Novel Diversity and Virulence Patterns Found in New Isolates of Cydia pomonella Granulovirus from China

Jiangbin Fan,a,b Jörg T. Wennmann,a Dun Wang,b Johannes A. Jehle,a

a Institute for Biological Control, Julius Kühn-Institut, Darmstadt, Germany
b State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, China

ABSTRACT Cydia pomonella granulovirus (CpGV) is successfully used worldwide as a biocontrol agent of the codling moth (CM) (Cydia pomonella). The occurrence of CM populations with different modes of resistance against commercial CpGV preparations in Europe, as well as the invasiveness of CM in China, threatening major apple production areas there, requires the development of new control options. Utilizing the naturally occurring genetic diversity of CpGV can improve such control strategies. Here, we report the identification of seven new CpGV isolates that were collected from infected CM larvae in northwest China. Resistance testing using a discriminating CpGV concentration and the determination of the median lethal concentration (LC50) were performed to characterize their levels of virulence against susceptible and resistant CM larvae. The isolates were further screened for the presence of the 2 × 12-bp-repeat insertion in CpGV gene pe38 (open reading frame 24 [ORF24]), which was shown to be the target of type I resistance. It was found that three isolates, CpGV-JQ, -KS1, and -ZY2, could break type I resistance, although delayed mortality was observed in the infection process. All isolates followed the pe38 model of breaking type I resistance, except for CpGV-WW, which harbored the genetic factor but failed to overcome type I resistance. However, CpGV-WW was able to overcome type II and type III resistance. The bioassay results and sequencing data of pe38 support previous findings that pe38 is the major target for type I resistance. The new isolates show some distinct virulence characteristics when infection of different CM strains is considered.

IMPORTANCE CpGV is a highly virulent pathogen of the codling moth (CM). It is registered and widely applied as a biocontrol agent in nearly all apple-growing countries worldwide. The emergence of CpGV resistance and the increasing lack of chemical control options require improvements to current control strategies. Natural CpGV isolates, as well as resistance-breaking isolates selected in resistant CM strains, have provided resources for improved resistance-breaking CpGV products. Here, we report novel CpGV isolates collected in China, which have new resistance-breaking capacities and may be an important asset for future application in the biological control of codling moths.

KEYWORDS Baculoviridae, codling moth, biological control, resistance test, median lethal concentration, pe38 diversity, PCR

The codling moth (CM), Cydia pomonella (L.), belongs to the lepidopteran family Tortricidae and has spread and established local populations in temperate zones worldwide. Its larvae cause considerable damage to apple, pear, apricot, plum, peach, nectarine, and walnut production (1–3). An efficient way to control coding moths without adverse effects to human health and the environment is the application of Cydia pomonella granulovirus (CpGV), a double-stranded DNA virus from the Betabaculovirus genus in the Baculoviridae family (4, 5). CpGV was first discovered in Mexico in


Editor Karyn N. Johnson, University of Queensland
Copyright © 2020 American Society for Microbiology. All Rights Reserved.
Address correspondence to Dun Wang, wanghande@yahoo.com, or Johannes A. Jehle, Johannes.Jehle@julius-kuehn.de.
Received 30 August 2019
Accepted 16 October 2019
Accepted manuscript posted online 1 November 2019
Published 7 January 2020
1963, and the isolate was named CpGV-M (4, 6). It is a fast-killing granulovirus that induces systemic infection in CM larvae, including the fat body, tracheal cells, and malpighian tubules, and typically results in larval death within 7 days of infection (7, 8). Two virion phenotypes of CpGV are produced during the infection process, the occlusion-derived virion (ODV) embedded in a viral occlusion body (OB) and the budded virus (BV), which are responsible for primary infection of midgut epithelial cells and secondary invasion of other larval tissues, respectively (9, 10). The circular, covalently closed DNA genome of CpGV ranges from 120.8 to 123.9 kbp in length and encodes 137 to 142 open reading frames (11–13). Natural isolates of CpGV have been grouped into five phylogenetic lineages, termed genome groups A to E, which were established by phylogenetic analysis using concatenated sequences of partial viral open reading frames (ORFs) granulin and lef-8 (14, 15), baculovirus core genes (13), or whole genomes (12) or via genomic fingerprints from specific single nucleotide polymorphisms (SNPs) (16).

