Novel types of resistance of codling moth to Cydia pomonella granulovirus

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Da es sehr förderlich für die Gesundheit ist,
habe ich beschlossen, glücklich zu sein.

(Voltair)

für meine Eltern, Edith Helena und Günter Friedrich Sauer

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List of Abbreviations

%	percent
#	number
× *g*	multiple of *g*
°C	degree Centigrade
µg	microgram
µm	micrometer
APRD	Arthropod Pesticide Resistance Database
BAC	bacterial artificial chromosome
BC	backcross
bp	base pair
BV	badded virus(es)
CL	cluster
CM	codling moth
D_x	dominance of resistance
DNA	desoxyribonucleic acid
dsDNA	double stranded desoxyribonucleic acid
EB	elution buffer
e.g.	for example
EPN	entomophathogenic nematode
etc.	et cetera
F_0	parental generation
F_1	first generation
F_2	second generation
FISH	fluorescence *in situ* hybridization
g	gram
GV	granulovirus
h	hour
ha	hectar
HC	hybrid cross
ICTV	International Committee on Taxonomy of Viruses
IPM	Integrated Pest Management
IRAC	Insecticide Resistance Action Committee
JKI	Julius Kühn Institute

kbp	kilobase pair
L1-L5	larval stage
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
m	meter
mM	millimolar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MNPV	multiple nucleopolyhedrovirus
µl	mikroliter
n	number of tested individuals
N	number of independent replicates
n.d.	not determinate
nm	nanometer
NPV	nucleopolyhedrovirus
nt	nucleotide
OB	occlusion body/ies
ODV	occlusion derived virus(es)
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pe38M	bacCpGVΔpe38$_M^{pe38M::eGFP}$
pe38S	bacCpGVΔpe38$_M^{pe38S::eGFP}$
pH	$-\log_{10}(a_H)$
p.i.	post infection/injection
pM	picomolar
PM	peritrophic membrane
PTA	phosphotungstic acid
qPCR	quantitative polymerase chain reaction
R-18	octadecyl rhodamine B chloride
RNA	ribonucleic acid
RT	room temperature
sec	seconds

SIT	sterile insect technique
SNPV	single nucleopolyhedrovirus
vs.	versus
w/v	weight per volume
WHO	World Health Organization

Viruses:

AdhoNPV	Adoxophyes honmai nucleopolyhedrovirus
AcMNPV	Autographa californica multiple nucleopolyhedrovirus
AgMNPV	Anticarsia gemmatalis multiple nucleopolyhedrovirus
BmNPV	Bombyx mori nucleopolyhedrovirus
CpGV	Cydia pomonella granulovirus
PhopGV	Phthorimaea operculella granulovirus
SfMNPV	Spodoptera frugiperda multiple nucleopolyhedrovirus
TnGV	Trichoplusia ni granulovirus
TnSNPV	Trichoplusia ni single nucleopolyhedrovirus

Summary

The Cydia pomonella granulovirus (CpGV, *Baculoviridae*) is an important biological control agent to control codling moth (CM; *Cydia pomonella*, L.) in organic and integrated pome fruit and walnut production. The CpGV is highly host-specific and supremely virulent for early larval stages of CM, additionally safe for the environment and other animals and humans. Since 2005, resistance against the widely used Mexican isolate (CpGV-M) has been reported from different countries in Europe. Until now, over 40 apple orchards with resistance to CpGV-M based products were identified. For several CM field populations in Europe a Z-linked, monogenetic and dominant inheritance was proposed suggesting a highly similar mode of resistance, termed type I resistance. Type I resistance is targeted only against the isolate CpGV-M and specific the 24 bp insertion in its viral gene *pe38*. Some other CpGV isolates collected from infected larvae of different geographical regions, lacking this 24 bp repetitive insertion in their *pe38* gene and caused virus infection in resistant larvae. Some of these isolates, e.g. CpGV-S, were eventually registered to re-establish the efficient control of CM larvae in the field. Recently, two CM field populations, called NRW-WE and SA-GO, with an untypically high resistance level against CpGV-M and other CpGV isolates, were identified and a novel resistance type II was proposed. This thesis focuses on the elucidation of their mode of inheritance and their cellular mechanism.

For generating genetically homogenous resistant strains out of the field population NRW-WE, larvae were selected by repeated mass crosses and selection under virus pressure, using the two isolates CpGV-M and CpGV-S, respectively. The resulting strains CpR5M and CpR5S showed a clear cross-resistance to both CpGV-M and CpGV-S. By crossing and backcrossing experiments between CpR5M or CpR5S and susceptible CM strain (CpS) an autosomal dominant and monogenetic inheritance of resistance was elucidated. The autosomal inheritance mode supported the evidence of a second type (type II) of resistance. Initially, an interchromosomal rearrangement involving the Z chromosome was hypothesized to explain the translocation from a Z-chromosomal to an autosomal inheritance. This hypothesis, however, could be clearly ruled out because a highly conserved synteny of all probed Z-linked genes was observed for different resistant CM strains when fluorescence *in situ* hybridization with marker genes (BAC-FISH) was applied.

Considering the cross-resistance in type II resistance, CM larvae were treated with single or mixtures of the isolates CpGV-M and CpGV-S. For these treatments no virus infection was observed but a recombinant of CpGV-M containing the *pe38* gene of CpGV-S caused high mortality. The results indicated that beyond the known *pe38* related mechanism of type I resistance against CpGV-M, a

second mechanism seemed to exist in type II resistance. With CpR5M and CpS budded viruses injections, circumventing initial midgut infection, gave further evidence that resistance against CpGV-S is midgut-related. A fluoresecence-quenching assay using rhodamin-18 labeled occlusion derived viruses could not fully elucidate whether receptor binding or an intercellular midgut factor is involved in type II resistance. The results led to the model of two different but genetically linked resistance mechanisms in the type II resistant CM larvae: resistance against CpGV-M is systemic and targeted against the *pe38* gene, whereas resistance against CpGV-S is based on an unidentified midgut factor, inhibiting initiation of infection.

A further CM field population, termed SA-GO, was also investigated for the biological and genetic background of CpGV resistance. Crossing experiments between CpS and field collected larvae of SA-GO, followed by resistance testing with two CpGV isolates revealed differences in the susceptibility and the mode of inheritance compared to the one found in type I or type II resistance of CM. Single-pair inbreeding generated the genetically more homogenous resistant strain CpRGO. Reciprocal hybrid crosses and backcrosses between individuals of CpRGO and susceptible CpS observed a dominant and polygenic inheritance of resistance in the majority of crosses. Resistance to CpGV-S appeared to be autosomal and dominant for larval survivorship but recessive when success of pupation of the hybrids was considered. Resistance of CpRGO to CpGV-M however, is proposed to be both autosomal and Z-linked inherited, since only male larvae were able to pupate, similar to the type I resistance. CpRGO was therefore termed type III resistance.

When the efficacy of different CpGV isolates classified to all known CpGV genome groups (A - E) was tested with neonates of all resistant strains. CpGV isolates of the genome groups B and C were able to cause significant mortality in larvae of all resistance types. In addition, CpGV of genome group D caused high mortality in type III resistant CM strain, whereas type I resistance was broken by all known CpGV genome groups, except group A. When isolates of commercial CpGV products were tested in the resistant CM strains, it was found that the commercially used CpGV isolates R5 and 0006 did break only type I and type III resistance, whereas isolate V15 was able to cause high mortality in all resistant types.

In conclusion, two types of CpGV resistance, type II and type III were identified and showed a high heterogeneity in their mode of inheritance, mode of action and response to CpGV isolates of different genome groups. The major finding of this thesis is that field resistance of CM to CpGV is genetically and functionally variable and needs to be carefully addressed when resistance management strategies are developed for CM control in the field.

Zusammenfassung

Das Cydia pomonella Granulovirus (CpGV) ist ein wichtiges Mittel zur biologischen Bekämpfung von Larven des Apfelwicklers (*Cydia pomonella*) im integrierten und ökologischen Apfelanbau. CpGV-Mittel stellen eine sehr wirksame und äußerst wirtsspezifische, nützlings- und umweltschonende Alternative zu chemischen Pflanzenschutzmitteln dar. Im Jahre 2005 wurde erstmals von einer Resistenz des Apfelwicklers gegen das damals ausschließlich eingesetzte Isolat CpGV-M berichtet. Bis heute sind mehr als 40 Apfelwickler-Populationen mit einer Minderempfindlichkeit gegen dieses Isolat im europäischen Obstbau bekannt. In Kreuzungsexperimenten wurde für einige dieser Freilandpopulationen eine monogenetische, dominante und geschlechtsgebundene (Z-chromosomale) Vererbung festgestellt. Dieser Befund führte zu der Annahme, dass eine ähnliche Resistenz in den betroffenen Populationen vorliegt, welche als Typ I-Resistenz bezeichnet wurde. Dieser Resistenz-Typus ist offensichtlich gegen eine 24 bp lange, repetitive Insertion im viralen Gen *pe38* von CpGV-M gerichtet, denn andere CpGV-Isolate, z.B. das Isolat CpGV-S, ohne diese Insertion zeigten weiterhin eine gute Wirkung gegen Apfelwickler mit Typ I-Resistenz.

Einige von ihnen, sind mittlerweile als Pflanzenschutzmittel zugelassen. Diese resistenzbrechenden Isolate gehören den phylogenetischen Genomgruppen B–E von CpGV an, während CpGV-M zur Genomgruppe A gehört.

Zwei resistente Freilandpopulationen des Apfelwicklers namens NRW-WE und SA-GO zeigten eine außergewöhnlich stark ausgeprägte Resistenz gegen CpGV-M und andere resistenzbrechende CpGV-Isolate. Es wurde daher angenommen, dass es sich bei diesen Populationen um einen weiteren Resistenztypus (Typ II) handelt.

Die beiden Populationen NRW-WE und SA-GO sind Gegenstand der vorliegend Arbeit und wurden hinsichtlich ihrer Resistenzvererbung und ihres zellulären Resistenzmechanismus gegen unterschiedliche CpGV-Isolate untersucht.

Über fünf Generationen hinweg wurden in Massenkreuzungen Individuen von NRW-WE vermehrt und anschließend jeweils durch die beiden Isolate CpGV-M und CpGV-S selektiert. Hieraus resultierten die Apfelwickler-Stämme CpR5M und CpR5S. In beiden Stämmen konnte eine Kreuzresistenz zu beiden CpGV-Isolaten nachgewiesen werden. Durch Rückkreuzungsexperimente zwischen CpR5M bzw. CpR5S mit einem empfindlichen Apfelwickler-Laborstamm (CpS) und anschließende Resistenztests der Nachkommen konnte eine monogenetische und dominante, in diesem Falle aber autosomale Vererbung der Resistenz nachgewiesen werden, welche die Annahme eines zweiten Resistenztypus (Typ II) bestätigte.

Als mögliche Erklärung für die Veränderung des festgestellten Resistenzlocus/gens von einem geschlechtsgebundenen zu einem autosomalen Chromosom wurde eine interchromosomale Umordnung unter Beteiligung des Z-Chromosoms vermutet. Diese Hypothese konnte durch Fluoreszenz-*in-situ*-Hybridisierungen mit 13 Z-chromosomalen Makergenen widerlegt werden, da die Z-Chromosme aller getesteten anfälligen und resistenten Apfelwickler-Stämme eine sehr ähnliche Architektur aufwiesen.

Um die Kreuzresistenz in CpR5M und CpR5S weiter zu untersuchen, wurden Resistenztests mit Mischungen von CpGV-M und CpGV-S durchgeführt, wobei keine Virusinfektion nachgewiesen werden konnte. Bei Infektionsversuchen mit einer Rekombinanten von CpGV-M, deren *pe38* Gen durch das *pe38* des Isolates CpGV-S substituiert war, konnte hingegen eine sehr hohe Mortalität von CpR5M- und CpR5S-Larven erzielt werden. Die beobachtete Infektiosität dieser Rekombinanten führte zu der Hypothese, dass zwei unterschiedliche Resistenzmechanismen in der Typ II-Resistenz vorliegen müssen: Ein Resistenzmechanismus basiert wie bei der Typ I-Resistenz auf dem *pe38* Gen für CpGV-M, ein zweiter Mechanismus muss gegen einen unbekannten Faktor von CpGV-S gerichtet sein. Zur weiteren Charakterisierung des unbekannten Resistenzmechanismus gegen CpGV-S wurden zwei vergleichende Untersuchungen in CpS- und CpR5M-Larven durchgeführt: (1) Injektionsversuche, zum Umgehen der Virusinfektion im Mitteldarm der Larven und (2) eine perorale Infektionen mit fluoreszenzmarkierten Viren, um deren Bindung und Fusion mit Mitteldarmepithelzellen zu quantifizieren. Ein signifikanter Unterschied zwischen Injektionen von CpGV-M und CpGV-S konnte festgestellt werden. Dies wies auf eine Resistenz gegen CpGV-S hin, die im Mitteldarm der Larve lokalisiert ist. Hingegen war kein signifikanter Unterschied in der Bindung von CpGV-M und CpGV-S an Mitteldarmepithelzellen von CpR5M festzustellen. Diese Ergebnisse lassen den Schluss zu, dass in CpR5M die Resistenz gegen CpGV-M sich von der Resistenz gegen CpGV-S in ihrem zellulären Mechanismus unterscheidet, der Eintritt des Virus in die Mitteldarmepithelzellen jedoch vermutlich nicht der direkte Zielort der Resistenz von CpGV-S ist.

Für die zweite resistente Apfelwickler-Freilandpopulation SA-GO wurde durch Einzelpaarkreuzungen mit CpS nachgewiesen, dass die Resistenz ebenfalls gegen CpGV-M und CpGV-S gerichtet ist, sich aber sowohl von der Typ I, als auch von der Typ II Resistenz in ihrer Vererbung unterscheidet. Der aus SA-GO selektierte Stamm CpRGO wurde im Labor durch Einzelpaarkreuzungen und Resistenztestung etabliert. In den meisten reziproken Hybridkreuzungen mit CpS zeigte CpRGO in einem siebentägigen Test eine polygenetische und dominante Vererbung der Resistenz. Für die Resistenz gegen CpGV-S wird angenommen, dass die Vererbung für das Überleben der Larven autosomal und dominant ist, jedoch einen rezessiven Einfluss besitzt, wenn man die Anzahl der überlebenden Puppen in Betracht zieht. Für die Resistenz gegen CpGV-M zeigte sich zusätzlich ein Z-

chromosmaler Einfluss der Vererbung, ähnlich der Typ I Resistenz, da ausschließlich männliche Larven die Virusbehandlung bis zur Verpuppung überlebten. Anhand dieser Ergebnisse wird für den resistenten Apfelwickler-Stamm CpRGO eine dritte (Typ III) Resistenz gegen verschiedene CpGV-Isolate angenommen.

In einem umfassenden systematischen Resistenztest wurden CpGV-Isolate aus den verschiedenen Genomgruppen A-E hinsichtlich ihrer Virulenz in allen verfügbaren anfälligen und resistenten Apfelwickler-Stämmen untersucht, mit dem Ergebnis, dass nur CpGV-Isolate der Genomgruppe B und C in allen resistenten Stämmen Virusinfektionen auslösen konnten. Zusätzlich wirkten Isolate der Genomgruppe D auch gegen Larven mit Typ III-Resistenz. Für Larven der Typ I-Resistenz bestätigte sich, dass diese nur gegen CpGV-M (Genomgruppe A) resistent sind. Getestete kommerzielle CpGV-Mittel mit den Isolaten R5 und 0006 waren resistenzbrechend für Larven des Typs I und III, wohingegen das Isolat V15 alle resistenten Stämme nach 14 Tagen erfolgreich abtötete.

Die Ergebnisse dieser Arbeit zeigen eine hohe Diversität der neuen CpGV-resistenten Apfelwickler-Stämme des Typs II und III gegen verschiedene CpGV-Isolate, sowohl in der Vererbung als auch im Resistenzmechanismus. Die genetische und funktionelle Vielfalt der Resistenzen des Apfelwicklers gegen CpGV-Produkte muss daher bei der Entwicklung geeigneter Resistenzmanagmentstrategien im Obstanbau sorgfältig beachtet werden.

1. General Introduction

Larvae of the codling moth (CM, *Cydia pomonella*) cause serious damage in pome fruit and walnut production almost all over the world (Lacey et al. 2008). In orchards with conventional plant protection this pest has been controlled with chemical insecticides that result in problems, such as effects to beneficial organisms, negative environmental implications and safety risks for applicators and consumers (Lacey and Shapiro-Ilan 2008). Thus, the demand for environmentally safe control agents to be applied in integrated and organic farming to control CM increased. The baculovirus Cydia pomonella granulovirus (CpGV), which is registered as biological control agent, is both highly specific for its host and virulent for early larval stages of CM. During the last decade more than 40 resistant field populations against the usually applied CpGV-M isolate have been reported in Europe (Schmitt et al. 2013). Some of these populations were transferred to the laboratory to understand the biological and genetic background of their CpGV resistance (Asser-Kaiser et al. 2007; Berling et al. 2009; Zichová et al. 2013). Next to the comprehensively described type I resistance of CM, evidence for further resistance types were noticed (Jehle et al. 2017). The addressed question of this thesis was therefore: Are there further types of resistance of CM and what are their inheritance modes and resistance mechanisms? The provided answers are expected to improve the understanding of CpGV resistance as well as the design of resistance management strategies.

1.1 The pest: *Cydia pomonella*

Cydia pomonella (L.) (Tortricidae: Lepidoptera) is an economically important and almost cosmopolitan insect pest of pome fruits. CM spreads to all major apple and pear production areas worldwide and eventually became a key pest in pome fruit production (Barnes 1991).

Figure 1 Appearance of codling moth. Adult moth of *Cydia pomonella* (left) (Foto S. Feiertag, JKI) and L5 larvae with damage inside an apple (right) (Foto JKI).

The geographic distribution of CM is restricted to temperate regions in the South and North hemisphere, i.e. Europe and Central Asia, Western China, Australia, New Zealand, South and North America, as well as South and North Africa with the exception of Japan, Taiwan, Korea, parts of Eastern China and Western Australia and possibly Brazil (Beers et al. 2003; Willett et al. 2009; Zhao et al. 2015) (Figure 2).

The prevalent hosts of CM are apple, European pear, nashi (Asian pear) and quince, where larvae cause economic loss of marketable fruits. For walnut and plum as well as peach, nectarine and apricot CM is a minor pest and causes significant damage only at high population size (Barnes 1991). In Europe, adult moths emerge from diapause when temperatures above 10 °C are achieved for more than 60 days (Neven 2013). The flight of the moths covers a period of approximately ten weeks (Harzer 2006). After copulation, female moths oviposits eggs on leaves or on the surface of developing fruits (Börner 1997). The fecundity of a single fertilized CM moth can highly deviate depending on the temperature, host plant, geographic area and rearing conditions (wild vs. laboratory); in average, a single female lays about 120 eggs (Blomefield and Giliomee 2011).

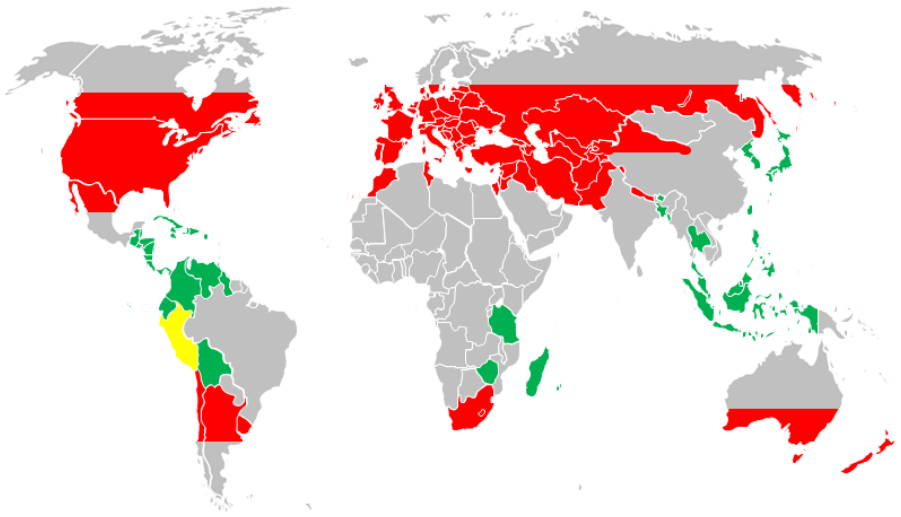


Figure 2 Occurrence of codling moth (CM) and its host plants. Red: CM host and CM present; green: CM host present but no CM; yellow: CM host present and CM possible present. Modified from Willett et al. (2009) and Zhao et al. (2015).

Shortly after hatching, neonate larvae locate apples on the base of the fruit volatile called E,E-α-fernescene (Sutherland and Hutchins 1972). The first instar larvae bore a hole into the small fruits and feed hidden inside the apple from the first to the fifth larval stage (L1-L5). Fifth instar larvae leave the fruit and move to protected habitats, such as bark scars on trunks and branches or the ground. There, the L5 larvae spin a cocoon (hibernaculum) for diapausing or pupate to start a further

generation in the same season (Lacey and Shapiro-Ilan 2008). Depending on the climate condition and the host plant, CM can generate one to four generations per year (Barnes 1991). The entrance of diapause is triggered by shorter day lengths (Neven 2013). The critical photoperiod of CM, the day length at which ≥50 % of the population enter diapause, varies from 13.5 h to 15.5 h of day light (Kumar et al. 2015).

The first generation of CM larvae causes damage to the unripe fruits, which may naturally drop from the tree or which may be discarded by the grower to increase the harvest quality. The second or following generations of CM induce much higher damage due to the larvae feeding on the ripening fruits. To avoid high infestation rates by following generations and older larvae, it is necessary to control already the first larvae and moths at the beginning of the growing season.

Control of *Cydia pomonella*

The extent of apple cultivation in Germany was 31,500 ha with 11 million tons of yield in 2014 (Statistisches Bundesamt 2015). Customers demand high-quality products when they buy fresh apples and pears. Fruits damaged or infested by CM are not marketable and can only be used for juice production. CM has the potential to cause 100 % infestation in untreated apple orchards (Beers et al. 2003). Because of the cryptic living behavior it is difficult to control CM to a satisfactory degree (Lacey and Shapiro-Ilan 2008). If CM control fails, further pest control (mites, leafrollers, etc.) is not relevant because the production of fruits with high quality is not ensured anymore. Thus, different control measures of CM are used as explained in the following.

Monitoring of CM prevalence and infestation rates

Before any action to control CM infestation is taken, it is necessary to monitor CM occurrence and early infestation of apples. Pheromone traps are used in orchards to determine the present amount of adult male moths (Joshi et al. 2016). For estimating the potential infestation risk of the second generation, it is recommended to examine 1,000 young apples in June for damage or presence of CM (Höhn et al. 2008). Spray thresholds are also based on the number of moths in the pheromone traps or on infestation rates detected in the harvest of current or last season. For apples, the economic threshold for the CM is 1 % of infested fruits (Beers et al. 2003).

Conventional and integrated control of CM

The control of CM in conventional orchards is mostly done by the application of insecticides that are registered for fruit growing areas. Chemical control of CM is still the main method in integrated pome fruit production. Most products are based on the active components of chlorantraniliprole, thiacloprid, tebufenozide or indoxcarb (BVL 2017). Resistance of CM to chemical compounds was first

reported in the USA (Hough 1928). Further evidence for a fast evolutionary adaption of CM to the applied chemicals resulting in a reduced susceptibility of CM was also reported (Bush et al. 1993; Dunley and Welter 2000; Sauphanor et al. 2000; Isci and Ay 2017).

Next to the development of resistances, many chemical products cause ecological problems. Not only CM, but also variety of other non-target insect species, including beneficial and natural predators are harmed by these insecticides (Epstein et al. 2000). Additional negative side effects of the intensive use of pesticides may be damage of non-target insect population, possible outbreak of secondary pests due to natural control disruptions, as well as safety issues with pesticide applicators and residues on food. Hence, the need of alternative control measures with a reduced impact on beneficial organisms and less detrimental effects on the environment compared to chemicals became obvious (Lacey and Shapiro-Ilan 2008). The concept of integrated pest management (IPM), firstly introduced in the late 1960s, has been developed to provide highly efficient plant protection measures and concurrently reducing its environmental adverse effects. IPM is a combination of preventive, biological and biotechnical methods to minimize the application of chemical insecticides. This combination of different control methods and products should prevent the development of resistance (Bajwa and Kogan 2002). In organic farming the use of chemical-synthetic compounds is prohibited, therefore mainly preventive cultural practices, mechanical and biological control methods are applied.

Biological control of CM

Mating disruption by applying the pheromone confusion technique is one alternative to suppress CM populations in an area-wide manner**.** Pheromones are normally released by female moths to attract male moths for mating. The pheromone confusion technique is based on chemically synthesized CM sex pheromones, such as (E,E)8,10-Dodecaienol, which are released by artificial dispensers hanging in the trees. As a consequence, the male moths get confused in the artificial emission of pheromones in an orchard and are not able to find female moths for mating (Knight et al. 2008). In 2006, this technique was implemented on more than 160,000 ha worldwide (Witzgall et al. 2008), while in Germany 17,900 ha were treated for CM control (Jehle et al. 2014a). Nevertheless, it is ineffective at high pest population densities and maybe also impaired by unfavorable weather conditions, such as strong wind and high temperature (Calkins and Faust 2003). The development of resistance against mating disruption has also been discovered in some pest insects (Spohn et al. 2003; Tabata et al. 2007). The efficiency of monitoring the CM population size by pheromone traps is also reduced by the concurrent use of mating disruption (Pringle et al. 2003).

Sterile insect technique (SIT) is used to control CM in integrated and organic plant production and essentially represents a birth control program for insects (Odendaal et al. 2015). Pest control is achieved by mass rearing of CM moths, which are then sterilized by radio-active irradiation and released *en masse* into the wild. The released sterile male moths mate with wild females, causing a reduction in fertility and a decrease in the pest population (Dyck et al. 2005). The mass-release of sterile insects for pest control is a relatively safe technique because the introduced organisms are native and cannot become established in the environment. In addition, it is exclusively species-specific and suitable to suppress pest numbers (Thorpe et al. 2016). SIT is successfully used against CM in isolated areas, e.g. in the Okanagan valley in Canada and certain areas in South Africa (Cartier 2014; Barnes et al. 2015) However, in general it is less cost-effective than other control methods (Barnes et al. 2015).

Parasitoids are insects that typically kill their associated arthropod host by laying eggs or larvae near, on or in the host for reproduction (Eggleton and Gaston 1990). Parasitoids species of the family Trichogrammatidae are naturally occurring antagonists which target eggs of Tortricidae (Cross et al. 1999). In Germany, a reduction of 53-84 % of CM and of the summer fruit tortrix, *Adoxophyes orana*, was achieved by the experimental release of two *Trichogramma* species in apple orchards (Hassan 1992). An additional benefit of the release of parasitoids is the simultaneous control of other pest species in apple orchards. The beneficial organism alone can play an effective role in integrated pest control but in general the effect for CM control is considered to be not sufficient (Thorpe et al. 2016).

Beyond the control of the neonate larvae of CM, the diapausing, hibernating cocooned larvae are a potential targets. After harvest, this larval stage should be eliminated to reduce the CM population in the following spring (Lacey and Shapiro-Ilan 2008). **Entomopathogenic nematodes** (EPNs) of the families Steinernematidae and Heterorhabditidae were shown to have a decimating effect on the population of hibernating CM (Kaya et al. 1984; Lacey et al. 2006; Odendaal et al. 2015).

Simple alternatives, such as **cultural control** are also helpful to reduce the population of CM. Removal of infested fruits, including fruits that remained after harvesting, and orchard sanitation are just some examples (Kienzle 2010). The usage of physical barriers to control pests is a well-established method. Sticky trunk barriers or **corrugated cardboards** around trunks are a simple control method with some efficiency. The L5 larvae will pupate or diapause inside the corrugated paper and the grower can discard the cardboards in fall (Barnes 1982). In addition, determining the number of diapausing L5 larvae inside the cardboards is also a good indicator to estimate the population size from year to year.

The most important biological method for CM control is the application of Cydia pomonella granulovirus (CpGV), a member of the insect specific, dsDNA virus family *Baculoviridae*.

1.2 Baculoviruses

Baculoviruses (family *Baculoviridae*) are a group of insect specific dsDNA viruses that have been isolated from larval stages of insects only (ICTV 2017). They were first accounted in the context of disease description in the rearing of the silkworm, *Bombyx mori*, in Spanish and Japanese manuals dating back to the sixteenth and eighteenth centuries (Steinhaus 1975). Baculoviruses based biological control agents were developed for different relevant lepidopteran pest species, e.g. *Helicoverpa* spp. (Rowley et al. 2011) and *Spodoptera* spp. (Nakai and Cuc 2005), which exhibited resistance to chemical insecticides, making their control more and more difficult (Moscardi 1999). Until now, at least 60 biocontrol products based on baculoviruses have been developed and commercially used in pest control (Beas-Catena et al. 2014). In total, the worldwide sale of biological control products with virus components was estimated to be 49.2 million US$ in 2010, representing about 12 % of the whole biocontrol product sales (Lacey et al. 2015). In addition to their application as biological control agents, baculoviruses are used as vectors for the experimental and commercial expression of proteins in insect and mammalian cells and for specific gene therapy of mammals (van Oers et al. 2015).

The most thoroughly studied baculovirus is Autographa californica multiple nucleopolyhedrovirus (AcMNPV), which was also the first baculovirus that was completely sequenced (Ayres et al. 1994). As of January 2017, the complete nucleotide sequences for 72 individual baculovirus genomes have been deposited on GenBank (https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid =10442). Baculovirus genomes vary in size from 81,755 base pairs (bp) (Neodiprion lecontei nucleopolyhedrovirus) to 178,733 bp (Xestia c-nigrum granulovirus) (Harrison and Hoover 2012). All baculoviruses share 37 orthologous core genes which encode conserved factors for some of the main biological functions, such as virus attachment and fusion, RNA transcription, DNA replication and structural components (Garavaglia et al. 2012).

The baculovirus DNA is packed into virions, which are embedded in a proteinaceous matrix and form occlusion bodies (OB), initiating the oral infection in the insect. Beyond OB, virions can occur as no-occluded (budded viruses, BV) when spreading infection from cell to cell (ICTV 2017).

Two types of OB can be distinguished (Figure 3): Granule shaped OB of granuloviruses (GV) contain a single virion with a single nucleocapsid or nucleopolyhedrovirus NPV containing several virions. Among NPVs, virions with one single nucleocapsid (SNPV) or virions with multiple nucleocapsids (MNPV) are distinguished and arranged randomly within the occlusion body matrix. The OB of NPV

range in size from 0.5 to 5 µm diameter and can be visualized by light microscope. In contrast, the smaller OB of GV are ovocylindrical in shape and are 120-350 nm wide and 300-500 nm long (ICTV 2017). The protein matrix of both OB is composed of the protein polyhedrin/granulin, which are homologous proteins. OB protects the virion(s) from damage by UV light, proteases and other environmental factors (Rohrmann 2008).

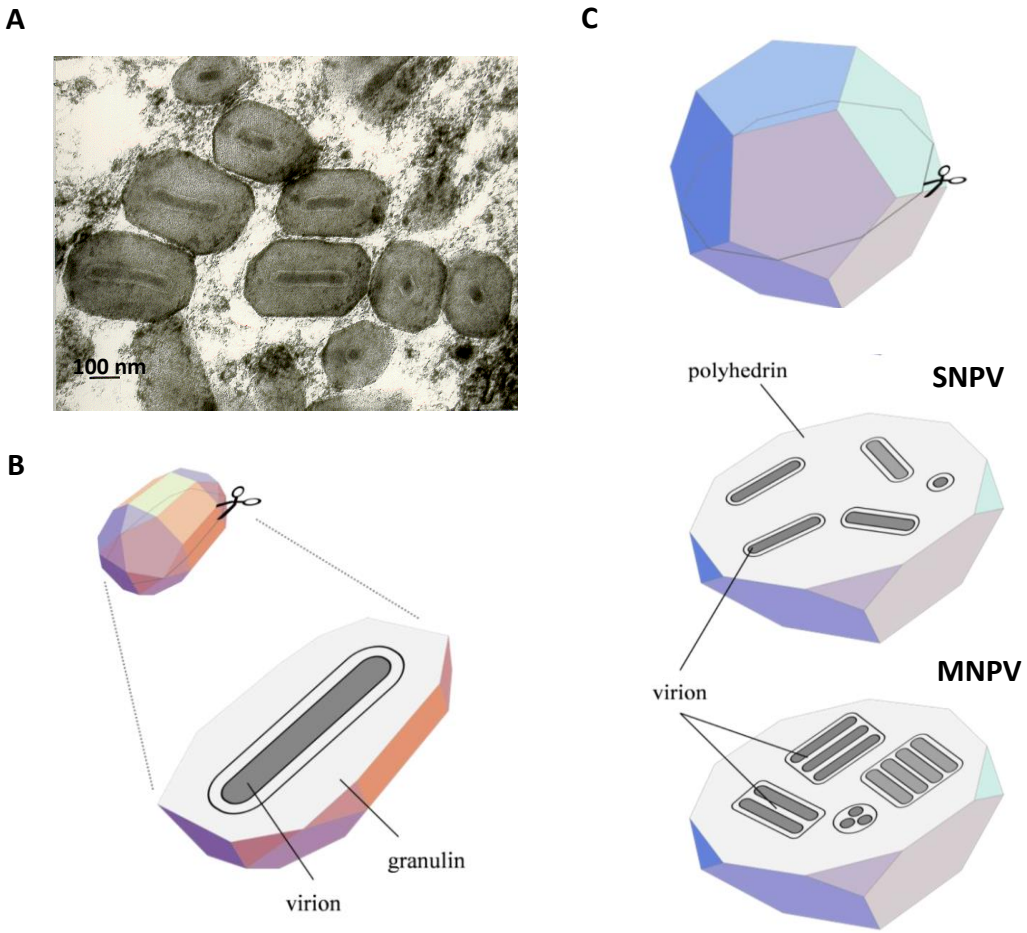


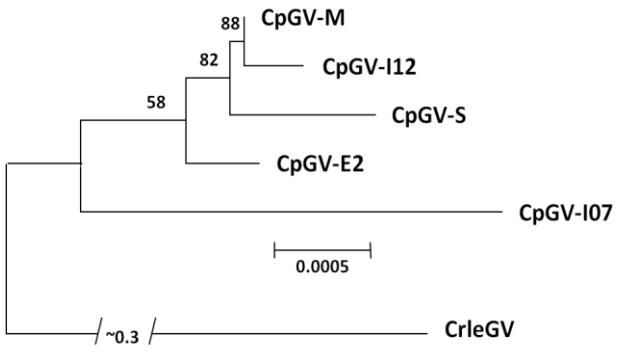
Figure 3 (A) Electron microscope image of Cydia pomonella granulovirus (CpGV) (Foto A. Huger, JKI). (B-C) Schematic illustrations of (B) occlusion bodies (OB) of granulovirus (GV), with a single virion and (C) OB of single nucleocapsid (SNPV) = several virions each containing only one nucleocapsid and multiple nucleocapsids (MNPV) = several virions with multiple nucleocapsids. GV and NPV OB are not true to scale and further information about structure and size are given in the text. (Illustrations modified from Wennmann (2014)).

Baculovirus have been reported from more than 600 insect species of the orders Lepidoptera, Hymenoptera and Diptera (Martignoni and Iwai 1986). Whereas baculovirus classification was previously mainly based on the OB morphology, a more natural classification using phylogenetic relationship was proposed by Jehle et al. (2006a) and finally accepted by the International Committee on Taxonomy of Viruses (ICTV). Currently, the family *Baculoviridae* is classified into the following four genera: *Alphabaculovirus* (consists of MNPV or SNPV, which are associated with Lepidoptera), *Betabaculovirus* (GV, only isolated from Lepidoptera), *Gammabaculovirus* (SNPV from Hymenoptera), and *Deltabaculovirus*: (SNPV, from the order Diptera).

Cydia pomonella granulovirus (CpGV)

One of the most important commercially used baculoviruses is the Cydia pomonella granulovirus (CpGV) which is also the type species of the genus *Betabaculovirus.* CpGV was originally discovered in infected CM larvae found near Chihuahua, Mexico (Tanada 1964); this isolate was termed CpGV-M. Further geographic isolates were found in Russia (Harvey and Volkman 1983), England (Crook et al. 1985), Canada, Iran and the Caucasian area (Rezapanah et al. 2008).

The first completely sequenced CpGV genome derived from an *in vivo* cloned genotype, termed CpGV-M1, that derived from the Mexican isolate, whose genome was determined to be 123.5 kbp and to have 143 open reading frames (ORFs) (Luque et al. 2001). The genome sequences of other geographic CpGV isolates varied between 124,269-120,858 bp, coding for 137-142 ORFs (Figure 4). On the basis of their phylogenetic relationship, CpGV isolates were clustered in five genome groups (A-E) (Eberle et al. 2009; Gebhardt et al. 2014)



CpGV isolate	Genome size, bp	Genome group
CpGV-M	123,529	A
CpGV-I12	124,269	D
CpGV-S	123,193	E
CpGV-E2	123,858	B
CpGV-I07	120,832	C

Figure 4 Phylogenetic tree of different isolates of Cydia pomonella granulovirus (CpGV) and their genome size. Genome grouping are given to the right (Eberle et al. 2009; Gebhardt et al. 2014).

Since the first registration in 1987 in Switzerland, CpGV products have been used as a biocontrol agents in virtually all apple growing areas worldwide (Huber 1998). Mass production of commercial CpGV products is still dependent on *in vivo* systems and rearing of CM larvae for OB production (Reid et al. 2014). Similar to other baculoviruses, the occluded phenotype of CpGV combines a number of main natural benefits as biocontrol agent: they are amenable to formulation, can be visualized by dark field light microscopy and are suitable for long-term storage (Lacey et al. 2015). In addition, growers are able to use their normal application equipment to spray the formulated OB in the field. On the other hand, inactivation by UV radiation, relatively slow speed of kill and high production costs are some drawbacks (Beas-Catena et al. 2014). Even so, it was estimated that CpGV products are applied on more than 100,000 ha per year in organic and integrated pome fruit production in Europe (Eberle and Jehle 2006). In Germany, they are used on about 10,000 ha per year, which represents 30 % of apple cultivation area (Jehle et al. 2014a)

Although different geographic CpGV isolates have been identified during the last decades, nearly all worldwide commercially available products were based on the Mexican isolate CpGV-M (Tanada 1964). CpGV products are registered and marketed by producers in Canada (BioTepp), France (Arysta Lifescience), Switzerland (Andermatt Biocontrol), Belgium (BioBest), Argentina (Agro Roco) and South Africa (River Bioscience) (Lacey et al. 2015). Currently, the registered CpGV products in Germany are Granupom, Madex 3, Madex MAX, Carpovirusine and Carpovirusine Evo2 (BVL 2017). CpGV is highly virulent to early larval stages of CM and infected neonates die within four to six days (Lacey et al. 2008). Virus infection caused by CpGV is highly host specific and is therefore safe for other non-target organisms or the environment. CpGV products can be used in the organic and integrated pome fruit production (Lacey et al. 2008).

Pathology

The infection and replication pathway of baculoviruses have been thoroughly studied for AcMNPV (*Alphabaculovirus*) and is assumed to be similar for other baculoviruses, such as CpGV (Figure 5).

The virus infection is initiated by peroral uptake of viral OB of the host larvae while feeding. The protein matrix of the OB is dissolved rapidly in the alkaline milieu (pH 8-11) of the midgut and ODV are released (Federici 1997). ODV pass the peritrophic membrane (PM) which lines the midgut epithelium cells as a physical barrier (Brandt et al. 1978; Granados 1980). The ODV bind to microvilli of the midgut columnar epithelial cells. Eight genes (*pif0 –pif7*) are known to encode for ODV envelope proteins, termed *per os* infection factors (PIFs) (Kuzio et al. 1989; Nie et al. 2012; Song et al. 2016). *Pif* genes are also present in betabaculoviruses. The PIF proteins are presumed to be necessary for binding and fusion of the ODV to the microvilli membrane and are essential to initiate the infection of the midgut. Deletion of these genes impairs the oral infectivity of ODV, but not the

systemic infectivity of BV (Harrison and Hoover 2012; Nie et al. 2012). In the midgut cell, nucleocapsids are transported driven by actin polymerization to the cell nucleus, where viral DNA is uncoated and virus replication is initiated (Summers 1971; Ohkawa et al. 2010). The transcription of the viral genes occurs in four temporal classes, starting with immediate early genes, followed by early, late and very late genes (van Oers and Vlak 2007). Viral DNA replication takes place in the host cell nucleus. As a consequence of virus infection, the nucleus becomes enlarged and a virogenic stroma is formed, where new nucleocapsids are assembled. Newly assembled nucleocapsids are released from the cell by budding from the cell membrane forming the second viral phenotype, the BV (see above) (Rohrmann 2008; Harrison and Hoover 2012). The BV spread the infection systemically through the trachea and hemolymph to other tissues, such as fat body and epidermal cells (Engelhard et al. 1994; Flipsen et al. 1995). The entry of BV into cells is assumed to occur by adsorptive endocytosis, fusion of BV membrane with endosomal membrane and release of the nucleocapsid into the cytoplasm. For betabaculoviruses, like CpGV, the BV envelope protein that initiates the binding to a membrane receptor is called F protein (Harrison and Hoover 2012). Very late in the infection the nuclear membrane breaks down and the production of new ODV is located in a mixed cytoplasmic-nuclear environment (Winstanley and Crook 1993). ODV are occluded by granulin protein matrix and are released as newly formed OB to the environment by break-down of the integument of the larval cadavers (Federici 1997).

