 1 LITRE)

48.4 g	Tris-base
11.42 g	Glacial Acetic Acid
20 ml	0.5 M EDTA (pH 8.0)

Components were filled up to 1 litre with deionized water and autoclaved at 120°C for 20 minutes.

A.I.6 6 X DNA LOADING DYE

0.25 %	Bromphenol blue
40 %	Sucrose (w/v) in sterile deionized water

A.I.7 YPD MEDIUM (1 LITRE)

20 g	Difco peptone
10 g	Yeast extract
(20 g	Agar-Agar for solid medium)
(15 ml	0.2% adenine hemisulfate solution for YPDA medium only)
950 ml	deionized water

pH was adjusted to 6.5 and medium was sterilized by autoclaving at 120°C for 20 minutes. After cooling down to ~55°C, 50 ml of a glucose stock solution (40%) was added. Final volume was adjusted to 1 litre by addition of sterile deionized water.

A.I.8 SD MEDIUM (1 LITRE)

6.7 g	Yeast nitrogen base without amino acids
(20 g	Agar-Agar for solid medium)
850 ml	deionized water
100 ml	of the appropriate sterile 10 x Dropout Solution

pH was adjusted to 5.8 and medium was sterilized by autoclaving at 120°C for 20 minutes. After the medium was cooled down to ~55°C, 50 ml of a glucose stock solution (40%) was added. In order to test the DNA-BD fusion for transcriptional activation 4 ml X-gal (20 mg/ml stock solution in DMF) was added. Final volume was adjusted to 1 litre by addition of sterile deionized water.

A.I.9 10 X DROPOUT (DO) SOLUTION (1 LITRE)

The appropriate dropout solutions were prepared by combination of all but one or more of the nutrients listed below. For example for SD/-His/-Trp does contain all nutrients but L-Histidine and L-Thryptophan.

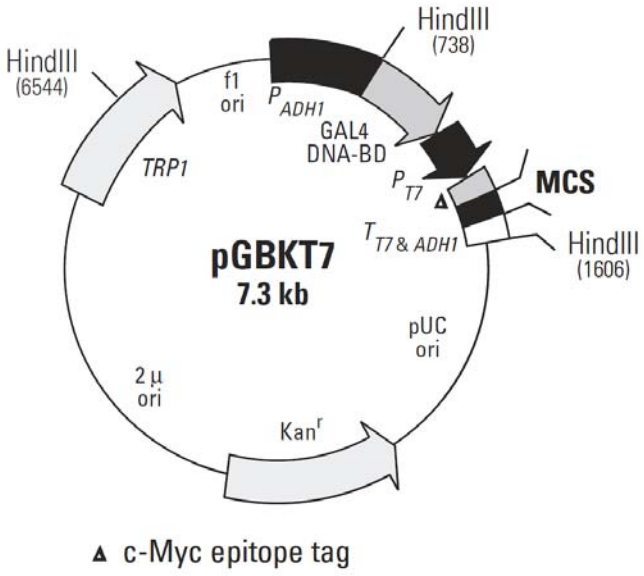
200 mg	L-Adenine hemisulfate salt
200 mg	L-Arginine HCl
200 mg	L-Histidine HCl monohydrate
300 mg	L-Isoleucine
1000 mg	L-Leucine
300 mg	L-Lysine HCl
200 mg	L-Methionine
500 mg	L-Phenylalanine
2000 mg	L-Threonine
200 mg	L-Thryphtophan
300 mg	L-Thyrosine
200 mg	L-Uracil
1500 mg	L-Valine

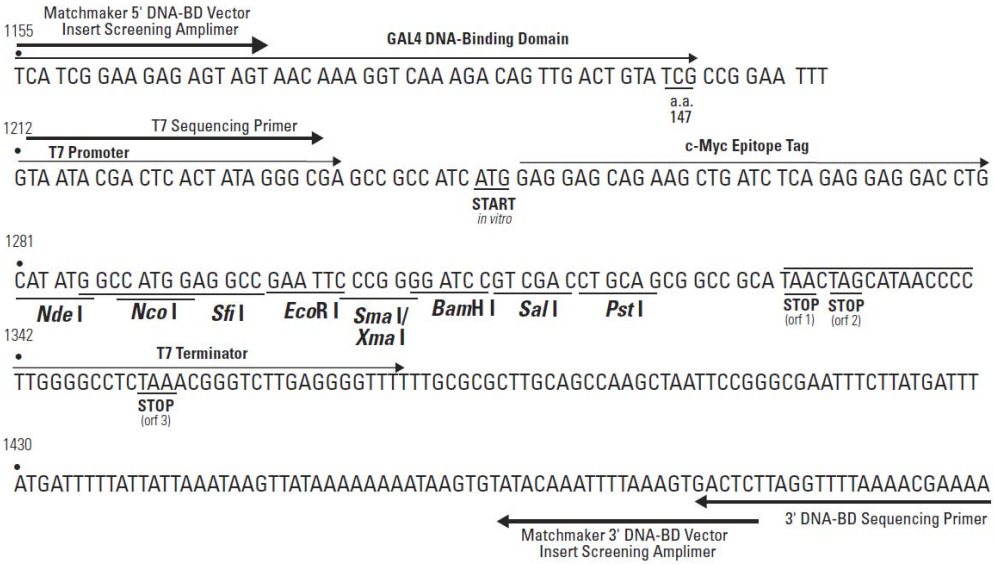
Components were filled up to 1 litre with deionized water and autoclaved at 120°C for 20 minutes.