Recently, diseased CM larvae showing typical signs of baculovirus infection, such as swollen bodies or fragile cadavers, were field collected in northwest China, and new CpGV isolates, namely, CpGV-ZY, -KS1, and -KS2, have been purified from the insect cadavers (17, 18). In China, the codling moth was first recorded as an invasive pest in Xinjiang in 1953 (19). In recent years, this CM population has spread eastward, and so far, the pest has been found in five provinces in northwest China, including Xinjiang, Gansu, Ningxia, and the western part of Inner Mongolia (20). In addition, a disjunct population, possibly from Russia, has become established in Jilin and Heilongjiang provinces in northeast China (21). As codling moths have started to spread in northern regions of China, where the major Chinese apple production area is located, this pest has become a possible threat to the largest apple production area in the world.

In Europe, after more than a decade of successful application of CpGV, over 40 orchards with CM populations resistant to commercial CpGV products have been recorded in seven European countries since 2005 (8, 22, 23). Three types (I to III) of CpGV field resistance with differing inheritance and susceptibility patterns have been identified (8, 24–26). Type I resistance, first discovered in Germany, is the most common one (27). Represented by the genetically homogeneous resistant CM strain CpRR1, inheritance of type I resistance is linked to the Z chromosome and follows a dominant mode (8, 28). This resistance is systemic and instar independent and causes an early block in virus replication (29, 30). Though inheritance of type I resistance was found to be highly stable, rearing of CpRR1 moths without selection pressure resulted in some loss of the strain’s resistance level (31). Type I resistance of CpRR1 is directed only against isolates from genome group A, such as CpGV-M, whereas isolates from other genome groups are able to break this type of resistance (13). Resistant CpRR1 females (carrying a single Z chromosome) or homozygous resistant males successfully developed to adulthood following inoculation with CpGV-M (8). Genome sequence comparisons and further molecular analyses of different CpGV isolates have demonstrated that type I resistance is targeted against a 2 × 12-bp repeat within the viral gene pe38 (ORF24), which is present in CpGV-M of genome group A (16). In contrast, other CpGV isolates from genome groups B, C, D, and E, which do not contain the additional repeat sequence, can break type I resistance (13, 32). This 2 × 12-bp difference in pe38 was also used to distinguish the replication of CpGV-M and the resistance-breaking isolate CpGV-R5 in susceptible and resistant (strain R geomet) CM strains from France (33). According to current knowledge, pe38 is a suitable genetic marker to identify whether a given CpGV isolate is able to break type I resistance in CM. Type II and type III resistances are less well studied but follow different inheritance patterns, and codling moths of these types display resistance to CpGV isolates from genome groups A and E, such as CpGV-M and -S (24–26).

Therefore, identifying and using new isolates of CpGV could be an effective approach to improve the control of this insect pest. Seven new CpGV isolates collected in China were tested for their activity against a susceptible and a resistant CM strain in resistance tests using a discriminating OB concentration of CpGV. Three CpGV isolates

---

"1963, and the isolate was named CpGV-M (4, 6). It is a fast-killing granulovirus that induces systemic infection in CM larvae, including the fat body, tracheal cells, and malpighian tubules, and typically results in larval death within 7 days of infection (7, 8). Two virion phenotypes of CpGV are produced during the infection process, the occlusion-derived virion (ODV) embedded in a viral occlusion body (OB) and the budded virus (BV), which are responsible for primary infection of midgut epithelial cells and secondary invasion of other larval tissues, respectively (9, 10). The circular, covalently closed DNA genome of CpGV ranges from 120.8 to 123.9 kbp in length and encodes 137 to 142 open reading frames (11–13). Natural isolates of CpGV have been grouped into five phylogenetic lineages, termed genome groups A to E, which were established by phylogenetic analysis using concatenated sequences of partial viral open reading frames (ORFs) granulin and lef-8 (14, 15), baculovirus core genes (13), or whole genomes (12) or via genomic fingerprints from specific single nucleotide polymorphisms (SNPs) (16).

Recently, diseased CM larvae showing typical signs of baculovirus infection, such as swollen bodies or fragile cadavers, were field collected in northwest China, and new CpGV isolates, namely, CpGV-ZY, -KS1, and -KS2, have been purified from the insect cadavers (17, 18). In China, the codling moth was first recorded as an invasive pest in Xinjiang in 1953 (19). In recent years, this CM population has spread eastward, and so far, the pest has been found in five provinces in northwest China, including Xinjiang, Gansu, Ningxia, and the western part of Inner Mongolia (20). In addition, a disjunct population, possibly from Russia, has become established in Jilin and Heilongjiang provinces in northeast China (21). As codling moths have started to spread in northern regions of China, where the major Chinese apple production area is located, this pest has become a possible threat to the largest apple production area in the world.