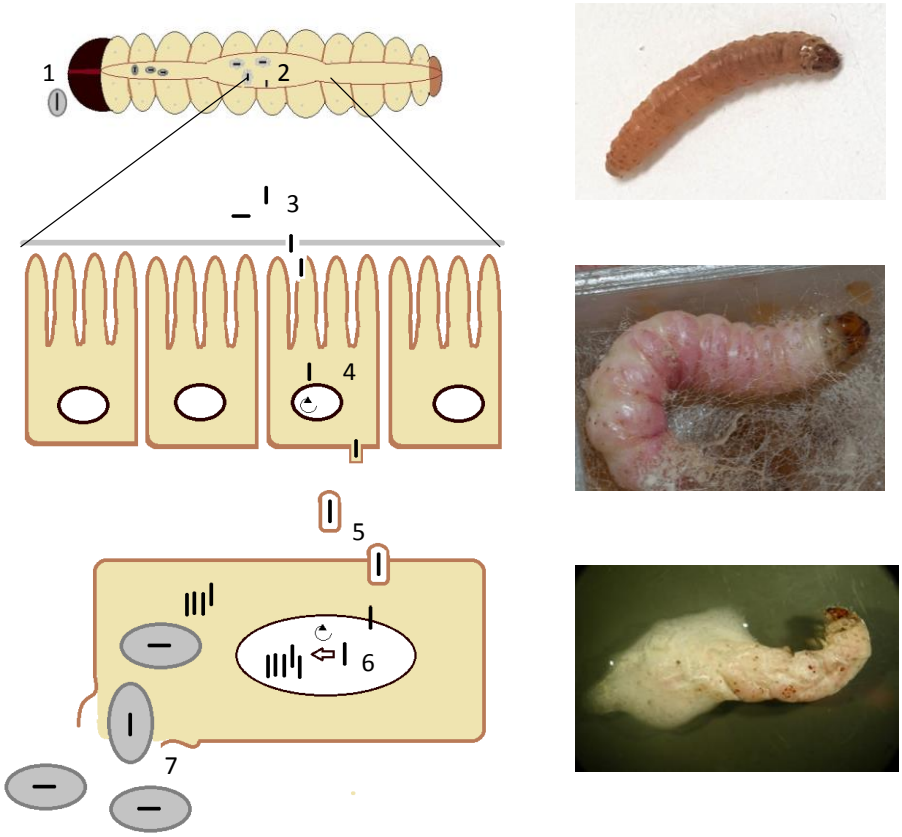


Figure 5 Schematic model of the infection pathway of the primary and secondary infection in CM caused by CpGV (left side) combined with pictures of the phenotypic transformation of infected CM larvae (right side). Occlusion bodies (OB) are ingested (1) by the larvae and are dissolved in the alkaline milieu of the midgut, where occlusion derived viruses (ODV) are released (2). ODV pass the peritrophic membrane (gray), and bind to the microvilli of midgut epithelial cells (3). Nucleocapsids are transported to the nucleus where the virus DNA is transcribed and replicated; new nucleocapsids are assembled in the nucleus (4). Nucleocapsids bud through the plasma membrane, forming budded virus (BV) and start secondary infections (5). BV bind to the cellular membrane and enter the cell and nucleus for replication (6). Later in the infection, new ODV are produced and embedded into OB, which are then released from larval cadavers (7). The phenotypic transformation of the infected larvae starts with swelling of the larval body and chance into a whitish, milky color of the cuticle. Larvae die by break-down of the cuticle and release of OB to the environment (Fotos: AJ Sauer and DLR Rheinpfalz).

1.3 Resistance

There are some disagreements in the literature on the definition of pesticide resistance, especially the term "field resistance" (Tabashnik et al. 2009). The definition of the world health organization (WHO) for resistance is: "*Resistance is 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*" (WHO 1957). The Insecticide Resistance Action Committee (IRAC) termed it: "*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*" (IRAC 2016). Based on this definition a repeated control failure of a product is the foundation of the field resistance definition (Huang et al. 2011).

The first documentation of cases of field resistance of San Jose scale (*Quadraspidiotus perniciosus*) to lime sulfur was in 1914 (Melander 1914). Today, emergence of resistance in pest populations is common in agriculture all over the world. According to the Arthropod Pesticide Resistance Database (APRD, http://www.pesticideresistance.org), 550 pest insects have developed resistance to one or more pesticides (status 2017). Insects have not only developed resistance to chemical insecticides. Also resistance against biological control agents, such as *Bacillus thuringiensis* products or baculoviruses has been reported in the literature (Siegwart et al. 2015).

Host resistance in baculovirus-host systems

The literature reports several cases of resistance in different baculovirus-host systems, which were mainly achieved by selection experiments under laboratory conditions (for review see Siegwart et al. 2015). Since in most cases no field insects or only those with a slightly reduced susceptibility to a baculovirus were the origin, these resistant laboratory strains were generally established by mass crosses accompanied by a repeated exposure to its selective agent.

When a susceptible laboratory strain of potato tuber moth *Phthorimaea operculella* was serially exposed to Phthorimaea operculella granulovirus (PhopGV) for six generations, a 140-fold increase of the median lethal dose (LD_{50}) was observed (Briese and Mende 1983). This report was one of the first examples of a selected resistance of an insect species to a baculovirus. However, the resistance decreased within a few generation when exposure of virus was discontinued (Briese 1986).

A population of the fall army worm *Spodoptera frugiperda* was reared under selection pressure in the laboratory and a three-fold resistance to SfMNPV based on the LC_{50} values, was achieved within seven generations (Fuxa et al. 1988). Because no difference of the mortality response of BV injection between the resistant and sensitive strain was observed, a midgut based block of virus infection was assumed (Fuxa and Richter 1990). Nevertheless, the resistant *S. frugiperda* strain also lost its reduced susceptibility to SfMNPV by rearing without virus (Fuxa and Richter 1989).

Abot et al. (1996) studied the occurrence of resistance to Anticarsia gemmatalis nucleopolyhedrovirus (AgMNPV) in the velvetbean caterpillar *Anticarsia gemmatalis* in Brazil, where AgMNPV has been used to control velvetbean caterpillars (Moscardi et al. 1999). Significant level of resistance in *A. gemmatalis* could be generated within 3-4 generations of exposure to AgMNPV and a resistance ratio of more than 1,000 could be established within 13-15 generations of selection. A final level of resistance was reached with a LC_{50} of 293,890 OB/ml, which was 17,000 times higher than that of the non-selected control (LC_{50} 17 OB/ml) (Abot et al. 1996). Levy et al. (2007) showed that the structure of the peritrophic membrane (PM) could function as a resistance barrier in this resistant *A. gemmatalis* strain. Though AgMNPV has been applied for more than two decades on very large areas of up to two million hectares per year to control the velvetbean caterpillars in soybean, its extensive application in the field did not induce reduced susceptibility, so far (Moscardi 2007).

A 22-fold increased LC_{50} could be generated in larvae of the cabbage looper *Trichoplusia ni*, which were exposed to Trichoplusia ni single nucleopolyhedrovirus (TnSNPV) for 27 generations (Milks and Myers (2000). Additionally, the selection was done on a recombinant AcMNPV, inducing two cross-resistances; one to TnSNPV (Milks and Theilmann 2000) and second to Trichoplusia ni granulovirus (TnGV) (Milks and Myers 2003).

An already less susceptible field population of the silkworm *Bombyx mori*, was highly resistant after eight generations of selection on Bombyx mori nucleopolyhedrovirus (BmNPV). Through classical genetic crossing experiments an autosomal dominant inheritance mode for this resistance was identified (Feng et al. 2012).

Recently, a laboratory strain of the tea tortrix *Adoxophyes honmai* was successfully selected by exposing a laboratory strain for more than 158 generations to Adoxophyes honmai nucleopolyhedrovirus (AdhoNPV). The LC_{50} values showed significant differences after nine generations and the resistance level based on LC_{50} increased linearly to a maximum factor of 20,700 compared to the unselected susceptible laboratory strain (Iwata et al. 2017). By a fluorescence-dequenching assay with ODV of AdhoNPV labeled with octadecyl rhodamine B chloride (R-18) a midgut-based resistance caused by a significantly reduced binding and fusion capacity of the ODV was identified (Iwata et al. 2017).

All these examples of resistance against baculoviruses were established by continuous selection under virus pressure in the laboratory; they clearly showed that selection of resistance against baculoviruses is generally possible. Field resistance of insects to commercially used baculovirus biocontrol products, however, has never been reported, until apple orchards with CM populations resistant to CpGV products were identified in Europe.

1.4 Field Resistance against CpGV

Resistance of CM field populations to CpGV products were first reported for two field populations in Germany in 2005 (Fritsch et al. 2005). In full-range bioassays performed with the offspring of overwintering larvae of these field populations, a 500 to 1,000 fold lower median lethal concentration (LC_{50}) than for the susceptible CM strain CpS was found. Later, further organic orchards with CM populations showing a reduced susceptibility to CpGV products were reported from Germany and Southern France (Fritsch et al. 2006; Sauphanor et al. 2006). Until now, 48 apple orchards with resistance to CpGV were identified in Austria (2 orchards), the Czech Republic (1), France (3), Germany (31), Italy (6), the Netherlands (3) and the Switzerland (2) (Sauphanor et al. 2006; Asser-Kaiser et al. 2007; Zichova et al. 2011; Gund et al. 2012; Schulze-Bopp and Jehle 2013; Schmitt et al. 2013) (Table 1). The resistance ratios, based on LC_{50} values, exceeded a factor of 1000 in the majority of the tested resistant field populations of CM (Asser-Kaiser et al. 2007). Genetic analyses with microsatellite and mitochondrial DNA markers of 33 CM populations could not identify significant differences between resistant and susceptible CM populations (Gund et al. 2012) arguing for a predominantly independent emergence of resistance in the analyzed CM populations. For further genetic and molecular investigations, some of these CpGV-resistant individuals were collected and reared in the laboratory to study of the underlying resistance mechanism(s) (Table 2).

Table 1 Summary of all reported resistant CM field populations in Europe (until January 2017). Median lethal concentration (LC_{50}) of treatment with CpGV-M, mortality of larvae treated with CpGV-M in a concentration higher than 10^4 OB/ml or mortality determined with the diagnostic concentration of 2 x 10^5 OB/ml (% mort-Cont.), all determined 14 days post infection.

No.	CM population	Country	LC_{50} [OB/ml] (95 % confidence limit)	% mort. >10^4 OB/ml	% mort-Cont.	source
1	A-NÖ-OB-07	Austria	6.02 x 10^6 (2.45 - 31.6)	24.2	10.0	1
2	A-WI-08	Austria	n.d.	n.d.	26.7	3
3	CH-VS-CO-06	Switzerland	2.33 x 10^5 (0.63 - 10.0)	73.3	24.7	1
4	CH-WTG-07	Switzerland	n.d.	n.d.	11.9	3
5	D-BW-BO-06	Germany	2.61 x 10^6 (0.39 - 121.0)	34.7	0	1
6	D-BW-BR-15	Germany	n.d.	n.d.	70.1*	unpublished
7	D-BW-DE-04	Germany	4.31 x 10^5 (1.71 - 12.70)	41.2	0	2
8	D-BW-FI-05	Germany	1.44 x 10^5 (0.17 - 6.82)	45.0	0	2
9	D-BW-FN-05	Germany	4.00 x 10^5 (2.19 - 6.83)	49.7	0	2
10	D-BW-FR-06 IP	Germany	n.d.	40.0	30	1
11	D-BW-HA-06	Germany	2.16 x 10^4 (0.03 – 507.0)	n.d.	n.d.	unpublished
12	D-BW-HI-08	Germany	6.71 x 10^7 (1.34 - 212.0)	17.5	0	1
13	D-BW-HI-06 IP	Germany	1.11 x 10^7 (0.33 - 9.69)	30.0	0	1
14	D-BW-HN-05	Germany	4.85 x 10^5 (0.77 - 44.5)	15.4	0	2
15	D-BW-KH-05	Germany	9.56 x 10^5 (2.32 - 44.10)	21.4	0	2
16	D-BW-KT-08	Germany	1.09 x 10^6 (0.26 - 3.03)	46.0	0	unpublished
17	D-BW-LF-06	Germany	2.17 x 10^6 (0.30 - 173.0)	31.1	0	1
18	D-BW-MA-06	Germany	1.31 x 10^6 (0,35 - 9,19)	44.2	0	1
19	D-BW-MN-06	Germany	1.40 x 10^7 (0.01 - 221.0)	23.1	0	1
20	D-BW-OD-05	Germany	8.89 x 10^5 (2.88 - 24.3)	25.0	0	2
21	D-BW-OK-04	Germany	7.62 x 10^4 (4.05 - 15.6)	51.9	0	unpublished
22	D-BW-OK-06 IP	Germany	1.56 x 10^5 (0.05 - 18.2)	76.5	39.0	1
23	D-BW-TU-08	Germany	2,25 x 10^7 (0.40 - 99.0)	32.4	5.3	1
24	D-HE-WI-05	Germany	8.02 x 10^5 (4.26 - 15.2)	26.5	0	2
25	D-NRW-WE-05	Germany	3.85 x 10^8 (0.98 - 64.0)	3.8	0	1
26	D-NS-EB-16	Germany	n.d.	n.d.	73.3*	unpublished
27	D-NS-HH-06	Germany	3.55 x 10^3 (0.40 - 17.0)	n.d.	72.0	4
28	D-NS-LJ-16	Germany	n.d.	n.d.	41.5*	unpublished
29	D-RP-MA-05	Germany	9.76 x 10^5 (3.28 - 27.3)	19.2	0	2
30	D-RP-NS-05	Germany	4.33 x 10^7 (1.20 - 42.4)	15.6	0	2
31	D-RP-NSG-08	Germany	n.d.	n.d.	38.2	3
32	D-RP-NSA-08	Germany	n.d.	n.d.	12.8	3
33	D-SA-GO-08	Germany	1.12 x 10^8 (0.23 - 27.4)	23.8	0	1
34	D-SA-HA-06 IP	Germany	1.37 x 10^5 (0.62 - 4.50)	45.5	8.6	1
35	D-SL-SA-04	Germany	4.79 x 10^5 (1.75 - 2.30)	43.2	0	2
36	F-13-ML-06	France	5.27 x 10^5 (1.31 - 13.7)	52.9	27.9	1

No.	CM population	Country	LC_{50} [OB/ml] (95 % confidence limit)	% mort. >10^4 OB/ml	% mort-Cont.	source
37	F-37-StA-06	France	4.6×10^6 (0.48 - 27.3)	53.6	43.3	1
38	F-26-StM-06-2	France	4.97×10^3 (0.004 - 27.2)	83.8	59.0	1
39	I-FC-CE-06	Italy	1.55×10^8 (0.28 -185)	31.2	18.5	1
40	I-FC-FO-06	Italy	n.d.	32.4	61.3	1
41	I-FE-MO-07	Italy	4.87×10^6 (2.88 - 9.85)	18.2	6.6	1
42	I-VR-SP-06-1	Italy	3.74×10^7 (1.04 -1470)	32.6	27.4	1
43	I-VR-SP-06-2	Italy	n.d.	32.4	10.5	1
44	I-GA	Italy	n.d.	n.d.	10.0	3
45	NL-GE-LO-06	Netherlands	3.14×10^6 (0.60 - 173)	27.7	10.0	1
46	NL-MA-09	Netherlands	n.d.	n.d.	38.9	3
47	NL-LO-07	Netherlands	n.d.	n.d.	50	4
48	Cp-VBII	Czech Republic	n.d.	16.7	n.d.	5

1= published in Schmitt et al. (2013); 2 = Asser-Kaiser et al. (2007); 3 = Schulze-Bopp and Jehle (2013); 4 = Gund et al. (2012); 5 = Zichova et al. (2011); unpublished = personal communication E. Fritsch and K. Undorf-Spahn

* Larvae were tested for resistance to CpGV-S but not CpGV-M

Type I resistance

For the field population CpR, also termed DE-BW-FI-03 = "Suedbaden", found in South Baden, Germany (Fritsch et al. 2005), an autosomal and incomplete dominant inherited resistance to CpGV-M was initially proposed (Eberle and Jehle 2006) (Table 2). The resistance level of CpR was stable for more than 60 generations, of rearing without virus. Additionally, no measurable fitness cost in terms of fecundity and fertility were exhibited under laboratory conditions (Undorf-Spahn et al. 2012). After it was found that this population was a mixture of susceptible and resistant individuals, CpR was selected by single pair inbreeding with simultaneous resistance testing for two consecutive generations, resulting in the CM strain CpRR1, for which a genetically fixed resistance was assumed (Asser-Kaiser et al. 2007). The karyotype of CM consists of 2n = 56 chromosomes, 54 autosomes and two sex chromosomes (W,Z), all of a holokinetic type (Fuková et al. 2005). CM, like most Lepidoptera, carries a ZW/ZZ sex chromosome system, the females being heterogametic (ZW) and males homogametic (ZZ) (Traut et al. 2007). For CpRR1 it was demonstrated by crossing and backcrossing experiments with susceptible CpS individuals that the resistance is inherited in a dominant and monogenetic but Z-linked mode (located on the Z chromosome) (Asser-Kaiser et al. 2007). Asser-Kaiser et al. (2010) also confirmed this finding later for CpR correcting the initial assumption of Eberle and Jehle (2006). Intra-hemocoelic injections of BV of CpGV-M into CpRR1 larvae elucidated a systemic and early block of resistance (Asser-Kaiser et al. 2010). Eberle et al. (2008) further showed that resistance in CpRR1 is expressed in all five larval stages (L1-L5). By screening other CpGV isolates

for resistance-breaking properties, CpGV-I12, -E2, -I07, and -S (genome group B-E) were identified to cause high mortality in CpR and CpRR1 larvae (Eberle et al. 2008; Gebhardt et al. 2014).

The field population St. Andiol from France was selected by mass crossing experiments subjected to virus pressure, resulting in the laboratory strain RGV with a genetically fixed resistance. Based on LC_{50} values, RGV showed a 60,000-fold resistance ratio for CpGV-M when compared to the susceptible strain. The isolate CpGV-I12 (genome group D) was also able to break the resistance in RGV (Berling et al. 2009). A strongly dominant and sex dependent inheritance of resistance was proposed also for RGV, although the detailed analysis of the crossing experiments between RGV and a susceptible strain suggested a more complex inheritance pattern (Berling et al. 2013).

Zichová et al. (2013) generated the strain CpR-CZ from the resistant CM field population Cp-VBII found in the Czech Republic (Zichova et al. 2011) by mass crosses combined with selection under CpGV-M pressure. Resistance testing of CpR-CZ indicated a dominant inheritance that is also linked to the sex chromosome Z. The isolates CpGV-E2, -I08, -I12, -S and -V15 (genome group B-E) were also able to break the resistance in CpR-CZ (Zichová et al. 2013).

The similarities of the mode of inheritance and the isolates that break the resistance led to the hypothesis that most of the resistant CM populations in Europe follow a similar mechanism, called type I resistance (Jehle et al. 2017).

Table 2 Origin of the resistant CM strains analyzed in the laboratory.

Field Population	Selected Laboratory Strain	Resistance Type	Reference
D-BW-FI-03	CpRR1	Type I	Asser-Kaiser et al. 2007
St. Andiol	RGV	Proposed Type I	Berling et al. 2009
Cp-VBII	CpR-CZ	Proposed Type I	Zichová et al. 2013
D-NRW-WE-08	-	Type II	Jehle et al. 2017
D-SA-GO-08	-	unknown	Schmitt et al. 2013, This study

Role of the CpGV gene *pe38* in type I resistance

The fact that several naturally occurring CpGV isolates from genome groups B-E were able to overcome type I resistance in CpRR1 stimulated the screening for a viral factor, which could be responsible for the resistance-breaking mechanism. Consequently, the genomes of all available resistance-breaking CpGV isolates were fully sequenced and the lack of a 24 bp repetitive insertion in the gene *pe38* of CpGV-M (group A) was identified as the only common genomic difference to all resistance-breaking isolates (group B-E). This insertion in *pe38* of CpGV-M leads to a predicted additional repeat of 2x4 amino acids in the encoded protein PE38. The biological function of PE38 in CpGV is unknown but it contains a predicted zinc finger and leucine zipper (Gebhardt et al. 2014). It is assumed that *pe38* belongs to the immediate early transcribed genes in AcMNPV (Krappa and Knebel-Mörsdorf 1991). It was found to play a crucial role in viral replication, budded virus production and virulence (Milks et al. 2003). A knock-out of *pe38* in a bacmid of CpGV-M (Hilton et al. 2008) resulted in a complete abolishment of virus infection in both, CpS and CpRR1, indicating the essential role of this gene in CpGV infection and replication (Gebhardt et al. 2014). Two recovery constructs of the pe38 knock-out bacmid, one repaired with the homologous *pe38* from CpGV-M bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M) and a second one repaired with *pe38* from resistance-breaking CpGV-S bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S) were established (Gebhardt et al. 2014). CpRR1 neonates subjected to OB derived from the two recombinants pe38M and pe38S could be infected with pe38S but not with pe38M, proposing that the viral gene *pe38* of CpGV-M is the target of type I resistance in CpRR1 (Gebhardt et al. 2014).

Evidence for new resistance types

Previous resistance tests could already prove that two field populations, namely D-NRW-WE-05 (=NRW-WE) and D-SA-GO-08 (=SA-GO), showed untypically high LC_{50} values of 3.85 x 10^8 OB/ml and 1.12 x 10^8 OB/ml, respectively (Table 1) (Asser-Kaiser et al. 2007; Schmitt et al. 2013). These LC_{50} values represented an about 1,000,000-fold increased level of resistance, compared to the LC_{50} of susceptible CpS and were the highest LC_{50} values ever detected in field populations (Schmitt et al. 2013). In addition, SA-GO showed as reduced susceptibility to isolate CpGV-I12 (genome group D), which was identified as resistance-breaking of type I resistance in CpRR1 (Schulze-Bopp and Jehle 2013).

Recently, Jehle et al. (2017) reported that NRW-WE was also resistant to isolates belonging to the resistance-breaking genome groups (C-E). Only the genome group B was able to infect larvae of this field population. By single pair crosses between field collected individuals of NRW-WE and susceptible CpS followed by resistance testing of the offspring, the Z-linked inheritance typical for

type I resistance could not be substantiated and further mode of CpGV resistance was postulated (Jehle et al. 2017).

1.5 Aim of this thesis

Whereas type I resistance, targeting CpGV-M, was in the focus of numerous studies mentioned above, a further novel type of resistance appeared to exist in the field populations NRW-WE and SA-GO. Hence the following questions have been addressed in this thesis:

1. What is the nature of the novel type of resistance in NRW-WE and SA-GO to CpGV isolates?
2. How differ their mode of inheritance from known type I resistance?
3. Which cellular and molecular factors are responsible for the resistance?
4. Which CpGV isolates and products are able to break the novel resistance type in NRW-WE and SA-GO?

In **Chapter 2** of this thesis the resistant field population NRW-WE was mass crossed and selected under virus pressure either on CpGV-M or on CpGV-S for five generations, resulting in the strains CpR5M and CpR5S with a genetically fixed resistance. Mass crosses between the two resistant strains and the susceptible CM strain CpS followed by resistance testing of the larval offspring elucidated a novel mode of inheritance of the resistance, termed type II resistance. In addition, the chromosomal architecture of the Z chromosome of CpR5M was investigated for rearrangements by fluorescence *in situ* hybridization (FISH).

The resistance mechanism of CpR5M was investigated in **Chapter 3**. Infection experiments with OB, ODV, BV and recombinant CpGV-bacmids were analyzed for their ability for initiating virus infection. Mixture infections of different resistance-prone and resistance-breaking CpGV isolates were performed to test the susceptibility of CpR5M/CpR5S; infection studies with different larval stages provide information on the resistance in particular larval stages; by BV injection and by ODV binding and fusion assays it was tested, whether the midgut is involved in type II resistance.

In **Chapter 4** the inheritance mechanism of SA-GO is analyzed by crossing experiments of diapausing field larvae with CpS. Selection for a genetically fixed resistance was done by single pair inbreeding of SA-GO and successive resistance testing for two generations, resulting in the CM strain CpRGO. The mode of inheritance of resistance in CpRGO was eventually examined by single pair crosses with CpS individuals and resistance testing of the offspring, revealing a further type III resistance in CM.

In **Chapter 5** analyses of the three CpGV resistance types I - III and their mortality response to different CpGV isolates representing the presently known genetic diversity of CpGV.

This final **Chapter 6** will discuss consequences of different types of CpGV resistance for development of appropriate management strategies to avoid development and spread of CpGV resistance in the field.

2. Novel resistance to Cydia pomonella granulovirus (CpGV) in codling moth shows autosomal and dominant inheritance and confers cross-resistance to different CpGV genome groups

Abstract

Commercial Cydia pomonella granulovirus (CpGV) products have been successfully applied to control codling moth (CM) in organic and integrated fruit production for more than 30 years. Since 2005, resistance against the widely used isolate CpGV-M has been reported from different countries in Europe. This so-called type I resistance is dominant and linked to the Z chromosome. Recently, a second form (type II) of CpGV resistance in CM was reported from a field population (NRW-WE) in Germany. Type II resistance confers reduced susceptibility not only to CpGV-M but to most known CpGV isolates and it does not follow the previously described Z-linked inheritance of type I resistance. To further analyze type II resistance, two strains, termed CpR5M and CpR5S, were generated from parental NRW-WE by repeated mass crosses and selection using the two isolates CpGV-M and CpGV-S, respectively. Both selection strains were considered to be genetically homogenous for the presence of the resistance allele(s). By crossing and backcrossing experiments with a susceptible CM strain, followed by resistance testing of the offspring, an autosomal dominant inheritance of resistance was elucidated. In addition, cross-resistance to CpGV-M and CpGV-S was ascertained in both CpR5M and CpR5S strains.

To test the hypothesis that the autosomal inheritance of type II resistance was caused by interchromosomal rearrangement involving the Z chromosome, fluorescence *in situ* hybridization with bacterial artificial chromosome probes (BAC-FISH) was used to physically map the Z chromosomes of different CM strains. Conserved synteny of the Z-linked genes in CpR5M and other CM strains rejects this hypothesis and argues for a novel genetic and functional mode of resistance in CM populations with type II resistance.

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Author contributions: E.F and K.U-S performed the resistance testing in NRW-WE, the selection process and the crossing and backcrossing experiment with CpS and CpR5M and CpR5S.

2.1 Introduction

Baculoviruses are insect pathogenic viruses, which are widely used as biological control agents of insect pests in agriculture and forestry. One of the most important commercially used baculovirus is the Cydia pomonella granulovirus (CpGV). CpGV belongs to the genus *Betabaculovirus* of the *Baculoviridae* family (Herniou et al. 2001); its genome size is 123.5 kbp and encodes 143 putative open reading frames (ORFs) (Luque et al. 2001). CpGV is highly virulent to early larval stages of the codling moth (CM) (*Cydia pomonella*, Lepidoptera: Tortricidae), whereas it is harmless to non-target insects and animals and it has no detrimental impact on the environment (Lacey et al. 2008).

CpGV products have been used since the late 1980s for the control of CM, which is one of the most destructive insect pests in apple, pear and walnut production. Without control, CM can cause severe damage and complete loss of marketable fruits. Pome fruit grower spray formulated viral occlusion bodies (OB) of CpGV, which are ingested by the CM larvae and cause larval death within four to six days (Lacey et al. 2008).

Although different geographic CpGV isolates have been detected during the last several decades, nearly all worldwide commercially available products contained one CpGV isolate, termed CpGV-M. This isolate was originally discovered in infected CM larvae in Mexico (Tanada 1964). After registration of CpGV products in more than 34 countries worldwide and their successful use in organic and integrated pome fruit production, first reports of resistance of CM populations to CpGV appeared in 2005 (Fritsch et al. 2005). Upon further investigations, 38 apple orchards with resistance to CpGV-M based products were identified in Austria (2 orchards), the Czech Republic (1), France (3), Germany (22), Italy (6), the Netherlands (2) and Switzerland (2) (Sauphanor et al. 2006; Asser-Kaiser et al. 2007; Schmitt et al. 2013; Zichová et al. 2013). Some of these CpGV-resistant field populations were collected and reared for genetic and molecular investigations, e.g. the strains CpRR1 (Germany), RGV (France) and CpR-CZ (Czech Republic) (Asser-Kaiser et al. 2007; Berling et al. 2009; Zichová et al. 2013).

The karyotype of CM consists of 2n = 56 chromosomes, 54 autosomes and two sex chromosomes (W,Z), all of a holokinetic type (Fuková et al. 2005). CM, like most Lepidoptera, carries a ZW/ZZ sex chromosome system, the females being heterogametic (ZW) and males the homogametic (ZZ) sex (Traut et al. 2007). Recently, it was shown by physical mapping that the Z chromosome in *C. pomonella* and other tortricids is the result of a fusion of the ancestral Z chromosome and the homeolog of chromosome 15 in the *Bombyx mori* reference genome (Nguyen et al. 2013). For CpRR1 it was demonstrated by crossing experiments that CpGV resistance is inherited in a dominant, Z-linked and monogenic mode (Asser-Kaiser et al. 2007; Asser-Kaiser et al. 2010). Whether the resistance allele of CpRR1 is located on the ancestral part of the Z chromosome or the part homeologous to the *B. mori* autosome 15 is unknown.

Based on the following two observations, it was hypothesized that most of the resistant CM populations in Europe follow a similar mechanism and inheritance mode. First, a Z-linked, dominant inheritance was also determined for other geographically distant CM populations in France and the Czech Republic (Berling et al. 2013; Zichová et al. 2013). Second, most of the resistant CM populations could be successfully controlled by the same resistance-breaking CpGV isolates (Jehle et al. 2006b; Eberle et al. 2008; Berling et al. 2009; Graillot et al. 2014). Phylogenetically, CpGV isolates can be classified into five different genome groups (A-E); the isolate CpGV-M belongs to genome group A (Eberle et al. 2009; Gebhardt et al. 2014). By comparing the genome sequences of the CpGV-M with resistance-breaking isolates belonging to CpGV genome group B-E, it was found that the genomes of all resistance-breaking isolates differed only in a single common difference from that of CpGV-M, namely an additional repeat of 24 nucleotides within the viral ORF *pe38* in CpGV-M (Gebhardt et al. 2014). ORF *pe38* encodes a zinc finger and leucine zipper containing protein and is supposed to be an early transcribed gene in Autographa californica multi nucleopolyhedrovirus (AcMNPV) (Krappa and Knebel-Moersdorf 1991). These results suggested that the resistance to CpRR1 described so far is isolate-dependent (Gebhardt et al. 2014). Several of these resistance-breaking isolates have been already tested in laboratory and field experiments. Most of them demonstrated good efficacy against sensitive and resistant CM strains and some are now registered and applied in different European countries for CM control (Eberle et al. 2008; Kienzle et al. 2008; Berling et al. 2009; Graillot et al. 2014). With the application of these novel resistance-breaking isolates, successful control of resistant CM field populations was possible.

However, recently some orchards were identified in which even these new resistance-breaking CpGV isolates failed to control CM sufficiently. One of these CM populations, called NRW-WE, was detected in north-west Germany (Jehle et al. 2014b). This population was not only resistant to CpGV-M belonging to the genome group A, but also to those of genome group C, D and E. Therefore, a second type of resistance (type II) was proposed for this CM population. So far, only CpGVs belonging to group B, such as the isolate CpGV-E2, is able to break type II resistance (Jehle et al. 2017).

Here, we report the establishment of two genetically homogenous inbred CM strains, termed CpR5M and CpR5S, which were generated from NRW-WE by a continuous selection procedure on either CpGV-M (genome group A) or CpGV-S (genome group E). Systematic crossing experiments revealed an autosomal dominant inheritance of resistance for both CpR5M and CpR5S; no Z-linked inheritance of resistance was detectable. We further tested the hypothesis that the difference between the Z-linked resistance in CpRR1 and the autosomal resistance in CpR5M could be a consequence of a large scale Z-chromosome rearrangement such as fission or translocation. Physical mapping of selected genes using fluorescence *in situ* hybridization (FISH) revealed a similar architecture of the Z chromosome in susceptible CM as well as in the resistant CpRR1 and CpR5M strains, rejecting the

rearrangement hypothesis and indicating a novel type of resistance and inheritance of CM populations with type II resistance.

2.2 Materials and Methods

Insects: The CpS strain of *C. pomonella* is susceptible to all CpGV isolates and has been reared at the Julius Kühn Institute (JKI) in Darmstadt (Germany) for many years (Asser-Kaiser et al. 2007). The CM strain CpS-Krym (= Krym-61) is also susceptible to all CpGV isolates and was used instead of CpS for the FISH technique in the Czech Republic (Fuková et al. 2005; Zichová et al. 2013). CpRR1 carries type I resistance against CpGV-M and arose from the resistant field population CpR (BW-FI-03, 'Sudbaden') by single pair crosses (Asser-Kaiser et al. 2007). The resistant field population NRW-WE descended from an apple orchard in North Rhine-Westphalia (Germany) and had a reduced susceptibility to CpGV-M and CpGV-S (Asser-Kaiser et al. 2007; Jehle et al. 2017). By collecting diapausing larvae, a laboratory rearing of NRW-WE was established in 2008/2009 at JKI. All CM strains were reared under laboratory conditions at 26 °C with 16/8 h light/dark photoperiod and 60 % relative humidity on modified diet of Ivaldi-Sender (Ivaldi-Sender 1974).

Viruses: Different Cydia pomonella granulovirus (CpGV) isolates were used in this study. CpGV-M (genome group A), the so-called "Mexican isolate" (Tanada 1964), was prepared from batch TPCpGVBTPS_02 and was part of the DLR Rheinpfalz virus stock (Gebhardt et al. 2014). The isolate CpGV-S (genome group E) was isolated from the commercial product Virosoft^{TM} (BioTEPP Inc., Canada) and propagated in CpS larvae. Purification of virus occlusion bodies (OB) was done as described before (Smith and Crook 1988); all samples were kept at -20 °C. Quantification of virus stocks was performed by OB counting in dark-field optics of a light microscope (Leica DMRBE) with the Petroff-Hauser counting chamber (depth 0.02 mm).

Resistance testing: The diet incorporation method was used to mix purified OB into the modified diet of Ivaldi-Sender (Ivaldi-Sender 1974) for resistance testing as described previously (Undorf-Spahn et al. 2012). Neonate CM larvae were placed on artificial diet with the final discriminating concentration of 5.8 x 10^4 OB/ml. This concentration caused 95-98 % mortality to neonate CpS larvae in seven day bioassays (Asser-Kaiser et al. 2007). Mortality was determined one, seven, 14 and 28 days post infection (p.i.) and only larvae surviving day one p.i. were introduced to the test. For each assay a minimum of 30 larvae were used and independently repeated four to seven times. The observed virus-induced mortality was corrected by the mortality of the untreated control group (Abbott 1925).

Establishing the virus selected strains CpR5M and CpR5S: For establishing genetically homogeneous strains from the field population NRW-WE, mass crosses under CpGV selective pressure were performed as described elsewhere (Berling et al. 2009; Zichová et al. 2013). Emerged first instar larvae of the mass reared NRW-WE were selected on artificial diet containing either CpGV-M or CpGV-S at a diagnostic concentration of 2 x 10^5 OB/ml. Larval mortality was recorded 16 days p.i. The surviving larvae were reared until pupation and the sex of the eclosed moths was documented and again mass crossed for the second round. The larval offspring were again subjected to the selection process and this procedure was repeated for five generations. The strain CpR5M was the outcome of the successive mass selection on CpGV-M, whereas CpR5S originated from continuous selection on CpGV-S. These two strains were reared separately under laboratory conditions without further exposure to CpGV.

Reciprocal crosses and backcrosses: To analyze the mode of inheritance of the resistance allele(s) in the selected strains CpR5M and CpR5S, reciprocal crosses between these two strains and CpS were performed. Pupae were separated by sex under the binocular microscope, according to the number of their abdominal segments (Eberle and Jehle 2006). Two genetic hybrid crosses were undertaken (Figure 6); in the female crosses, resistant female moths from either CpR5M or CpR5S were mated with males from the sensitive CpS strain. In the hybrid male crosses, resistant male moths were mass crossed with CpS females. Each cross consisted of eight to ten moths with a ratio of 1:1 males to females. Eggs were collected every second day. Neonate larvae of the first generation (F_1) were divided into three cohorts and subjected to artificial diet containing either (i) CpGV-M, or (ii) CpGV-S, each at a discriminating concentration of 5.8 x 10^4 OB/ml, or (iii) virus free diet as untreated control. Mortality in all three cohorts was scored as described in the resistance testing. The observed virus-induced mortality was corrected by the mortality of the untreated control group (Abbott 1925). The larvae of the control were further reared to adulthood and used for backcrossing experiments (see below). In addition, the sex of the surviving pupae in each treatment was recorded 28 days p.i. and determined as described before (Eberle and Jehle 2006).

Two hybrid backcrosses (BC) were carried out (Figure 6). For BC A, hybrid F_1 male moths were crossed with CpS females and in the BC B, hybrid F_1 males were mated with females of the parental strains CpR5M or CpR5S, respectively. Each backcross consisted of eight to ten F_1 male moths and an equal number of female moths. The neonate offspring were tested for resistance on CpGV-M or CpGV-S at a discriminating concentration of 5.8 x 10^4 OB/ml or were reared on untreated diet as described above. Hybrid crosses and backcrosses were independently repeated three to five times.

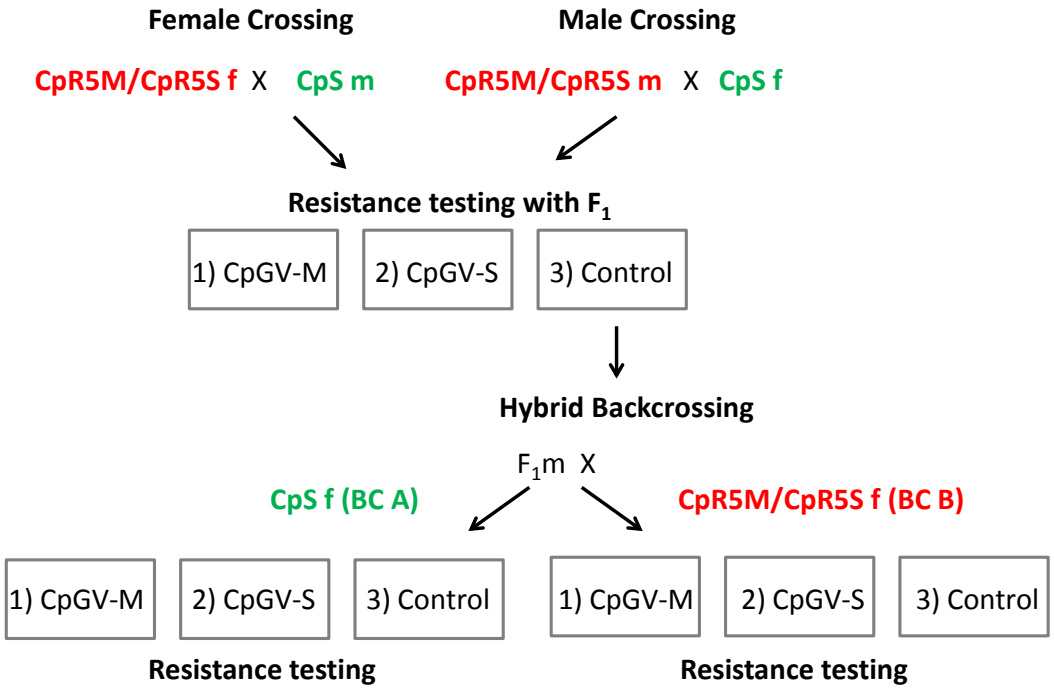

Figure 6 Schema of the crosses and backcrosses (BC) of the CM resistant strains (CpR5M or CpR5S) with the sensitive CpS. The female crosses started with female (f) CpR5M or CpR5S moths mated with CpS male (m) moths; the sex was reversed in the male crosses. Resulting neonate larvae of the first generation (F_1) were divided into three cohorts. Two cohorts were tested for resistance with the discriminating concentration 5.8×10^4 OB/ml of CpGV-M or CpGV-S. Mortality was observed seven, 14 and 28 days post infection. A third cohort of F_1 offspring was reared as control on artificial diet without virus until adulthood. Resulting male moths of the F_1 control group were mated with females of CpS (termed BC A) or CpR5M/CpR5S (BC B) and these offspring were tested for resistance as described before.

BAC-FISH mapping: To compare the genetic architecture of the Z chromosome of CpR5M and CpRR1 (with the susceptible CpS-Krym as a reference) fluorescence *in situ* hybridization (FISH) with probes prepared from DNA of *C. pomonella* bacterial artificial chromosome (BAC) clones, selected from the *C. pomonella* BAC library was used, as described in Nguyen et al. (2013). The BAC clones contained in total 13 genes located on the Z chromosome of *C. pomonella* (Nguyen et al. 2013). Three insecticide resistance genes (*ABCC2*, *ABCF2* and *Rdl*), three enzyme-coding genes (*Tpi*, *Idh-2* and *Pgd*) and seven genes encoding non-enzymatic proteins (*ap*, *Notch*, *kettin*, *RpS5*, *nan*, *per* and *RpP0*) were chosen as marker genes. Meiotic chromosomes were prepared from gonads of late fourth instar male larvae of the three CM strains, CpS-Krym, CpRR1 and CpR5M. Chromosomes were spread on slides using a heating plate and meiotic stage was checked under a phase contrast microscope (Fuková et al. 2005).