APPENDIX II (VECTORS)

DNA-BD vector pGBKT7

(Clontech Laboratories, Inc. USA)





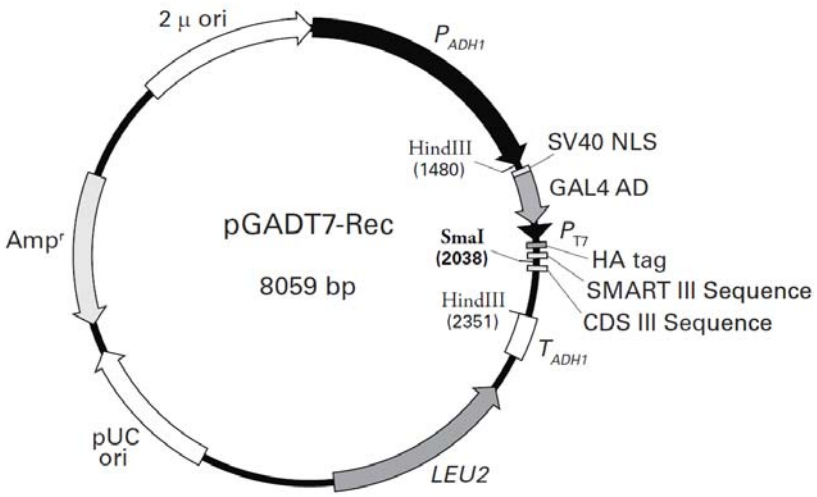
Restriction Map and Multiple Cloning Site (MCS) of pGBKT7. Unique restriction sites are in bold.

Vector description:

pGBKT7 expresses proteins that are fused to amino acids 1-147 of the GAL4 DNA binding domain (DNA-BD). In yeast, fusion proteins are expressed at high levels from the constitutive *ADH*1 promotor (P_{ADH1}) and transcription is terminated by the T7 and *ADH*1 transcription termination signals ($T_{T7\&ADH1}$). pGBKT7 contains the T7 promotor, a c-Myc epitope tag, and a multiple cloning site (MCS). The vector replicates autonomously in both *E. coli* and *S. cerevisiae* from the pUC and 2 μ ori, respectively. For selection in *E. coli*, the vector carries the Kan^r resistance gene. In yeast the nutritional marker *TRP1* is used for selection.

GAL-4 AD vector pGADT7-Rec

(Clontech Laboratories, Inc. USA)



SMART™ III terminus

MATCHMAKER 5' AD LD-Insert Screening Amplimer — GAL4 Activation Domain (a.a. 881) — T7 Sequencing Primer / T7 Promoter

1858 CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG ATC TTT AAT ACG ACT

START *in vitro* — HA Epitope Tag

1915 CAC TAT AGG GCG AGC GCC GCC ATG GAG TAC CCA TAC GAC GTA CCA GAT TAC GCT

SMART III Oligonucleotide Sequence

1969 CAT ATG GCC ATG GAG GCC AGT GAA TTC CAC CCA AGC AGT GGT ATC AAC GCA GAG TGG

2026 CCA TTA TGG CCC

Partial SmaI site

CDS III terminus

CDS III Primer Sequence

2038 GGG AAA AAA CAT GTC GGC CGC CTC GGC CTC TAG AGG GTG GGC ATC GAT ACG GGA TCC

Partial SmaI site

STOP

2095 ATC GAG CTC GAG CTG CAG ATG AAT CGT AGA TAC TGA AAAACCCCGCAAGTTCACTTC

MATCHMAKER 3' AD LD-Insert Screening Amplimer

2152 AACTGTGCATCGTGCACCATCT

3' AD Sequencing Primer

Vector description:

pGADT7-Rec is developed for the construction of GAL4AD/cDNA libraries by homologous recombination in yeast. In yeast the vector pGADT7-Rec expresses a protein of interest as a GAL4 activation domain (GAL4 AD) fusion. Transcription starts with the constitutive *ADH1* promotor (P_{ADH1}) and ends with the ADH1 termination signal (T_{ADH1}). The GAL4 AD sequence includes the SV40 nuclear localisation signal (SV40 NLS; 1) so that fusions translocate to the nucleus. GAL4 fusions also contain a hemagglutinin epitope tag (HA tag).

The T7 promotor in pGADT7-Rec allows in vitro transcription and translation of the hemagglutinin (HA)-tagged fusion protein. It also provides a binding site for the T7 Sequencing Primer. In its circular form, pGADT7-Rec replicates autonomously in both *E. coli* and *S. cerevisiae* from the pUC and 2 µ ori, respectively. For selection in *E. coli* the vector carries Amp^r and for selection in yeast a *LEU2* nutritional marker.