In Europe, after more than a decade of successful application of CpGV, over 40 orchards with CM populations resistant to commercial CpGV products have been recorded in seven European countries since 2005 (8, 22, 23). Three types (I to III) of CpGV field resistance with differing inheritance and susceptibility patterns have been identified (8, 24–26). Type I resistance, first discovered in Germany, is the most common one (27). Represented by the genetically homogeneous resistant CM strain CpRR1, inheritance of type I resistance is linked to the Z chromosome and follows a dominant mode (8, 28). This resistance is systemic and instar independent and causes an early block in virus replication (29, 30). Though inheritance of type I resistance was found to be highly stable, rearing of CpRR1 moths without selection pressure resulted in some loss of the strain’s resistance level (31). Type I resistance of CpRR1 is directed only against isolates from genome group A, such as CpGV-M, whereas isolates from other genome groups are able to break this type of resistance (13). Resistant CpRR1 females (carrying a single Z chromosome) or homozygous resistant males successfully developed to adulthood following inoculation with CpGV-M (8). Genome sequence comparisons and further molecular analyses of different CpGV isolates have demonstrated that type I resistance is targeted against a 2 × 12-bp repeat within the viral gene pe38 (ORF24), which is present in CpGV-M of genome group A (16). In contrast, other CpGV isolates from genome groups B, C, D, and E, which do not contain the additional repeat sequence, can break type I resistance (13, 32). This 2 × 12-bp difference in pe38 was also used to distinguish the replication of CpGV-M and the resistance-breaking isolate CpGV-R5 in susceptible and resistant (strain R geomet) CM strains from France (33). According to current knowledge, pe38 is a suitable genetic marker to identify whether a given CpGV isolate is able to break type I resistance in CM. Type II and type III resistances are less well studied but follow different inheritance patterns, and codling moths of these types display resistance to CpGV isolates from genome groups A and E, such as CpGV-M and -S (24–26).

Therefore, identifying and using new isolates of CpGV could be an effective approach to improve the control of this insect pest. Seven new CpGV isolates collected in China were tested for their activity against a susceptible and a resistant CM strain in resistance tests using a discriminating OB concentration of CpGV. Three CpGV isolates
were further used to determine their median lethal concentration (LC₅₀). Sequence analyses of the 2 × 12-bp-repeat insertion of pe38 among these isolates revealed that not all isolates followed the current type I resistance model, suggesting that additional genetic factors are likely involved in CpGV resistance.

RESULTS

Virulence of CpGV isolates. Seven new CpGV isolates, designated CpGV-ZY, -JQ, -ALE, -KS1, -KS2, -ZY2, and -WW, all collected from northwest China (Fig. 1), as well as the reference isolates CpGV-M, -S, and -E2, were tested to compare their levels of virulence against neonate larvae of four CM strains, namely, CpS (CpGV susceptible), CpRR1 (type I resistance), CpR5M (type II resistance), and CpRGO (type III resistance). Neonate larvae were exposed to a discriminating concentration of 5.8 × 10⁴ OBs/ml, which would cause ≥95% virus-induced mortality in susceptible CM larvae at 7 days postinfection (dpi) (8, 26).