BAC DNA was extracted using the Qiagen Plasmid Midi Kit (Qiagen, Düsseldorf, Germany) and labeled by Cy3-dUTP (GE Healthcare, Buckinghamshire, UK) or ChromaTide Fluorescein-12-dUTP (Invitrogen, Paisley, UK) using a Nick Translation Kit (Abbott Molecular, Des Plaines, IL, USA). Labeled BAC probes were hybridized to chromosome preparations using two-color BAC-FISH as described in Nguyen et al. (2013). BAC-FISH preparations were counterstained with DAPI and observed in a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) equipped with appropriate fluorescence filter sets. In total, more than 1100 images of clearly visible and complete pachytene ZZ bivalents were recorded using an Olympus CCD monochrome camera XM10 equipped with cellSens 1.9 digital imaging software (Olympus Europa Holding, Hamburg, Germany) pseudocolored and superimposed with Adobe Photoshop CS3. Due to the absence of the primary striction, the centromere, in holokinetic chromosomes of Lepidoptera (Wolf 1996; Carpenter et al. 2005), the *apterous (ap)* gene was selected as the common marker for calculating the relative distance of different gene loci along the Z chromosome. By probing different pairs of genes while keeping *ap* as a reference, six different BAC-FISH experiments were performed to complete the mapping of all 13 marker genes in each CM strain. The images were analyzed using the freeware ImageJ (National Institutes of Health). The relative position of hybridization signals was measured three times and normalized for the whole length of the Z chromosome. The tip of the Z chromosome at the *ap* end was defined as the zero point.

Statistical methods: Dominance of resistance (D_x) was calculated for the hybrid crosses as described by Bourguet et al. (2000). $D_X = (x_{RS} - x_{SS})/(x_{RR} - x_{SS})$, where x_{SS}, x_{RS} and x_{RR} were the observed mortality of the susceptible homozygous, heterozygous and resistant homozygous individuals, respectively. Values of D_X ranged from 0 to 1. D values close to 0 were considered to represent a completely recessive inheritance, whereas values close to 1 represent a completely dominant resistance (Bourguet et al. 2000).

Statistical analyses of backcrosses were performed with a χ^2 test of fit (df = 1) between the expected and observed backcross mortality at the discriminating concentration for monogenetic inheritance, as described by Tabashnik (1991). The expected number of deaths was calculated as n_iM_i, where n_i is the mortality scored at the discriminating concentration i and $M_i = 0.5 \times (W_{RS} + W_{SS})$ or $M_i = 0.5 \times (W_{RS} + W_{RR})$ depending on the type of backcross and the CM strain. W_{RS}, W_{RR} and W_{SS} are the mortality of the tested hybrid F_1 (RS) and homozygous parental (RR) or (SS) strains at the discriminating concentration i.

Statistical differences in the summarized mortality data between hybrid crosses, BC A and BC B were analysed by ANOVA post-hoc Tukey HSD test comparisons of the means (RStudio edition 2.3.4.4.).

The relative positions of hybridization signals of BAC probes on the Z chromosome were statistically analysed by ANOVA, Scheffé test (RStudio edition 2.3.4.4.).

2.3 Results

Establishment of genetically homogenous strains from NRW-WE: To establish genetically homogeneous strains from the field population NRW-WE, five successive inbreeding crosses (F_1-F_5), each followed by an exposure of the larval offspring to selection pressure by either CpGV-M or CpGV-S, were performed. This selection procedure resulted in two resistant strains CpR5M (selected on CpGV-M) and CpR5S (selected on CpGV-S) (Figure 7). During selection on CpGV-M the mortality decreased within the five generations from 51.3 % (F_1) to 6.7 % (F_5), assessed 16 days p.i. When selection was performed on CpGV-S, the virus-induced mortality decreased from 32.3 % (F_1) to 11.4 % (F_5). Thus, the selection procedure caused decreases of mortality by a factor of 7.7 when selected on CpGV-M and by a factor of 2.8 on CpGV-S, respectively. The percentages of surviving male moths were balanced in all generations (Figure 7). Since the last two generations (F_4 and F_5) showed a similar mortality, the resistance in generation F_5 was considered as genetically fixed and the offspring were reared without further selection pressure.

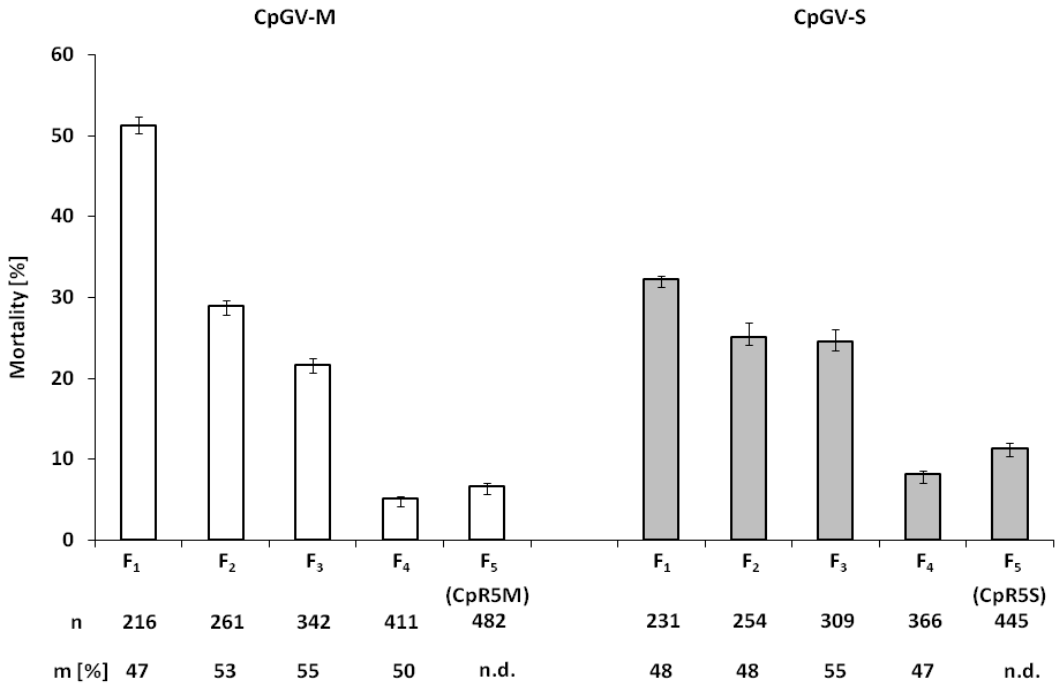

Figure 7 Establishment of genetically homogenous strains from NRW-WE. Mortality of first instar larvae of the mass rearing of NRW-WE, selected on artificial diet containing either CpGV-M (white bars) or CpGV-S (gray bars) at a diagnostic concentration of 2 x 10^5 OB/ml. Surviving larvae from each generation were reared to adulthood and the moths were used for the subsequent mass crosses; resulting neonate larvae were treated as described previously for five generations, ending with the strains CpR5M and CpR5S. The mean Abbott-corrected mortality and standard errors (bars) were determined 16 days post infection, generation number (F_1-F_5), the number of tested individuals (n) and the percentage of surviving male moths (m [%]) are given below the chart; n.d. stands for not determined.

Resistance testing in CpR5M, CpR5S and CpS: Neonate larvae of CpR5M, CpR5S and CpS were exposed to the discriminating concentration of 5.8 x 10^4 OB/ml of CpGV-M and CpGV-S, respectively, to compare the level of resistance. For CpS, virus-induced mortality was 96 % on CpGV-M and 92 % on CpGV-S after seven days (Figure 8). CpR5M neonates showed mortality of 1 % on both CpGV-M and CpGV-S. Mortality of CpR5S neonates was 1 % on CpGV-M and 0 % on CpGV-S after seven days. After 14 days, the mortality of CpS increased to 100 % for both CpGV-M and CpGV-S. In contrast, mortality of the two resistant strains varied between 2 % and 6 % (Figure 8). Thus, both CpR5M and CpR5S strains selected either on CpGV-M (group A) or CpGV-S (group E) showed clear cross-resistance to both CpGV genome groups.

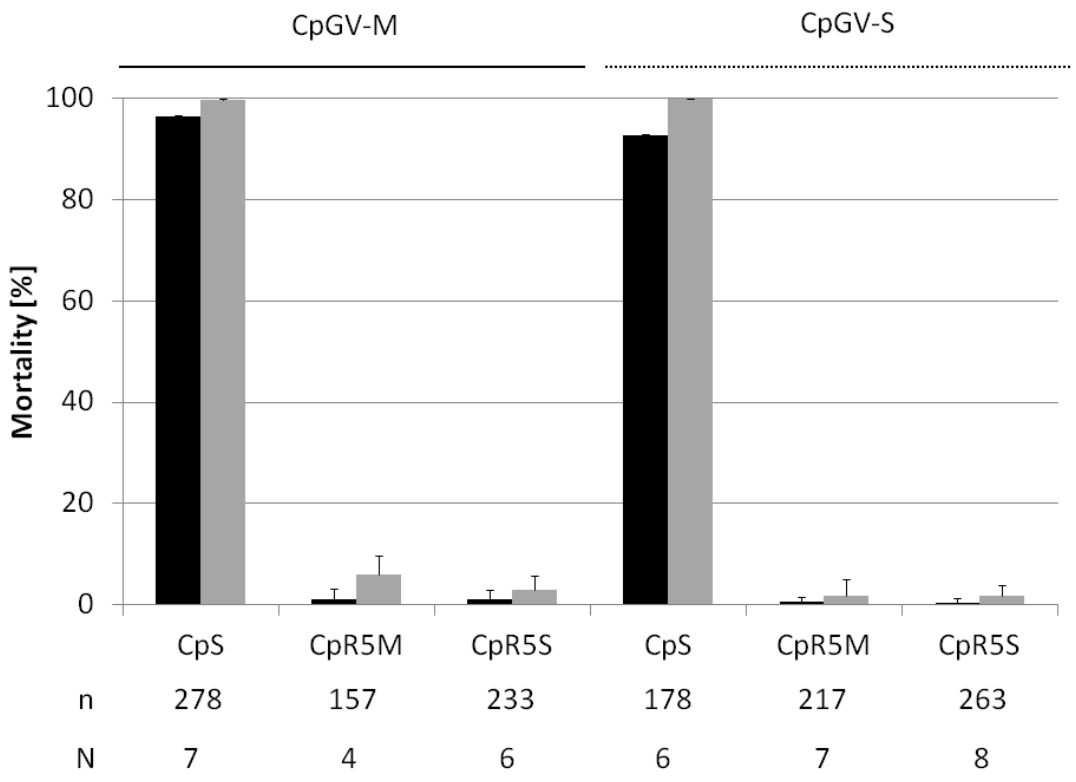

Figure 8 Resistance testing in CpR5M, CpR5S and CpS. Mortality of neonate larvae of CpR5M, CpR5S or CpS tested for resistance on artificial diet containing either CpGV-M (continuous line) or CpGV-S (broken line) at a discriminating concentration of 5.8×10^4 OB/ml. The mean Abbott-corrected mortality and standard errors (error bars) were determined seven (black bars) and 14 days (gray bars) post infection. The total numbers of tested individuals (n) and number of independent replicates (N) are given below the chart.

Crosses and backcrosses of CpR5M or CpR5S with CpS: Hybrid mass crosses and backcrosses with CpS individuals were performed to examine the mode of inheritance of resistance to CpGV-M and CpGV-S in the CM strains CpR5M and CpR5S (Figure 6). Following either an autosomal or a Z-linked, dominance hypothesis, differences in the mortality rates should become obvious in six out of twelve hybrid crosses and backcrosses (highlighted in Table 3). The mortality rates of the F_1 offspring of the female hybrid crosses CpR5Mf x CpSm were 0.01 and 0 on CpGV-M or CpGV-S, respectively, after seven days. When F_1 male (F_1m) moths, resulting from the control group were backcrossed with CpS female moths (BC A) and their offspring were exposed to CpGV-M and CpGV-S, the mortality rates were 0.49 and 0.39, respectively. BC B progeny of F_1m with CpR5Mf showed mortality rates of 0.01 for CpGV-M and 0 for CpGV-S in the seven day bioassays (Table 3).

Table 3 Crosses and backcrosses of CpR5M or CpR5S with CpS. Mortality rates of CM larvae determined in bioassays with neonate larvae seven days post infection (p.i.), exposed to CpGV-M or CpGV-S at a discriminating concentration of 5.8 X 10^4 OB/ml. The mortality was ascertained with the offspring of the different strains CpS, CpR5M, and CpR5S, of the hybrid crosses between CpR5M or CpR5S with CpS and of the backcrosses BC A and BC B. Progeny genotypes are shown for two hypotheses: a single Z-linked, dominant resistance gene (Z) or a single autosomal, dominant resistance gene (A). BC stands for backcross; F_1, offspring of the first generation; R, resistant; S, susceptible; f, female; m, male, M, mean; SD, standard deviation; N, number of independent replicates and n, total number of tested larvae. The indicated expected (Exp) mortality represents a dominant Z-linked (Z) or autosomal (A) inheritance hypothesis of the F_1 generation or of the backcrosses. Differences in the expected mortality between Z-linked and autosomal inheritance of the resistance are highlighted in green. Expected mortality was compared to the observed mortality from the bioassay. Mortality data of the same crosses determined at 14 days and 28 days p.i. are given in Table 4.

Strain	Crosses	Progeny genotypes, by hypothesis		Mortality rates, 7 days p.i.			
		(Z)	(A)	Exp (Z)	Exp (A)	Observed with CpGV-M M (SD) [N, n]	Observed with CpGV-S M (SD), [N, n]
CpS		Z^SZ^S, Z^SW	A^SA^S	1.00	1.00	0.97 (0) [7, 278]	0.93 (0) [6, 178]
CpR5M		Z^RZ^R, Z^RW	A^RA^R	0.00	0.00	0.01 (0.02) [4, 157]	0.01 (0) [7, 217]
CpR5S		Z^RZ^R, Z^RW	A^RA^R	0.00	0.00	0.01 (0.02) [6, 233]	0.00 (0) [8, 263]
CpR5M	Female crosses: CpR5Mf X CpSm	Z^RZ^S, Z^SW	A^RA^S	0.50	0.00	0.01 (0.02) [3, 234]	0.01 (0.01) [3, 168]
	BC A: F_1m X CpSf	Z^RZ^S, Z^SZ^S, Z^SW, Z^RW	A^SA^S, A^SA^R	0.50	0.50	0.49 (0.13) [3, 289]	0.39 (0.09) [3,259]
	BC B: F_1m X CpR5Mf	Z^RZ^S, Z^RZ^R, Z^SW, Z^RW	A^RA^R, A^SA^R	0.25	0.00	0.01 (0.02) [2, 201]	0.00 (0) [2, 155]
	Male crosses: CpR5Mm X CpSf	Z^RZ^S, Z^RW	A^RA^S	0.00	0.00	0.02 (0.03) [4, 425]	0.00 (0) [4, 430]
	BC A: F_1m X CpSf	Z^RZ^S, Z^SZ^S, Z^SW, Z^RW	A^SA^S, A^SA^R	0.50	0.50	0.41 (0.09) [3, 233]	0.44 (0.04) [3, 169]
	BC B: F_1m X CpR5Mf	Z^RZ^S, Z^RZ^R, Z^SW, Z^RW	A^RA^R, A^SA^R	0.25	0.00	0.00 (0.01) [2, 343]	0.00 (0) [2, 261]

Strain	Crosses	Progeny genotypes, by hypothesis (Z)	Progeny genotypes, by hypothesis (A)	Mortality rates, 7 days p.i. Exp (Z)	Exp (A)	Observed with CpGV-M M (SD) [N, n]	Observed with CpGV-S M (SD), [N, n]
CpR5S	Female crosses: CpR5Sf X CpSm	Z^RZ^S, Z^SW	A^RA^S	0.50	0.00	0.04 (0.07) [4, 294]	0.05 (0.11) [4, 327]
	BC A: F_1m X CpSf	Z^RZ^S, Z^SZ^S, Z^SW, Z^RW	A^SA^S, A^SA^R	0.50	0.50	0.40 (0.09) [3, 223]	0.43 (0.03) [3, 239]
	BC B: F_1m X CpR5Mf	Z^RZ^S, Z^RZ^R, Z^SW, Z^RW	A^RA^R, A^SA^R	0.25	0.00	0.00 (0) [3, 221]	0.00 (0) [3, 241]
	Male crosses: CpR5Sm X CpSf	Z^RZ^S, Z^RW	A^RA^S	0.00	0.00	0.01 (0.01) [4, 298]	0.04 (0.03) [4, 413]
	BC A: F_1m X CpSf	Z^RZ^S, Z^SZ^S, Z^SW, Z^RW	A^SA^S, A^SA^R	0.50	0.50	0.48 (0.06) [3, 181]	0.50 (0.12) [3, 202]
	BC B: F_1m X CpR5Mf	Z^RZ^S, Z^RZ^R, Z^SW, Z^RW	A^RA^R, A^SA^R	0.25	0.00	0.00 (0) [3, 267]	0.06 (0.02) [3, 233]

The reciprocal male crosses CpR5Mm x CpSf showed mortality rates of 0.02 and 0 on the different CpGV treatments. Mortality rates of the offspring of the F_1m x CpSf backcross (BC A) on CpGV-M were 0.41 and 0.44 on CpGV-S after seven days. In BC B (F_1m x CpR5Mf) a mortality rate of 0 was ascertained for the offspring larvae treated with the two CpGV isolates. Analogous crossing experiments were done with the CM line CpR5S with similar results (Table 3).

When evaluated after seven days, the results of all twelve crossing and backcrossing experiments supported a dominant, autosomal inheritance pattern, whereas six out of twelve crosses and backcrosses contradicted a Z-linked inheritance of resistance.

The mortality response in the resistance tests was further determined after 14 and 28 days. In the hybrid crosses, mortality rates varied between 0.54 and 0.82 depending on the virus isolate and the CM strain. In the BC A, mortality of both strains after 28 days increased to 0.96 on CpGV-M and 0.91 on CpGV-S, whereas in the BC B the mortality rates were 0.47 on CpGV-M and 0.65 on CpGV-S, respectively (Table 4).

As these data suggested an influence of the allele numbers on the survival rate in both CpR5M and CpR5S, the mortality rates of the hybrid crosses (A^RA^S), BC A (A^SA^S, A^SA^R) and BC B (A^RA^R, A^SA^R) were combined and analyzed for statistical differences (Figure 9). At 28 days, the mortality of all larval offspring of the crosses differed significantly. Furthermore, significant differences were assessed

after seven and 14 days for the hybrid crosses and the BC A and within the two BC (ANOVA, post-hoc Tukey HSD test, $P < 0.05$), indicating an increased survival rate with increasing number of resistance alleles in the progenies of different crosses. In contrast, seven days ($P = 0.71$) and 14 days ($P = 0.06$) post infection, BC B differed from the hybrid cross, which can be attributed to the presence of susceptible (A^SA^S) individuals in the progeny (Figure 9). These results indicate an incomplete dominance when the mortality at day 14 and 28 is considered.

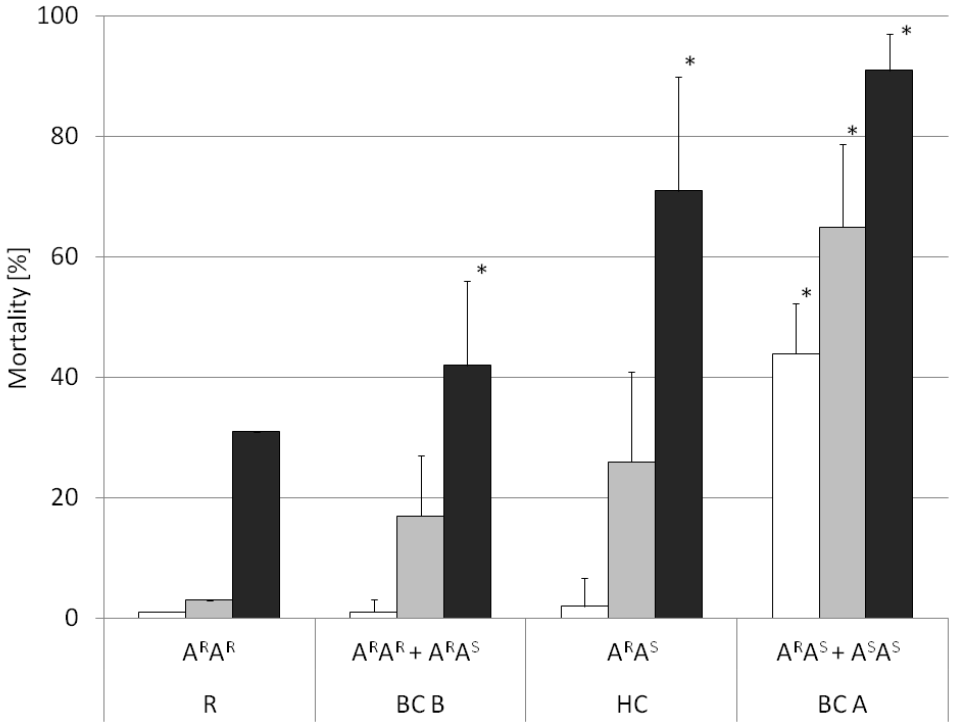

Figure 9 Comparison of the combined mortality data of all hybrid crosses (HC) and backcrosses (BC B and BC A). Combined mortality was determined of neonate larvae of HC, BC B and BC A subjected to artificial diet containing the discriminating concentration of 5.8×10^4 OB/ml of CpGV-M or CpGV-S. The mean Abbott-corrected mortality and standard deviation (error bars) were determined seven days (white bars), 14 days (gray bars) or 28 days (black bars) post infection. The asterisk indicates a statistically significant difference between mortality at the three different time points (ANOVA, post-hoc Tukey HSD test, $P < 0.05$). The result for R (CpR5M) is based on a single test and was excluded from the statistical analysis. The hypothetical progeny genotypes are given at the bottom; A^RA^R stands for homozygous resistant, A^SA^S homozygous sensitive and A^RA^S for heterozygous.

The sex proportion of surviving male pupae after day 28 of the offspring of female hybrid crosses (CpR5Mf x CpS) was 0.45 on both CpGV-M and CpGV-S. Similarly, for the strain CpR5S the fraction of males among surviving pupae subjected to CpGV-M was 0.51, and 0.56 when subjected to CpGV-S. In the male hybrid crosses of CpR5M and CpR5S the sex ratio was also equal. Thus, when considering the survivors after 28 days, both the crosses and backcrosses indicated a similar number of male and female survivors around 50 % and no indication for Z-linkage (Table 4).

The dominance values were calculated for the mortality rates observed at seven days p.i. in the female and male hybrid crosses of CpR5M or CpR5S. The values ranged between 0.94 and 1.01, and were thus very close to 1 (Table 5). Therefore, resistance of CpR5M and CpR5S to CpGV-M or CpGV-S was considered to be fully dominant after seven days.

The mortality of the offspring of BC A and BC B were also analyzed for monogenic inheritance applying the Chi-squared (χ^2) test. Statistically significant differences were observed for each CM resistant strain, BC A and BC B and the two virus treatments at the discriminating concentration after seven days. Six out of 16 backcrosses revealed a significant difference from the monogenic model. In five of these backcrosses, the observed mortality was significantly lower than the expected mortality. The other ten backcrosses were in line with the expected values for a monogenic inheritance model of the type II resistance against CpGV-M and CpGV-S (Table 5).

Table 4 Mortality rates of crosses and backcrosses of CpR5M or CpR5S with CpS determined after 14 and 28 days. Mortality rates of CM larvae determined in bioassays with neonates exposed to CpGV-M or CpGV-S at a discriminating concentration of 5.8×10^4 OB/ml at 14 and 28 days post infection (p.i.). The mortality was determined with offspring of hybrid crosses or backcrosses between CpS and CpR5M or CpR5S. For more details, see legend of Table 3. The observed mortality was corrected following the formula of Abbott. The rate of surviving male pupae was determined by the number of abdominal segments of male and female pupae (Eberle and Jehle 2006).

		Mortality rates, 14 days p.i.		Mortality rates 28 days p.i.			
Strain	**Cross**	**CpGV-M M (SD)**	**CpGV-S M (SD)**	**CpGV-M**	**CpGV-S**	**Mean rate of surviving male on CpGV-M[§]**	**Mean rate of surviving male on CpGV-S[§]**
CpS		1.00 (0)	1.00 (0)	1.00 (0)	1.00 (0)	n.a.	n.a
CpR5M		0.06 (0.04)	0.02 (0.03)	0.36* (n.d.)	0.25* (n.d.)	n.d.	n.d.
CpR5S		0.03 (0.03)	0.02 (0.02)	n.d.	n.d.	n.d.	n.d.
CpR5M	Female crosses: CpR5Mf X CpSm	0.22 (0.26)	0.17 (0.11)	0.74 (0.25)	0.54 (0.28)	0.45	0.45
	BC A: F_1m X CpSf	0.65 (0.25)	0.48 (0.15)	0.96 (0.01)	0.89 (0.05)	0.57	0.56
	BC B: F_1m X CpR5Mf	0.24 (0.07)	0.21 (0.03)	0.46 (0)	0.48 (0.11)	0.49	0.61
	Male crosses: CpR5Mm X CpSf	0.29 (0.06)	0.12 (0.05)	0.79 (0.14)	0.54 (0.14)	0.62	0.51
	BC A: F_1m X CpSf	0.72 (0.03)	0.64 (0.17)	0.94 (0.04)	0.89 (0.08)	0.65	0.54
	BC B: F_1m X CpR5Mf	0.15 (0.04)	0.04 (0.01)	0.47 (0.03)	0.34 (0.07)	0.51	0.59
CpR5S	Female crosses: CpR5Sf X CpSm	0.39 (0.17)	0.27 (0.20)	0.80 (0.09)	0.71 (0.15)	0.51	0.56
	BC A: F_1m X CpSf	0.68 (0.09)	0.59 (0.04)	0.91 (0.06)	0.84 (0.09)	0.60	0.42
	BC B: F_1m X CpR5Mf	0.17 (0.10)	0.20 (0.19)	0.29 (0.09)	0.65 (0.19)	0.49	0.56
	Male crosses: CpR5Sm X CpSf	0.38 (0.14)	0.23 (0.07)	0.82 (0.19)	0.70 (0.20)	0.51	0.48
	BC A: F_1m X CpSf	0.75 (0.11)	0.65 (0.12)	0.96 (0.08)	0.91 (0.02)	0.57	0.50
	BC B: F_1m X CpR5Mf	0.09 (0.08)	0.23 (0.05)	0.29 (0.16)	0.46 (0.10)	0.41	0.44

Table 5 Dominance values and direct test for monogenic inheritance in CpR5M and CpR5S calculated for mortality rates at seven days p.i. Dominance values according to Bourguet (Bourguet et al. 2000) were calculated with the mortality rates observed in the female or male hybrid crosses of the CM strains CpR5M or CpR5S subjected to CpGV-M and CpGV-S, respectively. A direct test of monogenic inheritance for CpGV-M or CpGV-S resistance (Tabashnik 1991) was conducted by comparing expected and observed mortality of the backcrosses subjected to CpGV-M and CpGV-S, respectively. Asterisks indicate significant differences (df = 1; $^{*}P$ value < 0.05).

		Dominance value		**Direct test for monogenic inheritance**			
Strain	**Crosses**	**CpGV-M**	**CpGV-S**	**CpGV-M**		**CpGV-S**	
				X^2	*P* value	X^2	*P* value
	Female crosses: CpR5Mf X CpSm	1.00	1.00				
	BC A: F_1m X CpSf			0	1.00	6.65	0.01*
CpR5M	BC B: F_1m X CpR5Mf			0	1.00	1.57	0.21
	Male crosses: CpR5Mm X CpSf	0.99	1.01				
	BC A: F_1m X CpSf			6.73	0.01*	0.42	0.51
	BC B: F_1m X CpR5Mf			1.72	0.19	1.31	0.25
	Female crosses: CpR5Sf X CpSm	0.97	0.95				
	BC A: F_1m X CpSf			9.84	0.01*	3.44	0.06
	BC B: F_1m X CpR5Mf			5.67	0.02*	6.18	0.01*
CpR5S							
	Male crosses: CpR5Sm X CpSf	1.00	0.96				
	BC A: F_1m X CpSf			0.07	0.79	0.10	0.21
	BC B: F_1m X CpR5Mf			2.70	0.10	19.02	<0.01*

BAC-FISH mapping of the Z chromosome of CM strains CpS, CpRR1 and CpR5M: After the crossing experiments of CpR5M and CpR5S had demonstrated an autosomal inheritance of type II resistance, which differed from the Z-chromosoal inheritance of CpRR1 (type I), we examined the possibility that a polymorphic chromosomal rearrangement could explain the difference. As previously mentioned, BAC-FISH studies have shown that the large CM Z chromosome consists of a fusion between a smaller Z chromosome and an autosome of an ancestral lepidopteran. If the type I resistance gene was located on the ancestral autosome portion, and if fission of the large Z chromosome had occurred in CM populations with type II resistance, then the same resistance gene could be responsible, but appear to be sex-linked in type I populations and autosomal in type II populations. To examine whether such a fission had occurred in type II populations the Z chromosomes of CpRR1 and CpR5M were mapped using BAC-FISH technique for 13 marker genes located on the Z chromosome of susceptible CM (CpS-Krym strain). The relative positions of hybridization signals of BAC probes on the Z chromosome were measured in at least seven separate and properly spread pachytene nuclei on each slide (Table 6). In CpS-Krym, CpRR1 and CpR5M all 13 BAC probes, each containing a different marker gene, effectively hybridized to the Z chromosome in pachytene nuclei. The vast majority of analyzed images showed synteny of all marker genes (i.e. location to the same chromosome) and a conserved gene order along the Z chromosome (Figure 10 and Figure 11), except minor local translocations observed for *ABCC2* and *per* in CpRR1 but with statistically inconclusive relative distances of the genes (Table 7; Figure 11).

Some statistically significant differences of the mean relative distances were detected for *ap* and *ABCF2* genes in CpR5M compared to CpS-Krym and CpRR1; significant differences between CpRR1 and CpR5M were also found for *ABCC2*, *per* and *nan* (ANOVA, Scheffé test, $P < 0.05$) (Table 7; Figure 11). In conclusion, BAC-FISH mapping ruled out fission of the large Z chromosome in the CpR5M strain, which could have rendered the location of the resistance allele(s) to CpGV-M and CpGV-S autosomal.

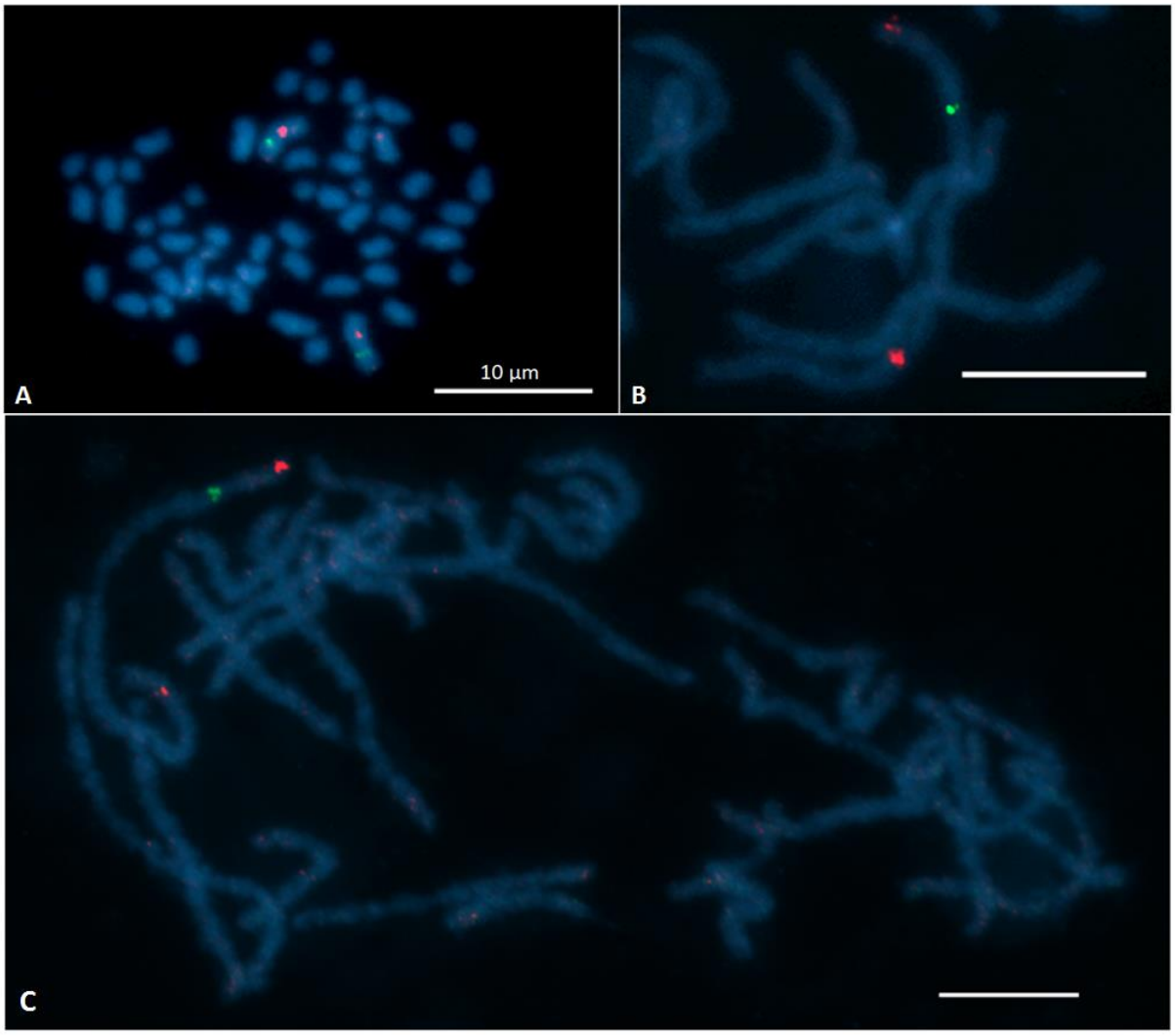

Figure 10 Examples of BAC-FISH mapping of Z-linked genes in the *Cydia pomonella* Z chromosome. Cy3-dUtP-labeled (red hybridization signals) and Fluorescein-12-dUTP-labeled (green) BAC probes were used for BAC-FISH mapping in spread chromosome preparation from testes, counterstained with DAPI (blue). (A) CpS mitotic spermatogonial metaphase showing 2n = 56 chromosomes; two Z chromosomes marked with hybridization signals of BAC probes containing *per* (green) and *nan* (red) genes, respectively. (B) Detailed view of pachytene ZZ bivalent of CpRR1 with hybridized *ap* (terminal red), *Tpi* (green) and *Idh-2* (interstitial red) probes. (C) CpR5M pachytene complement showing a long ZZ bivalent with hybridized with *ap* (terminal red), *ABCF2* (green) and *Notch* (interstitial red) probes.

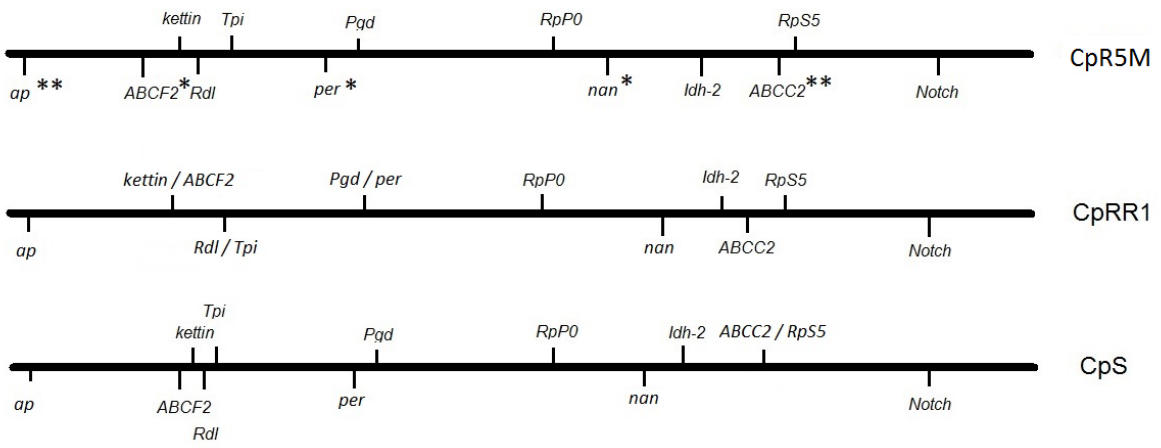

Figure 11 Gene-based scheme of the Z chromosome of the codling moth strains CpR5M, CpRR1 and CpS, integrating all BAC-FISH mapping results. The relative positions of 13 marker gene loci were generated by measuring the physical distance between hybridization signals and the *ap*-labeled chromosome end of at least seven ZZ bivalents of each strain. The measured distances were normalized to the total length of the ZZ bivalent. Genes of CpR5M labeled with one asterisk showed a statistically significant difference in their mean position of the normalized gene loci to CpRR1; genes with two asterisks are significantly different to both CpRR1 and CpS (ANOVA, Scheffé test, $P = 0.05$).

Table 6 Means of the relative position of 13 marker genes located on the Z chromosome of codling moth strains CpS, CpRR1 and CpR5M. The relative positions of the marker gene loci were generated by measuring the physical distance between hybridization signals and the *ap*-labelled chromosome end of ZZ bivalents in the strains. The measured distances were normalized to the total length of the ZZ bivalent; N stands for total number of ZZ bivalents that were measured and SD for standard deviation. (Continued see next page)

Marker gene	CpS			CpRR1			CpR5M		
	N	mean	±SD	N	mean	±SD	N	mean	±SD
ABCC2	9	0.731	0.023	13	0.722	0.043	15	0.754	0.024
ABCF2	11	0.163	0.039	9	0.162	0.010	15	0.131	0.014
ap	68	0.018	0.005	67	0.019	0.005	75	0.015	0.004
Idh-2	7	0.656	0.093	12	0.697	0.027	10	0.679	0.055
kettin	12	0.176	0.021	9	0.160	0.034	12	0.168	0.017
nan	17	0.618	0.032	11	0.639	0.024	11	0.587	0.040
notch	11	0.898	0.037	9	0.901	0.030	15	0.911	0.014
per	17	0.334	0.034	11	0.353	0.026	11	0.310	0.029

Marker gene	CpS			CpRR1			CpR5M		
	N	mean	±SD	N	mean	±SD	N	mean	±SD
Pgd	12	0.356	0.031	13	0.345	0.045	12	0.343	0.048
Rdl	9	0.188	0.013	13	0.205	0.289	15	0.186	0.019
PpP0	12	0.529	0.050	13	0.521	0.058	12	0.533	0.064
RpS5	12	0.740	0.040	9	0.760	0.033	12	0.770	0.031
Tpi	7	0.200	0.038	12	0.216	0.025	10	0.219	0.032

Table 7 Overview of BAC-FISH probes and summary of results of statistical analyses of 13 marker genes located on the Z chromosome of different codling moth (CM) strains. The relative position of hybridization signals to the total length of the Z chromosome in at least seven ZZ bivalents was calculated in each CM strain. Different letters indicate statistical differences in the mean relative position of gene loci (following ANOVA, Scheffé test, $P = 0.05$).

		CM strain		
Gene name	**Symbol**	**CpS**	**CpRR1**	**CpR5M**
ABC transporter family C protein ABCC2	*ABCC2*	AB	A	B
ABC transporter family F protein ABCF2	*ABCF2*	A	A	B
apterous	*ap*	A	A	B
Isocitrate dehydrogenase 2	*Idh-2*	A	A	A
kettin	*kettin*	A	A	A
nanchung	*nan*	AB	A	B
Notch	*Notch*	A	A	A
period	*per*	AB	A	B
Phosphogluconate dehydrogenase	*Pgd*	A	A	A
Resistant to dieldrin	*Rdl*	A	A	A
Ribosomal protein P0	*RpP0*	A	A	A
Ribosomal protein 55	*RpS5*	A	A	A
Triosephosphate isomerase	*Tpi*	A	A	A

2.4 Discussion

Resistance to CpGV isolates in CM field populations has been reported several times (Zichová et al. 2013; Schmitt et al. 2013; Asser-Kaiser et al. 2007; Fritsch et al. 2006). One of the main factors for a successful resistance management is knowledge of the inheritance pattern of the resistance. For NRW-WE, an atypical resistance to CpGV isolates has recently been described with a hint for a second type of resistance that differs from the previously reported Z-linked and *pe38*-related type I resistance in CpRR1 (Asser-Kaiser et al. 2007; Gebhardt et al. 2014; Jehle et al. 2017).