All isolates showed similar levels of activity in CpS larvae at 7 and 14 dpi. For CpRR1 larvae, the average rates of mortality caused by CpGV-JQ, -KS1, and -ZY2 at 7 dpi were 62.7%, 67.7%, and 77.9%, respectively, similar to or higher than the 61.4% mortality induced by the resistance-breaking isolate CpGV-S (Fig. 2A). As the CpRR1 mortality (40.6%) induced by CpGV-M was higher than expected from previous experiments and some decline of the resistance level of the CpRR1 colony was suggested (31), CpGV-JQ, -KS1, and -ZY2 were considered resistance-breaking because they caused higher mortality than the type I resistance-breaking isolate CpGV-S in the current experiments. In contrast, CpGV-ALE, -KS2, and -WW resulted in average mortality rates of only 15.2%, 42.2%, and 43.3%, respectively. The mortality of CM larvae inoculated with CpGV-ZY was intermediate compared to the average mortality rates in CpGV-M- and -S-treated insects. At 14 dpi, all isolates except CpGV-ALE and -M induced >80% mortality in CpRR1 larvae (Fig. 2B). When the mortality rates at 7 and 14 dpi were compared for CpS and CpRR1 larvae, delayed mortality was observed in CpRR1 larvae (Fig. 2). Interestingly, virus-induced mortality varied considerably in CpR5M larvae (resistance type II), not only at 7 dpi but also at 14 dpi. The mean mortality rates induced by CpGV-ZY and -WW in CpR5M larvae at 7 dpi were 64.1% and 72.3%, respectively, which was even higher than for CpRR1 larvae, with 55.9% and 43.3%, respectively (Fig. 2A). A similar trend could be observed at 14 dpi, though at a higher mortality level. This finding indicated that CpGV-ZY and -WW were more virulent to CpR5M larvae than to CpRR1 larvae. Strikingly, both CpGV-JQ and -ZY2 caused high mortality in CpRR1 larvae but not in CpR5M larvae, whereas CpGV-ZY overcame resistance in CpR5M larvae but not in CpRR1 larvae. Both CpRR1 and CpR5M larvae showed low susceptibility to CpGV-ALE, with average mortality values of less than 25% and 55% at 7 and 14 dpi, respectively. For CpRGO larvae (type III resistance), some variability in mortality was observed, although the results for all isolates except CpGV-M at 7 dpi were statistically not different from the result for the most virulent isolate, CpGV-E2 (post hoc test with
Tukey’s method, *P > 0.05*. In summary, these comparative tests suggested that isolates CpGV-JQ, -KS1, and -ZY2 were type I resistance breaking, because their mortality values were similar to or higher than that of CpGV-S, whereas CpGV-ZY and -WW were able to overcome type II resistance.

To further corroborate some of these findings, the median lethal concentrations (LC50s) of three isolates (CpGV-ZY, -ALE, and -WW) were determined for CpS and CpRR1 larvae. At 7 dpi, the LC50s of CpGV-ZY and -WW were 11.7- and 2.7-fold higher for CpRR1 than for CpS larvae, respectively. The LC50 of CpGV-ALE could not even be determined because of the lack of response of CpRR1 larvae (Table 1).

**TABLE 1** LC50s of three CpGV isolates tested on susceptible and resistant codling moth strains

<table>
<thead>
<tr>
<th>CpGV isolate</th>
<th>CM strain</th>
<th>No. of larvae tested</th>
<th>Value for indicated parameter at:</th>
<th>Probit line and fit&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Value for indicated parameter at:</th>
<th>Probit line and fit&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>7 dpi</strong></td>
<td><strong>14 dpi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LC50 (10&lt;sup&gt;3&lt;/sup&gt; × no. of OBs/ml) (95% CI)</td>
<td>Probit line and fit&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LC50 (10&lt;sup&gt;3&lt;/sup&gt; × no. of OBs/ml) (95% CI)</td>
<td>Probit line and fit&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ZY</td>
<td>CpS</td>
<td>914</td>
<td>2.99 (1.81–4.77)</td>
<td>1.33 ± 0.16</td>
<td>11.87 (4)</td>
<td>1.04 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>CpRR1</td>
<td>971</td>
<td>34.84 (28.42–42.71)</td>
<td>1.40 ± 0.20</td>
<td>2.30 (4)</td>
<td>1.35 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>ALE</td>
<td>1,022</td>
<td>19.63 (16.05–24.01)</td>
<td>1.27 ± 0.16</td>
<td>4.64 (4)</td>
<td>1.65 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>CpS</td>
<td>923</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.70 ± 0.17</td>
<td>16.16 (4)</td>
<td>1.35 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>CpRR1</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WW</td>
<td>1,255</td>
<td>14.65 (9.55–22.47)</td>
<td>1.71 ± 0.17</td>
<td>19.19 (4)</td>
<td>1.61 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>CpS</td>
<td>1,624</td>
<td>39.60 (15.31–102.38)</td>
<td>0.74 ± 0.10</td>
<td>22.82 (4)</td>
<td>1.14 ± 0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup>CpS, susceptible codling moth (CM) strain; CpRR1, type I resistant CM strain.

<sup>b</sup>The slope of the probit line and its standard error (SE) and the χ² value of the fit to the probit analysis and the degrees of freedom (df) are given.

<sup>c</sup>ND, not determined because the calculated value was beyond the tested concentrations by more than a factor of 1,000.
phenomenon was also recorded at 14 dpi, though to a lower degree. Only CpGV-WW induced similar mortality responses in both CpS and CpRR1 larvae (Table 1), underscoring its delayed activity as suggested by the resistance test (Fig. 2).