Resistant field populations of insects normally consist of a mixture of homozygous and heterozygous, resistant and susceptible individuals. Two selection methods are typically used for establishing laboratory insect strains with a genetically fixed resistance. One option is continuous inbreeding by single pair crosses followed by resistance testing of the offspring and selecting families with a uniform resistance response (Asser-Kaiser et al. 2007). The other option is mass crosses and selection under virus pressure followed by rearing of the survivors of the treatment (Berling et al. 2009; Zichová et al. 2013). The method of single pair crosses was attempted several times for NRW-WE, however without success. Two main aspects were considered to be a reason for this failure: (i) the number of progeny was too low, especially for the second round of inbreeding, which did not give enough individuals to be tested on CpGV-M, CpGV-S and for the untreated control which is used for the next round of selection, and (ii) the families did not show an uniform response from one generation to the next generation. These two aspects made it impossible to select individual resistant families. Eventually, the establishment of genetically homogeneous strains from the NRW-WE field population was achieved by five rounds of mass crosses each followed by selection under CpGV virus pressure. Though the number of surviving individuals increased during the subsequent selections from F_1 to F_4, the sex ratio of the surviving moths was balanced in each generation, pointing to inheritance of resistance that is independent from of the Z chromosome.

According to Jehle et al. (2017) mortality of the ancestral NRW-WE field strain was 6.7 % on CpGV-M and 8.9 % on CpGV-S after seven days and 45.6 % and 26.8 %, after 14 days, when exposed to the discriminating concentration of 5.8×10^4 OB/ml. For CpR5M and CpR5S, mortality decreased to <6 % on any of the virus treatments, indicating that the resistance in the field population NRW-WE was indeed not genetically fixed. Genetic fixation of resistance was apparently only achieved in CpR5M and CpR5S after the selection procedure.

Hybrid crosses and backcrosses between the resistant CM strains CpR5M and CpR5S with the susceptible CpS in combination with testing the resistance levels of the offspring revealed the mode of inheritance of the resistance. Four findings are of importance:

(i) The observed mortality in all crossing and backcrossing experiments fit perfectly to a dominant, autosomal inheritance of resistance to both viruses, CpGV-M and CpGV-S, in both CpR5M and CpR5S strains. These findings explained the experimental indications against a Z-linked inheritance in NRW-WE reported previously (Jehle et al. 2017). In addition, when the sex of surviving pupae in the hybrid female crosses was determined, a more or less balanced 1:1 ratio of male to female survivors was noticed. This observation is also consistent with an autosomal but not with a Z-lined inheritance. Assuming that resistant females carrying either Z^RW or A^RA^R were mated with susceptible males (Z^SZ^S or A^SA^S), then the F_1 offspring would be either Z^RZ^S for males and Z^SW for females or A^RA^S for both males and females. In this case Z-linkage would eliminate females among the survivors, which was not observed.

As shown by BAC-FISH experiments, the autosomal location of the resistance allele in CpR5M (and analogously in CpR5S) cannot be explained by large scale chromosome rearrangements such as fission of the Z chromosome or translocation of a Z-chromosomal segment to an autosome. The architecture of Z chromosomes in the susceptible strain Cp-Krym, CpRR1 (type I resistance) and CpR5M (type II resistance) was virtually the same; all marker genes showed synteny to a single chromosome in all three CM strains. Some minor variations in the distance of marker genes to each other might be due to various degree of chromatin condensation, artifacts of chromosome spreading technique or measurement error (Yoshido et al. 2005). Furthermore, we cannot rule out that distances between gene markers were altered by fine scale intrachromosomal rearrangements such as inversions which were shown to be common in Lepidoptera (d'Alencon et al. 2010) despite the otherwise conserved organization of lepidopteran genomes (Van't Hof et al. 2013; Ahola et al. 2014). Yet none of these would explain why resistance in CpRR1 is Z-linked, whereas it is autosomal in CpR5M.

(ii) Statistical testing supported a dominant mode of inheritance. This finding is similar to type I resistance of strain CpRR1 and its ancestor population CpR (Eberle and Jehle 2006; Asser-Kaiser et al. 2007). A dominant inheritance pattern was also described in the French population RGV (Berling et al. 2009) and the CM strain CpR-CZ from the Czech Republic (Zichová et al. 2013). When the larval mortality until 28 days is considered, a strong correlation with the number of resistance alleles is apparent and only about 30 % of the heterozygous offspring survive exposure to the discriminating concentration of CpGV-M and CpGV-S.

(iii) The majority of the backcrossing results supported a monogenic mode of inheritance. Nevertheless, in six backcrosses, independent of the CM strain or the BC procedure, monogenic inheritance was rejected in favor of an alternative hypothesis. The latter result would imply that

more than one gene is involved in type II resistance of CM. For a more comprehensive picture of the number of alleles involved in the inheritance of type II resistance, a comparison of concentration-mortality response over a range of different virus concentrations needs to be assessed. Nevertheless, the assumption of a monogenic inheritance model is conceivable according to the single concentration responses detected in this study.

(iv) Selection of NRW-WE either on CpGV-M (genome group A) or CpGV-S (genome group E) resulted in two strains, CpR5M and CpR5S, which were both cross-resistant to CpGV-M and CpGV-S. This observation would argue in the first instance for a functional cross-resistance mechanism against both CpGV-M and CpGV-S that must be different from the mechanism of type I resistance. As shown for CpRR1, type I resistance is targeted against a 24 bp repeat in *pe38* of genome group A CpGVs, such as CpGV-M, but not in other CpGVs (Gebhardt et al. 2014). Since CpGV-S also lacks this repeat in *pe38*, this locus cannot be the major target of type II resistance (Gebhardt et al. 2014; Jehle et al. 2017). Therefore, a novel resistance mechanism must be involved in type II resistance, which would also explain its autosomal inheritance. Though our experiments provide clear evidence for a dominant and autosomal cross- resistance to CpGV-M and CpGV-S in CpR5M and CpR5S, they do not fully explain the results of hybrid crosses obtained with the parental CM strain NRW-WE (Jehle et al. 2017). There, single pair hybrid crosses between individuals of NRW-WE and susceptible CpS resulted in a highly heterogeneous response of different hybrid families, with highly variable mortality rates in different families, not supporting a simple model of cross-resistance to CpGV-M and CpGV-S. Hence, additional factors or phenotypic plasticity may be involved in the resistance observed in the field populations, which may become lost during the inbreeding and selection procedure to generate genetically homogenous strains.

Another important factor is the temporal expression of resistance and the time point of observation. Since virus resistance is generally measured in mortality rates at certain time points after infection, it may make a difference in data interpretation, whether the virus-caused mortality is scored at the larval or pupal stage. As shown for type I resistance in CpRR1, Z^RW females and homozygous Z^RZ^R males were less susceptible and survived longer than heterozygous Z^RZ^S males (Asser-Kaiser et al. 2007). In this case, surviving heterozygous male larvae did not pupate when exposed to high virus concentrations; hence, resistance measured as larval mortality appeared to be dominant, whereas resistance measured as the probability of successful pupation appeared recessive at high virus concentrations. A similar situation seems to exist in CpR5M and CpR5S, where mortality also increased in heterozygous offspring of hybrid crosses when virus exposure was extended to 14 and 28 days (pupation), respectively. The offspring of the BC A theoretically will consist of a mixture of

heterozygous and homozygous susceptible individuals (A^RA^S and A^SA^S). The hybrid crosses will result in offspring which carry A^RA^S and are therefore heterozygous. In contrast, BC B consists of heterozygous and homozygous resistant individuals (A^RA^S and A^RA^R). The indicated mortalities of BC A hybrid crosses and BC B were significantly different, at least after 28 days. Therefore, a dose effect in terms of numbers of resistance alleles as well as in the response to a certain virus concentration seems to exist in both type I and type II resistance.

The discovery of type II resistance with a mechanism and inheritance that are different to that of type I resistance underlines the high capacity of adaptation of CM to CpGV isolates, posing a continuous threat to the successful use of CpGV products in organic and integrated pome fruit production. Therefore, further optimization of resistance management strategies of CM towards the sustainable use of the genetic diversity of CpGV is an imperative for research and extension services.

3. Cross-resistance of coding moth against different isolates of Cydia pomonella granulovirus is caused by two different but genetically linked resistance mechanisms

Abstract

Different types of field resistance of codling moth (CM) larvae against the baculovirus Cydia pomonella granulovirus (CpGV) have been observed and pose a severe threat on the successful control of CM in organic and integrated pome fruit production. Type I resistance is systemic and targeted against the viral *pe38* gene of isolate CpGV-M and is dominant Z-linked inherited. It can be broken by CpGV-S and other CpGV isolates, lacking a 24-bp repeat in their *pe38*. Here, we report that cross-resistance to CpGV-M and CpGV-S of type II resistance in CM, which is dominant autosomally inherited, cannot be broken by mixtures of CpGV-M and CpGV-S but by a recombinant of CpGV-M containing the *pe38* gene of CpGV-S. This finding indicates that beyond the known *pe38* related mechanism of type I resistance against CpGV-M, a second mechanism must exist, which can be broken by a CpGV-M related genomic factor, although only in *cis* but not in *trans* function. Budded virus injection experiments circumventing initial midgut infection provided further evidence that resistance against CpGV-S is indeed midgut related, though fluorescence-dequenching assay using rhodamin-18 labeled occlusion derived viruses (ODV) could not fully elucidate whether receptor binding or an intercellular midgut factor is involved in type II resistance. We conclude, that two different but genetically linked resistance mechanisms exist in CM with type II resistance: resistance against CpGV-M is systemic and targeted against the *pe38* gene, similar to type I resistance, and a second resistance mechanism targeted against CpGV-S with an unidentified midgut factor that interferes with virus entry or BV replication at a very early time of infection.

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3.1 Introduction

Baculoviruses form a large group of dsDNA viruses, which are specific for the larval stages of insects of the orders Lepidoptera, Hymenoptera and Diptera (Herniou et al. 2003). Because of their narrow host range and high virulence to early insect instars they are widely used as commercial biocontrol agents replacing the use of environmentally detrimental chemical pesticides (Moscardi et al. 2011; Lacey et al. 2015). Being registered in virtually all pome fruit growing countries worldwide, the Cydia pomonella granulovirus (CpGV) (type species of the genus *Betabaculovirus*) is the most widely applied and supremely successful bio-control agent in integrated and organic apple, pear and walnut production. CpGV was first discovered in Mexico but further geographic isolates have been found in the Caucasian area, Europe and North America (Tanada 1964; Harvey and Volkman 1983; Crook et al. 1985; Rezapanah et al. 2008). The genome of CpGV is 120.8-124.3 kbp in size and encodes between 137-142 open reading frames (ORFs) depending on the isolate (Luque et al. 2001; Gebhardt et al. 2014). Phylogenetically, CpGV isolates can be classified into five genome groups A-E (Eberle et al. 2009; Gebhardt et al. 2014). The CpGV infection pathway in the insect larva is initiated by an oral uptake of viral occlusion bodies (OB), containing a single viral nucleocapsid. After OB have dissolved in the alkaline environment of the larval midgut, the thereby released occlusion-derived viruses (ODV) initiate infection of midgut epithelial cells (Federici 1997). After the initial infection of the midgut, a second phenotype, termed budded virus (BV) is produced and released through the basal midgut membrane. The BV is responsible for the systemic spread of the viral infection to other larval tissues via trachea and the haemolymph system of the host insect (Engelhard et al. 1994; Flipsen et al. 1995). At the end of the infection process, new virus OB are produced and eventually released from the larval cadavers to the environment to infect other caterpillars (Federici 1997).

The Mexican isolate CpGV-M has been used for several decades in commercial CpGV products in Europe and most other countries worldwide (Huber 1998; Lacey et al. 2008) until first cases of field resistance of CM against CpGV-M were discovered in 2005 (Fritsch et al. 2005; Sauphanor et al. 2006). More than 40 apple plantations with CM populations resistant to CpGV-M have been identified since then in Austria, the Czech Republic, Germany, France, Italy, the Netherlands and Switzerland (Berling et al. 2009; Zichova et al. 2011; Schmitt et al. 2013). Because most of these resistant CM populations could be successfully controlled by newly registered resistance-breaking CpGV isolates it was assumed that resistance to CpGV follows a wildly spread, common mechanism, called type I resistance (Jehle et al. 2017). It is assumed that type I resistance is inherited by a single, dominant allele, located on the Z chromosome (Asser-Kaiser 2009; Berling et al. 2009; Zichová et al. 2013). Type I resistance is targeted only against CpGV-M (genome group A), whereas other CpGV isolates from genome group B-E were still virulent in resistant CM populations (Jehle et al. 2006b; Kienzle et al. 2008; Eberle et al. 2008; Berling et al. 2009; Zichová et al. 2013; Graillot et al. 2014;

Graillot et al. 2016). The viral gene *pe38* of CpGV-M was proposed as the main target of type I resistance in the laboratory-selected CM strain CpRR1, because replacement of this gene in CpGV-M by a *pe38* gene from the resistant-breaking isolate CpGV-S enabled the recombinant CpGV-M also to overcome resistance (Gebhardt et al. 2014). Type I resistance is further characterized by a systemic and early block of CpGV replication, which occurs in all larval instars (Eberle et al. 2008; Asser-Kaiser et al. 2010).

Recently, a further type of CpGV resistance was detected in an apple plantation in Germany. This so-called type II resistance is also dominant but inherited autosomally (Jehle et al. 2017)(Chapter 2) and appears to be targeted against CpGV isolates from genome groups A, C, D and E. Only isolate CpGV-E2 (genome group B) was able to overcome this resistance (Jehle et al. 2017). Selection of this field population for five generations on either CpGV-M (genome group A) or CpGV-S (genome group E) rendered two CM strains, namely CpR5M and CpR5S, which were cross-resistant to both genome groups, suggesting in first instance a different resistance mechanism as found for type I resistance (Chapter 2). Notably, a further CM field population combining characteristics of type I and type II resistance was also identified (Chapter 4).

The occurrence of insect populations with different degrees of baculovirus resistance, as found for CM field populations, is unprecedented and poses a severe threat to the successful and sustainable application of baculoviruses in biological insect control.

To elucidate the apparently novel resistance mechanism in CM larvae with type II resistance, we conducted *in vivo* experiments using different isolates and recombinants of CpGV. Surprisingly, our experiments revealed clear evidence for two independent but genetically linked resistance mechanisms, one was still targeted against *pe38*, as previously found for the systemic type I resistance, but its underlying genetic factor is apparently translocated from the Z chromosome to an autosome; the second mechanism was located in the midgut of caterpillars. The results obtained argue not only for genetic link of both resistances but also for a mobility of the genetic factor of resistance I between the Z chromosome and an autosome.

3.2 Material and Methods

Wild-type and recombinant viruses: Different wild-type isolates and recombinants of Cydia pomonella granulovirus (CpGV) were used; isolate CpGV-M (genome group A) (Tanada 1964), isolate CpGV-S (group E) and isolate CpGV-E2 (group B) (Gebhardt et al. 2014). In addition, two different CpGV recombinants based on the CpGV bacmid (bacCpGV$^{hsp\text{-}eGFP}$) of the *in-vivo* cloned genotype CpGV-M1 (Hilton et al. 2008) with modifications in the *pe38* locus were applied; (1) recovery construct containing the homologous *pe38M* from the CpGV-M (bacCpGVΔpe38$_M^{pe38M::eGFP}$ = pe38M) and (2) heterologous *pe38* from the CpGV-S (bacCpGVΔpe38$_M^{pe38S::eGFP}$ = pe38S) (Gebhardt et al.

2014). Occlusion bodies (OB) of the two modified bacmids pe38M and pe38S were produced in cell culture by transfection of *C. pomonella* cell line Cp14R (Winstanley and Crook 1993) followed by OB production in CM larvae (Smith and Crook 1988; Jehle et al. 1992; Gebhardt et al. 2014).
All OB were stored at -20°C until usage. Quantification of virus stocks was performed by OB counting with a light microscope (Leica DMRBE) in dark-field optics with the Petroff-Hauser counting chamber (depth 0.02 mm).

Insects: The codling moth (CM) strain CpS originated from a colony fully susceptible to CpGV treatments (Asser-Kaiser et al. 2007). The two CM strains CpR5M and CpR5S originated from the field population NRW-WE, which was selected on either CpGV-M or CpGV-S for five generations; CpR5M and CpR5S show cross-resistance against both CpGV-M and CpGV-S (Chapter 2). The different CM strains were reared in the laboratory at 26 °C with 16/8 h light/dark photoperiod and 60 % relative humidity; larvae were kept on semi-artificial, modified diet of Ivaldi-Sender (Ivaldi-Sender 1974).

Resistance testing: Neonates of CpS or CpR5M were tested for resistance with different virus CpGV isolates and recombinants as described before (Undorf-Spahn et al. 2012). The applied discriminating concentration of 5.8×10^4 OB/ml diet caused >95 % mortality in CpS neonates (L1) in bioassays after seven days (Asser-Kaiser et al. 2007). Mortality of larvae was determined one, seven and 14 days post infection; only larvae surviving day one past inoculation were introduced to the test. For the resistance testing with different CpGV isolates, or bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M) or bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S), purified OB with the discriminating concentration were incorporated into the diet. For co-infection tests, CM larvae were exposed to different ratios of CpGV-M:CpGV-S (90:10, 50:50 and 10:90) at a final concentration of 5.8×10^4 OB/ml diet. Mortality was corrected with control mortality as described by Abbott (1925). For each assay a minimum of 30 larvae were used and independently repeated at least three times with CpR5M and one to four times with CpS.

Instar-specific assays: First to fifth instars (L1 to L5) were exposed to virus concentration of 2.0×10^5 OB/ml of CpGV-M, CpGV-S and bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S) incorporated into diet. This concentration caused >95 % mortality in all larval stages of CpS after seven days (Eberle et al. 2008). For each assay about 20-35 larvae of CpR5M, CpR5S or CpS were used and the virus-induced larval mortality of three independent replicates was assessed daily for 14 days; all mortality data were corrected with control mortality as described by Abbott (1925).

Budded virus production: Budded virus (BV) of CpGV isolates was produced as described by Asser-Kaiser et al. (2010), with some modifications. Fourth instar (L4) larvae of the susceptible strain CpS were orally infected with 1 x 10^4 OB of CpGV-M or CpGV-S, which were added to a small piece of diet. Larvae that ingested the piece of diet within 12 h were transferred to virus free diet for three days. Hemolymph was collected by cutting off the second proleg of anesthetized larvae and pooled in IZD04 cell culture medium (Winstanley and Crook 1993) containing a small crystal of N-Phenylthiourea (Sigma-Aldrich, St. Louis, USA). Hemolymph of uninfected L4 larvae of CpS was included as a negative control. After centrifugation at 1,000 x *g* at 4 °C for 5 min, the supernatant containing the BV was stored at 4 °C for a maximum of one month. BV concentration of CpGV-M and CpGV-S was estimated by quantitative PCR (qPCR).

Quantitative PCR: BV concentration was determined by qPCR, using an internal OB standard of three-fold dilutions between 7.5 x 10^4 and 7.5 x 10^8 OB/ml (Wennmann et al. 2015). OB standard suspensions were dissolved in 100 mM Na_2CO_3 at 37 °C for 30 min. 100 µl of each, OB standard suspensions and BV hemolymph were purified by Ron´s Tissue DNA Mini Kit® (Bioron GmbH, Ludwighafen am Rhein, Germany). DNA was eluted in 100 µl elution buffer (EB) provided with the kit and used as template for qPCR. The qPCR reaction was performed according to the protocol of Maxima SYBR Green qPCR® (Thermo Scientific, Waltham, USA). Briefly, for a single qPCR sample 2 µl of standard or sample DNA template was mixed with 1 µl 0.2 pM of each of the granulin gene specific oligonucleotides 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‘) (Asser-Kaiser et al. 2010), 12.5 µl 1 x Maxima SYBR Green qPCR® MasterMix and 8.5 µl bidest. H_2O to a total reaction volume of 25 µl. Negative control contained 2 µl of bidest. H_2O instead of DNA template. All qPCR reactions were started with a denaturation step of 5 min at 95 °C, followed by 44 cycles of denaturation (95 °C for 30 sec), primer annealing (60 °C for 30 sec) and elongation (72 °C for 30 sec) and a final elongation step (72 °C for 7 min). Melting curve analysis was performed from 50 °C to 95 °C with an increment of 0.5 °C each 10 sec. The amount of PCR product copies in the BV samples were calculated and extrapolated on the basis of the OB derived DNA standard with the Bio-Rad CFX Manager (3.1) software according to the theory 1 CpGV OB = 1 CpGV-BV.

Intra-hemocoelic BV injections: Five microliters of diluted BV suspension containing 1 x 10^6 OB/ml of either CpGV-M or CpGV-S were injected into the hemocoel of anesthetized L4 larvae of CpS or CpR5M using a Hamilton syringe. Hemolymph of uninfected CpS larvae was injected as control. After recovering from the injection, larvae were transferred to virus free diet and virus-induced mortality was recorded 14 days post injection. Larvae, which died within five days because of injection

treatment and without virus symptoms, were excluded from the experiment. Each variant consisted of 10-15 L4 larvae and at least three independent replicates were performed.

Occlusion-derived virus production and labeling with R-18: Occlusion-derived virus (ODV) of CpGV-M and CpGV-S were prepared according to Haas-Stapleton et al. (2004)and Iwata et al. (2017) with some modifications. 500 µl of 1 x 10^{10} OB/ml of either CpGV-M or CpGV-S suspension were centrifuged at 20,800 x *g* for 10 min. The pellet containing the OB was resuspended and incubated in 450 µl DAS Buffer (alkaline saline, 100 mM Na_2CO_3, 100 mM NaCl, pH 11.5) for 30 min at 37 °C to release the ODV from OB. The ODV suspension was neutralized by adding 100 µl 1 M Tris-HCl (pH 6.5) and incubated at room temperature for 20 min. After another centrifugation step at 2,060 x *g* for 10 min, a droplet of the supernatant and of the pellet, respectively, was used to visualize the ODV production by transmission electron microscopy as a control (see below). The ODV concentration in the supernatant was estimated by using the BCA Protein Assay® (Thermo Fisher Scientific, Waltham, USA) and immediately used for labeling. For binding and fusion assays, the ODV were labeled with the self-quenching fluorescent probe octadecyl rhodamine B chloride (R-18) (Thermo Fisher Scientific, Waltham, USA) as described in Nussbauer and Loyter (1987). Labeled ODV were kept at 4 °C in the dark for a maximum of one month until using.

Transmission electron microscopy with negative staining: For a visual control of ODV preparation, transmission electron microscopy with negative staining was conducted as described elsewhere (Adams and Bonami 1991; Lacey 2012). Briefly, a droplet of both the supernatant and the resuspended pellet was placed on a formvar-coated grid and incubated for approximately 1 min. Then, excess of applied droplet was carefully removed by carefully soaking it with a filter paper. Then a drop of 2 % (w/v) aqueous phosphotungstic acid (PTA, pH 7.5) was added for 1 min and the grid was allowed to dry prior examination with a transmission electron microscope (Zeiss TEM 902, Oberkochen, Germany).

ODV and fluorescence-dequenching assay: For the fluorescence-dequenching assays (for details see Haas-Stapleton et al. 2005 and Iwata et al. 2017), CpS and CpR5M larvae were reared on virus-free diet until they reached L4. Then, larvae were starved over-night and then orally inoculated with small pieces of diet supplied with 2 µl of labeled ODV at a concentration of 2.4 µg ODV/larvae or with water as a negative control. After the larvae had ingested the piece of diet, they were divided into two cohorts. Because no differences were assessed in binding and fusion efficacy between 30 min and 120 min post infection in ODV assays before, a time interval of 1 h post infection was chosen before midgut was dissected. One cohort of larvae was anesthetized and used for dissection of the

midgut for the fluorescence-dequenching assay. Midgut epithelium of each larva was separated from the basal lamina as described previously Haas-Stapleton et al. (2005) and incubated in 50 µl separation buffer (100 mM KCl, 100 mM EGTA, 100 mM Na_2CO_3, pH 9.5). Determination of ODV binding was done immediately or samples were kept at -70 °C in the dark until measurement of binding and fusion as described below. At least six larvae were used for each replicate and five to six replicates were realized for the fluorescence-dequenching assay.

To confirm the infectivity of the ODV preparation in CpS and CpR5M larvae, the second cohort of infected larvae was transferred to virus-free diet and incubated at 26 °C with 16/8 h light/dark photoperiod and 60 % relative humidity. Dead larvae were recorded seven days post infection and at least 10 individuals were used for each replicates. Five to six independent replicates were done for the ODV bioassay.

Determination of ODV binding and fusion by fluorescence-dequenching assay: Collected midgut epithelial cells suspended in 50 µl of separation buffer (100 mM NaCl, 100 mM EDTA, 100 mM Na_2CO_3, pH 9.5) were transferred to a 8-tube strip (BioRad, Hercules, USA), and fluorescence was measured for relative fluorescence units (RFUs) for 10 sec at 22 °C and 560 nm (excitation) and 610 nm (emission) using a CFX96 Touch™ Real-Time PCR Detection System (Biorad). To quantify the total amount of labeled ODV, Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) with the final concentration of 1 % was added to the samples and incubated over night at 4 °C in the dark to allow solubilization of R-18. To determine ODV fusion, RFUs was measured again as described. Fifty µl of labeled ODV of CpGV-M or CpGV-S were measured as a positive control to calculate the total amount of ODV (for details see Iwata et al. 2017). Measured RFUs were corrected for background fluorescence associated with the midgut epithelial cells from control larvae fed with water. Four to ten larvae were examined for each replicate and four to five replicates were performed.

Statistical analysis: Statistical analyses were done with ANOVA Scheffé test of the Agricolae Package of RStudio (RStudio edition 2.3.4.4.). Box-plots analyses were done using RStudio.

3.3 Results

Mortality of CpS and CpR5M larvae on different CpGV isolates and recombinants: Neonates of the susceptible CpS strain were exposed to a discriminating concentration of 5.8 x 10^4 OB/ml of either, CpGV-M, CpGV-S, CpGV-E2, recombinants or co-infection of CpGV-M and CpGV-S. Minimum mortality was 73.5 % after seven days and 84.5 % after 14 days and most of the virus combinations caused mortality to a maximum of 100 % after 14 days (Figure 12).

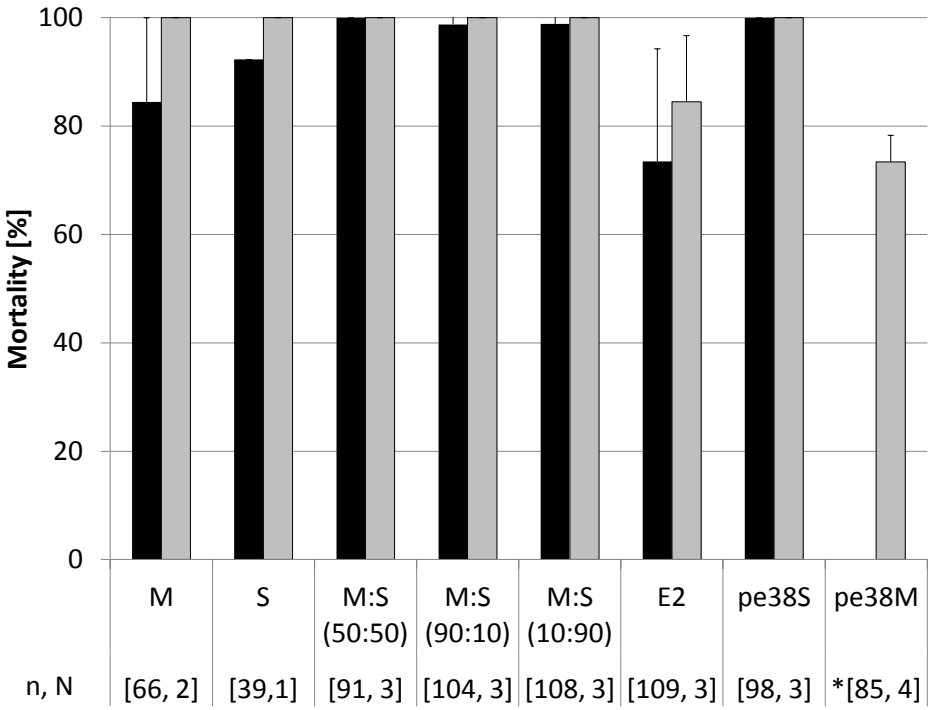


Figure 12 Resistance testing in CpS larvae with different CpGV isolates and recombinants. Mortality of CpS neonates tested for resistance on artificial diet containing different CpGV isolates, recombinants and CpGV mixtures, all at a final concentration of 5.8 x 10^4 OB/ml. CpGV-M (M), CpGV-S (S), CpGV-E2 (E2), bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M), bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38S) or co-infections with compositions of 50:50, 90:10 or 10:90 of the different isolates were applied. Abbott-corrected mean mortality and standard deviations (error bars) were determined seven (black bars) and 14 days (gray bars) post infection. Total number of tested individuals (n) and number of independent replicates (N) are indicated below the charts.

* previously reported by Gebhardt et al. (2014) where CpS L3 larvae were fed with 1 x 10^3 OB per larvae of the recombinant bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M).

Mean mortality of neonates of the CM strain CpR5M subjected to CpGV-M or CpGV-S varied between 3.4 % and 2.0 % after seven days and did not exceed 11.5 % after 14 days (Figure 13). Co-infections with both CpGV-M and CpGV-S at different ratios did not increase mortality compared to single infections and achieved a maximum of 10.3 % mortality after 14 days. In contrast, isolate CpGV-E2 caused mortality of 87.4 % after seven and 98.8 % after 14 days in CpR5M neonates. Resistance testing of CpR5M neonates exposed to OB derived from recombinants bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M) and bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S) (Gebhardt et al. 2014) resulted for pe38M in a very low mortality of 2.2 % after seven days and 6.7 % after 14 days. Strikingly, OB of pe38S caused 98.8 % mortality after seven and 14 days (Figure 14).

Thus, these results indicated that CpGV-E2, but neither CpGV-M nor CpGV-S alone, nor mixtures of CpGV-M and CpGV-S were able to cause significant mortality in CpR5M larvae, whereas the recombinant between CpGV-M and CpGV-S, pe38S, was able to break resistance in CpR5M

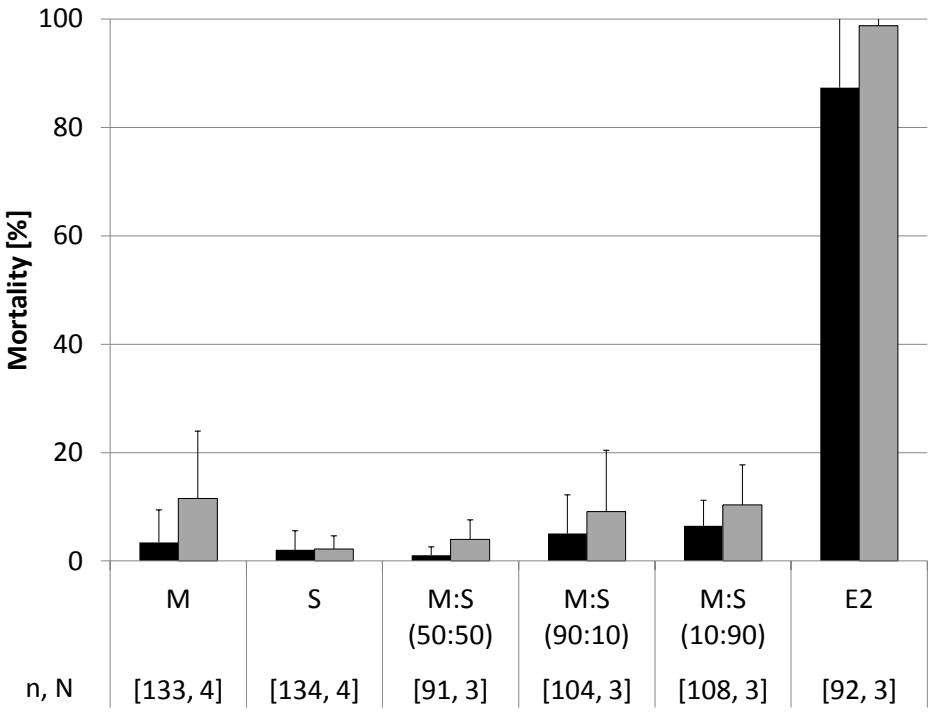


Figure 13 Resistance testing in CpR5M with different virus combinations. Mortality of neonates of CpR5M tested for resistance on artificial diet containing different CpGV isolates and CpGV mixtures, all at a final concentration of 5.8 x 10^4 OB/ml. The isolates CpGV-M (M), CpGV-S (S), CpGV-E2 (E2) or co-infections with compositions of 50:50, 90:10 or 10:90 of the different isolates were applied. The Abbott-corrected mean mortality, standard deviations (error bars) were determined seven (black bars) and 14 (gray bars) post infection. The total number of tested individuals (n) and number of independent replicates (N) are given below the charts.

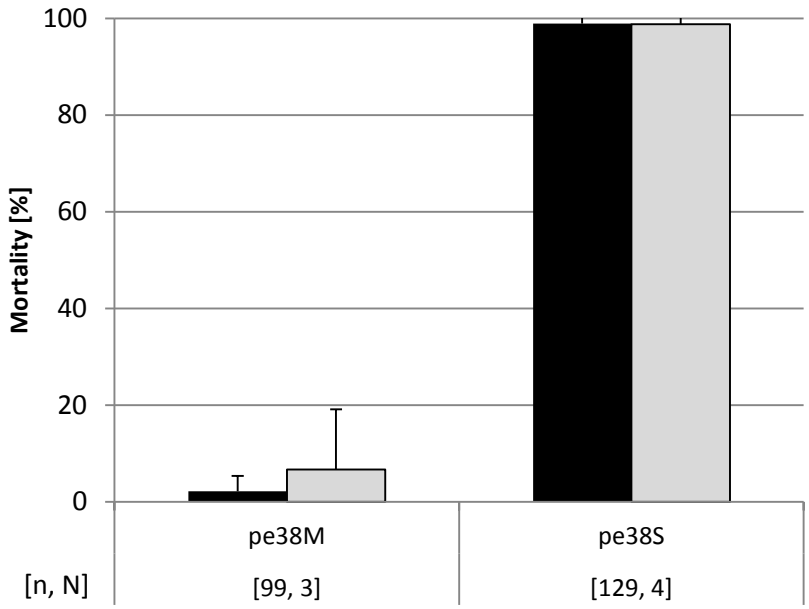


Figure 14 Resistance testing in CpR5M with recombinants. Mortality of neonates of CpR5M exposed to OB of bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M) or bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38S) at the discriminating concentration of 5.8×10^4 OB/ml. Abbott-corrected mean mortality and standard deviation (error bars) were determined seven (black bars) and 14 days (gray bars) post infection. The total number of tested individuals (n) and number of independent replicates (N) are given below the chart.

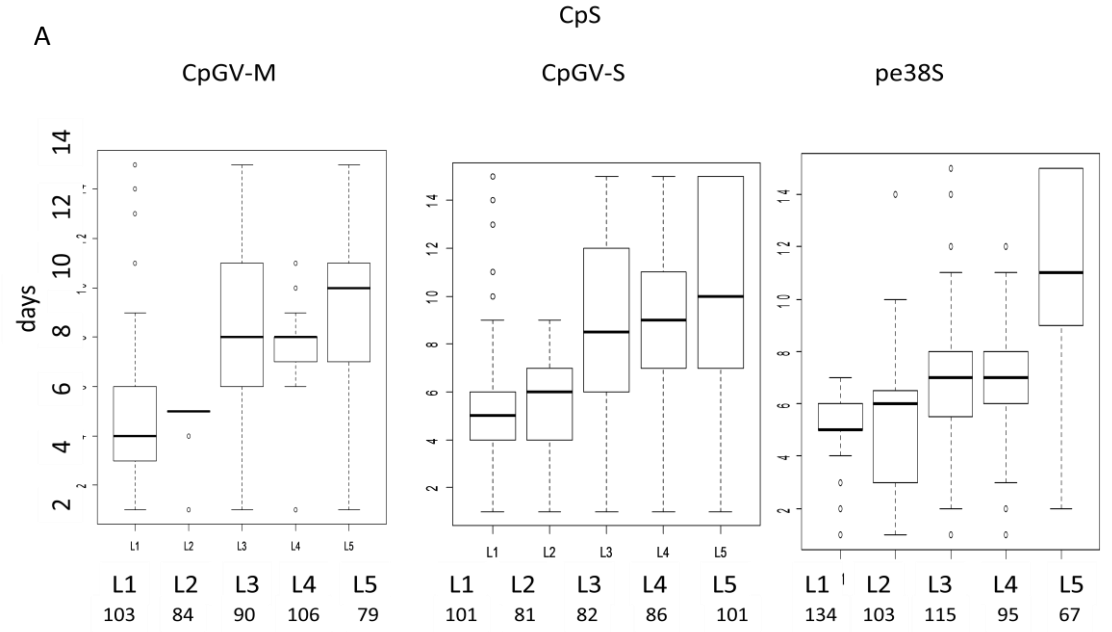
Instar-specific assay: To investigate whether resistance to CpGV-M and CpGV-S or the resistance-breaking characteristic of pe38S is related to a particular larval age, different instars (L1-L5) of CpS, CpR5M and CpR5S were infected using a single virus concentration of 2×10^5 OB/ml. The virus-induced mortality was recorded daily and subjected to a box-blot analysis showing the 25 %, 50 % and 75 % percentiles of the time to death (Figure 15A-C).

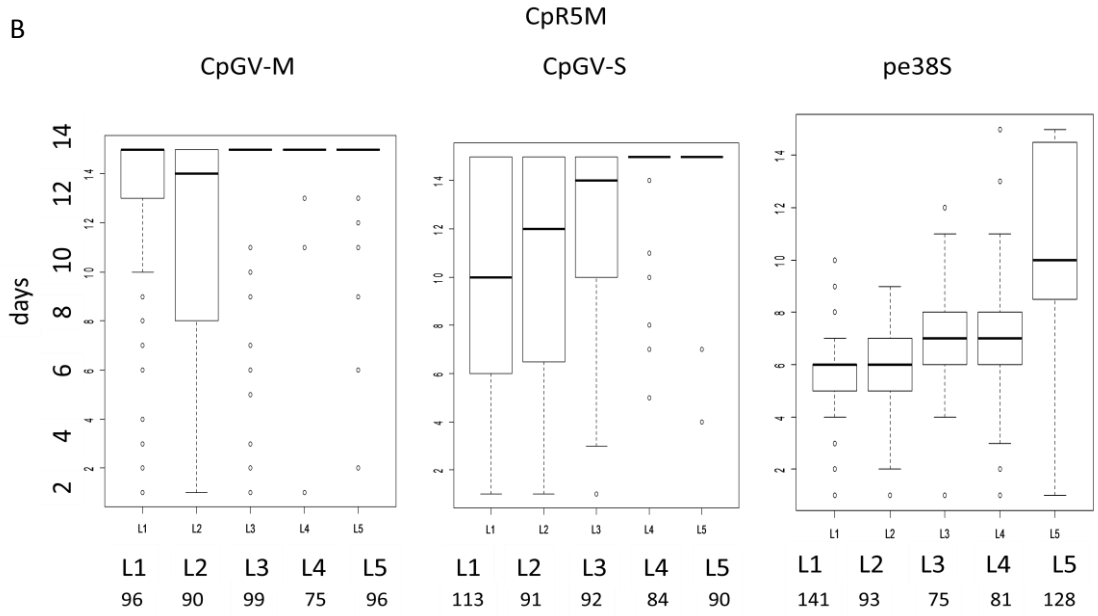
At the chosen virus OB concentration a mortality of >95 % is expected for all instars in a 14-day test when larvae have full susceptibility (Eberle et al. 2008) and was reached for CpGV-M, -S and pe38S in CpS (Figure 15A). The median mortality of CpS in all virus treatments was between 4 days for L1 and 11 days for L5 larvae and almost all larvae died within the test period.

In all larval stages of CpR5M and CpR5S a reduced susceptibility to CpGV-M and CpGV-S was detectable (Figure 15B and C). Mortality of >50 % could only be observed for CpGV-M in L2 larvae of CpR5M after 14 days. In all other larval stages of CpR5M, virus-induced mortality was lower than 50 % (Figure 15B). For treatment with CpGV-S, median mortality for CpR5M was achieved at day 10 (L1), day 12 (L2) and day 14 (L3) post infection. For the instars L4 and L5 subjected to CpGV-S, more

than 50 % of all tested larvae survived the virus treatment. Different larval stages of CpR5M subjected to pe38S showed a clear difference: 50 % mortality was achieved between 6 days (L1 and L2) and 10 days (L5) and almost all larvae died during the test (Figure 15B). The other selected strain CpR5S showed a similar response to CpGV-M and CpGV-S with similar results as CpR5M, pe38S was not tested on this strain (Figure 15C).

In summary, these experiments indicated a certain age-dependent increase of median time to death, which was visible for all strains CpS, CpR5M and CpR5S and all tested viruses. In addition, resistance of CpR5M and CpR5S against CpGV-M and CpGV-S was expressed in all instars, whereas the resistance-breaking property of pe38S was also detectable through all larval stages of CpR5M.





For description of **Figure 15A** and **Figure 15B** see next page

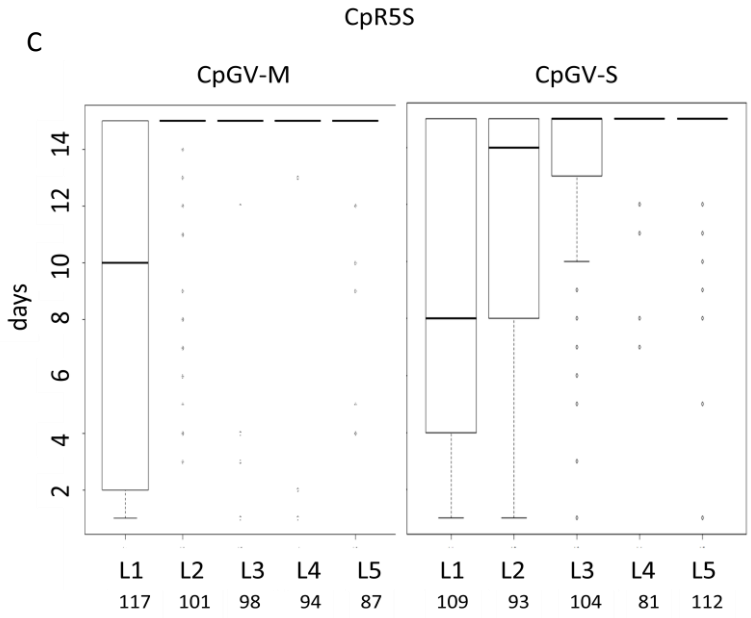


Figure 15 Box-plot analysis of instar-specific (L1-L5) mortality of (A) CpS, (B) CpR5M or (C) CpR5S larvae subjected to CpGV-M, CpGV-S or the recombinant bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S) at a concentration of 2.0 x 10^5 OB/ml for 14 days. Mortality was scored daily, open box indicates the 25 % - 75 % percentile of time to death, bold horizontal lines in the box give the day when 50 % of test animals died, vertical lines indicate the days when >0 % (lower end, excluding outliners) and 100 % (upper end, excluding outliers) mortality were observed, points stand for outliners. Larval stage (L1 – L5) and total number of tested individuals (n) of three independent replicates are given under the box-plot.

BV injection assay: To investigate if resistance of CpR5M against CpGV-M and CpGV-S is systemic or related to oral infection, BV were injected into the hemocoel of L4 instar larvae of CpR5M and CpS to bypass the *per os* infection pathway. To determine the appropriate amount of BV applied to the larvae different concentrations of CpGV-M were injected into L4 larvae of CpS, resulting in mortality between 19.6 % for 50 BV/larvae and 100 % for 5 x 10^5 BV/larvae after 14 days (Table 8). Based on this pre-test, the concentration of 5,000 BV/larvae was chosen for the following BV injection assays, because it caused high mortality of more than 70 % at a moderate number of BV to be used.

Table 8 Mortality of L4 larvae of CpS injected with BV of CpGV-M after 14 days.

Concentration [BV/larvae]	Number of CpS larvae	Mortality [%]
50	14	19.6
500	15	58.3
5,000	19	73.6
50,000	17	92.7
500,000	14	100.0

For the susceptible strain CpS, mortality of 73.2 % and 94.0 % were obtained with BV injection of 5,000 BV/larvae of CpGV-M and CpGV-S, respectively, and did not differ statistically from each other (Table 9). In contrast, mean mortality of resistant CpR5M injected with BV of CpGV-M was only 33.4 % and differed significantly from mortality caused by CpGV-S (83.4 %) or the mortality observed with CpS injections with CpGV-M and CpGV-S (ANOVA, Scheffé test, $P < 0.05$) (Table 9), suggesting that resistance of CpR5M is midgut-based for CpGV-S but not for CpGV-M. Mortality in the control group with injection of uninfected hemolymph from CpS was 31.1 % for CpS and 8.0 % for CpR5M (data not shown).

Table 9 Mortality of L4 larvae of CpS and CpR5M after BV injection of CpGV-M and CpGV-S. Larvae were injected with 5×10^3 BV/larvae into the hemocoel and given are the Abbott-corrected mean mortality 14 days post injection (p.i.), standard deviation (± SD), number of tested individuals (n) and number of independent replicates (N). Different letters indicates statistical differences in the means following ANOVA, Scheffé test ($P < 0.05$).

CM strain	BV treatment	n, N	Mortality % [± SD] 14 days p.i.	Test for significant statistical differences
CpR5M	CpGV-M	61, 4	33.4 [± 6.4]	A
CpR5M	CpGV-S	42, 3	83.4 [± 7.5]	B
CpS	CpGV-M	57, 4	73.2 [± 10.9]	B
CpS	CpGV-S	41, 3	94.0 [± 5.9]	B

ODV infection test and fluorescence-dequenching assay: To prove whether the observed difference of midgut related resistance to CpGV-M and CpGV-S depends on differences in the binding and fusion of ODV to the midgut epithelial cells in CpR5M fluorescence-dequenching test were performed. R-18 labeled ODV originated from CpGV-M and CpGV-S were used for the fluorescence-dequenching test with L4 larvae of CpR5M and CpS. The quality of the ODV preparations derived from OB by alkaline treatment was tested by electron microscopy; it showed that the used supernatant contained ODV free from OB (Figure 16).

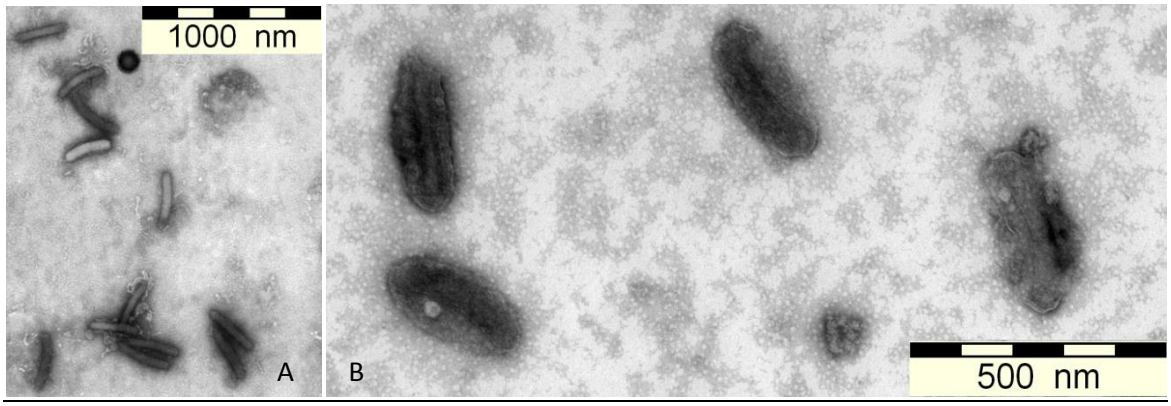


Figure 16 Transmission electron microscopy images of ODV preparation with negative staining. (A) Released ODV in the supernatant and (B) halfway dissolved OB and ODV in the pellet after incubation in DAS buffer and centrifugation; bars indicate magnification.

When the ODV preparations were fed to CpS larvae mortality of 62 % was observed for both CpGV-M and CpGV-S after seven days, whereas in CpR5M larvae 0 % and 15 % mortality were recorded for CpGV-M and CpGV-S, respectively (Figure 17A). The results showed that *per os* infection of larvae with the labeled ODV caused high mortality in CpS but very low mortality in the resistant strain CpR5M.

By applying the fluorescence-dequenching assay it was found that in CpS larvae 0.046 µg ODV of CpGV-S and 0.037 µg ODV of CpGV-M bound to the midgut membrane; 0.022 µg ODV of each virus fused with the midgut membrane (Figure 17B). In assays with CpR5M, lower binding and fusion mean values were calculated for CpGV-S (0.024 µg in the binding and 0.010 µg in the fusion) than CpGV-M (0.037 µg in binding and 0.017 µg in the fusion). Thus, whereas each binding and fusion of CpGV-M and CpGV-S appeared similar, a tendency of a reduced ODV binding and fusion of CpGV-S compared to CpGV-M was observed in CpR5M, suggesting a reduced binding and fusion capacity of CpGV-S in CpR5M. However, these differences were not significant due to a high variation between the single measurements in the test replicates (Scheffé Test, $P > 0.05$) (Figure 17B).

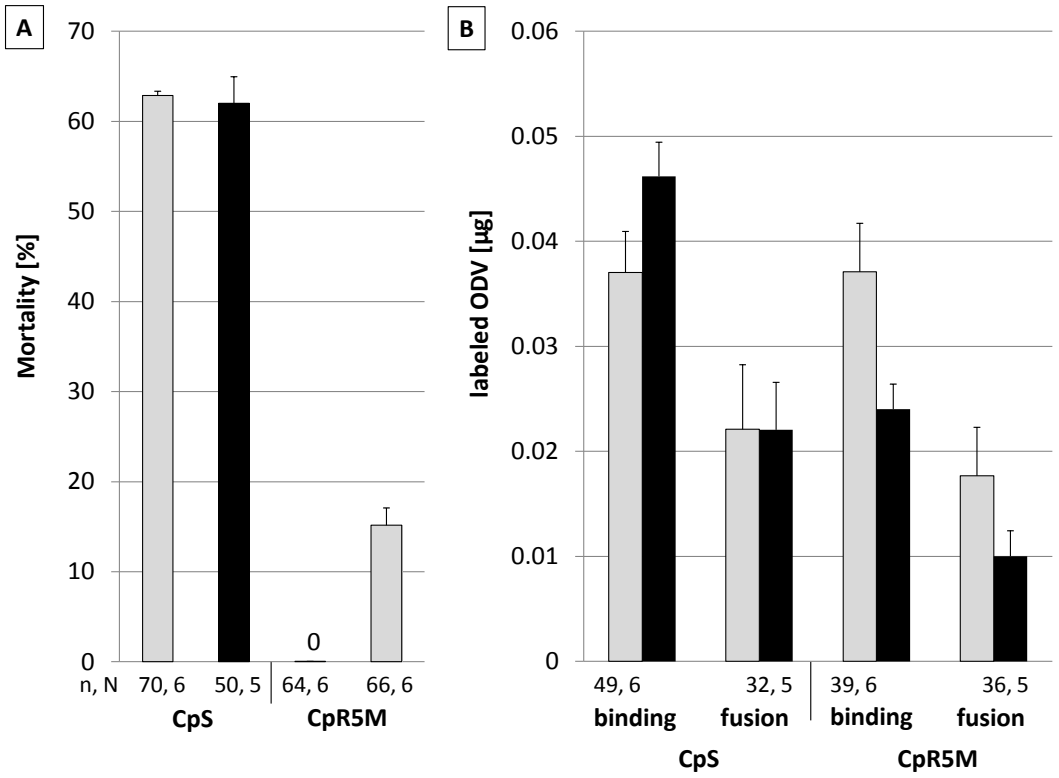


Figure 17 (A) Mortality and (B) results of fluorescence-dequenching assays of CpS and CpR5M *per os* infected with labeled ODV. L4 larvae were orally infected with 2.4 µg of labeled ODV of CpGV-M (gray) or CpGV-S (black). (A) Abbott-corrected mean mortality and standard errors (error bars) were recorded seven days post infection. (B) Amount of labeled ODV bound or fused with midgut epithelial cells, dissected of midgut epithelial cells was undertaken 1 h post infection. The total number of tested individuals (n) and number of independent replicates (N) are given below the chart.

3.4 Discussion

In the present study, infection assays with OB, ODV, BV of CpGV-M, CpGV-S alone, mixtures of both and a recombinant between CpGV-M and CpGV-S were carried out to elucidate the mechanism of resistance in CpR5M (and likewise in CpR5S).

Per os infections with OB of wild-type isolates showed that only the isolate CpGV-E2 was able to cause productive infections in CpR5M and CpR5S larvae. CpGV-E2 had already been described to be resistance-breaking in the ancestral field population NRW-WE (Jehle et al. 2017), and it also infected a further resistant strain CpRGO (Chapter 4). Thus, CpGV-E2 is proposed to be an important isolate to control CM, especially those CM populations with type II resistance.

Mixed infections of CpR5M with CpGV-M and CpGV-S did not result in a notable break of resistance or an increase of virulence compared to single isolate infections. Thus, no synergistic interaction of

CpGV-M and CpGV-S could be noticed in CpR5M. This finding is in contrast to the type I resistance, where some synergistic effect of CpGV-M and the resistant-breaking CpGV-R5 was noticed in infection experiments of larvae of the French resistant colony RGV (Graillot et al. 2016).

Cross-resistance to CpGV-M and CpGV-S observed for CpR5M and CpR5S after their selection process (Chapter 2) initially suggested a novel common mechanism against both isolates. Surprisingly, the resistance testing with OB of the recombinants bacCpGVΔpe38$_M^{pe38M::eGFP}$ (pe38M) and bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S) showed that the heterologous recombinant pe38S but not the homologous construct pe38M was able to cause high mortality in CpR5M (Figure 14)

This finding evidenced that resistance of CpR5M against CpGV-M can be broken by the same recombinant which overcomes resistance in CpRR1 (Gebhardt et al., 2014). For CpRR1 it was shown that type I resistance is targeted against *pe38* of CpGV-M (or its product) and can be broken when it was replaced by *pe38* of CpGV-S. Since pe38S can also overcome type II resistance in CpR5M the same resistance mechanism in CpR5M as already observed in CpRR1 must be assumed, though its genetic factor is now autosomal and not Z-linked. The recombinant pe38S showed a very high virulence to CpR5M in all larval stages (L1-L5) causing similar time to death rates as CpGV-M or CpGV-S in CpS larvae (Figure 15).

On the other hand, *pe38* of CpGV-S cannot be the only resistance-breaking factor for type II resistance in pe38S; otherwise wild-type CpGV-S alone or other genome groups of CpGV with an identical *pe38* should be infective for CpR5M, which is not the case. Only CpGV-E2 was able to break resistance in CpR5M (and CpR5S). Therefore, it is predicted that a second factor, which is located on the backbone of CpGV-M in pe38S, must be involved in resistance-breaking mechanism in CpR5M. Only the combination of both allows resistance-breaking of pe38S. This second resistance factor is targeted against a CpGV-S specific character. It is further hypothesized that the two factors cannot interact in *trans* to beak type II resistance, because mixed infection with CpGV-M and CpGV-S did not increase larval mortality of CpR5M (Figure 13). Based on this scenario we developed the hypothesis that one factor is targeted against *pe38* of CpGV-M, whereas a second factor is specifically directed to an unknown factor of CpGV-S located in the midgut of CpR5M larvae (Figure 18).

If this assumption is true, (i) BV of CpGV-S but not those of CpGV-M should be infective for CpR5M larvae when the midgut infection is bypassed, and (ii) the binding and fusion capacity of ODV of CpGV-S to the midgut epithelium should be reduced when compared to ODV of CpGV-M. Two experiments were performed to prove these assumptions: (i) BV injection into the hemocoel to bypass midgut transfer, and (ii) fluorescence-dequenching assays with ODV to determine differences in the binding and fusion of ODV with midgut cells.

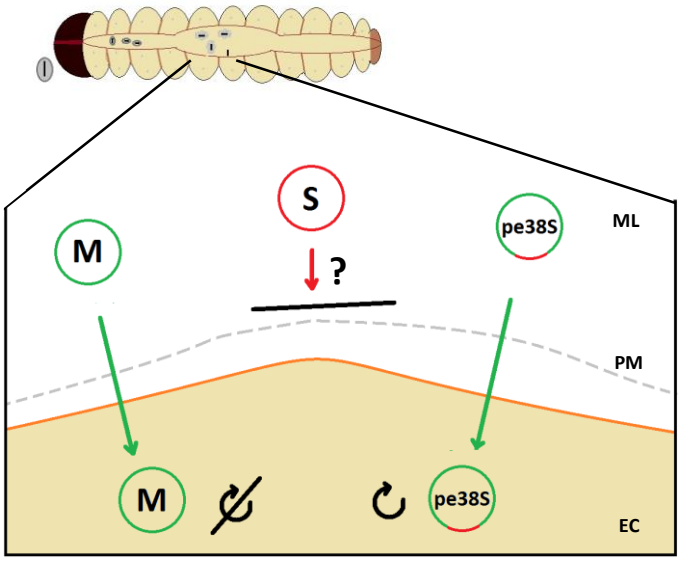


Figure 18 Schematic illustration of the assumption of two different, resistance mechanisms in CpR5M larvae for resistance to CpGV-M and CpGV-S. Green circle indicates occlusion derived viruses (ODV) of CpGV-M isolate (M) inside the midgut lumen (ML). M is able to pass the peritrophic membrane (PM) and enter the midgut epithelial cell (EC). Intercellular the virus replication is blocked due to resistance mechanism is targeted against the 24 bp insertion in the *pe38* gene of CpGV-M. Red circle indicates ODV of CpGV-S (S), which is prevented to integrate into the EC by an unknown factor (?). Green circle with red section indicates ODV of the recombinant bacCpGVΔpe38$_M^{pe38S::eGFP}$ (pe38S), containing the CpGV-M isolate with the *pe38* of CpGV-S. The recombinant is able to integrate into the midgut cells, because of the backbone of CpGV-M and circumvent the block of replication by the *pe38* gene of CpGV-S, following by productive virus infection in CpR5M larvae.

BV injections are a very powerful method to discriminate midgut-based blocks of infection from systemic ones (Grove and Hoover 2007; Asser-Kaiser et al. 2010; Jakubowska et al. 2010; Chikhalya et al. 2013). Injections of BV of CpGV-S into CpR5M larvae caused high mortality, similar to that of BV injections of CpGV-S and CpGV-M into susceptible CpS larvae, clearly indicating that the midgut alone is the barrier of CpGV-S infection in CpR5M. Mortality of CpR5M larvae injected with BV of CpGV-M was significantly lower. From this difference in the susceptibility of CpR5M to BV injections of CpGV-M and CpGV-S it can be concluded that resistance to CpGV-M and CpGV-S follows indeed different mechanisms, a midgut based mechanism for CpGV-S and a systemic mechanism for CpGV-M that cannot (or only to a low extent) be circumvented by BV injections. Asser-Kaiser et al. (2010) showed by BV injections experiments that type I resistance against CpGV-M in CpRR1 is also not midgut-based but is systemic. Thus, the BV infection experiment supported the hypothesis of two different

resistance mechanisms in CpR5M and that resistance against CpGV-S is indeed located in the midgut of CpR5M.

One conceivable explanation for a midgut related resistance could be, that the peritrophic membrane (PM), which acts as a physical barrier of the ODV passage from the gut lumen to the midgut epithelial cells (Wang and Granados 1998) is changed in CpR5M. This possibility, however, would be very unlikely to explain midgut-based resistance against CpGV-S because it would require an isolate-dependent, selective sieving capacity of the PM.

Midgut-based resistance could be the consequence of a isolate-specific disturbance of ODV attachment to midgut epithelial cells (Haas-Stapleton et al. 2005). ODV binding and fusion is highly complex process, which involves numerous baculovirus binding and fusion proteins as well as host receptors, which are so far not fully identified (Kuzio et al. 1989; Ohkawa et al. 2005; Sparks et al. 2011; Mu et al. 2014). A mutation of a receptor molecule in the insect midgut resulting in an isolate-specific change of ODV binding may impair the ODV entry into the midgut cell and eventually impair larval susceptibility. Fluorescence-dequenching assays with ODV derived from CpGV-M and CpGV-S, however, did not reveal statistically significant differences between the binding and fusion of CpGV-M and CpGV-S ODV in both CpR5M and CpS strains. Though in CpR5M larvae the mean binding and fusion capacity of ODVs from CpGV-S was about 40 % lower than those of CpGV-M ODV, this weak and statistically not confirmed difference alone appears not to be strong enough to explain the midgut-based resistance to CpGV-S. By using the same method, Iwata et al. 2017 recently succeeded to demonstrate a laboratory-selected midgut-based resistance of the tea tortrix *Adoxophyes honmai* larvae to Adoxophyes honmai nucleopolyhedrovirus (AdhoNPV).

If ODV binding and fusion cannot explain midgut-based resistance to CpGV-S, another so far unknown block of virus infection must be located in the midgut of CpR5M. Such a block may have an intracellular location in the midgut cells which does not play a role in the BV driven systemic infection and which cannot be rescued in the midgut by co-infecting CpGV-M.

The discovery of two separate resistance mechanisms against CpGV-M and CpGV-S in CpR5M poses another question on the observed cross-resistance of CpR5M (and CpR5S) to both viruses (Chapter 2). If the two mechanisms are functionally not related but apparently co-inherited, it is predicted that the underlying genetic factors must be located in close vicinity on the same autosome of the genome of CpR5M.

4. A third type of codling moth resistance against Cydia pomonella granulovirus (CpGV) shows a mixture of a Z-linked and autosomal inheritance pattern

Abstract

Different isolates of Cydia pomonella Granulovirus (CpGV) are being used worldwide for the control of codling moth larvae (*Cydia pomonella*; CM) in organic and integrated apple and pear production. Two different types of dominant inherited field resistances of CM to CpGV products have been recently identified; Z-chromosomal type I resistance and autosomal type II resistance.

In the present study a CpGV-resistant CM field population (termed SA-GO) originating from North-East of Germany was investigated. Similar to the type II resistance, SA-GO individuals showed cross-resistance to CpGV isolates of genome group A (CpGV-M) and genome group E (CpGV-S), whereas genome group B (isolates CpGV-E2 and V15) were still infective. Crossing experiments with individuals from a CM strain CpS susceptible to CpGV indicated differences in susceptibility and inheritance found in CM type I or type II resistance. By single-pair inbreeding of SA-GO individuals for two generations, the genetically more homogenous strain CpRGO was developed.

Reciprocal hybrid crosses and backcrosses between individuals of resistant CpRGO and susceptible CpS revealed a dominant and polygenic inheritance of resistance in the majority of crosses. Resistance to CpGV-S appeared to be autosomal and dominant for larval survivorship but recessive when success of pupation of the hybrids was considered. Resistance to CpGV-M however, is proposed to be both autosomal and Z-linked inherited, since Z-linkage of resistance was needed for pupation. Hence we propose a further type III resistance to CpGV in CM, which appears to have mixed characteristics of type I and type II resistance.

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Author contributions: E.F and K.U-S. provided resistance testing in SA-GO. S.S-B. managed single pair crossing and resistance testing between individuals of the field population SA-GO and CpS larvae.

4.1 Introduction

The codling moth (CM; *Cydia pomonella*) is a Lepidoptera (family: *Totricidae*), which larvae cause serious damage in pome fruit production, mainly in apple and pear (Lacey et al. 2008). CM is worldwide distributed mostly through the temperate fruit growing regions (Barnes 1991). In conventional plant protection this pest has been controlled with chemical insecticides that result in a variety of problems, such as detrimental effects to beneficial organisms, negative environmental implications and safety risks for applicators and consumers (Lacey and Shapiro-Ilan 2008). Thus, the demand for environmentally safe control agents of CM increased. Cydia pomonella granulovirus (CpGV), first described from diseased CM larvae found in Mexico (Tanada 1964), has a very narrow host range and is highly virulent for early larval stages of CM. CpGV belongs to the genus *Betabaculovirus* of the *Baculoviridae* family. Its genome comprises double-stranded circular DNA of about 120.8-123.9 kb (Luque et al. 2001; Gebhardt et al. 2014). Because CpGV is highly effective against early larval stages and harmless to non-target insects, mammals or the environment, CpGV-based biocontrol agents were developed for biological CM control (Lacey et al. 2008).

Since its first registration in 1987 in Switzerland, CpGV products have been used as bio-pesticide in virtually all apple growing areas worldwide (Huber 1998). The CpGV occlusion bodies (OB) can be applied in their natural form, CM larvae taking up the OB during feeding and die within a few days after ingestion (Lacey et al. 2008). Most of the commercial CpGV products of the first generation contained only the Mexican isolate, termed CpGV-M (Huber 1998; Tanada 1964). In 2005, after years of general usage several organic apple orchards in South-West Germany were located, where CpGV treatment had failed to control CM satisfactorily (Fritsch et al. 2005). In laboratory bioassays with progeny of these populations, a 1000 to 100,000-fold reduced susceptibility to CpGV-M was confirmed (Asser-Kaiser et al. 2007). Similar observations of field resistance of CM populations against CpGV products were also reported from France (Sauphanor et al. 2006); this was the first time that occurrence of resistance in insect populations against commercial baculovirus products had been reported. Meanwhile resistance to CpGV-M has been noted in more than 40 apple plantations in Austria, the Czech Republic, France, Germany, Italy, the Netherlands and Switzerland (Zichová et al. 2013; Schmitt et al. 2013; Jehle et al. 2017).

The codling moth exhibits a karyotype of 2n = 56 chromosomes, with 54 autosomes (A) and a WZ/ZZ (female/male) sex chromosome system (Fuková et al. 2005). For the laboratory strain CpRR1, derived from the resistant field population CpR, a dominant, monogenic and Z-linked mode of inheritance for the resistance could be demonstrated (Asser-Kaiser et al. 2007; Asser-Kaiser et al. 2010). In further genetic analyses of the resistant CM strains RGV (France) and CpR-CZ (Czech Republic) the Z-linked and dominant inheritance of the resistance against CpGV-M was confirmed (Berling et al. 2013; Zichová et al. 2013). Hence, a single main inheritance type has been initially assumed in CM

populations over Europe. This so-called type I resistance appeared to be targeted only against CpGV-M (group A), whereas other CpGV isolates (genome group B-E) where able to overcome resistance (Eberle et al. 2008; Gebhardt et al. 2014).

For CpRR1, it was demonstrated that resistance-breaking properties of the CpGV isolates depended on the lack of an insertion of 24 bp in the ORF *pe38* in CpGV-M (group A), which is missing in all resistance-breaking isolates of genome group B-E CpGVs (Gebhardt et al. 2014). Based on some of these isolates, successful commercial control of the type I resistance in CM was re-established in Europe (Jehle et al. 2006b; Eberle et al. 2008; Kienzle et al. 2008; Berling et al. 2009; Graillot et al. 2014; Graillot et al. 2016).

More recently, CM field populations were identified, where the use of novel resistance-breaking CpGV products also failed. Resistance of one of these populations, termed NRW-WE, is targeted not only to CpGV-M but furthermore to isolates belonging to the genome groups C-E (Jehle et al. 2017), suggesting an alternative mode (= type II) of resistance in NRW-WE (Jehle et al. 2017). Indeed, when the laboratory strains CpR5M and CpR5S were generated by selection experiments starting with NRW-WE individuals, it was demonstrated that type II resistance followed a dominant, monogenic and autosomal inheritance pattern. Furthermore, a cross-resistance to at least two CpGV isolates, CpGV-M and CpGV-S, was observed (Chapter 2).

A further CM field population from North-East Germany, called SA-GO, showed also a reduced susceptibility to both CpGV-M and CpGV-S. SA-GO was first reported in a study concerning the pan-European distribution of CpGV resistance and showed a lethal concentration (LC_{50}) of 1.12×10^8 ($0.23–27.4 \times 10^8$) OB/ml with CpGV-M. This LC_{50} represented an about 1,000,000-fold increased level of resistance, compared to the level of susceptible CpS. It was one of the highest detected LC_{50} values in field populations which strongly differed from the resistance response of other tested populations following the type I resistance (Schmitt et al. 2013).

The aim of the current study was to investigate the genetic basis and inheritance mode of CpGV resistance in SA-GO. Resistance tests to identify CpGV isolates with resistant-breaking properties for SA-GO were conducted. Single-pair crosses between the susceptible strain CpS and field collected individuals of SA-GO were carried out to obtain first hints on the inheritance mode and frequency of resistance in this field population. A two step inbreeding of SA-GO individuals combined with simultaneous resistance testing of the offspring resulted in a genetically homogenous strain CpRGO with low mortality against CpGV-M and CpGV-S. Further crosses and backcrosses between CpRGO and CpS unveiled the genetic basis, the inheritance mechanisms, dominance level and the chromosomes (sex-linked or autosomal) involved in this CpGV resistance. These investigations allow allocating resistance of CpRGO neither to the type I nor type II but to a further type of a combined of autosomal and Z-linked inheritance, which we termed type III resistance against CpGV.

4.2 Material and Methods

Insects: The codling moth (CM) strain CpS is susceptible to all known CpGV isolates and has been reared at the Julius Kühn Institute (JKI), Institute for Biological Control in Darmstadt (Germany), for many years (Asser-Kaiser et al. 2007). The CM field population SA-GO derived from an organic orchard in Saxony, Germany, where CpGV-M products had been applied in the past. In autumn 2008, cardboard stripes were stripped around tree trunks to collect diapausing larvae, which were reared in the laboratory henceforward. Resistance to CpGV-M in this orchard was already demonstrated by full-range bioassays (Schmitt et al. 2013). All CM strains were reared under laboratory conditions at 26 °C with 16/8 h light/dark photoperiod and 60 % relative humidity on modified artificial diet (Ivaldi-Sender 1974).

Viruses: Four different Cydia pomonella granulovirus (CpGV) isolates were used in this study: CpGV-M (Tanada 1964), belonging to the genome group A, isolate CpGV-E2 (genome group B) (Crook et al. 1985), CpGV-S (genome group E) (Gebhardt et al. 2014) and CpGV-V15 (a semi-commercial CpGV preparation from Andermatt Biocontrol, Switzerland). CpGV occlusion bodies (OB) were purified as described previously (Smith and Crook 1988; Jehle et al. 1992) and all samples were stored at -20 °C. Quantitation of virus stocks was performed by OB counting with a light microscope (Leica DMRBE) in dark-field optics with the Petroff-Hauser counting chamber (depth 0.02 mm).

Resistance testing: To differentiate between resistant and susceptible individuals, first instar larvae were tested in bioassays as described elsewhere (Undorf-Spahn et al. 2012). All larval offspring were separated into three cohorts (i-iii); (i) neonate larvae were tested for resistance on artificial diet containing CpGV-M, (ii) larvae were exposed to diet with CpGV-S, and (iii) for untreated control, larvae were reared on diet without virus. For resistance testing, purified OB were incorporated into modified diet of Ivaldi-Sender (1974) to obtain a final discriminating concentration of 5.8×10^4 OB/ml, which causes > 95 % mortality in susceptible CpS neonates after seven days (Asser-Kaiser et al. 2007). Mortality of larvae was determined one, seven, 14 and 21 days post infection (p.i.). Only larvae surviving day one p.i. were introduced to the test. All mortality data were corrected for control mortality following Abbott´s Formula (Abbott 1925). After 21 days surviving pupae were sexed according to the number of their abdominal segments described previously (Eberle and Jehle 2006).

Single-pair crosses: For single-pair crosses (families) pupae of SA-GO were sexed as described before (Eberle and Jehle 2006) and one female pupa and one male pupa were placed together for emergence and mating. The obtained eggs were collected daily and stored at 4 °C until end of

oviposition. Then all eggs of a family were incubated at 26 °C until hatching of the neonates. Single-pair families producing less than 20 neonates were excluded from the resistance testing. For details of single-pair crosses see Asser-Kaiser et al. (Asser-Kaiser et al. 2007).

Reciprocal single-pair crosses: In order to obtain information about the mode of inheritance in both, SA-GO or CpRGO, reciprocal single-pair crosses between one of the two resistant strains and the susceptible strain CpS were conducted. Two different crosses were performed; female crosses represent the mating of one virgin female (f) moth of SA-GO or CpRGO with a male (m) moth of CpS (SA-GOf or CpRGOf x CpSm). Male crosses means the crosses of a male moth of SA-GO or CpRGO mated with a virgin female moth of CpS (SA-GOm or CpRSOm x CpSf). The resulting offspring of the reciprocal male or female crosses (F_1) were divided in three cohorts and subjected to resistance tests on CpGV-M, CpGV-S and untreated control as described above. If the number of F_1 neonates was lower than 90 individuals only one virus (CpGV-M or CpGV-S) treatment and the untreated control group were included.

Backcrossing experiments: The F_1 control group of the male or female crosses were reared to pupae and separated by sex. The hatching male moths were individually backcrossed with female CpS moths (backcross A; BC A) or with a virgin female moth of CpRGO (backcross B; BC B). The resulting offspring (F_2) of BC A or BC B were tested for resistance as described above.

Statistical analysis: Statistical differences in the resistance testing were analysed using ANOVA pairwise t-Test with the Program RStudio (RStudio edition 2.3.4.4.). Dominance of resistance (D_x) was calculated as described by Bourguet et al. (Bourguet et al. 2000). $D_X = (x_{RS} - x_{SS})/(x_{RR} - x_{SS})$, where x_{SS}, x_{RS} and x_{RR} are the determined mortality of the susceptible homozygote, heterozygote and resistant homozygote individuals, respectively. Values of D_X range from 0 to 1, D_x values close to 0 are considered to represent a completely recessive inheritance, whereas values close to 1 represent a completely dominant resistance (Bourguet et al. 2000). Statistical analyses of backcrosses were performed by using a χ^2 test for goodness of fit (df = 1) between the expected and observed mortality data at the discriminating concentration for monogenic inheritance, as described by Tabashnik (Tabashnik 1991). The expected number of dead larvae was calculated as $n_i M_i$, where n_i is the number of larvae tested at the discriminating concentration i and $M_i = 0.5(W_{RS} + W_{SS})$ or $M_i = 0.5(W_{RS} + W_{RR})$ depending on the backcrosses. W_{RS} W_{RR} and W_{SS} are the mortality of the tested hybrid F_1 (RS) and homozygous parental (SS) or (RR) strains at the discriminating concentration i. Mortality data in this study were corrected for control mortality (Abbott 1925).

4.3 Results

Resistance testing in SA-GO and CpS with different CpGV isolates: The susceptibility of neonates of SA-GO to the isolates CpGV-M, S, E2 and V15 was tested with a discriminating concentration of 5.8×10^4 OB/ml, causing >95 % virus-induced mortality in susceptible strains after seven days (Asser-Kaiser et al. 2007). CpS was included as a control (Figure 19). After seven days, mortality on CpGV-M and CpGV-S was only 2 % and 3 %, respectively. It increased to 9 % for both viruses after 14 days. In contrast, when CpGV-E2 and V15 where applied, mortality was 28 % and 30 % after seven days and 81 % and 92 % after 14 days, respectively. For susceptible CpS, virus-induced mortality of the different CpGV isolates varied between 87 % and 97 % after seven days and was more than 99 % after 14 days. Statistical analyses between the different CM strains and all CpGV isolates verified significant differences between SA-GO and CpS after seven days (ANOVA, pairwise t-Test, $P < 0.05$). For SA-GO, the larval mortality caused by CpGV-E2 or -V15 differed statistically significant from the mortality caused by CpGV-M or -S after seven and 14 days, respectively. However, mortality determined on SA-GO larvae challenged with CpGV-V15 and -E2 showed no significant differences to the mortality data assessed with the different isolates on CpS after 14 days. No statistical differences were identified between the isolate treatments in CpS after seven and 14 days (Figure 19).
The results revealed a resistance of SA-GO against both isolates, CpGV-M and CpGV-S, whereas both CpGV-V15 and -E2 expressed full resistance-breaking characteristics in SA-GO only after 14 days.

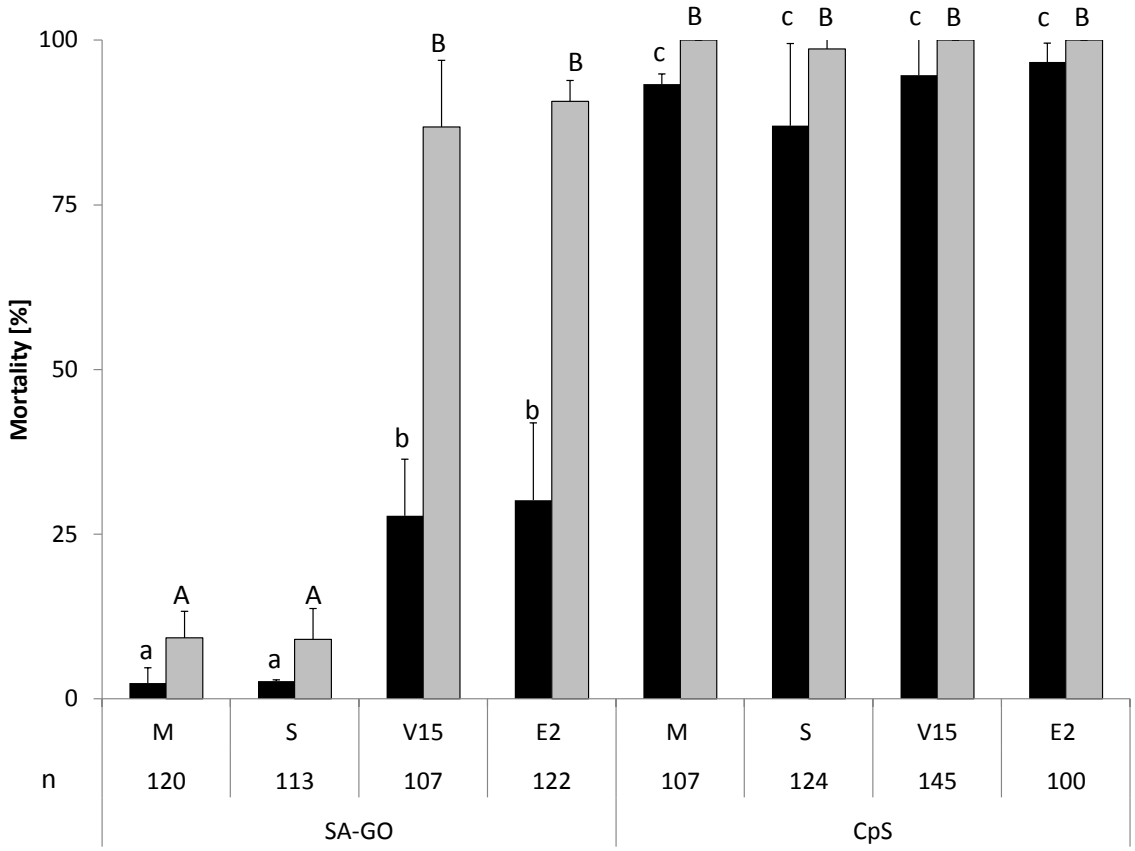
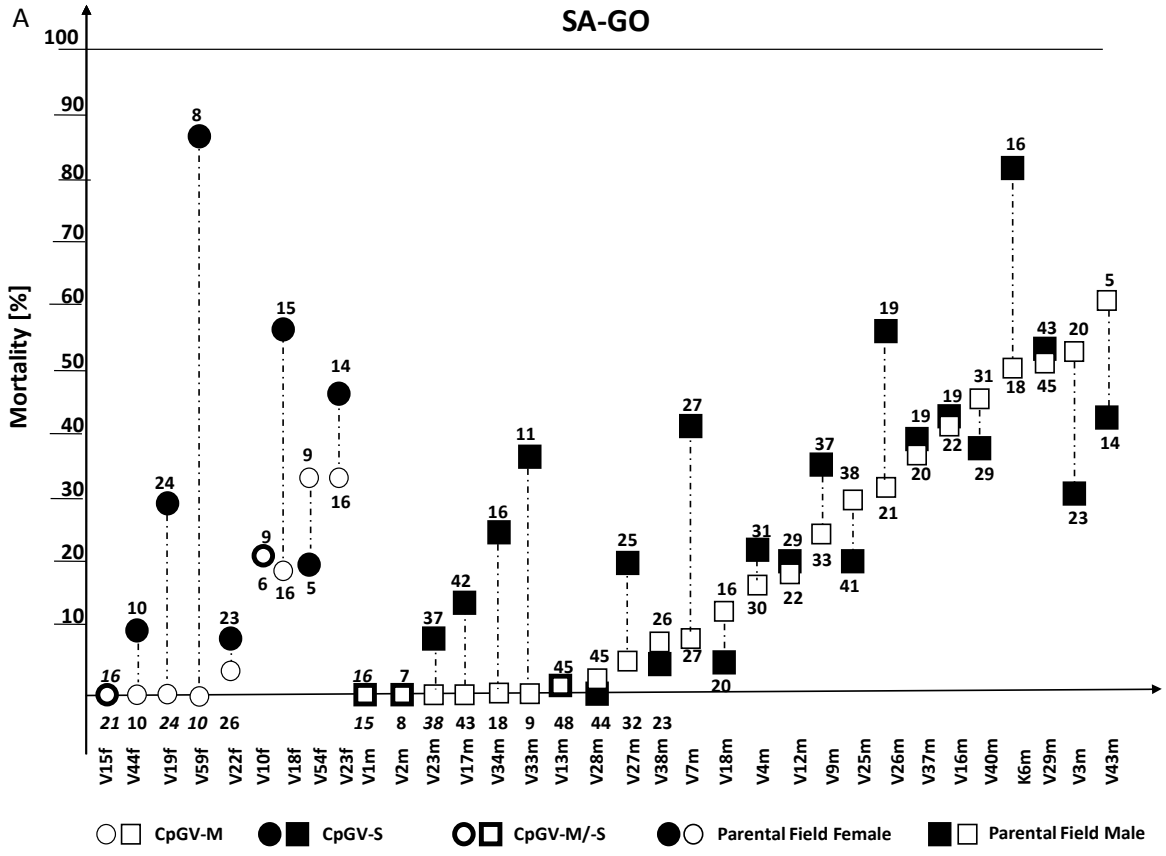


Figure 19 Resistance testing in SA-GO and CpS with different CpGV isolates. Mortality of neonate larvae of SA-GO and CpS tested for resistance on artificial diet containing either CpGV-M (M), CpGV-S (S), CpGV-V15 (V15) or CpGV-E2 (E2) at a discriminating concentration of 5.8×10^4 OB/ml. Abbott-corrected mean mortality and standard deviation (error bars) were determined seven (black bars) and 14 days (gray bars) post infection. The total number of tested individuals (n) of three independent replicates is given below the chart. Columns marked by different letters differ significantly (ANOVA, pairwise t-Test, $P < 0.05$).