**Analysis of pe38 PCR products.** To determine whether the new CpGV isolates harbor the 2×12-bp insertion, which was proposed in previous studies to be the target of the type I resistance in CpRR1 larvae (13), a partial pe38 (ORF24) fragment covering the 2×12-bp insertion region was amplified by PCR from all seven Chinese CpGV isolates and from CpGV-M as the reference sequence. Agarose gel electrophoresis revealed that the PCR fragments of CpGV-ZY, -ALE, -KS1, and -M were within the same size range of approximately 304 bp (Fig. 3). However, the PCR fragments of isolates CpGV-JQ, -ZY2, and -WW showed smaller bands with estimated sizes of around 280 bp. Interestingly, the CpGV-KS2 fragment showed a slightly larger size than that found in CpGV-M. The CpGV-ZY2 fragment and also those of CpGV-KS1 and -ZY showed submolar bands similar in size to the fragment of CpGV-KS2, suggesting that these isolates are mixtures of genotypes carrying heterogeneous versions of pe38.

**Multiple sequence alignment of pe38 segments.** Sanger sequencing of cloned PCR fragments revealed some unexpected heterogeneity of the pe38 repeat region. Only CpGV-ALE and -KS1 contained the 2×12-bp-repeat insertion (2×DTVD [aspartic acid-threonine-valine-aspartic acid] amino acid motif) that is typical for CpGV-M and related isolates, whereas isolates CpGV-JQ and -WW lacked the insertion, as would be characteristic of isolates that can break type I resistance (Fig. 4A). The other isolates showed mixed versions of pe38; CpGV-ZY and -ZY2 contained pe38 types with and without the 2×12-bp repeat, whereas CpGV-KS2 had a 3×12-bp insertion and a unique 12-bp-repeat unit. In summary, there were three types of pe38, representing no insertion (CpGV-WW and -JQ), a 2×12-nucleotide insertion (CpGV-ALE), or a 3×12-nucleotide insertion (CpGV-KS2), resulting in one, three, and four repeats of the DTVD amino acid motif, respectively (Fig. 4B).

**DISCUSSION**

The capacity of seven new CpGV isolates to infect susceptible and resistant CM strains was determined by resistance testing and LC50 determination. All isolates were highly infectious for susceptible CM larvae, and several of them were able to break different types of CpGV resistance. During the experiments it was noted that the mean mortality rates of CpRR1 larvae induced by CpGV-M were 40.6% at 7 dpi and 79.2% at 14 dpi. Though a reduced susceptibility of CpRR1 larvae to CpGV-M was clearly notable compared to the susceptibility of CpS larvae, the observed mortality of CpRR1 larvae was considerably higher than the mortality values of 5% and 11% obtained under similar conditions in previous studies (26). This finding provided evidence that CpRR1 may have lost some degree of its high level of resistance during the last couple of years of rearing without further virus selection, as was recently analyzed in more detail by Fan et al. (31). A similar phenomenon was reported for CpR (34), which showed a decline but not a complete loss of resistance during 60 generations of rearing without viral selection pressure. Strain CpRR1 has been reared since 2011 without virus selection pressure. For CpR5M and CpRGO larvae also, mortality caused by CpGV-M and CpGV-S
appeared to be slightly higher, though the general pattern of resistance was clearly confirmed (26). In relation to CpRR1 (type I resistance), CpGV-M is a target of resistance, whereas CpGV-S is a resistance-breaking isolate (13, 25). Therefore, the rates of mortality induced by CpGV-M and CpGV-S were set as fixed points to determine the resistance-breaking activities of the new isolates. CpGV-ALE, -KS2, and -WW were not able to efficiently kill CpRR1 larvae, as they induced low rates of mortality similar to that of CpGV-M (genome group A) at 7 dpi (13). The mortality of CpRR1 larvae caused by CpGV-ALE was lower than that of any other isolate at both 7 and 14 dpi, even lower than that induced by CpGV-M. Other CpGV isolates, such as CpGV-R (Russia), CpGV-I66 and -I68 (Iran), and CpGV-G01 and -G02 (Georgia) showed lower levels of virulence than CpGV-M in bioassays using susceptible CpS neonates, suggesting considerable variability in the virulence of naturally occurring CpGV isolates (15, 35).