Resistance testing in F_1 progeny of the reciprocal single-pair crosses between field-derived SA-GO moths and susceptible CpS moths: To obtain immediate information on the character of the resistance inheritance in SA-GO, reciprocal hybrid crosses between CpS moths and adults that had emerged from diapausing field larvae of SA-GO were performed. Neonates of the F_1 progeny were then subjected to resistance testing with a discriminating concentration of 5.8×10^4 OB/ml. Nine female crosses (SA-GOf x CpSm) yielded virus-induced offspring mortality between 0 % and 34 % when subjected to CpGV-M for seven days. CpGV-S caused mortality between 0 % and 87 % in the offspring of the different single-pair families. In total, 24 single pairs of the male crosses (SA-GOm x CpSf) were obtained and yielded CpGV-M induced mortality of 0 % to 59 % after seven days, whereas CpGV-S caused mortality between 0 % and 81 %, (Figure 20A).

After 14 days, the F_1 progeny of the female crosses showed CpGV-M-induced mortality between 0 % and 75 % and mortality between 49 % and 100 % on CpGV-S. Mortality of the progeny of the male crosses ranged from 0 % and 84 % on CpGV-M and 0 % to 93 % on CpGV-S (Figure 20B). The male crosses generated in total 1.7 times more offspring larvae (ø = 75.3/cross) compared to the female crosses (ø = 42.8).

The results of the single-pair crosses between CpS and SA-GO suggested a dominant resistance against both genetic groups A (CpGV-M) and E (CpGV-S) in SA-GO. In addition, a Z-linkage could be excluded because four female crosses (v15f, v44f, v19f and v59f) showed 0 % mortality. Compared to the resistance of NRW-WE (Jehle et al. 2017) these experiments indicated already that resistance in SA-GO is dominant inherited and followed more type II than type I resistance.



For description of **Figure 20A** see next page

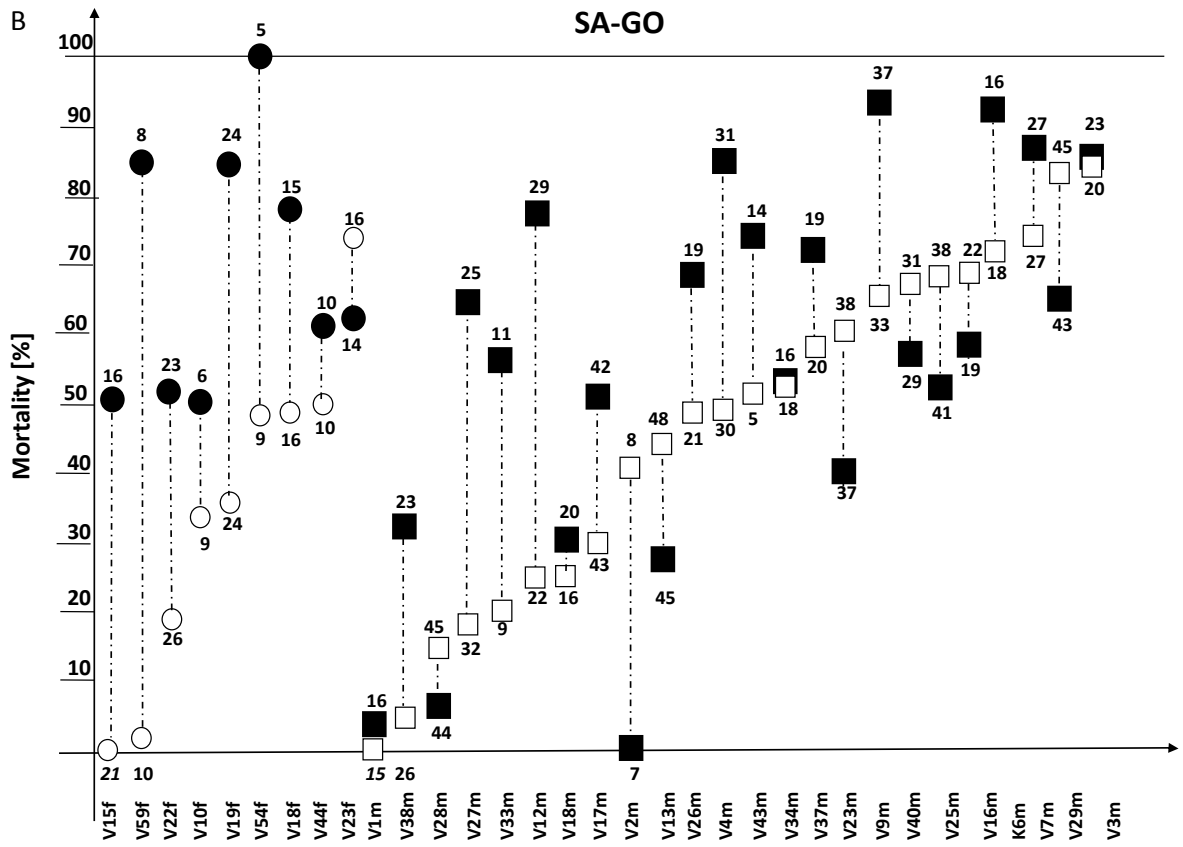


Figure 20 Resistance testing in F_1 progeny of the reciprocal single-pair crosses between SA-GO and susceptible CpS. Mortality of neonate L1 larvae from single-pair hybrid crosses between individuals of field-collected SA-GO and the laboratory strain CpS tested for resistance after seven days (A) and 14 days (B) on diet containing 5.8 x 10^4 OB/ml of either CpGV-M or CpGV-S. A third, untreated cohort of each family was used for mortality correction according to the method of Abbott (Abbott 1925). Each column represents the mortality of a single family. Squares indicate field males x CpS females; circles represent field females x CpS males; open circles and squares represent mortality on CpGV-M, filled circles and squares represent CpGV-S treatments; circles and squares in bold indicate the same results achieved with both viruses. Numbers of tested larvae in each cohort are given next to circles and squares. For example, family V15f derived from a parental field female crossed with a male CpS; 16 larvae were tested on CpGV-S resulting in 0 % and 50 % mortality after seven and 14 days, respectively, whereas 21 larvae were subjected to CpGV-M causing 0 % mortality after seven and 14 days. When control mortality exceeded mortality with treatment, the Abbott correction would result in negative mortality values. In this case, mortality was set to zero and sample number of the relevant families is shown in italics. The codes of tested families are given at the bottom; all families were ordered on *x* axis according to increasing mortality when treated with CpGV-M.

Establishment of a genetically homogenous strains from SA-GO: To further evaluate the genetics of CpGV resistance in SA-GO a genetically more homogeneous strain was established by two consecutive rounds of inbreeding using single-pair crosses (families) of SA-GO individuals combined with resistance testing of the offspring of the families. Starting with 20 single-pair crosses, only seven families rendered sufficient F_1 offspring (20-60 neonates) to be further used for resistance testing and breeding. Mortality of the F_1 larvae exposed to CpGV-M and CpGV-S for seven days was 0 % and 33.3 %, respectively (Table 10). The mean mortality of all tested siblings was 7.2 % on CpGV-M and 9.3 % on CpGV-S. After 14 days, the mortality increased in some families to 32.5 % for CpGV-M and 47.9 % for CpGV-S.

Table 10 Establishment of genetically homogenous strains from SA-GO. Mortality of neonate larvae of the single-pair inbreeding of SA-GO, tested for resistance on artificial diet containing CpGV-M or CpGV-S at the discriminating concentration of 5.8 x 10^4 OB/ml seven and 14 days post infection (p.i.) (Family #5–#15). Individuals of the untreated control cohort of #12 were crossed to obtain F_2 offspring (family #12.1-12.4). Untreated control cohorts of #12.2 and #12.4 were pooled to strain CpRGO. Mean mortality are corrected following Abbott Formula with mortality <6 % (7 days) and <16 % (14 days) in untreated control cohorts; standard deviations (SD); number of tested individuals (n); number of independent replicates (N).

	Mortality [%] on CpGV-M					Mortality [%] on CpGV-S				
Family/ strain	n [N]	7 days p.i.	Mean (SD)	14 days p.i.	Mean (SD)	n	7 days p.i.	Mean (SD)	14 days p.i.	Mean (SD)
#5	11	0.0	7.2 (11.2)	22.1	19.0 (11.9)	12	8.3	9.3 (10.6)	1.9	11.2 (16.6)
#8	14	0.0		1.4		15	13.3		22.1	
#9	15	1.5		1.5		16	1.1		1.1	
#10	12	33.3		31.1		10	10.0		5.5	
#12	22	5.3		19.5		20	32.3		47.9	
#14	14	10.0		32.5		12	0.0		0.0	
#15	4	0.0		25.0		8	0.0		0.0	
#12.1	29	13.8	6.2 (6.3)	20.7	13.8 (4.2)	29	10.3	4.1 (4.4)	20.7	13.4 (8.0)
#12.2	**28**	**0.0**		**13.5**		**34**	**6.1**		**17.6**	
#12.3	18	11.1		11.1		17	0.0		15.4	
#12.4	**7**	**0.0**		**10.0**		**4**	**0.0**		**0.0**	
CpRGO	40	3.1		42.4		37	0.9		26.4	
CpRGO (2013-2016)	273 [10]		11.3 (17.2)		28.0 (25.7)	330 [10]		19.6 (23.8)		33.9 (35.7)

All control groups of the different families were reared to pupae and separated by sex to undertake a second round of single-pair inbreeding within the siblings. Only family number 12 (#12) provided enough F_1 moths to perform further four single-pair crosses termed #12.1, #12.2, #12.3 and #12.4. At seven days post infection, mortality of the F_2 neonates was between 0 % and 13.8 % on CpGV-M and 0 % and 10.3 % on CpGV-S (Table 10). All offspring of the family #12.2 and #12.4 survived the treatment on CpGV-M and mortality of 0 % and 6.1 % were determined on CpGV-S, respectively. The mortality of these two families increased after 14 days to 17.6 % when subjected to CpGV-M or CpGV-S. Since these two families #12.2 and #12.4 showed lowest mortality, they were combined; their offspring was mass-reared and termed CpRGO. Virus-induced mortality of CpRGO inbreeding was 3.1 % on CpGV-M and 0.9 % on CpGV-S after seven days but increased to 42.4 % on CpGV-M and 26.4 % on CpGV-S after 14 days. CpRGO was reared in laboratory without selection pressure and further used for crossing and backcrossing experiments with CpS. Resistance testing of CpRGO was done frequently through three years of rearing with mean mortality values of 11.3 % on CpGV-M and 19.6 % on CpGV-S after seven and 28.0 % and 33.9 % after 14 days, respectively (Table 10).

Genetic inheritance studies: Female and male single-pair crosses and backcrosses between CpRGO and CpS individuals were performed to determine (i) the mode of inheritance of the resistance against CpGV-M and CpGV-S, (ii) the degree of dominance, (iii) the composition of the sex of the surviving pupae, and (iv) to estimate the number of genes that are involved in conferring resistance. Following an autosomal or Z-linked, dominant or recessive inheritance hypothesis, differences in the mortality rates in the crosses and backcrosses were expected following Figure 21.

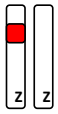
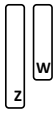
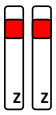
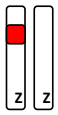
Female crosses	**F_0: Parental chromosomal genotypes, by hypothesis**		**F_1: Progeny chromosomal genotypes, by hypothesis**		**Expected mortality**		**Ratio of male pupae**
	female	male	female	male	**7 days p.i.**	**21 days p.i.**	
Z-linked, dominant and monogenetic					50 %	>50 %	1.00
Autosomal, dominant and monogenetic					0 %	>0 %	0.5
Male crosses	♀	♂	♀	♂			
Z-linked, dominant and monogenetic					0 %	>0 %	0.5
Autosomal, dominant and monogenetic					0 %	>0 %	0.5

Figure 21 Hypothetical inheritance schema for Z-linked and autosomal inheritance of resistance against CpGV. Hypothetically expected mortality after seven and 21 days post infection (p.i.) and ratio of surviving male pupae after 21 days p.i. of the offspring originating from male or female crosses of resistant and susceptible individuals of CM exposed to a discriminating concentration of CpGV-M. Parental and F_1 progeny of the chromosomal-genotypes are shown for a dominant, monogenic, autosomal or Z-linked inheritance for resistance against CpGV. A, autosome; Z and W, sex chromosomes; p.i., post infection; F_0, parental moth; F_1, progeny of the crosses; red square, resistance gene on the Z chromosome; blue square, resistance gene on the autosome.

Female crosses: In total, 38 single female crosses (CpRGOf x CpSm) were performed, nine crosses rendered sufficient offspring for resistance testing. The female crosses showed low mortality between 16 % (#52) and 44 % (#37) after seven days and increased after 21 days to 47 % (#38) till 76 % (#49) after infection with CpGV-M. All these crosses were grouped in one cluster (A) according to their mortality response observed in the CpGV-M treatment. A single cross #46 showed mortality of 100 % after seven days; it was therefore supposed to be a susceptible individual and was not clustered. When the sex of the surviving pupae was determined, it was found that only male but no female pupae developed on CpGV-M (Table 11). Neonate offspring of three female crosses (#35, #37

and #52) were simultaneously tested on CpGV-M and CpGV-S, whereas cross #17 was only tested on CpGV-S. The mortality ranged between 25 % and 81 % after seven days and between 88 % and 100 % after 21 days, rendering only a very few surviving male and female pupae. Hence, in contrast to the CpGV-M treatment the rate of surviving pupae on CpGV-S was much lower and an influence of the sex on the pupation success was not given.

Table 11 Mortality of neonate larvae subjected to CpGV-M or CpGV-S at the discriminating concentration of 5.8 x 10^4 OB/ml determined after seven and 21 days post infection (p.i.). The mortality was ascertained with the offspring of the female or male crosses between CpRGO and CpS. The male surviving rate was determined in pupae after 21 days. f, female moth; m, male moth; n, number of tested larvae; n.d., not determinate; n.s., no surviving pupae; D, degree of dominance of resistance to CpGV-M or CpGV-S calculated according to Bourguet et al. (Bourguet et al. 2000).

			Subjected to CpGV-M				Subjected to CpGV-S				
			7 days p.i.		21 days p.i.			7 days p.i.		21 days p.i.	
	Crossing Number	n	Mortality [%]	D	Mortality [%]	Surviving male rate [n male : n female]	n	Mortality [%]	D	Mortality [%]	Surviving male rate [n of male : n of female]
Cluster	**Female crosses CpRGOf x CpSm**										
A	#3	12	42	0.58	58	1.00 [5:0]	0	n.d	n.d	n.d	n.d
	#27	44	23	0.78	61	1.00 [17:0]	0	n.d	n.d	n.d	n.d.
	#35	46	20	0.82	63	1.00 [17:0]	16	25	0.76	88	0.50 [1:1]
	#37	45	44	0.55	62	1.00 [17:0]	39	36	0.64	96	0.00 [0:1]
	#38	31	42	0.58	47	1.00 [10:0]	0	n.d	n.d	n.d	n.d
	#49	21	29	0.71	76	1.00 [5:0]	0	n.d	n.d	n.d	n.d.
	#52	45	16	0.86	53	1.00 [21:0]	6	67	0.30	100	n.s.
	#17	0	n.d.	n.d.	n.d.	n.d.	36	81	0.15	100	n.s.
	#46	23	100	-0.05	100	n.s.	0	n.d	n.d	n.d	n.d
mean			39.5	0.60	65.0	1.00 [92:0]		52.3	0.46	96	0.33 [1:2]
	Male crosses CpRGOm x CpSf										
B	#21	45	9	0.93	n.d.	n.d.	40	28	0.73	n.d.	n.d.
	#22	46	4	0.99	4	n.d.	30	31	0.70	91	0.50 [2:2]
	#31	49	4	0.99	8	0.42 [19:26]	50	8	0.95	90	0.25 [1:4]
	#32	39	10	0.92	18	0.44 [14:18]	31	26	0.75	61	0.58 [7:5]
C	#4	47	43	0.57	51	0.30 [7:16]	47	91	0.04	100	n.s.
	#16	49	4	0.98	47	0.43 [10:13]	45	4	0.89	96	0.50 [1:1]
	#20	47	2	1.01	45	0.38 [10:16]	46	28	0.73	87	0.50 [3:3]
D	#6	32	50	0.49	88	0.75 [3:1]	33	13	0.89	76	0.38 [3:5]
mean			15.8	0.9	37.3	0.41 [63:90]		28.6	0.7	0.9	0.46 [17:20]

Male crosses: In total, 19 single-pair male crosses (CpRGOm x CpSf) were performed; eight single pairs produced sufficient offspring for resistance testing. According to their mortality response against CpGV-M the male crosses were grouped into three clusters (B-D). The virus-induced mortality on CpGV-M of the F_1 larvae grouped in cluster B was low after seven days (between 4 % and 10 %) and increased slightly to 18 % (#32) after 21 days. In cluster C, the mortality was between 2 % (#20) and 43 % (#4) after seven days and increased to 45 % (#20) and 51 % (#4) after 21 days. The offspring mortality of the crosses in cluster C raised not higher than 51 % after 21 days, therefore cross #6 was grouped in cluster E with 50 % mortality after seven days and 88 % after 21 days. Sex ratio of the surviving pupae was nearly equal (mean male rate 0.41). The mortality of the neonate larvae subjected to CpGV-S after seven days was between 4 % (#16) and 91 % (#4). After 21 days, mortality increased to 100 %. The mean ratio of male of the surviving pupae after 21 days was 0.46 (Table 11). The fertility of the male and the female crosses was also compared; again the male crosses generated 1.8 times more offspring larvae (ø =129.7/cross) than the female crosses (ø =71.6).

Dominance of inheritance: Degree of dominance was calculated for the mortality rates in the male and female crosses after seven days (Bourguet et al. 2000). The dominance values ranged between -0.05 and 0.86 with a mean of 0.60 for the female crosses subjected to CpGV-M, whereas for CpGV-S the dominance values were between 0.15 and 0.76, with a mean of 0.46. In the male crosses, all dominance values for CpGV-M were close to 1, except for crosses #4 and #6 (Cluster C and D). On CpGV-S the dominance values ranged between 0.04 and 0.95 with a mean of 0.7 (Table 11).

Backcross A: In total, 37 families of the backcrosses A (BC A; F_1m x CpSf) were set-up, of which 19 single pairs generated sufficient neonates for resistance testing (Table 3). On CpGV-M, virus-induced mortality of F_2 offspring of these families varied between 29 % (#52.1) and 100 % after seven days and increased to 57 % (#4.3) and 100 % after 21 days. Strikingly, all F_2 families derived from F_1m cluster A and half of cluster C (#4.3, #4.5 and #4.6) showed a similar variation (29-75 %) after seven and (57-96 %) after 21 days. The mean ratio of surviving male pupae in these families was 0.37. In contrast, the other three families of cluster C (#4.1, #4.2 and #4.4) as well as progeny of cluster D showed 100 % mortality already after seven days, without any surviving pupae (Table 12)

When cohorts of neonates of the same BC A crosses were subjected to CpGV-S the mortality ranged between 6 % (#27.1) and 88 % (#52.3) after seven days and 44 % to 100 % after 21 days. Mean mortality increased after seven to 21 days from 65 % to 81 % on CpGV-M and from 57 % to 91 % on CpGV-S to 16 % for CpGV-M and 34 % for CpGV-S, suggesting a higher but delayed efficacy of CpGV-S. Male ratio of the few surviving pupae after 21 days was 0.37 and 0.54 for CpGV-M and CpGV-S, respectively (Table 12).

Table 12 Mortality [%] of the offspring of the back crosses BC A and BC B exposed to diet containing CpGV-M or CpGV-S at the discriminating concentration of 5.8 x 10^4 OB/ml seven and 21 days post infection (p.i.). The F_1 male moth used for backcrosses originated of the control group of the female or male single-pair crosses between CpRSO and CpS (Table 11). Resulting F_1 males were reared to adulthood and moths were backcrossed by single-pair crosses with female CpS moths (BC A; F_1m x CpSf) or CpRSO female moths (BC B; F_1m x CpRSOf). The rates of surviving male pupae were assessed after 21 days. For more detailed information see Table 11.

	CpGV-M					CpGV-S			
	Crossing number	n	Mortality [%] 7 days p.i.	Mortality [%] 21 days p.i.	Rate of surviving males	n	Mortality [%] 7 days p.i.	Mortality [%] 21 days p.i.	Rate of surviving males
F_1 from cluster	**BC A; F_1m x CpSf**								
A	#27.1	22	32	73	0.33	16	6	44	0.55
	#27.2	9	56	78	0.50	27	33	56	0.58
	#35.1	46	67	96	0.50	45	53	89	0.20
	#35.5	44	48	84	0.16	39	26	77	0.30
	#37.1	41	56	68	0.38	22	45	100	n.s.
	#37.5	43	49	70	0.53	46	48	93	0.66
	#38.1	36	42	72	0.10	n.d.	n.d.	n.d.	n.d.
	#38.5	40	65	90	0.25	n.d.	n.d.	n.d.	n.d.
	#49.1	35	63	82	0.50	n.d.	n.d.	n.d.	n.d.
	#52.1	48	29	60	n.d.	23	48	95	1.00*
	#52.3	42	55	71	0.50	24	88	100	n.s.
C	#4.1	48	100	100	n.s.	47	77	100	n.s.
	#4.2	46	100	100	n.s.	46	80	96	1.00*
	#4.3	42	50	57	0.55	39	59	100	n.s.
	#4.4	32	100	100	n.s.	45	58	98	0.00*
	#4.5	49	53	67	0.25	46	85	100	n.s.
	#4.6	20	75	75	0.20	34	53	100	n.s.
D	#6.1	49	100	100	n.s.	45	76	100	n.s.
	#6.2	44	100	100	n.s.	45	73	100	n.s.
mean			65.3	81.2	0.37		56.8	90.5	0.54
	BC B; F_1m x CpRGOf								
A	#27.3	16	13	44	0.77	18	17	100	n.s.
	#35.4	37	27	70	0.45	17	59	100	n.s.
	#37.2	46	22	43	0.50	13	69	100	n.s.
	#52.2	42	62	76	0.80	n.d.	n.d.	n.d.	n.d.
B	#32.1	25	8	48	0.40	16	38	n.d.	n.d.
	#32.2	7	29	29	0.60	13	8	n.d.	n.d.
mean			31.0	56.7	0.59		38.2	100	n.d.

* only one/two surviving pupae

Table 13 Direct test of monogenic inheritance for CpGV-M and CpGV-S resistance by comparing expected and observed number of dead larvae of neonates obtained from single-pair backcrosses (BC A or BC B). Neonate larvae were subjected to of CpGV-M or CpGV-S for seven days at the discriminating concentration of 5.8×10^4 OB/ml. m, male moth; f, female moth; n, total number of tested individuals. Expected number of dead larvae was calculated according to Tabahnik (Tabashnik 1991) and compared with the observed number by χ^2 test for goodness of fit (*$P < 0.05$).

	CpGV-M						CpGV-S				
			number that died					number that died			
	Crossing number	n	expected	observed	χ^2	*P*	n	expected	observed	χ^2	*P*
F_1 from cluster	**BC A; F_1m x CpSf**										
A	#52.1	48	26.2	14	12.423	<0.05*	23	17.71	11	11.053	<0.05*
	#27.1	22	12.8	7	6.191	<0.05*	n.d.	n.d.	n.d.	n.d.	n.d.
	#35.5	44	24.9	21	1.378	0.24	39	21.8	10	14.588	<0.05*
	#52.3	42	22.9	23	0.001	0.97	24	18.5	21	1.494	0.22
	#27.2	9	5.2	5	0.022	0.88	n.d.	n.d.	n.d.	n.d.	n.d.
	#49.1	35	21.4	22	0.051	0.82	n.d	n.d	n.d	n.d	n.d
	#35.1	46	26.0	31	2.220	0.14	45	25.2	24	0.130	0.72
	#38.1	36	24.3	15	10.952	<0.05*	n.d	n.d	n.d	n.d	n.d
	#37.5	43	29.5	21	7.705	<0.05*	46	28.3	22	3.633	0.06
	#37.1	41	28.1	22	4.185	<0.05*	22	13.5	10	2.392	0.12
	#38.5	40	27.0	26	0.114	0.74	n.d	n.d	n.d	n.d	n.d
E	#4.3	42	28.6	21	6.254	<0.05*	39	34.7	23	35.914	<0.05*
	#4.5	49	33.3	26	5.025	<0.05*	46	40.9	39	0.836	0.36
	#4.6	20	13.6	15	0.450	0.50	34	30.3	18	45.156	<0.05*
	#4.1	48	32.6	48	22.588	<0.05*	47	41.8	36	7.387	<0.05*
	#4.2	46	31.3	46	21.647	<0.05*	46	40.9	37	3.447	0.06
	#4.4	32	21.8	32	15.059	<0.05*	45	40.1	26	44.808	<0.05*
F	#6.1	49	35.0	49	19.531	<0.05*	45	27.0	34	4.537	<0.05*
	#6.2	44	31.5	44	17.538	<0.05*	45	27.0	33	3.333	0.07
	BC B; F_1m x CpRGOf										
A	#27.3	16	2.1	2	0.004	0.95	n.d.	n.d.	n.d.	n.d.	n.d.
	#35.4	37	4.3	10	8.765	<0.05*	17	2.2	10	31.562	<0.05*
	#52.2	42	20.1	26	0.376	0.54	n.d.	n.d.	n.d.	n.d.	n.d.
	#37.2	46	10.8	10	15.142	<0.05*	13	2.4	9	22.190	<0.05*
C	#32.1	25	1.4	2	0.301	0.58	16	2.0	6	9.143	<0.05*
	#32.2	7	0.4	2	7.169	<0.05*	13	1.6	1	0.275	0.60

Backcross B: In the backcross B (BC B; F_1m x CpRGOf) only six out of 18 single-pair families rendered sufficient offspring for resistance testing. Mortality rates of these F_2 neonates on CpGV-M deviated between 8 % (#32.1) and 62 % (#52.2) after seven days and increased to a maximum of 76 % after 21 days. The mean ratio of male and female surviving pupae was slightly shifted (m:f= 0.59:0.41) Five BC B families also could be tested on CpGV-S. Here, mortality ranged between 8 % (#32.2) and 69 % (#37.2) after seven days and increased to 100 % after 21 days for all tested BC B families. Again the increase of mortality from seven days determination to 21 days was clearly higher on CpGV-S (38 % to 100 %) than on CpGV-M (31 % to 56 %) (Table 12)

Determination of monogenic and polygenic inheritance of resistance: The results of all backcrosses (BC A and BC B) were used to test for a monogenic inheritance model of resistance (Tabashnik 1991). The monogenic hypothesis was rejected in 15 out of 25 BC A and BC B crosses for CpGV-M and in 10 out of 18 crosses tested on CpGV-S (Table 13). Hence, a highly heterogeneous signal was found again among the single families, yet supporting a polygenic inheritance in their majority.

4.4 Discussion

Development of field resistance against commercial products containing CpGV-M has been well documented in more than 40 apple plantations in Europe (Fritsch et al. 2005; Sauphanor et al. 2006; Asser-Kaiser et al. 2007; Berling et al. 2009; Schmitt et al. 2013; Zichová et al. 2013; Jehle et al. 2017). In the present study, resistance of SA-GO was tested by using CpGV isolates from different genome groups. The results showed that SA-GO larvae were not susceptible to both, CpGV-M and CpGV-S, concluding that the resistance in SA-GO is directed against two different genome groups A and E of CpGV (Eberle et al. 2008; Gebhardt et al. 2014). However, the isolates CpGV-E2 and CpGV-V15 exhibited resistance-breaking characteristics. The detection of resistance against CpGV-S supports the hint of a new type of resistance, which is different to the type I resistance reported previously for CM in Europe (Asser-Kaiser et al. 2007; Berling et al. 2009; Schmitt et al. 2013; Zichová et al. 2013) but similar to type II resistance described for NRW-WE and CpR5M/CpR5S (Jehle et al. 2017) (Chapter 2).

The results of the single-pair crosses between individuals of SA-GO and CpS showed a clear picture of dominant inheritance through all crosses. Otherwise higher mortality of the hybrid progeny should become obvious. Besides this, also a Z-linkage of resistance factor as it is typical for type I resistance in CM can be rejected. Otherwise, crosses between resistant females and susceptible males should result in death of F_1 females because they have inherited their only Z chromosome from the

susceptible CpS male. However, 0 % mortality after seven days was observed with CpGV-M and CpGV-S for several female crosses (Figure 20A). Even after 14 days two female crosses showed mortality values under 5 %, which is not compatible with a Z-linkage of the resistance gene (Figure 20B). The results from the crosses of SA-GO and CpS give a clear hint for an autosomal inheritance in SA-GO, similar to type II resistance (Chapter 2).

To gain more insight into the resistance in SA-GO, a two-step inbreeding process with single-pair crosses, each followed by resistance testing of the offspring, was conducted. By using this selection procedure, the resulting laboratory strain CpRGO was obtained from a single SA-GOf x SA-GOm pair (#12). The low mortality on CpGV-M and CpGV-S observed in the resistance testing during the selection process of CpRGO suggested that the parental individuals exhibited a low genetic variation in their resistance response to both CpGV isolates.
Nevertheless, when the resulting strain CpRGO was repeatedly tested on either CpGV-M or CpGV-S during three years of rearing, low mortality occurred after seven days but considerable increase to about 30 % mortality on both viruses was observed after 14 days. This finding contrasts type II resistance of the strains CpR5M and CpR5S, where low mortality was detected in bioassays even after 14 and 21 days (Chapter 2).

By generating female and male single-pair crosses and backcrosses with CpS and CpRGO, several conclusions on the inheriting factors of the resistance to CpGV-M and CpGV-S can be drawn. (i) A dominant inheritance trait for the resistance against both CpGV-M and CpGV-S was supported for CpRGO after seven days in the majority of crosses (Table 11). A dominant inheritance was also reported for type I resistance in the strains CpRR1 in Germany (Asser-Kaiser et al. 2007), RGV in France (Berling et al. 2009) and CpR-CZ in the Czech Republic (Zichová et al. 2013) and for the type II resistance of CpR5M and CpR5S (Chapter 2). (ii) Statistical tests supported a polygenic inheritance pattern in the majority of the backcrosses for the resistance against both viruses (Table 13). This observation is in clear contrast to the monogenic mode of inheritance suggested for type I resistance against CpGV-M in CpRR1 (Asser-Kaiser et al. 2007; Berling et al. 2009; Zichová et al. 2013) and for type II resistance in CpR5M (Chapter 2), for which a monogenic mode of inheritance was also identified in the majority of crosses. (iii) Only male pupae survived the CpGV-M treatment in the female crosses after 21 days, suggesting a Z-linked inheritance survivorship similar to type I resistance but clearly different to the autosomal inheritance in the type II resistance (Asser-Kaiser et al. 2007) (Chapter 2). Only a very few male and female pupae developed on CpGV-S, indicating a variation in the efficacy of CpGV-M and CpGV-S in both infection process and sex selection. (iv) No or a very low correlation in the mortality response between treatment of CpGV-M and CpGV-S were

detected in the crosses and backcrosses, also suggesting a somehow different and heterogeneous action of resistance in CpRGO for these two CpGV genome groups. Such a picture of heterogeneity found for CpRGO was also visible in hybrid crosses between the field population SA-GO and CpS (Figure 20A and 20B), but was never observed in type I nor in type II resistance.

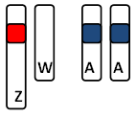
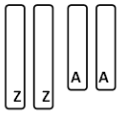
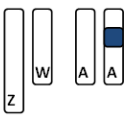
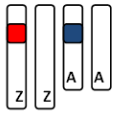
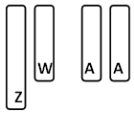
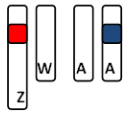
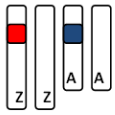
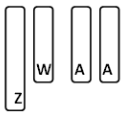
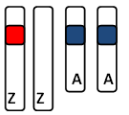
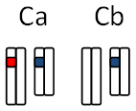
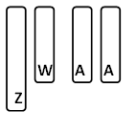
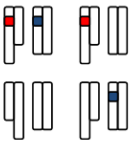
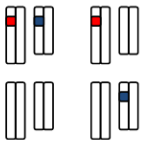
Considering that CpRGO originated from only a single pair (#12) of CM individuals, the heterogeneity in the F_1 crosses as well as in the backcrossing experiments is striking and suggests that resistance is not fully genetically fixed in the genome of this strain. In the following we are trying to interpret most of the observed crossing results by a combination of at least two genome loci or some quantitative aspects of resistance. We propose an inheritance mechanism for the resistance of CpRGO against CpGV-M that is based on at least two loci on an autosome and the Z chromosome and therefore depends on a sex and dose effect (two-gene hypothesis). The advantage of using single-pair crosses is the possibility of generating hypothesis for the chromosomal genotypes for each individual family. By taking all results together it is proposed that survivorship after seven days of the crosses needs at least one resistance gene either on an autosome (blue symbol) or on the Z chromosome (red symbol) (Figure 22). Individuals with only one resistance gene on the autosome but missing a second resistance gene on the Z chromosome are able to survive seven days but do not develop to pupae. This is the case for F_1 females from the female crosses, which obtained their Z chromosome from a susceptible CpS male. Our hypothesis further includes a certain increase of the number of dead larvae until day 21 caused by long-term virus exposure (Figure 12). This is comparable with the observed increased long-term mortality of heterozygous males compared to homozygous resistant males in type I resistance (Asser-Kaiser et al. 2007; Asser-Kaiser et al. 2010).

Our two-gene hypothesis is also compatible with the hypothetical mortality of the F_1 progeny of homogeneous resistant female moths crossed with susceptible CpS male moths ($Z^RWA^RA^R$ x $Z^SZ^SA^SA^S = Z^SWA^SA^R$, $Z^RZ^SA^RA^S$) which fits to all observed mortality of cluster A. For the male crosses, F_1 offspring were clustered in three groups (B-D). The results for cluster B can be explained by selecting a homogeneous male moth ($Z^RZ^RA^RA^R$) and C and D for two different genotypes of heterogeneous individuals ($Z^RZ^SA^RA^R$ or $Z^RZ^SA^RA^S$).

Because clear chromosomal genotypes were generated for the F_1 progeny of the crosses, most of the backcrossing results are explainable: The mortality of BC A, F_2 offspring originating from clusters A, B and Ca, fits to the expected mortality because the selected F_1 male moth had a homogeneous genotype ($Z^RZ^SA^RA^S$). If randomly a F_1 male moth with just one resistance gene ($Z^SZ^SA^RA^S$) for example from cluster Cb was selected, mortality up to 100 % were determined as shown in Figure 22. The hypothesis also matched for the most of the observed results for the BC B.

On the basis of these results, the laboratory strain CpRGO does not exist of only fully genetically homogeneous resistant individuals. At least one female moth was fully susceptible and

heterogeneous males were also randomly chosen for the crosses. Therefore, the two-step inbreeding clearly helped to identify some genetic traits of the resistance in CpRGO but it was not successful to generate a genetically fully fixed resistance and caused high variability in the male and female crosses, as well as in the backcrosses. If such a trait is dominant and polygenic as proposed for CpRGO, it will be very intricate to generate such a genetically homogenous line as it might be difficult to separate heterogeneous from homogeneous individuals.

Female crosses	**F_0: Parental chromosomal genotypes, by hypothesis**		**F_1: Progeny chromosomal genotypes, by hypothesis**		**Expected mortality**		**Ratio of male pupae**	**CL**
	female	male	female	male	**7 days p.i.**	**21 days p.i.**		
Z-linked and autosonal, dominant and polygenetic, homogeneous					0-50 %	>50 %	1.00	A
Male crosses								
Z-linked and autosonal, dominant and polygenetic, homogeneous					0 %	>0 %	0.5	B
Z-linked and autosomal, dominant and polygenetic, heterogeneous					0-50 %	>50 %	0.5	C
Z-linked and autosomal, dominant and polygenetic, heterogeneous					25-75%	>75%	0.5	D

For description of **Figure 22** see next page

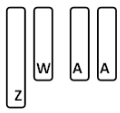
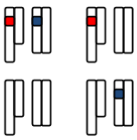
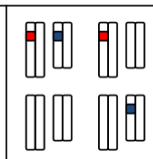
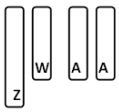
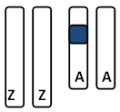
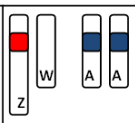
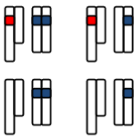
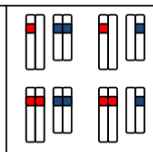
BC A	F_1: Parental chromosomal genotypes, by hypothesis		F_2: Progeny chromosomal genotypes, by hypothesis		Expected mortality		Ratio of male pupae
	female	male	female	male	7 days p.i.	21 days p.i.	
Heterogeneous F_1 males from cluster A, B, Ca					25-75 %	>75 %	0.5
Heterogeneous F_1 males from cluster Cb, D					50-100%	100 %	0.5
BC B							
Heterogeneous F_1 males from cluster A, B, Ca					0-25 %	>25 %	0.75

Figure 22 Hypothetical inheritance schemas for the two-gene hypothesis for the resistance against CpGV-M in CpRGO. Hypothetically expected mortality of the offspring originating from male or female crosses and backcrosses of CpRGO and CpS exposed to the discriminating concentration of CpGV-M. Given are the expected mortality after seven and 21 days post infection (p.i.) and ratio of surviving male pupae. Parental and progeny of the chromosomal genotypes are shown for a dominant, polygenic, mixed (Z-linked and autosomal) inheritance for resistance against CpGV-M. A, autosome; Z and W, sex chromosomes; CL, cluster; F_0, parental moth; F_1, progeny of the crosses; F_2, progeny of crosses of the second generation; red square, resistance gene on the Z chromosome; blue square, resistance gene on the autosome. Expected mortality are estimated for the hypothesis that offspring with one resistance gen survive at least the seven day bioassay but die till 21 days p.i. Progeny with one resistance gene on the autosome and one resistance gene on the Z chromosome mostly survive 21 days p.i. and develop to pupae. Cluster represents crosses from Table 11 and Table 12) related to the hypothetical chromosomal genotypes.

For the CpGV-S treatments low mortality after seven days but a strong increase in mortality until 21 days was detected for nearly all crosses and backcrosses. These findings were independent of the number of resistance allele(s) suggesting a more recessive nature of inheritance of the resistance in CpRGO against CpGV-S, when pupation success of the hybrid offspring is considered. In heterozygous offspring, CpGV-S appeared to be infective though the death of the larvae was highly delayed compared to susceptible larvae. Taking together, neither an autosomal nor a Z-linked inheritance alone can explain the highly complex mortality patterns of the crosses and backcrosses observed in

this study. Therefore, we propose a third type of CpGV resistance in CpRGO which may represent a combination of Z-linked type I resistance and autosomal type II resistance. Crossing experiments of type I and type II resistant individuals may provide a proof of this hypothesis. Some circumstantial evidence for an autosomal and Z-linked inheritance was also suggested for French strain field population St-Andiol, though this trait was also lost during selection experiments rendering only Z-linkage of the descendent strain RGV (Berling et al. 2013). Since it cannot be fully ruled out that some genetic traits become lost during the crossing and selection events, our study also poses some critical questions on the generation of genetically homogenous strains as performed for CpRR1 (type I resistance) and CpR5M (type II resistance). High genetic variability in field populations necessitates generating genetically more homogenous insect strains as a basis for crossing experiments and elucidating inheritance patterns. However, even the selection process, single-pair crosses with parallel resistance testing vs. mass crossing experiments under selection pressure, may result in different fixation scenarios. Therefore, the combination of resistance testing of hybrid crossing experiments from field-collected individuals as well as of laboratory-selected strains with fixed resistance are important to explain the perhaps more complex picture of baculovirus resistance in the field than hitherto anticipated.

5. Comparative analyses of different Cydia pomonella granulovirus (CpGV) isolates in codling moth strains with type I - III CpGV resistance

Abstract

The development of resistance against commercially used Cydia pomonella granulovirus (CpGV) isolates has been challenging the successful control of codling moth (CM) in organic apple plantations. Three types of resistance (type I - III) have been identified and differ strongly in their susceptibility to CpGV isolates belonging to different genome groups.

Systematic resistance testing with CpGV of all genome groups (A-E) was performed with neonates of the susceptible CM strain CpS and the resistant strains CpRR1 (type I resistance), CpR5M and CpR5S (type II resistance), and CpRGO (type III resistance). In addition, isolates of commercial products were tested for their activity in the different susceptible and resistant CM strains. The results show that only CpGV isolates of the genome groups B and C were able to cause significant mortality of larvae of all resistant types. In addition, genome group D (CpGV-I12) caused high mortality in type III resistant CM, whereas type I resistance was broken by all known CpGV genome groups, except genome group type A (CpGV-M).

The commercially used CpGV isolates R5 and 0006 did not break type II resistance, but type I and type III resistance; they caused similar mortality in all tested CM strains. V15 caused high mortality in all resistant strains.