CpGV-WW killed CpR5M and CpRGO larvae as efficiently as the resistance-breaking CpGV-E2, which has been considered to be the most powerful CpGV isolate so far, capable of breaking all three types of resistance (16, 26). On the other hand, the mortality of CpRR1 larvae induced by CpGV-WW was significantly lower than that induced by CpGV-E2 at 7 dpi (Fig. 2A), leaving CpGV-WW as the first CpGV isolate able to break type II and III resistance but not type I resistance. The speed of killing by CpGV-WW in CpRR1 larvae appeared to be delayed, as mortality at 7 dpi but not at 14 dpi was lower than that of several other isolates. The results of resistance testing at the discriminating concentration were followed up in selected full-range bioassays of CpGV-ZY, -ALE, and -WW on CpS and CpRR1 larvae. The LC50 at 7 dpi could not be determined for CpGV-ALE due to the very low mortality, whereas the LC50 value at 14 dpi was significantly higher than those of CpGV-ZY and -WW (Table 1).

FIG 4 Sequence analysis of pe38 repeat region obtained from PCR products of new Chinese CpGV isolates. Sequences of CpGV-M, -S, and -E2 are given as reference sequences (13). (A) Nucleotide sequence of randomly picked clone from each isolate. (B) Amino acid sequences corresponding to nucleotide sequences in panel A. For isolates CpGV-ZY and -ZY2, two types of pe38 (indicated by suffixes -1 and -2), with and without a 12-bp insertion, were found; CpGV-KS2 showed a 12-bp insertion or no insertion; and CpGV-JQ and -WW contained no 212-bp insertion in pe38. Isolates CpGV-ALE and -KS1 showed the same repeat pattern of pe38 as CpGV-M. The number of sequenced colonies belonging to each type is given in parentheses at the end of the isolate’s name.
The viral gene *pe38* was confirmed as a target of type I resistance (13), and this gene has also been used as a genetic marker to distinguish between CpGV isolates prone to this type of resistance or able to break it (13, 33), depending on the presence of a 2 × 12-bp-repeat insertion that is found in CpGV-M and other CpGVs of genome group A but not in resistance-breaking isolates like CpGV-E2, -S, and others.

PCR and sequence analysis of the 2 × 12-bp-repeat insertion region in *pe38* of the seven new CpGV isolates showed that there were three types of *pe38*: those without the insertion (CpGV-JQ and -WW), those with a 2 × 12-bp-repeat insertion (CpGV-ZY, -ZY2, -ALE, and -KS1), and one with a 3 × 12-bp-repeat insertion (CpGV-KS2) (Fig. 3 and 4). Gel electrophoresis of PCR products indicated that some isolates, such as CpGV-ZY, -ZY2, and likely -KS1, contained double bands of the 2 × 12-bp-repeat area. Sanger sequencing of cloned PCR fragments further corroborated this finding for CpGV-ZY, -ZY2, and -KS2 but not for CpGV-KS1 (Fig. 4). Thus, based on the *pe38* heterogeneity, at least some of the isolates clearly represented genotype mixtures. This finding may further explain the high variability observed in the resistance testing. Field mixtures of baculovirus provide the genetic source for genome diversity, giving the possibility for baculovirus to keep and obtain variable genotypes (36). This also appears to be the case for CpGV, for which field isolates have been identified as mixtures of genotypic variants which may have the potential to adapt more efficiently to different ecological habitats (37, 38). On the other hand, laboratory selection on resistant CM populations (R<sub>GV</sub>) allowed CpGV to adapt to the resistant host and resulted in increased virulence against type I resistance, e.g., progeny virus of isolate NPP-R1 propagated on R<sub>GV</sub> larvae for either 4 or 16 generations caused a decrease of CpGV-M-like genotypes and an increase of its virulence for type I resistant R<sub>GV</sub> larvae (39, 40).

In previous studies, CpGV-E2, a minor variant of CpGV-E, could not be obtained by in vivo passage experiments using CpGV-E as the inoculum on susceptible CM larvae (35). Restriction endonuclease analyses and whole-genome sequencing of CpGV-E2 revealed that this isolate is a mixture of genome group A and B isolates (13, 35). As CpGV-E2 showed high virulence against the CpRR1 strain, it is conceivable that genotypes from genome group A may positively influence the virulence of resistance-breaking genotypes, though genome group A viruses alone cannot break resistance. This hypothesis is supported by the observation of an LC<sub>50</sub> of isolate CpGV-ZY which was lower than that of CpGV-WW on CpRR1 at 7 and 14 dpi (Table 1). A mixture of CpGV-M and -R5 (each at 50%) induced mortality in resistant CM strain R<sub>GV</sub> (type I resistance strain) larvae that was similar to that caused by the resistance-breaking isolate CpGV-R5 alone (33). Interestingly, although *pe38* of CpGV-WW comprised only one type of *pe38* containing no 2 × 12-bp-repeat insertion, its virulence against CpRR1 was reduced or at least delayed (Fig. 2 and 4A). This finding contradicts the previously established model, which proposed that the lack of a 2 × 12-bp-repeat insertion of *pe38* results in high virus-induced mortality of type I resistant CM larvae. This inconsistent result implies that the structure of *pe38* alone may not fully explain whether an isolate breaks type I resistance or not. Berling et al. (41) proposed that more than one gene is involved in type I resistance; the observed response of CpGV-WW provides a hint in the same direction.

In conclusion, resistance tests and the partial sequences of *pe38* of seven new CpGV isolates from northwest China revealed novel patterns of virulence toward different CM strains. CpGV-ALE showed consistently low efficacy in all three resistant CM colonies, whereas CpGV-WW had a superior effect in CpR5M larvae (type II resistance) and CpRGO larvae (type III resistance) but not in CpRR1 larvae (type I resistance). Even though there is no CpGV resistance reported in China as of now, new isolates could be used as biocontrol agents for codling moths. Some of these isolates can break CM resistance, and the genetic differences related to resistance breaking may provide new genetic resources for future resistance management strategies.
MATERIALS AND METHODS

Insects. Four strains of codling moth (CM) (Cydia pomonella L.), CpS, CpRR1, CpRSM, and CpRGO, were used to perform resistance tests under laboratory conditions. CpS (susceptible to all CpGV isolates), CpRR1 (type I resistance), CpRSM (type II resistance), and CpRGO (type III resistance) larvae were reared in light chambers at 26°C under a 16-h/8-h light/dark photoperiod. Strain CpRR1 was established in 2007 and was reared since 2011 without selection pressure (8, 34). Strains CpRSM and CpRGO were established as described previously (25, 26). Larvae were reared on a semisemifluid diet at the Institute for Biological Control of the Julius Kühn-Institut (JKI), Darmstadt, Germany (42). Fifth-instar larvae were removed from the diet to allow pupation in wrinkled cardboard strips. Adults were kept under the same rearing conditions and were allowed to mate and to produce eggs. Recently hatched neonates (7 to 12 h after hatching) were used directly for bioassays.

CpGV isolates and propagation. Seven geographical isolates of CpGV were collected from northwest China during 2006 to 2014 and designated CpGV-ZY, -JQ, -ALE, -KS1, -KS2, -ZY2, and -WW according to the locations where specimens were collected (ZY, Zhangye; JQ, Juquan; ALE, Aral; KS, Kashgar; and WW, Wuwei) (Fig. 1). All viruses were isolated from individual dead larvae showing typical signs of baculovirus infection. Infected larvae normally died inside apples or were found under the cracks of bark on the trunks of apple trees. Occlusion bodies (OBs) were isolated from CM cadavers using the method described by Smith and Crook (43). Propagation of these isolates was carried out by feeding purified OBs to fourth-instar larvae of the susceptible CM strain CpS. OBs were purified using glycerol gradient centrifugation (44). Virus stocks of CpGV-M, -S, and -E2 were derived from 10°C stocks held at JKI (13, 15). Enumeration of viral OBs was performed by dark-field microscopy using a Petroff-Hauser counting chamber (Hauser Scientific, Horsham, PA, USA) and a light microscope (Leica DM RBE, Leica, Wetzlar, Germany) (29).

Virulence determination. A discriminating concentration of 5.8 × 10^4 OBs/ml, which would result in >95% mortality in susceptible CpS larvae at 7 dpi (8), was used to differentiate the levels of virulence of CpGV-ZY, -JQ, -ALE, -KS1, -KS2, -ZY2, and -WW, as well as CpGV-M, -S, and -E2, on CpS, CpRR1, CpRSM, and CpRGO neonates. Infection experiments were conducted in autoclavable 50-well plates containing 45 ml of diet mixed with 5 ml of OB suspension as described previously (29). For untreated controls, the virus suspension was replaced with 5 ml of water. Larvae that died from handling were recorded on the first day and were excluded from the experiment. Mortality of neonates was recorded at 7 and 14 dpi. Each treatment was independently performed three to five times. Recorded virus-induced mortality was corrected for mortality of the untreated control group using the formula of Abbott (45). The Abbott-corrected mortality values were analyzed using a quasibinomial generalized linear model (GLM). Mean values were compared by Tukey's test using the emmeans package 1.3.2 (R version 3.5.2 in RStudio 1.0).