5.1 Introduction

Cydia pomonella granulovirus (CpGV), first described in Mexico (Tanada 1964), belongs to the genus *Betabaculovirus* of the *Baculoviridae* family. Its genome comprises double-stranded circular DNA of 120.8-123.9 kb (Luque et al. 2001; Gebhardt et al. 2014). CpGV isolates are phylogenetically classified into five genome groups A-E (Eberle et al. 2009; Gebhardt et al. 2014). CpGV has a very narrow host range and is highly virulent to early larval stages of the lepidopteran *Cydia pomonella* (codling moth, CM), which is a serious pest in pome fruits almost all over the world (Lacey et al. 2008). CpGV has been successfully used as biological control agent of CM in virtually all apple growing areas worldwide since the early 1990s (Huber 1998).

In 2005, however, CM populations resistant to CpGV products containing the isolate CpGV-M (genome group A) were reported (Fritsch et al. 2005). Since then, more than 40 orchards with a

reduced efficiency of CpGV-M treatments have been identified (Sauphanor et al. 2006; Asser-Kaiser et al. 2007; Schmitt et al. 2013; Zichová et al. 2013). The first described resistance, termed type I, is targeted against the isolate CpGV-M and follows a dominant, Z-linked and monogenetic mode of inheritance (Asser-Kaiser et al. 2007; Berling et al. 2009; Zichová et al. 2013). To control type I resistance in the field, new CpGV isolates were identified, tested and eventually registered as novel biocontrol agent (Jehle et al. 2006b; Eberle et al. 2008; Kienzle et al. 2008; Berling et al. 2009; Graillot et al. 2014; Graillot et al. 2016). Gebhardt et al. (2014) showed that type I resistance could be broken by the isolates CpGV-E2 (genome group B), -I07 (genome group C), -I12 (genome group D) and -S (genome group E). By further molecular studies it was proposed that the resistance-breaking capacity of genome group B-E CpGVs was due to one common trait, a missing repetitive insertion of 24 bp in the ORF *pe38* that was found in CpGV-M (genome group A). The type I resistance is therefore based on the insertion in this ORF (Eberle et al. 2009; Gebhardt et al. 2014).

More recently, two further types of field resistance against CpGV were observed; resistance of the field population NRW-WE was targeted not only against CpGV-M but also against isolates of the genome groups C, D and E (Jehle et al. 2017), suggesting an alternative mode (type II resistance) (Jehle et al. 2017). Accordingly, the laboratory strains CpR5M and CpR5S, derived from NRW-WE, demonstrated that type II resistance followed a dominant, monogenic but autosomal inheritance pattern. Furthermore, a cross-resistance to at least two CpGV isolates, CpGV-M and CpGV-S, was observed (Chapter 2, Chapter 3).

Finally, the CM field population SA-GO was used to generate the laboratory strain CpRGO by single pair inbreeding followed by resistance testing of the offspring and selecting families with a uniform resistance response. CpRGO showed a further type III of resistance to CpGV isolates of the genome groups A and E with a highly complex inheritance and resistance pattern. Type III resistance seemed to be a mixture of type I and type II resistance (Chapter 4).

To analyse the emerging picture of the diversity of CpGV resistance, systematic resistance testing of all currently known CM resistance types I - III with a wide range of CpGV isolates was performed. In addition, the commercially used CpGV isolates R5, 0006 and V15 were tested for their capacity to infect the susceptible and resistance laboratory strains.

5.2 Material and Methods

Insects: The codling moth (CM) strain CpS is susceptible to all CpGV isolates and the resistant strains CpRR1 (type I resistance), CpR5M and CpR5S (type II), and CpRGO (type III) were reared in the Julius Kühn Institute (JKI) under laboratory conditions (for more information about CM strains see Chapter 2 and Chapter 4). All CM strains were reared on modified artificial diet of Ivaldi-Sender

(Ivaldi-Sender 1974) at 26°C with 16/8 h light/dark photoperiod and 60 % relative humidity (Asser-Kaiser et al. 2007).

Virus: Five different Cydia pomonella granulovirus isolates (CpGV-M, -E2, -I07, -I12 and -S) were used for the resistance testing in the CM laboratory strains. Both Isolates CpGV-I07 (genome group C) and CpGV-I12 (genome group D) originated from Iran (Rezapanah et al. 2008; Eberle et al. 2008) (for further information about CpGV isolates see Chapter 2-4). The isolate 0006 from the CpGV products Madex® MAX, V15 from Madex® TOP (Andermatt Biocontrol, Switzlerland) and R5 from the product Carpovirusine® Evo2 (Arysta Lifescience, France) were also used for resistance testing. The number of OB/ml of the commercial products and CpGV isolates were scored using Petroff-Hauser counting camber (depth 0.02 mm) in the dark field optic of a Leica light microscope (DMRBE).

Resistance testing with different CpGV isolates: To test the activity of different CpGV isolates in resistant and susceptible CM strains, first instar larvae were subjected to a resistance testing as described in Undorf-Spahn et al. (2012) and Chapter 2-4. All larval offspring were separated into two to six cohorts, depending on the number of available neonate larvae. The first cohort was the untreated control group, without virus; second to sixth cohort were neonate larvae exposed to artificial diet containing the isolates CpGV-M, -S, -E2, -I12 and -I07, respectively. Purified OB of these isolates were incorporated into modified diet of Ivaldi-Sender (1974) to obtain a final discriminating concentration of 5.8×10^4 OB/ml, which causes >95 % mortality in susceptible CpS neonates after seven days (Asser-Kaiser et al. 2007). Mortality of larvae was determined one, seven and 14 days post infection (p.i.). Each assay consisted of at least 30 individuals and three to nine independent replicates were conducted, all mortality data were corrected for control mortality following Abbott´s Formula (Abbott 1925).

Resistance testing with CpGV products: The susceptibility of neonate larvae of CM laboratory strains was elucidated with the isolates R5, 0006 and V15. OB of the CpGV products as described above, or diluted according to the OB concentration given on the product. Resistance testing was done with the discriminating concentration of 5.8×10^4 OB/ml as described before. All products were tested concurrently in all five CM strains; water instead of viral OB was included in the untreated controls. Each assay consisted of at least 30 individuals and three to five independent replicates were conducted, all mortality data were corrected for control mortality following Abbott´s Formula (Abbott 1925).

Statistical analysis: Differences between strains and treatments were statistically evaluated using ANOVA and post-hoc Tukey HSD test of the means (RStudio edition 2.3.4.4.).

5.3 Results

Resistance testing in different CM strains with CpGV isolates: Neonate larvae of resistant and susceptible CM laboratory strains were tested for resistance by using the discriminating concentration of 5.8×10^4 OB/ml of five different CpGV isolates. For the susceptible strain CpS mortality between 76 % (for CpGV-E2) and 97 % (CpGV-I07) after seven days and between 97 % and 100 % after 14 days were achieved in all CpGV treatments (Figure 23). All tested CpGV isolates were infective for CpS.

For CpRR1 (type I resistance) low mortality in the bioassay with CpGV-M after seven (5 %) and 14 days (11 %) were determined, which differed significantly from the treatments with the isolates CpGV-S, -E2, -I12 and -I07 (ANOVA, post-hoc Tukey HSD test $P < 0.05$). These isolates caused high mortality after seven and 14 days. Therefore, the type I resistance is targeted only against CpGV-M (Figure 23).

CpGV-M caused 3 % mortality in CpR5M and 6 % in CpR5S, and CpGV-S caused 2 % and 6 %, respectively, after seven days. After 14 days, the virus-induced mortality of CpGV-M and CpGV-S increased to a maximum of 12 % (CpGV-M in CpR5S) in CpR5M and CpR5S. The isolate CpGV-I12 caused mortality of 23 % in CpR5M and 1 % in CpR5S after seven days and increased after 14 days to 50 % and 19 %, respectively (Figure 23).The isolates CpGV-E2 and -I07 induced high mortality up to 100 % in CpR5M and 98 % in CpR5S after 14 days. Therefore, the two isolates CpGV-E2 and -I07 were classified as resistance-breaking for the type II resistance.

For neonates of CpRGO, the virus-induced mortality of CpGV-M and CpGV-S was 12 % and 19 %, respectivetly, after seven days. The mortality increased to a maximum of 37 % (CpGV-S) afte 14 days, which differed significantly (ANOVA, post-hoc Tukey HSD test $P < 0.05$) from the determined mortality of the treatments achieved with CpGV-E2, -I12 and -I07.

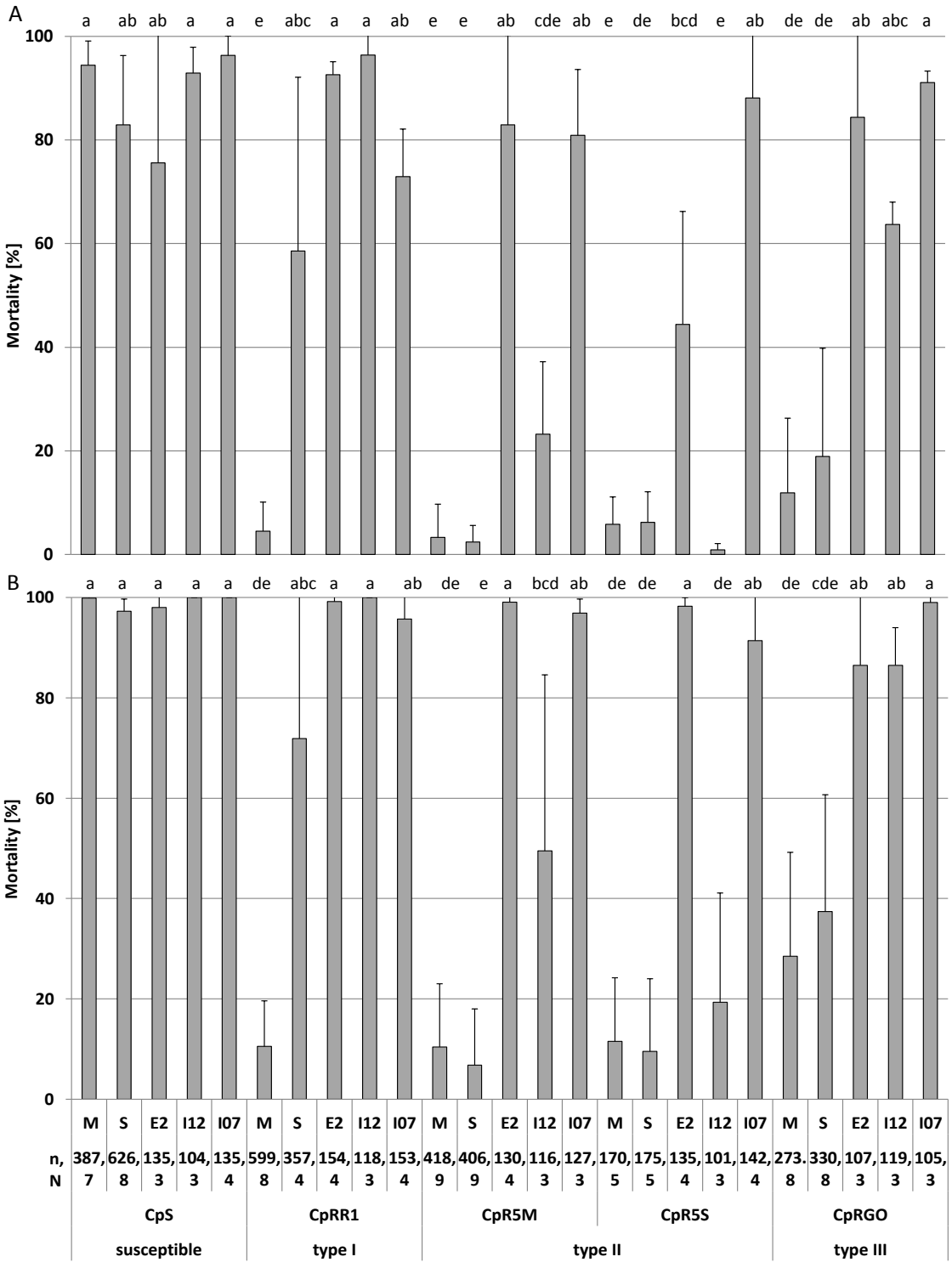
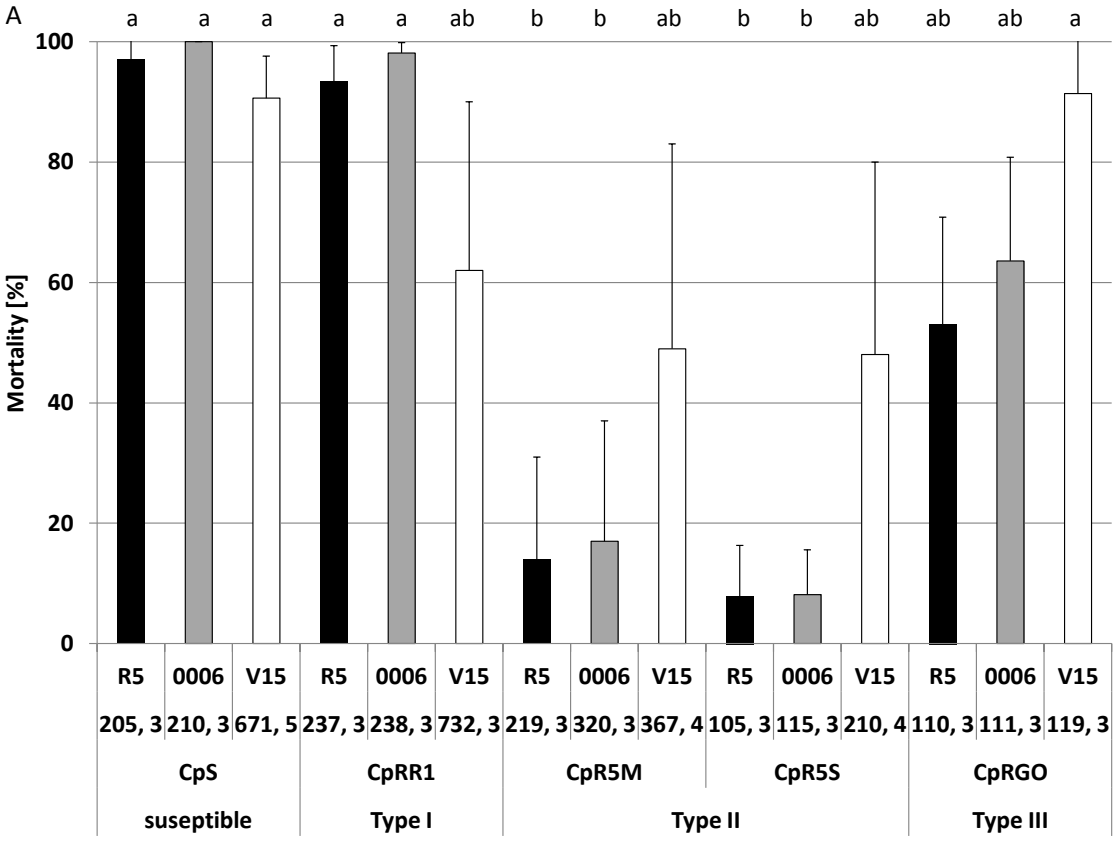


Figure 23 Resistance testing in different CM strains with CpGV isolates. Mortality of neonate larvae of CpS, CpRR1, CpR5M, CpR5S and CpRGO (Type I - III resistance) tested for resistance on artificial diet containing either CpGV-M (M), CpGV-S (S), CpGV-E2 (E2), CpGV-I12 (I12) or CpGV-I07 (I07) at a discriminating concentration of 5.8 x 10^4 OB/ml. Abbott-corrected mean mortality and standard deviations (error bars) were determined seven days (A) and 14 days (B) post infection, total number of tested individuals (n) and numer of independent replicates (N) are given below the chart. Columns marked by different letters differ significantly (ANOVA, post-hoc Tukey HSD test, $P < 0.05$).

Resistance testing with commercial CpGV products: Virus-induced mortality of the commercial isolates R5, 0006 and V15 ranged between 91 % (V15) and 100 % (0006) in CpS neonate larvae after seven days, and increased to 99 % (R5) and 100 % (0006 and V15) after 14 days (Figure 24). No differences were determined in CpS between the different CpGV isolates (ANOVA, post-hoc Tukey HSD test, $P < 0.05$).

For CpRR1, virus-induced mortality of the CpGV isolates R5 and 0006 was high (93 % and 98 %, respectively) after seven days and increased to a maximum of 99 % after 14 days. Mortality caused by V15 was 62 % in CpRR1 larvae after seven days and increased to 86 % (V15) and 98 % (0006 and R5) after 14 days (Figure 24). The determined mortality of the neonates of the strains CpR5M and CpR5S was between 8 % and 17 % for the isolates R5 and 0006 after seven days and increasing to a maximum of 37 % after 14 days. V15 caused moderate mortality (48 % and 49 %, respectively) after seven days and increased to 85 % after 14 days in both CpR5M and CpR5S. For Neonates of CpRGO mortality of 53 % subjected to R5 was determined. The isolate 0006 caused mortality of 64 % and V15 of 91 % in CpRGO larvae after seven days. The mortality in CpRGO larvae increased to a maximum of 100 % (V15) after 14 days (Figure 24).

The results clearly indicated a high virulence of the isolates R5 and 0006 against the strains CpS, CpRR1 and also an effect on CpRGO, but low infectivity for CpR5M and CpR5S. Only the isolate V15 was able to cause >80 % mortality in all tested CM strains after 14 days.



For description of **Figure 24A** see next page

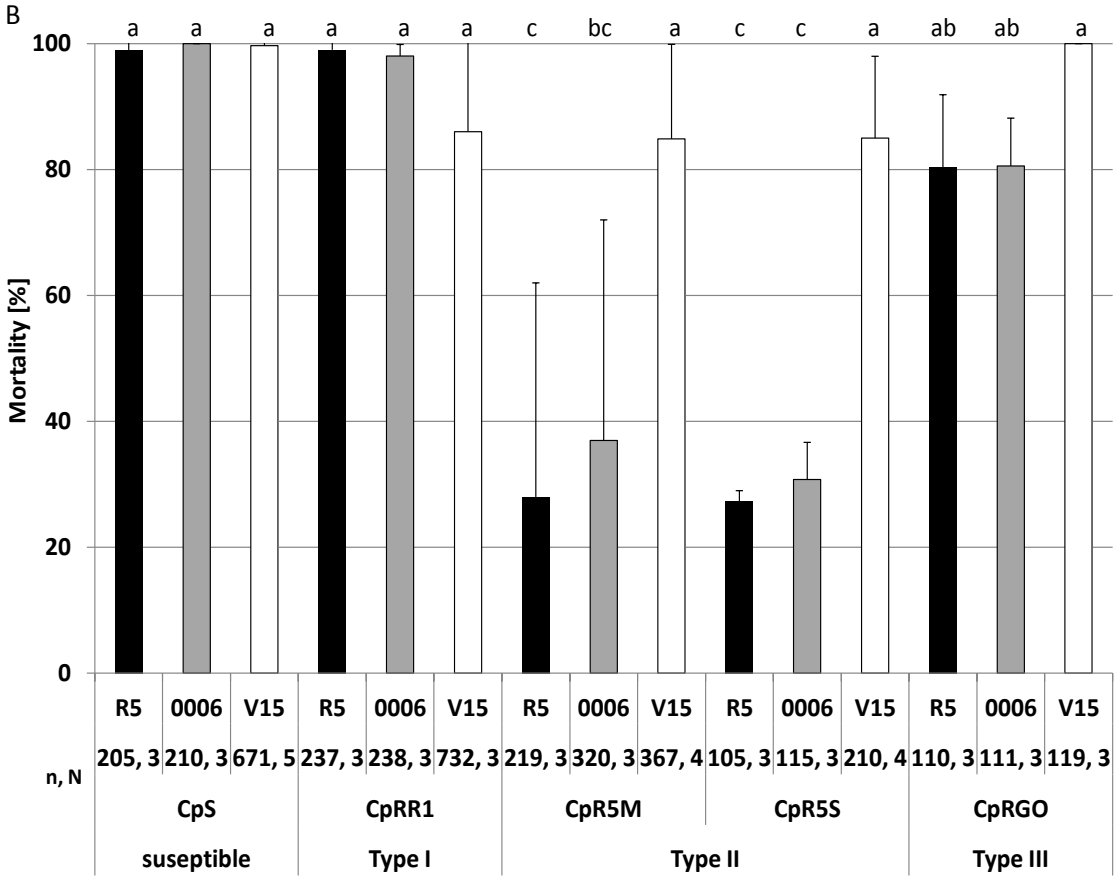


Figure 24 Resistance testing in different CM strains with commercial isolates. Mortality of neonates of CpS, CpRR1, CpR5M, CpR5S and CpRGO (type I - III resistance) tested for resistance on artificial diet containing the commercial CpGV isolates R5 (black bars), 0006 (gray bars) or V15 (white bars) at the discriminating concentration of 5.8 x 10^4 OB/ml. Abbott-corrected mean mortality and standard deviations (error bars) were determined seven (A) and 14 days (B) post infection. The total number of tested individuals (n) and independent replicates (N) are given below the chart. Columns marked by different letters differ significantly (ANOVA, post-hoc Tukey HSD test, $P < 0.05$).

5.4 Discussion

Highly virulent CpGV isolates and products are indispensible for a successful control of CM for organic and integrated pome fruit production (Lacey et al. 2008). With the first observation of CpGV field resistance, the screening for resistance-breaking CpGV isolates as well as the investigation of the CM resistance mechanisms in different host populations has been initiated. A comprehensive comparison of the infectivity of a wide range of CpGV isolates and novel CpGV products to the known CM resistance types I - III is reported in this chapter and summarized in Table 14.

Table 14 Resistance-breaking characteristics of CpGV genome groups and commercial isolats tested in different resistant CM strains (type I - III). Red O stands for observed resistance, mortality <40 %; orange X/O stands for larvea not full susceptible, mortality 40-80 %; and green X stands for genome groupe is highly virulent, mortality >80 %.

7 days p.i.	Genome group(s)					Isolates		
Type of CM resistance	A	B	C	D	E	R5	0006	V15
Type I	O	X	X/O	X	X/O	X	X	X/O
Type II	O	X	X	O	O	O	O	X/O
Type III	O	X	X	X/O	O	X/O	X/O	X
14 days p.i.								
Type I	O	X	X	X	X/O	X	X	X
Type II	O	X	X	X/O	O	O	O	X
Type III	O	X	X	X	O	X	X	X

As expected, CpS larvae were susceptible to all genome groups (A-E), causing similar high mortality after seven and 14 days. In a previous study it was reported that isolates belonging to the genome group C (CpGV-I07) induced lower mortality compared to those of group A and D in CpS larvae, suggesting a lower virulence of CpGV-I07 in CpS (Eberle et al. 2009). We could not confirm this result, since mortality of 96 % was already achieved after seven days in CpS larvae treated with CpGV-I07 (genome group C). Eberle et al. (2009) applied a CpGV concentration of 2000 OB/ml, which was 29 times lower than the discriminating concentration used in this study. Hence, further comparative tests will be necessary to prove whether there is a concentration-dependent effect, causing a disproportionate reduction in mortality at low concentrations of CpGV-I07 as observed by Eberle et al. (2009).

Previous studies proposed that type I resistance in CpRR1 is only directed against genome group A, whereas other genome groups of CpGVs break this type of resistance (Gebhardt et al. 2014). Resistance testing with the ancestor strain CpR of CpRR1 identified high mortality subjected to CpGV-I12 (genome group D) and low mortality to CpGV-M (genome group A) (Eberle and Jehle 2006; Eberle et al. 2008). For CM resistance strain CpR-CZ from the Czech Republic, high virus-induced mortality

was determined in infections with CpGV of the genome groups B, C and E, but very low for genome group A (Zichová et al. 2013). Another CM resistance strain from France also appeared to be highly resistant to genome group A (Graillot et al. 2014).

In a previous study by Jehle et al. (2017), the field population NRW-WE showed low susceptibility to isolates CpGV-M, -I12, -S and -I07 (genome groups A, C-E), but could be infected by CpGV-E2 and V15. The same results, except for CpGV-I07 (genome group C), were now obtained for the two resistant strains CpR5M and CpR5S. In CpR5M and CpR5S the isolate CpGV-I07 caused a similar mortality as CpGV-E2 and differed in its virulence from that for NRW-WE. Since CpR5M and CpR5S originated from NRW-WE, it is possible that the resistance of NRW-WE against CpGV-I07 disappeared during the selection process resulting in CpR5M and CpR5S. CpRGO responded differently to type I and type II resistance. Next to group B and C, also the genome group D (CpGV-I12) CpGVs were highly infective to larvae of CpRGO after seven and 14 days. This difference in the resistance-response to the type I and type II resistance also supports the classification of the strain CpRGO into a third resistance group (Chapter 4).

The commercial isolates R5 and 0006 showed a very similar response in the tested strains: CpS and CpRR1 were fully susceptible; CpR5M and CpR5S were highly resistant, whereas CpRGO showed a moderate susceptibility after seven days and full susceptibility after 14 days. CpGV-V15 was highly infective for all strains, though a delayed efficacy became obvious in CpRR1, CpR5M and CpR5S, as suggested by the moderate mortality caused after seven days p.i.

Genome sequencing and quantification of single nucleotide polymorphism (SNPs) specific to different genome groups of CpGV revealed that the commercial products containing compositions of two isolates: Both R5 and 0006 consist of a mixture of genome group A (for R5 35 % and for 0006 25 %) and E (65 % and 74 %, respectively) whereas V15 consists of the genome group B (41 %) and E (57 %) (G. Gueli Alletti; A. J. Sauer; J. A. Jehle, unpublished).

The results in the resistance testing in type I resistance with R5 and 0006 (98 % mortality) compared to the resistance testing with single genome groups A and E (11 % and 72 %, respectively) revealed a synergistic effect, because higher mortality rates were identified for the already mixed commercial isolates. Similar effects were also identified in larvae of the resistance CM strain RGV from France, where some synergy of mixed application of CpGV-M and the resistant-breaking CpGV-R5 in infection experiments were noticed (Graillot et al. 2016).

Nevertheless, synergistic effects of R5 or 0006 could not be indicated for type II resistance. This was also determined in previous resistance testing in both single applications and co-infections of CpGV-M and CpGV-S (Chapter 3).

On the other hand, V15 (mixture of genome group B and E), induced lower mortality (85 % after 14 days) than the single application of CpGV-E2 (maximum of 99 %) in larvae of the type II resistance, therefore a synergistic effect for this mixture type was not assumed.

The similarity of R5 and 0006 was identified in both their bioactivity in different CM strains and their composition of the genome groups A and E. The rotation of these commercial isolates in the field for resistance management on the basis of applying different genome groups is neither beneficial nor harmful. For the resistance testing of V15 all types of resistance in CM were susceptible, at least after 14 days and this commercial isolate is therefore a good biocontrol agent for controlling resistant CM populations in the field.

6. General Discussion and Conclusion

The purpose of this thesis was to elucidate the inheritance and mechanisms of resistance to different Cydia pomonella Granulovirus (CpGV) isolates observed in naturally occurring codling moth (CM) field populations. Based on previous investigations, it was suggested that CpGV resistance in the populations NRW-WE und SA-GO differed from type I resistance, originally found in CpRR1 (Asser-Kaiser et al. 2007; Jehle et al. 2017). In depth analyses of the resistance in NRW-WE and SA-GO resulted in the discovery of two novel resistance types: type II resistance in the CM strains CpR5M and CpR5S, which derived from NRW-WE, and type III resistance in CpRGO, which was obtained from SA-GO. Both type II and type III resistances show specific characteristics which depend on different factors including the genetic inheritance pattern, cross-resistance, the cellular and molecular resistance mechanism, fitness cost as well as the specificity against certain CpGV isolates or genome groups. In the following, these aspects are recapped and discussed particularly in regard to possible resistance management strategies.

With the identification of type II (**Chapter 2** and **Chapter 3**) and type III (**Chapter 4**) resistance, three different types of resistance against CpGV are now identified in German apple plantations. It appeared that CpGV resistance is genetically highly variable in both the mode of inheritance and the resistance mechanism. All currently located field populations resistant to CpGV and those field populations which were the origin to the different laboratory-selected CM strains analyzed in this thesis are depicted in Figure 25.

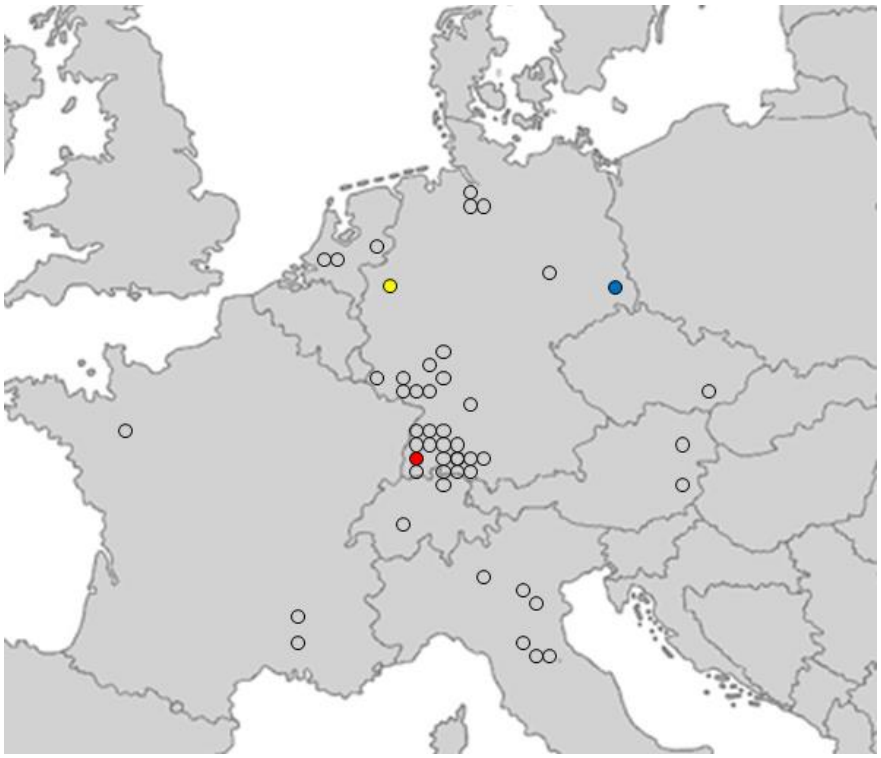


Figure 25 Currently known apple plantations with field resistance to CpGV in Europe (as of January 2017). Every circle indicates an orchard with resistance against CpGV. Colored dots indicate the original locations of plantations with type I resistance (CpR, CpRR1) (red), type II resistance (NRW-WE, CpR5M and CpR5S) (yellow) and type III resistance (SA-GO; CpRGO) (blue) (Data from Table 1).

The high number of already documented resistant field populations of CM strongly suggests that resistance against CpGV can emerge rapidly in populations under selection pressure. Several findings support this hypothesis:

Fitness cost can affect a wide range of biological properties of the pest, such as fecundity, fertility, weight and survival rates under exposition to stress. For resistance against chemicals, alleles responsible for resistance are rare before exposure and have been commonly presumed to have negative effects on fitness in the absent of control agent (Groeters et al. 1994). For some baculoviruses resistance achieved by selection experiments, reversion of resistance was shown when selection was discontinued for several generations (Briese 1986; Fuxa and Richter 1989). In contrast, resistance to CpGV-M in CpR (type I resistance) remained stable for at least 30 generations without exposure to the virus, concluding that at least under laboratory conditions the resistance to CpGV-M in CpR is not associated with fitness costs (Undorf-Spahn et al. 2012). Occasionally repeated resistance testing of individuals of CpR5M (type II resistance) and CpRGO (type III resistance) during the last years gave no hint for an increase in susceptibility to the virus (**Chapter 2-5**).

Furthermore, the rapid spread of resistance in one population is due to the pest behavior and the orchard design. CM has a flight range of 150-200 m depending on the climate condition (Wildbolz and Baggiolini 1959) and it is likely that they do not spread between different orchards.

The CM populations described in this thesis had been controlled in the field for many years with CpGV products based on the CpGV-M isolate only. The populations NRW-WE and SA-GO attracted attention because their resistance level to CpGV-M treatments was untypically high (Asser-Kaiser et al. 2007; Schmitt et al. 2013) and application of novel resistance-breaking CpGV isolates failed to reduce damage caused by CM. By further crossing and infection experiments in the laboratory an additional resistance to CpGV-S was identified for these field populations (Jehle et al. 2017) (**Chapter 2** and **Chapter 4**). Resistance to CpGV-S appeared to have established in these orchards even without selection pressure by this specific isolate.

Before the resistance mechanism and inheritance in the two field populations NRW-WE and SAGO could be unveiled, genetically homogeneous resistant strains had to be established. Two selection methods were used in this thesis. This involved continuous inbreeding by single pair crosses followed by resistance testing of the offspring and selecting families with a uniform resistance response as described for the type III resistance in CpRGO (**Chapter 4**). The other method was mass crosses and selection under virus pressure followed by rearing of the survivors of the treatment as described for the type II resistance strains (**Chapter 2**). These two methods have been commonly used in other studies to generate CM strains with genetically fixed resistances (Asser-Kaiser et al. 2007; Berling et al. 2009; Zichová et al. 2013)

Hybrid crossing experiments with susceptible and resistant individuals are commonly used to disclose the mode of inheritance to an autosomal or sex-linked pattern. CM, like most Lepidoptera, carries a ZW/ZZ sex chromosome system, females being heterogametic (ZW) and males the homogametic (ZZ) sex (Traut et al. 2007). In case of type I resistance of CpRR1, the resistance allele was located on the Z chromosome (Asser-Kaiser et al. 2007). Here, female with only one copy of the resistance allele are fully resistant and will pass the resistance faster through the population than an autosomally inherited resistance (Asser-Kaiser et al. 2007). For type II resistance, the inheritance mechanism was verified as autosomal (**Chapter 2**), indicating a clear difference to type I resistance. Fluorescence *in situ* hybridization (FISH) was used to rule out the hypothesis that a break of the Z chromosome of CpR5M caused the shift from Z-linked resistance as found in CpRR1 to the autosomal inheritance typical for CpR5M. The FISH experiments showed a highly similar arrangement of marker genes on the Z chromosome of susceptible and type I and type II resistant CM strains, clearly indicating

separate mechanisms involved in the different resistances types. The hypothesis that the type II resistance could be explained by a translocation of a Z-chromosomal segment to an autosome and might therefore be based on the same trait as type I resistance was rejected at first.
Nevertheless, CpR5M and CpR5S were generated by selection under virus pressure; it could be possible that additional inheritance factors or phenotypic plasticity become lost during the selective inbreeding while generating genetically homogenous strains (**Chapter 2**).
In contrast, the strain CpRGO was established through single pair crosses with subsequent resistance testing of the offspring and screening for resistant families. Here, the rearing on virus pressure was deliberately avoided for retaining unclear heterogenic resistance mechanism or unknown inheritance factors.
For the resulting type III resistance, an autosomal and Z-linked inheritance pattern is proposed, since Z-linkage of resistance was needed for pupation in CpRGO larvae infected with CpGV-M (**Chapter 4**). The two-gene hypothesis for CpRGO was developed on the basic of clear parental and progeny chromosomal genotypes, which only could be generated through the using of single pair crosses. This diversity and heterogeneity in resistance of CpRGO against CpGV-M and CpGV-S may only be visible because of the selection without virus pressure.

Mass crosses of NRW-WE and selection either on CpGV-M or on CpGV-S resulted in two strains, CpR5M and CpR5S, which were both cross-resistant to CpGV-M and CpGV-S. Thus, resistance against CpGV-M and CpGV-S appear to be somehow genetically linked and an identical functional resistance mechanism against both CpGV-M and CpGV-S was assumed for the type II resistance. By using different combinations of wild-type and recombinant CpGV viruses, it was determined that the resistance mechanisms against CpGV-M and CpGV-S are not similar (**Chapter 3**). Furthermore, resistance against CpGV-M is systemic and targeted against the *pe38* gene and resistance to CpGV-S is the consequence of an unidentified midgut factor. This finding implies that the mechanism of the resistance to CpGV-M is similar in both type I and type II resistance.

All three resistance types I - III varied in their susceptibility to CpGV isolates of different genome groups (**Chapter 5**). Type I resistance is targeted against genome group A only, type II resistance is targeted against genome group A, D and E, and type III resistance is targeted against genome group A and E only. These findings show that only CpGV isolates of the genome group B and C and the commercially available isolate V15 were able to cause significant mortality in all resistance types.
It must be noted, however, that the resistance testing was evaluated after seven and 14 days. Some resistance-breaking isolates caused only moderate mortality (lower than 80 %) in CM larvae after seven days and developed full mortality only after 14 days of virus exposure (**Chapter 5**). Thus, a

delayed efficacy of resistance-breaking isolates is observable in some of the resistance CM strains. Applying these findings to the situation in the field could mean that a sufficient control of resistance CM may be delayed and that larvae continue to feed for several days. In these cases, the feeding damage will be higher, although the larvae become virus-infected and die in the last consequence.

All investigations on CpGV resistance presented in this thesis were performed under laboratory conditions. Hence, some quantitative aspects, such as time to death or mortality rates at specific OB concentrations, may be hardly extendable to the field situation. Nevertheless, the compiled knowledge of the complex inheritance patterns, differences in susceptibility to different CpGV genome groups, as well as the investigations concerning the mechanisms of type I - III resistance provide a comprehensive understanding and allow important conclusions, as summarized above, to improve resistance management of CpGV in CM field populations.

Resistance management of CpGV

A combination of different control techniques, following the concept of integrated pest management (IPM) (Bajwa and Kogan 2002), is the most promising solution to minimize the development of resistance of pests against a specific control agent.

The basis for biological control of CM is good sanitation in the orchard with a frequent removal of infested fruits (Kienzle 2010). Apart from that, storage of wooden piles and billets or other hidden places where CM larvae can hide and pupate should be avoided in the surroundings of the orchards. Especially for organic growers only a small variety of biological control measures are available for CM control. Pheromone-based mating disruption, use of beneficial organism and insect pathogens, as described in **Chapter 1**, are some of the few alternatives to CpGV application for the biological control of CM. All of them are more sensitive to environmental conditions, such as temperature, wind or humidity, their handling is more complex and their efficacy of CM is often lower. Therefore, grower of organic and integrated fruit production rely on an effective CpGV-based control against CM.

Novel CpGV products with resistance-breaking isolates were successfully commercialized and their application succeeded to control resistant CM populations in most cases. While applying CpGV isolates that are still resistance-breaking, some aspects have to be considered:

The novel commercial isolates CpGV-R5 and 0006 are supposed to consist of isolates with a highly similar composition of genome group A and E CpGVs. A very similar efficacy to each other was shown when larvae from different resistance types (I - III) were infected with the two isolates (**Chapter 5**). Therefore, it is unlikely that rotation of the commercial products containing these isolates will improve the success of CM control in the field or will contribute to a favorable resistance

management for CpGV. In contrast, rotation of products with CpGV from different genome groups is desirable.

Maybe the application of one genome group to control the first generation and rotation to product based on a further genome group can be effective. An alternative would be rotation from different CpGV genome groups from one year to another. The best application and rotation strategy to sustain the efficacy of novel CpGV isolates as long as possible needs to be designed and evaluated. Next to that, the screening for new resistance-breaking isolates needs to be continued. Since diversity of current CpGV is restricted, an improvement of efficacy by mixing resistance-breaking isolates with resistance-prone ones could generate a better control effect. Some synergistic effects were shown in the laboratory (Graillot et al. 2016) and also mixed genome group characters of the commercial isolates R5 and 0006 seemed to have slightly better efficacy than the pure viruses (**Chapter 5**). Nevertheless, monitoring of the effectiveness of field treatments is highly recommended; by doing this, emergence of resistance can be detected early and an adaption to alternative control strategies can be realized.

CpGV resistance is the most thoroughly investigated example of host resistance against a baculovirus. The identification of two novel types of CpGV resistance in CM significantly expanded the current picture of adaptation of the insect host to baculovirus infection. This body of knowledge together with rather simple rearing conditions of CM in the laboratory and the availability of CM cell lines for molecular analyses and functional genetics of CpGV genes make CM resistance to CpGV an excellent model organism for investigating host-baculovirus adaption. The understanding of the cellular and molecular basis of resistance, initiated in this thesis, is essential to optimize further biocontrol products with baculoviruses as active ingredients. Additionally, the investigations on the resistance mechanism may be useful when Lepidoptera are commercially mass reared for economic purposes, such as *Bombyx mori* for silk production in sericulture, CM for sterile insect technique and caterpillars for feed and food production where baculovirus infections are undesired and a considerable economic threat. Beyond the significant economic importance of baculovirus resistance for agricultural practice and the solutions provided by this thesis, investigating CM resistance to CpGV is a fascinating field of research which allows understanding the complexity of virus-host interaction in insects and to study the adaptability of Lepidoptera to baculovirus infections in general.

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. Journal of Economic Entomogy 18:265-267.

Abot AR, Moscardi F, Fuxa JR, SosaGomez DR, Richter AR (1996) Development of resistance by *Anticarsia gemmatalis* from Brazil and the United States to a nuclear polyhedrosis virus under laboratory selection pressure. Biological Control 7 (1):126-130.

Adams JR, Bonami JR (1991) Atlas of Invertebrate Viruses. In: Atlas of Invertebrate Viruses. CRC Press, Boca Raton, USA.