The results were plotted in R using ggplot2 (46). Full-range bioassays of isolates CpGV-ZY and -ALE were done to determine the median lethal concentration (LC50) using six different virus concentrations (3 × 10^1, 1 × 10^1, 3 × 10^1, 1 × 10^2, 3 × 10^2, and 1 × 10^3 OBs/ml) (29). Six OB concentrations (5 × 10^4, 1 × 10^4, 5 × 10^4, 1 × 10^5, 5 × 10^5, and 1 × 10^6 OBs/ml) were used to determine the LC50 of CpGV-WW against CM larvae. For untreated controls, the virus suspension was replaced with 5 ml water. Bioassays were processed as described for the discriminating test. Mortality data were determined at 7 and 14 dpi and were corrected for control mortality (45). Full range bioassay data were analyzed by Probit analysis using ToxRat software (version 2.10) (ToxRat Solutions GmbH, 2005).

Comparison of pe38 among CpGV isolates. Virus DNA extraction was performed as described by Arends and Jehle (44). Briefly, viral OBs were first dissolved in 100 mM Na2CO3. Then, the solution was neutralized using 1.0 M HCl and 1% SDS was added to disrupt the virions. After two washings with a mixture of Tris-EDTA (TE)-saturated phenol/chloroform/isoamyl alcohol (25:24:1 [vol/vol/vol]), the viral DNA was precipitated in 96% ethanol, dried, and solubilized in double distilled water. A pair of PCR oligonucleotide primers, based on the CpGV-M genome (GenBank accession no. KM217575.1) (13) and covering the region of the 2-bp-repeat insertion of pe38, was designed using Primer Premier 5 software (pe38F_18881, 5'-TGGATAAGAAGGAATTGGAGG-3', and pe38R_18578, 5'-TTAATGGGTTTTTGGTGCGC-3'). The fragment of each CpGV isolate was amplified in a reaction mixture volume of 50 μl containing 2 μl of each primer (10 pmol/μl), 100 ng of viral DNA, 5 μl of 10× PCR buffer, 4 μl of MgCl2 (25 mM), 0.5 μl of deoxynucleoside triphosphate (dNTP) (10 mM), and 0.5 μl of Taq DNA polymerase and adding distilled water to a final volume of 50 μl. The amplification conditions were set as follows: 3 min predenaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension period of 72°C for 10 min. Small amounts of 5 μl of PCR products were applied on 2% agarose gel electrophoresis gels stained with midori green advance (Nippon Genetics Europe GmbH, Düren, Germany) and visualized using the ChemoCam Imager ECL UV transilluminator and software (INTAS Science Imaging Instruments GmbH, Göttingen, Germany). The major PCR products were purified using the DNA Clean & Concentrator kit (Zymo Research, Freiburg, Germany). Total purified DNA was cloned into the pGEM-T Easy vector (Promega Corporation, Madison, USA) as described by the manufacturer. Two to four white colonies selected with ampicillin were picked randomly for purification and Sanger sequencing at StarSEQ GmbH (Mainz, Germany). All sequenced fragments were aligned using MUSCLE alignment in Geneious version 9 (Biomatters, New Zealand) and with pe38 of CpGV-M as the reference sequence.

Data availability. Newly determined sequences were deposited in GenBank under accession numbers MN605990 to MN605999.
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

J.F. received financial support from the China Scholarship Council (grant no. 201506300113) to carry out the study at the Institute for Biological Control, Julius Kühn-Institut. The part of the study performed in China was supported by NSFC (grant number 31670659).

Excellent technical assistance in codling moth rearing by Doris El Mazouar, Birgit Ruoff, and Eckhard Gabrys is acknowledged. We thank Doreen Gabriel (JKI) for excellent statistical advice and Peter Krell (University of Guelph, Canada) for critically reading the manuscript.

REFERENCES


4. Tanada YA. 1964. Granulosis virus of codling moth, Caropaca pome


19. Zhang XZ. 1957. Taxonomic notes on the codling moth, Caropaca pome