Ahola V, Lehtonen R, Somervuo P, Salmela L, Koskinen P, Rastas P, Valimaki N, Paulin L, Kvist J, Wahlberg N, Tanskanen J, Hornett EA, Ferguson LC, Luo SQ, Cao ZJ, de Jong MA, Duplouy A, Smolander OP, Vogel H, McCoy RC, Qian K, Chong WS, Zhang Q, Ahmad F, Haukka JK, Joshi A, Salojarvi J, Wheat CW, Grosse-Wilde E, Hughes D, Katainen R, Pitkanen E, Ylinen J, Waterhouse RM, Turunen M, Vaharautio A, Ojanen SP, Schulman AH, Taipale M, Lawson D, Ukkonen E, Makinen V, Goldsmith MR, Holm L, Auvinen P, Frilander MJ, Hanski I (2014) The Glanville fritillary genome retains an ancient karyotype and reveals selective chromosomal fusions in Lepidoptera. Nature Communications 5.

Asser-Kaiser S (2009) On the inheritance and mechanism of baculovirus resistance of the codling moth, *Cydia Pomonella* (L.). Dissertation, Fachbereich Biologie, Johannes Gutenberg-Universität, Mainz.

Asser-Kaiser S, Fritsch E, Undorf-Spahn K, Kienzle J, Eberle KE, Gund NA, Reineke A, Zebitz CPW, Heckel DG, Huber J, Jehle JA (2007) Rapid emergence of baculovirus resistance in codling moth due to dominant, sex-linked inheritance. Science 317 (5846):1916-1918.

Asser-Kaiser S, Radtke P, El-Salamouny S, Winstanley D, Jehle JA (2010) Baculovirus resistance in codling moth (*Cydia pomonella* L.) caused by early block of virus replication. Virology 410 (2):360-367.

Ayres MD, Howard SC, Kuzio J, Lopezferber M, Possee RD (1994) The complete DNA-Sequence of *Autographa californica* nuclear polyhedrosis virus. Virology 202 (2):586-605.

Bajwa WI, Kogan M (2002) Compendium of IPM Definitions (CID). What is IPM and how is it defined in the worldwide literature. Integrated Plant Protection Center (IPPC) Oregon State University, Online: http://rl.zf.jcu.cz/GGTSPU-eridanus.jki.bund.de-795-5138847-XMEPYVbvphrVf1Tb-DAT/docs/ruzne/ruz-IOR-IPM-definice-58e12d8762.pdf (23.2.2017).

Barnes BN (1982) Snout Beetles - guidelines for the optimal use of cardboard trunk banding in apple orchards. Deciduous Fruit Grower 32:324-328.

Barnes BN, Hofmeyr JH, Groenewald S, Conlong DE, Wohlfarter M (2015) The sterile insect technique in agricultural crops in South Africa: a metamorphosis .... but will it fly? African Entomology 23 (1):1-18.

Barnes MM (1991) Codling moth occurrence, host race formation, and damage. In: By Van der Geest LPS, Evenhuis HH (Eds),Tortricid pests: their biology, natural enemies and control. Elsevier, Amsterdam, The Netherlands 313-327.

Beas-Catena A, Sanchez-Miron A, Garcia-Camacho F, Contreras-Gomez A, Molina-Grima E (2014) Baculovirus biopesticides: An overview. Journal of Animal and Plant Sciences 24 (2):362-373.

Beers EH, Stuckling DM, Prokopy RJ, Avila J (2003) Ecology and management of apple arthropod pests. In: Ferree DC, Warrington IJ (Eds.), Apples: botany, production and uses, CABI Publishing, Wallingford, UK 489-514.

Berling M, Blachere-Lopez C, Soubabere O, Lery X, Bonhomme A, Sauphanor B, Lopez-Ferber M (2009) *Cydia pomonella granulovirus* genotypes overcome virus resistance in the codling moth and improve virus efficiency by selection against resistant hosts. Applied Environmental Microbiology 75 (4):925-930.

Berling M, Sauphanor B, Bonhomme A, Siegwart M, Lopez-Ferber M (2013) A single sex-linked dominant gene does not fully explain the codling moth's resistance to granulovirus. Pest Management Science 69 (11):1261-1266.

Blomefield TL, Giliomee JH (2011) Effect of temperature on the oviposition, longevity and mating of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). African Entomology 19 (1):42-60.

Börner H (1997) Krankheitserreger und Schädlinge: Lepidoptera. In: Pflanzenkrankheiten und Pflanzenschutz. Ulmer, Stuttgart, Germany 394-401.

Bourguet D, Genissel A, Raymond M (2000) Insecticide resistance and dominance levels. Journal of Economic Entomology 93 (6):1588-1595.

Brandt CR, Adang MJ, Spence KD (1978) Peritrophic membrane – Ultrastructural analysis and funktion as a mechanical barrier to microbial infection in *Orgyia pseudotsugata*. Journal of Invertebrate Pathology 32 (1):12-24.

Briese DT (1986) Insect resistance to baculoviruses. In: Granados RR, Federici BA (Eds.), Biology of baculoviruses. Practical application for insect control. vol. 2, CRC Press, Boca Raton, USA 238-263.

Briese DT, Mende HA (1983) Selection for increased resistance to a granulosis-virus in the potato moth*, Phthorimaea operculella* (Zeller) (Lepidoptera, Gelechiidae). Bulletin of Entomological Research 73 (1):1-9.

Bush MR, Abdelaal YAI, Rock GC (1993) Parathion resistance and esterase-activity in codling moth (Lepidoptera, Tortricidae) from North-Carolina. Journal of Economic Entomology 86 (3):660-666.

BVL Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (2017) List of authorised plant protection products in germany with information on terminated authorisations. Online: https://www.bvl.bund.de/DE/04_Pflanzenschutzmittel/01_Aufgaben/02_ZulassungPSM/01_ZugelPSM/psm_ZugelPSM_node.html (23.2.2017).

Calkins CO, Faust RJ (2003) Overview of area-wide programs and the program for suppression of codling moth in the western USA directed by the United States Department of Agriculture Agricultural Research Service. Pest Management Science 59:601–604.

Carpenter JE, Bloem S, Marec F (2005) Inherited sterility in insects. In: Dyck VA, Hendrichs J, Robinson AS (Eds), Sterile insect technique. principles and practice in area-wide integrated pest management, Springer, Dordrecht, The Niederlands 115-146.

Cartier L (2014) A benefit-cost analysis of the okanagan kootenay sterile insect release program. Online: http://www.oksir.org/GGTSPU-eridanus.jki.bund.de-795-2632101-nVVE4SYscutBmxtK-DAT/wp-content/uploads/2016/05/SIR-Regular-Board-Minutes-06-20-2014.pdf (22.2.2017).

Chikhalya A, Stephens KD, Archie JW, Haas-Stapleton EJ (2013) Virulence and pathogenesis of the baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV) in *Pseudoplusia includens* larvae. Biological Control 65 (1):101-108.

Crook NE, Spencer RA, Payne CC, Leisy DJ (1985) Variation in *Cydia pomonella* granulosisvirus isolates and physical maps of the DNA from three variants. Journal of General Virology 66:2423-2430.

Cross JV, Solomon MG, Babandreier D, Blommers L, Easterbrook MA, Jay CN, Jenser G, Jolly RL, Kuhlmann U, Lilley R, Olivella E, Toepfer S, Vidal S (1999) Biocontrol of pests of apples and pears in northern and central Europe: 2. Parasitoids. Biocontrol Science and Technology 9 (3):277-314.

d'Alencon E, Sezutsu H, Legeai F, Permal E, Bernard-Samain S, Gimenez S, Gagneur C, Cousserans F, Shimomura M, Brun-Barale A, Flutre T, Couloux A, East P, Gordon K, Mita K, Quesneville H, Fournier P, Feyereisen R (2010) Extensive synteny conservation of holocentric chromosomes in Lepidoptera despite high rates of local genome rearrangements. Proceedings of the National Academy of Sciences of the United States of America 107 (17):7680-7685.

Dunley JE, Welter SC (2000) Correlated insecticide cross-resistance in azinphosmethyl resistant codling moth (Lepidoptera : Tortricidae). Journal of Economic Entomology 93 (3):955-962.

Dyck VA, Hendrichs J, Robinson AS (2005) Steril Insect Technique. In: Principles and Practice in Area-Wide Integrated Pest Management. Springer, Dordrecht, The Niederlands.

Eberle KE, Jehle JA (2006) Field resistance of codling moth against *Cydia pomonella* granulovirus (CpGV) is autosomal and incompletely dominant inherited. Journal of Invertebrate Pathology 93 (3):201-206.

Eberle KE, Asser-Kaiser S, Sayed SM, Nguyen HT, Jehle JA (2008) Overcoming the resistance of codling moth against conventional *Cydia pomonella* granulovirus (CpGV-M) by a new isolate CpGV-I12. Journal of Invertebrate Pathology 98 (3):293-298.

Eberle KE, Sayed S, Rezapanah M, Shojai-Estabragh S, Jehle JA (2009) Diversity and evolution of the *Cydia pomonella* granulovirus. Journal of General Virology 90:662-671.

Eggleton P, Gaston KJ (1990) Parasitoid species and assemblages – Convenient definitions or misleading compromises. Oikos 59 (3):417-421.

Engelhard EK, Kammorgan LNW, Washburn JO, Volkman LE (1994) The insect tracheal system – A conduit for the systemic spread of *Autographa californica* M nuclear polyhedrosis virus. Proceedings of the National Academy of Sciences of the United States of America 91 (8):3224-3227.

Epstein DL, Zack RS, Brunner JF, Gut L, Brown JJ (2000) Effects of broad-spectrum insecticides on epigeal arthropod biodiversity in Pacific Northwest apple orchards. Environmental Entomology 29 (2):340-348.

Federici BA (1997) Baculovirus pathogenesis. In: LK Miller (Ed), The Baculoviruses, New York Plenum Press, USA.

Feng F, Fu J, Hu P, Zahang X, Yao Q, Chen K (2012) Genetic analysis of baculovirus resistance in lepidopteran model insect *Bombyx mori* L. African Journal of Biotechnology 11 (79):14417-14421.

Flipsen JTM, Martens JWM, Vanoers MM, Vlak JM, Vanlent JWM (1995) Passage of *Autographa californica* nuclear polyhedrosis virus through the midgut epithelium of *Spodoptera exigua* larvae. Virology 208 (1):328-335.

Fritsch E, Undorf-Spahn K, Kienzle J, Zebitz CPW, Huber J (2005) Apfelwickler-Granulovirus: Erste Hinweise auf Unterschiede in der Empfindlichkeit lokaler Apfelwickler-Populationen. Nachrichtenblatt Deutscher Pflanzenschutzbund 57 (2):29-34.

Fritsch E, Undorf-Spahn K, Kienzle J, Zebitz CPW, Huber J (2006) Codling moth granulovirus: variations in the susceptibility of local codling moth populations. Ecofruit. 12th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing. Proceedings of the Conference, Weinsberg, Germany, 31 January - 2 February 2006. Fordergemeinschaft Okologischer Obstbau e.V. (FOKO), Weinsberg, Germany.

Fuková I, Nguyen P, Marec F (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. Genome 48 (6):1083-1092.

Fuxa JR, Mitchell FL, Richter AR (1988) Resistance of *Spodoptera frugiperda* [Lep. : Noctuidae] to a nuclear polyhedrosis virus in the field and laboratory. Entomophaga 33 (1):55-63.

Fuxa JR, Richter AR (1989) Reversion of resistance by *Spodoptera frugiperda* to a nuclear polyhedrosis virus. Journal of Invertebrate Pathology 53 (1):52-56.

Fuxa JR, Richter AR (1990) Response of nuclear polyhedrosis virus-resistance *Spodoptera frugiperda* larvae to other pathogens and to chemical insecticides. Journal of Invertebrate Pathology 55 (2):272-277.

Garavaglia MJ, Miele SAB, Iserte JA, Belaich MN, Ghiringhelli PD (2012) The *ac53*, *ac78*, *ac101*, and *ac103* genes are newly discovered core genes in the family *baculoviridae*. Journal of Virology 86 (22):12069-12079.

Gebhardt MM, Eberle KE, Radtke P, Jehle JA (2014) Baculovirus resistance in codling moth is virus isolate-dependent and the consequence of a mutation in viral gene *pe38*. Proceedings of the National Academy of Sciences of the United States of America 111 (44):15711-15716.

Graillot B, Berling M, Blachere-Lopez C, Siegwart M, Besse S, Lopez-Ferber M (2014) Progressive adaptation of a CpGV isolate to codling moth populations resistant to CpGV-M. Viruses-Basel 6 (12):5135-5144.

Graillot B, Bayle S, Blachere-Lopez C, Besse S, Siegwart M, Lopez-Ferber M (2016) Biological characteristics of experimental genotype mixtures of Cydia pomonella granulovirus (CpGV): Ability to control susceptible and resistant pest populations. Viruses-Basel 8 (5).

Granados RR (1980) Infectivity and mode of action of baculoviruses. Biotechnology and Bioengineering 22 (7):1377-1405.

Groeters FR, Tabashnik BE, Finson N, Johnson MW (1994) Fitness costs of resistance to *Bacillus thuringiensis* in the diamondback moth (*Plutella xylostella*). Evolution 48 (1):197-201.

Grove MJ, Hoover K (2007) Intrastadial developmental resistance of third instar gypsy moths (*Lymantria dispar* L.) to *L. dispar* nucleopolyhedrovirus. Biological Control 40 (3):355-361.

Gund NA, Wagner A, Timm AE, Schulze-Bopp S, Jehle JA, Johannesen J, Reineke A (2012) Genetic analysis of *Cydia pomonella* (Lepidoptera: Tortricidae) populations with different levels of sensitivity towards the Cydia pomonella granulovirus (CpGV). Genetica 140 (4-6):235-247.

Haas-Stapleton EJ, Washburn JO, Volkman LE (2004) P74 mediates specific binding of *Autographa californica* M nucleopolyhedrovirus occlusion-derived virus to primary cellular targets in the midgut epithelia of *Heliothis virescens* larvae. Journal of Virology 78 (13):6786-6791.

Haas-Stapleton EJ, Washburn JO, Volkman LE (2005) *Spodoptera frugiperda* resistance to oral infection by *Autographa californica* multiple nucleopolyhedrovirus linked to aberrant occlusion-derived virus binding in the midgut. Journal of Virology 86:1349-1355.

Harrison RL, Hoover K (2012) Baculoviruses and other occluded insect viruses. In: Vega FE, Kaya HK (Eds), Insect Pathology, Academic Press, London, England 73-131.

Harvey JP, Volkman LE (1983) Biochemical and biological varation of *Cydia pomonella* (codling moth) granulosis virus. Virology 124 (1):21-34.

Harzer U (2006) Lebenszyklus des Apfelwicklers. Obstbau 31:261.

Hassan SA (1992) Erfahrungen bei der Anwendung von *Trichogramma* spp. zur Bekämpfung des Apfelwicklers *Cydia pomonella* L. und des Apfelschalenwicklers *Adoxophyes orana* F.R: Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 8:70-74.

Herniou EA, Luque T, Chen X, Vlak JM, Winstanley D, Cory JS, O'Reilly D (2001) Use of whole genome sequence data to infer Baculovirus phylogeny. Journal of Virology 75 (17):8117-8126.

Herniou EA, Olszewski JA, Cory JS, O'Reilly DR (2003) The genome sequence and evolution of baculoviruses. Annual Review of Entomology 48:211-234.

Hilton S, Kemp E, Keane G, Winstanley D (2008) A bacmid approach to the genetic manipulation of granuloviruses. Journal of Virological Methods 152 (1-2):56-62.

Höhn H, Naef A, Hollinger E, Widmer A, Neuweiler R, Linder C, Viret O, Kehrli P, Delabays N (2008) Pflanzenschutzempfehlungen für den Erwerbsobstbau 2008/2009. Schweizerische Zeitung für Wein- und Obstbau (2):4-5.

Hough WS (1928) Relative resistance to arsenical poisoning of two codling moth strains. Journal of Economic Entomology 21 (2):325-329.

Huang FN, Andow DA, Buschman LL (2011) Success of the high-dose/refuge resistance management strategy after 15 years of Bt crop use in North America. Entomologia Experimentalis et Applicata 140 (1):1-16.

Huber J (1998) Western Europe In: Hunter-Fujita FR, Entwistle PhF, Evans HF, Cook NE (Eds.), Insect viruses and pest management. Wiley & Sons, New York, USA 201-215.

ICTV International Committee on Taxonomy of Viruses (2017) Virus Taxonomy: The classification and nomenclature of viruses. ICTV Report 10. Online: https://talk.ictvonline.org/ictv-reports/ictv_online_report/ (22.2.2017).

IRAC Insecticides Resistance Action Committee (2016) IRAC mode of action classification schema. 8.1:1-26. Online: http://www.irac-online.org/ (10.2.2017).

Isci M, Ay R (2017) Determination of resistance and resistance mechanisms to thiacloprid in *Cydia pomonella* L. (Lepidoptera: Tortricidae) populations collected from apple orchards in Isparta Province, Turkey. Crop Protection 91:82-88.

Ivaldi-Sender C (1974) Techniques simples our un élévage permanent de la tordeuse orientale, *Grapholita molesta* (Lepidoptera Tortricidae) sur milieu artificiel. Annales de Zoologie, Ecologie Animale 6 (2):337-343.

Iwata K, Haas-Stapleton EJ, Kunimi Y, Inoue MN, Nakai M (2017) Midgut-based resistance to oral infection by a nucleopolyhedrovirus 1 in the laboratory-selected strain of the smaller tea tortrix, *Adoxophyes honmai* (Lepidoptera: Tortricidae). Journal General Virology doi:10.1099/jgv.0.000684

Jakubowska AK, Lynn DE, Herrero S, Vlak JM, van Oers MM (2010) Host-range expansion of *Spodoptera exigua* multiple nucleopolyhedrovirus to *Agrotis segetum* larvae when the midgut is bypassed. Journal of General Virology 91:898-906.

Jehle JA, Backhaus H, Fritsch E, Huber J (1992) Physical map of the *Cryptophlebia leucotreta* granulosis virus genome and its relationship to the genome of *Cydia pomonella* granulosis virus. Journal of General Virology 73:1621-1626.

Jehle JA, Blissard GW, Bonning BC, Cory JS, Herniou EA, Rohrmann GF, Theilmann DA, Thiem SM, Vlak JM (2006a) On the classification and nomenclature of baculoviruses: A proposal for revision. Archives of Virology 151 (7):1257-1266.

Jehle JA, Sayed S, Wahl-Ermel B (2006b) What do we (need to) know about low-susceptibility of codling moth against *Cydia pomonella* granulovirus (CpGV)! Ecofruit. 12th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing. Proceedings of the conference, Weinsburg, Germany, 31 January - 2 February 2006. Fordergemeinschaft Okologischer Obstbau e.V. (FOKO), Weinsberg, Germany.

Jehle JA, Herz A, Keller B, Kleespies RG, Koch E, Larem A, Schmitt A, Stephan D (2014a) Status Report: biological plant protection. Berichte aus dem Julius Kuhn-Institut (173):117.

Jehle JA, Sauer A, Fritsch E, Undorf-Spahn K (2014b) Resistance to *Cydia pomonella* granulovirus: novel findings on its distribution and diversity. Ecofruit. 16th International Conference on Organic-Fruit Growing: Proceedings of the Conference, 17-19 February 2014, Hohenheim, Germany. Fordergemeinschaft Okologischer Obstbau e.V. (FOKO), Weinsberg, Germany.

Jehle JA, Schulze-Bopp S, Undorf-Spahn K, Fritsch E (2017) Evidence for a second type of resistance against Cydia pomonella granulovirus (CpGV) in field populations of codling moths. Applied and Environmental Microbiology 83 (2):1-16.

Joshi NK, Rajotte EG, Naithani KJ, Krawczyk G, Hull LA (2016) Population dynamics and flight phenology model of codling moth differ between commercial and abandoned apple Orchard Ecosystems. Frontiers in Physiology 7:13.

Kaya HK, Joos JL, Falcon LA, Berlowitz A (1984) Suppression of the codling moth (Lepidoptera, Olethreutidae) with the entomogenous nematod, *Steinernema feltiae* (Rhabiditida, Steinernematidae). Journal of Economic Entomology 77 (5):1240-1244.

Kienzle J (2010) Erfahrungen mit der Einführung biologischer Pflanzenschutzferfahren im Ökologischen Obstbau. Journal für Kulturpflanzen 62:81-83.

Kienzle J, Zimmer J, Volk F, Zebitz CPW (2008) Field tests with Madex Plus against CpGV-resistant codling moth populations in organic orchards in 2006. Ecofruit. 13th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing. Proceedings of the conference, Weinsberg, Germany, 18-20 February 2008. Fordergemeinschaft Okologischer Obstbau e.V. (FOKO), Weinsberg, Germany.

Knight AL, Larson TL, Ketner K, Hilton R, Hawkins L (2008) Field evaluations of concentrated spray applications of microencapsulated sex pheromone for codling moth (Lepidoptera : Tortricidae). Environmental Entomology 37 (4):980-989.

Krappa R, Knebel-Mörsdorf D (1991) Identification of the very early transcribed Baculovirus gene *pe-38*. Journal of Virology 65 (2):805-812.

Kumar S, Neven LG, Zhu HY, Zhang RZ (2015) Assessing the global risk of establishment of *Cydia pomonella* (Lepidoptera: Tortricidae) using CLIMEX and MaxEnt niche models. Journal of Economic Entomology 108 (4):1708-1719.

Kuzio J, Jaques R, Faulkner P (1989) Identification of *p74*, a gene essential for virulence of baculovirus occlusion bodies. Virology 173 (2):759-763.

Lacey LA, Arthurs SP, Unruh TR, Headrick H, Fritts R (2006) Entomopathogenic nematodes for control of codling moth (Lepidoptera : Tortricidae) in apple and pear orchards: Effect of nematode species and seasonal temperatures, adjuvants, application equipment, and post-application irrigation. Biological Control 37 (2):214-223.

Lacey LA, Shapiro-Ilan DI (2008) Microbial control of insect pests in temperate orchard systems: Potential for incorporation into IPM. Annual Review of Entomology 53:121-144.

Lacey LA, Thomson D, Vincent C, Arthurs SP (2008) Codling moth granulovirus: a comprehensive review. Biocontrol Science and Technology 18 (7):639-663.

Lacey LA (2012) Manual of techniques in invertebrate pathology. Academic Press, Academic Press, London, England 461-465.

Lacey LA, Grzywacz D, Shapiro-Ilan DI, Frutos R, Brownbridge M, Goettel MS (2015) Insect pathogens as biological control agents: Back to the future. Journal of Invertebrate Pathology 132:1-41.

Levy SM, Falleiros AMF, Moscardi F, Gregorio EA (2007) Susceptibility/resistance of *Anticarsia gemmatalis* larvae to its nucleopolyhedrovirus (AgMNPV): Structural study of the peritrophic membrane. Journal of Invertebrate Pathology 96 (2):183-186.

Luque T, Finch R, Crook N, O'Reilly DR, Winstanley D (2001) The complete sequence of the *Cydia pomonella* granulovirus genome. Journal of General Virology 82:2531-2547.

Martignoni ME, Iwai PJ (1986) A catalog of viral diseases of insects, mites and ticks. Washington, DC: USPTO: USDA Forest Service. Online: https://www.fs.fed.us/pnw/publications/pnw_gtr195/pnw_gtr195a.pdf (20.2.2017)

Melander AL (1914) Can Insects Become Resistant to Sprays? Journal of Economic Entomogy 7:167-173.

Milks ML, Myers JH (2000) The development of larval resistance to a nucleopolyhedrovirus is not accompanied by an increased virulence in the virus. Evolutionary Ecology 14 (7):645-664.

Milks ML, Theilmann DA (2000) Serial selection for resistance to a wild-type and to a genetically modified nucleopolyhedrovirus in *Trichoplusia ni*. Biological Control 19 (3):283-289.

Milks ML, Myers JH (2003) Cabbage looper resistance to a nucleopolyhedrovirus confers cross-resistance to two granuloviruses. Environmental Entomology 32 (2):286-289.

Milks ML, Washburn JO, Willis LG, Volkman LE, Theilmann DA (2003) Deletion of *pe38* attenuates AcMNPV genome replication, budded virus production, and virulence in *Heliothis virescens*. Virology 310 (2):224-234.

Moscardi F (1999) Assessment of the application of baculoviruses for control of Lepidoptera. Annual Review of Entomology 44:257-289.

Moscardi F (2007) Development and use of the nucleopolyhedrovirus of the velvetbean caterpillar in soybeans. In: Vincent C, Goettel MS, Lazarovits G (Eds), Biological Control: A Global Perspective, CAB International, Wallingford, UK 344-353.

Moscardi F, de Souza ML, de Castro MEB, Moscardi ML, Szewczyk B (2011) Baculovirus pesticides: present state and future perspectives. In: Ahmad I, Ahmad F, Pichtel J (Eds), Microbes and microbial technology. Springer, Dordrecht, The Neatherlands 415-445.

Mu JF, van Lent JWM, Smagghe G, Wang Y, Chen XW, Vlak JM, van Oers MM (2014) Live imaging of baculovirus infection of midgut epithelium cells: a functional assay of *per os* infectivity factors. Journal of General Virology 95:2531-2539.

Nakai M, Cuc NTT (2005) Field application of an insect virus in the Mekong Delta: Effects of a Vietnamese nucleopolyhedrovirus on *Spodoptera litura* (Lepidoptera : Noctuidae) and its parasitic natural enemies. Biocontrol Science and Technology 15 (5):443-453.

Neven LG (2013) Effects of short photoperiod on codling moth diapause and survival. Journal of Economic Entomology 106 (1):520-523.

Nguyen P, Sýkorová M, Šíchovaá J, Kůta V, Dalíková M, Čapková Frydrychová R, Neven LG, Sahara K, Marec F (2013) Neo-sex chromosomes and adaptive potential in tortricid pests. Proceedings of the National Academy of Sciences of the United States of America 110 (17):6931-6936.

Nie YC, Fang MG, Erlandson MA, Theilmann DA (2012) Analysis of the *Autographa californica* multiple nucleopolyhedrovirus overlapping gene pair *lef3* and *ac68* reveals that AC68 is a *per os* infectivity factor and that LEF3 is critical, but not essential, for virus replication. Journal of Virology 86 (7):3985-3994.

Odendaal D, Addison MF, Malan AP (2015) Control of codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) in South Africa with special emphasis on using entomopathogenic nematodes. African Entomology 23 (2):259-274.

Ohkawa T, Washburn JO, Sitapara R, Sid E, Volkman LE (2005) Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of *Heliothis virescens* larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. Journal of Virology 79 (24):15258-15264.

Ohkawa T, Volkman LE, Welch MD (2010) Actin-based motility drives baculovirus transit to the nucleus and cell surface. Journal of Cell Biology 190 (2):187-195.

Pringle KL, Eyles DK, Brown L (2003) Trends in codling moth activity in apple orchards under mating disruption using pheromones in the Elgin area, Western Cape Province, South Africa. African Entomology 11 (1):65-75.

Reid S, Chan L, van Oers MM (2014) Production of entomopathogenic viruses. In: Rojas MG, Shapiro-Ilan DI (eds), Mass production of beneficial organisms. Academic Press, San Diego, USA 437-482.

Rezapanah M, Shojai-Estabragh S, Huber J, Jehle JA (2008) Molecular and biological characterization of new isolates of *Cydia pomonella* granulovirus from Iran. Journal of Pest Science 81 (4):187-191.

Rohrmann GF (2008) Baculovirus Molecular Biology. Bethesda (MD): National Libaray of Medicine (US). National Center for Biotechnology Information; Online: https://www.ncbi.nlm.nih.gov/books/NBK1736/ (23.2.2017)

Rowley DL, Popham HJR, Harrison RL (2011) Genetic variation and virulence of nucleopolyhedroviruses isolated worldwide from the heliothine pests *Helicoverpa armigera*, *Helicoverpa zea*, and *Heliothis virescens*. Journal of Invertebrate Pathology 107 (2):112-126.

Sauphanor B, Berling M, Toubon J-F, Reyes M, Delnatte J (2006) Carpocapse des pommes cas de résistance aux virus de la granulose dans le Sud-Est [Codling moth: Cases of resistance to granuloviruses in organic orchards]. Phytoma — La Défense des Végétaux 590:24-27.

Sauphanor B, Brosse V, Bouvier JC, Speich P, Micoud A, Martinet C (2000) Monitoring resistance to diflubenzuron and deltamethrin in French codling moth populations (*Cydia pomonella*). Pest Management Science 56 (1):74-82.

Schmitt A, Bisutti IL, Ladurner E, Benuzzi M, Sauphanor B, Kienzle J, Zingg D, Undorf-Spahn K, Fritsch E, Huber J, Jehle JA (2013) The occurrence and distribution of resistance of codling moth to *Cydia pomonella* granulovirus in Europe. Journal of Applied Entomology 137 (9):641-649.

Schulze-Bopp S, Jehle JA (2013) Development of a direct test of baculovirus resistance in wild codling moth populations. Journal of Applied Entomology 137 (1-2):153-160.

Siegwart M, Graillot B, Lopez CB, Besse S, Bardin M, Nicot PC, Lopez-Ferber M (2015) Resistance to bio-insecticides or how to enhance their sustainability: a review. Frontiers in Plant Science 6:19.

Smith IRL, Crook NE (1988) *In vivo* isolation of baculovirus genotypes. Virology 166 (1):240-244.

Song JJ, Wang X, Hou DH, Huang HC, Liu XJ, Deng F, Wang HL, Arif BM, Hu ZH, Wang ML (2016) The host specificities of baculovirus *per os* infectivity factors. Plos One 11 (7):12.

Sparks WO, Harrison RL, Bonning BC (2011) *Autographa californica* multiple nucleopolyhedrovirus ODV-E56 is a *per os* infectivity factor, but is not essential for binding and fusion of occlusion-derived virus to the host midgut. Virology 409 (1):69-76.

Spohn BC, Zhu JW, Chastain BB, Haynes KF (2003) Influence of mating disruptants on the mating success of two strains of cabbage loopers, *Trichoplusia ni* (Hubner) (Lepidoptera : Noctuidae). Environmental Entomology 32 (4):736-741.

Statistisches Bundesamt (2015) Wachstum und Ernte -Baumobst-. Land- und Forstwirtschaft, Fischerei, Fachserie 3 (Reihe 3.2.1), Wiesbaden, Germany 1-18.

Steinhaus EA (1975) Disease in a minor chord. Ohio State University Press, USA.

Summers MD (1971) Elektron microscopic observations on granulosis virus entry uncoating, and replication process during infection of the midgut cells of *Trichoplusia ni*. Journal of Ultrastructure Research 35 (5-6):606.

Sutherland OR, Hutchins RF (1972) α-Farnesene, a natural attractant for codling moth larvae. Nature 239 (5368):170.

Tabashnik BE (1991) Determining the mode of inheritance of pesticide resistance with backcrossing experiments. Journal of Economic Entomology 84 (3):703-712.

Tabashnik BE, Van Rensburg JBJ, Carriere Y (2009) Field-evolved insect resistance to Bt crops: Definition, theory, and data. Journal of Economic Entomology 102 (6):2011-2025.

Tabata J, Noguch H, Kainoh Y, Mochizuki F, Sugie H (2007) Behavioral response to sex pheromone-component blends in the mating disruption-resistant strain of the smaller tea tortrix, *Adoxophyes honmai Yasuda* (Lepidoptera : Tortricidae), and its mode of inheritance. Applied Entomology and Zoology 42 (4):675-683.

Tanada Y (1964) Granulosis virus of codling moth, *Carpocapsa pomenella* (Linnaeus) (Olethreutidae, Lepidoptera) Journal of Insect Pathology 6 (3):39-47.

Thorpe PT, Pryke JS, Samways MJ (2016) Review of ecological and conservation perspectives on future options for arthropod management in Cape Floristic Region pome fruit orchards. African Entomology 24 (2):279-306.

Traut W, Sahara K, Marec F (2007) Sex chromosomes and sex determination in Lepidoptera. Sexual Development 1 (6):332-346.

Undorf-Spahn K, Fritsch E, Huber J, Kienzle J, Zebitz CPW, Jehle JA (2012) High stability and no fitness costs of the resistance of codling moth to *Cydia pomonella* granulovirus (CpGV-M). Journal of Invertebrate Pathology 111 (2):136-142.

Van't Hof AE, Nguyen P, Dalikova M, Edmonds N, Marec F, Saccheri IJ (2013) Linkage map of the peppered moth, *Biston betularia* (Lepidoptera, Geometridae): a model of industrial melanism. Heredity 110 (3):283-295.

van Oers MM, Vlak JM (2007) Baculovirus genomics. Current Drug Targets 8 (10):1051-1068.

van Oers MM, Pijlman GP, Vlak JM (2015) Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. Journal of General Virology 96:6-23.

Wang P, Granados RR (1998) Observations on the presence of the peritrophic membrane in larval *Trichoplusia ni* and its role in limiting baculovirus infection. Journal of Invertebrate Pathology 72 (1):57-62.

Wennmann JT (2014) Diversity of Baculoviruses Isolated from Cutworms (*Agrotis* spp.). Dissertation, Fachbereich Biologie, Johannes Gutenberg-Universität, Mainz.

Wennmann JT, Kohler T, Alletti GG, Jehle JA (2015) Mortality of cutworm larvae is not enhanced by Agrotis segetum granulovirus and Agrotis segetum Nucleopolyhedrovirus B coinfection relative to single infection by either virus. Applied and Environmental Microbiology 81 (8):2893-2899.

WHO World Health Organization (1957) Expert committee on malaria World Health Organization, technical report vol.7. Online: http://apps.who.int/iris/bitstream/10665/40380/1/WHO _TRS_125.pdf (20.2.2017).

Wildbolz T, Baggiolini M (1959) Über das Mass der Ausbreitung des Apfelwicklers während der Eiablageperiode. Mitteilungen der Schweizischen Entomologischen Gesellschaft 32:241-257.

Willett MJ, Neven L, Miller CE (2009) The Occurrence of codling moth in low latitude countries: Validation of pest distribution reports. Horttechnology 19 (3):633-637.

Winstanley D, Crook NE (1993) Replication of *Cydia pomonella* granulosis virus in cell-culture. Journal of General Virology 74:1599-1609.

Witzgall P, Stelinski L, Gut L, Thomson D (2008) Codling moth management and chemical ecology. In: Annual Review of Entomology 53:503-522.

Wolf KW (1996) The structure of condensed chromosomes in mitosis and meiosis of insects. International Journal of Insect Morphology & Embryology 25 (1-2):37-62.

Yoshido A, Bando H, Yasukochi Y, Sahara K (2005) The *Bombyx mori* karyotype and the assignment of linkage groups. Genetics 170 (2):675-685.

Zhao L, Hou P, Zhu GP, Li M, Xie TX, Liu Q (2015) Mapping the disjunct distribution of introduced codling moth *Cydia pomonella* in China. Agricultural and Forest Entomology 17 (2):214-222.

Zichova T, Falta V, Kocourek F, Stara J (2011) Differences in the susceptibility of codling moth populations to *Cydia pomonella* granulovirus in the Czech Republic. Horticultural Science 38 (1):21-26.

Zichová T, Stará J, Kundu JK, Eberle KE, Jehle JA (2013) Resistance to *Cydia pomonella* granulovirus follows a geographically widely distributed inheritance type within Europe. Biocontrol 58 (4):525-534.

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Ehrenwörtliche Erklärung:

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Darmstadt, den 24.2.2017

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Professional experience and Studies

12/2012 – 10/2016 **Ph.D. student** at Technische Universität Darmstadt, working at the Julius Kühn Institute, Federal Institute for Biological Control Darmstadt, Germany. DFG-funded project: "Adaptations and counter-adaptations in the co-evolutionary arms race of a baculovirus (CpGV) and its insect host (*Cydia pomonella*)."

- Analysis of genetic variability of *Cydia pomonella*, novel CpGV resistance mechanism, inheritance pattern in the host and resistance management strategies
- Bioassay, molecular, cell cultural, insect-virology and proteomic working in the lab
- Presentation of study results at international meetings and preparing of reports
- Tutoring of master students

11/2011 – 05/2012 **Traineeship** with Glades Crop Care Inc. Agricultural Consultants, Florida (USA), funded by CA Education Programm and „Deutsche Bauernverband"

- Scouting, monitoring and consulting in agricultural crop by identifying and reporting diseases and pests.

05/2010 – 10/2011 **Scientific assistant** at Laboratory of Biotechnological Crop Protection (DLR Rheinpfalz) Neustadt an der Weinstraße, Germany. Member of the BMBF-funded cooperation project "Biological safety research into genetically modified plants".

- Seasonal expression monitoring in transgenic corn
- Protein chemical methods and establishing of specific ELISA test systems

06/2009 – 02/2010 **Diploma thesis** at the Julius Kühn Institute, Federal Institute for Biological Control, Darmstadt (Germany). Title: The vegetative insecticide protein (VIP) of *Bacillus thuringiensis* – production and biocide impact against *Agrotis segetum* larvae.

10/2005 – 02/2009 **Study of Biotechnology** at the University of Applied Sciences Darmstadt (Germany)

Congress Participation & Stay Abroad (Extract)

July 2016	Society for Invertebrate Pathology (SIP) congress in **Tours (France)** with oral presentation
April-Mai 2016	Cooperation Exchange with **Tokyo University** of Agriculture and Technology (Japan) for six weeks
February 2016	Scientific talk at the Georg-August-University in **Göttingen (Germany)**
August 2015	SIP congress in **Vancouver (Canada)** with oral presentation
June 2015	International Organisation for Biological and Integrated Control (IOBC) congress in **Riga (Latvia)** with oral presentation
January 2015	Research project at the Institute of Entomology in **České Budějovice (Czech Republic)**
August 2014	SIP congress in **Mainz (Germany)** with oral presentation
July 2013	IOBC congress in **Zagreb (Croatia)** with oral presentation
June 2011	EIGMO congress in **České Budějovice (Czech Republic)** with oral presentation
August 2010	ICIRD congress in **Prague (Czech Republic)** with poster presentation
October 2009	Research project at the Julius Kühn Institute, **Quedlinburg (Germany)** for five weeks
April 2002	School exchange with **Melbourne (Australia)** for six weeks

Honors and Awards

Travel award 2016 at the SIP congress in Tours (France). Title: Identification of a novel mode of resistance against Cydia pomonella granulovirus (CpGV) in codling moth indicates a highly dynamic adaptation in the host population

Travel award 2015 at the IOBC congress in Riga (Latvia). Title: A novel mode of resistance of codling moth against Cydia pomonella granulovirus with a dominant and autosomal inheritance pattern

Poster price 2010 at the ICIRD congress in Prague (Czech Republic). Title: Quantification of Cry1A.105, Cry2Ab2 and Cry3Bb1in transgenic corn MON 89034 x MON 88017

Publication and Previous Presentations (Extract)

Sauer, A.; Fritsch, E.; Undorf-Spahn, K.; Jehle, J.A. (2015): A novel mode of resistance of codling moth against Cydia pomonella granulovirus with a dominant and autosomal inheritance pattern. In: 15th Meeting of the IOBC-WPRS Working Group "Microbial and Nematode Control of Invertebrate Pests": New Challenges for Biological Control, Riga, 48-48.

Sauer, A.J.; Gebhardt, M.M.; Fritsch, E.; Undorf-Spahn, K.; Jehle, J.A. (2015): A novel type of resistance of the codling moth against Cydia pomonella granulovirus shows two different resistance mechanisms. In: SIP (eds.): International Congress on Invertebrate Pathology and Microbial Control and 48th Annual Meeting of the Society of Invertebrate Pathology : Program and Abstract book, 64-65.

Ben Hamadou-Charfi, D.; Sauer, A.J.; Abdelkefi-Mesrati, L.; Tounsi, S.; Jaoua, S.; Stephan, D. (2014): Susceptibility of *Agrotis segetum* (Noctuidae) to *Bacillus thuringiensis* and analysis of midgut proteinases. Preparative biochemistry & biotechnology 45(5): 411-420.

Sauer, A. J., Fritsch, E., Undorf-Spahn, K., Jehle, J. A. (2014): A novel mode of resistance of codling moth against Cydia pomonella granulovirus. (Berichte aus dem Julius Kühn-Institut 174), 51.

Sauer, A.J.; Fritsch, E.; Undorf-Spahn, K.; Jehle, J.A. (2013): Elucidation of a novel mode of resistance of codling moth against Cydia pomonella granulovirus by homogenization experiments. In: Jehle, J.A. et al. (ed.): Insect pathogens and entomopathogenic nematode: proceedings of the meeting "Biological Control, its unique role in organic and integrated production" at Zagreb (Croatia), 16 20 June, 2013 (IOBC WPRS bulletin 90), Darmstadt, 161-165.

Sauer A.J.; Nguyen H.T.; Nagel D.; Jehle J.A. (2011): Establishing of protein standards for Cry3Bb1, Cry2Ab2 and Cry1A.105 and quantification of multi-Cry proteins in transgenic corn. 5th Ecological Impact of Genetically Modified Organisms (EIGMO) congress in České Budějovice in June 2011, Abstract Book Page 42.

Sauer A.J.; Nguyen H.T.; Nagel D.; Jehle J.A. (2010): Quantification of Cry3Bb1, Cry2Ab2 and Cry1A.105 in transgenic corn MON89034 x MON 88017. ICIRD congress in Prague, August 2010 with poster presentation.

Hamadou, D.B.; Sauer, A.; Jaoua, S.; Stephan, D. (2010): Screening of *Bacillus thuringiensis* isolates against *Agrotis segetum* with specific focus on vegetative insecticidal proteins. In: 43rd Annual Meeting of the Society for Invertebrate Pathology, Trabzon, Turkey, 146-147.

Darmstadt, 24 February 2017