Isolation of an *Adoxophyes orana* granulovirus (AdorGV) occlusion body morphology mutant: biological activity, genome sequence and relationship to other isolates of AdorGV

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A granulovirus (GV) producing occlusion bodies (OBs) with an unusual appearance was isolated from *Adoxophyes* spp. larvae in the field. Ultrastructural observations revealed that its OBs were significantly larger and cuboidal in shape, rather than the standard ovo-cylindrical shape typical of GVs. N-terminal amino acid sequence analysis of the OB matrix protein from this virus suggested that this new isolate was a variant of *Adoxophyes orana* granulovirus (AdorGV). Bioassays of this GV (termed AdorGV-M) and an English isolate of AdorGV (termed AdorGV-E) indicated that the two isolates were equally pathogenic against larvae of *Adoxophyes honmai*. However, AdorGV-M retained more infectivity towards larvae after irradiation with UV light than did AdorGV-E. Sequencing and analysis of the AdorGV-M genome revealed little sequence divergence between this isolate and AdorGV-E. Comparison of selected genes among the two AdorGV isolates and other Japanese AdorGV isolates revealed differences that may account for the unusual OB morphology of AdorGV-M.

INTRODUCTION

Baculoviruses are dsDNA viruses belonging to the family *Baculoviridae*, an insect-specific virus family whose members have been identified in lepidopteran, dipteran and hymenopteran insects. Viruses of this family are distinguished by rod-shaped virions that are packaged in proteinaceous occlusion bodies (OBs) (Rohrmann, 2011). Baculoviruses generally produce two physically and biochemically distinguishable virion phenotypes during infection: occlusion-derived viruses (ODVs), which are liberated from the OBs in the host midgut lumen and establish primary infection of midgut epithelial cells, and budded viruses, which are released from infected host cells and serve to establish secondary infection of other host tissues.

The family *Baculoviridae* is divided into four genera on the basis of morphology, host taxonomy and phylogenetic relationships (Herniou et al., 2011). These genera comprise: *Alphabaculovirus*, the lepidopteran nucleopolyhedroviruses (NPVs); *Betabaculovirus*, the granuloviruses (GVs); and *Gammabaculovirus* and *Deltabaculovirus*, which consist of NPVs from Hymenoptera and Diptera, respectively. The GVs have been isolated exclusively from insects of Lepidoptera but are often distinguished from lepidopteran NPVs by the size and shape of their OBs and the number of virions occluded per OB. While alphabaculovirus OBs are usually polyhedral in shape and 0.4–2.5 µm in diameter, betabaculovirus OBs are smaller, ovo-cylindrical structures 0.12–0.35 µm in width by 0.3–0.5 µm in length (Tanada & Hess, 1991). While alphabaculovirus OBs generally contain multiple ODVs, betabaculovirus OBs contain one or two ODVs at most.

GVs have been formulated for control of insect pests around the world. Isolates of *Adoxophyes orana* granulovirus

The GenBank/EMBL/DDBJ accession number for the AdorGV-M genome sequence of the occlusion body morphology mutant is KM226332. The nucleotide sequences for additional AdorGV sequences from other isolates are available under accession numbers KM234090–KM234092 (p47), KM234093–KM234095 (orf112/113), KM234096–KM234098 (orf30), KM234099–KM234101 (p10 and orf14), KM234102–KM234104 (pep), KM234105–KM234107 (dbp), KM234108–KM234110 (desmoplakin) and KM234111–KM234113 (pep-p10).

Four supplementary tables are available with the online Supplementary Material.
(AdorGV) are used to control *Adoxophyes* spp. (Lepidoptera: Tortricidae) in a variety of crop systems. Initially, AdorGV was isolated from the apple pest *A. orana* (Aizawa & Nakazato, 1963), and the field trials were done in apple orchards in Japan (Shiga *et al*., 1973). Since the 1980s, AdorGV isolates have been developed to control *Adoxophyes honmai* on tea (Nakai, 2009). AdorGV was registered in 2003 and commercialized to control *A. honmai* and *A. orana* in tea fields and apple orchards, respectively. There are three *Adoxophyes* spp. in Japan, including *Adoxophyes dubia*, which also attacks tea in southern Japan. These species are closely related (Sakamaki & Hayakawa, 2004; Yasuda, 1998). Similarly, GVs isolated from *A. orana* and *A. honmai* yielded identical restriction fragment patterns by restriction endonuclease analysis, suggesting they are variants of the same species (Hilton & Winstanley, 2008).

In this study, an OB morphology variant of AdorGV from Miyazaki, Japan (AdorGV-M) was identified and characterized. In addition to ultrastructural examination of OBs and evaluation of biological activity, the genome of this variant was sequenced and compared with that of an English isolate of AdorGV (AdorGV-E) (Wormleaton *et al*., 2003) and with sequences of individual genes from three other Japanese AdorGV isolates.

## RESULTS AND DISCUSSION

### Identification of a GV morphology variant in Miyazaki Prefecture

In a screen for pathogens of *A. honmai*, 102 *A. honmai* larvae were collected from a tea field in Miyazaki Prefecture, Japan. Collected larvae were reared on artificial diet and monitored for pathogenesis and mortality. While approximately one-third of the larvae survived to pupation, 12 exhibited typical symptoms of GV infection (Table 1). Observation of these larvae by light microscopy revealed that one of the cadavers contained cube-shaped OBs that were larger than expected for a GV (Fig. 1a). The OBs were isolated from the cadaver. In transmission electron micrographs of larvae infected with a stock of this virus isolate, the OBs deviated significantly from the ovo-cylindrical shape normally seen with GV OBs (Fig. 1b–d). Rarely, tetrahedral OB sections were observed (Fig. 1b). The cuboidal OBs ranged from 0.5 to 2 μm in diameter, but no more than one virion containing a singly enveloped nucleocapsid was observed in any given OB section (Fig. 1c).

Purified OBs from larvae infected with this OB morphology variant were loaded and run on an SDS-PAGE gel. A major band migrating at 25 kDa was observed that corresponded to the presumptive OB matrix protein (data not shown). This band was cut out of the gel and subjected to Edman degradation to determine the amino acid sequence at the N terminus. This procedure yielded a 36 aa sequence (GYNKSLRYSRHGFTCVIDNHHLKSLGSLVNLIDVI) that was 100% identical to residues 2–37 of the granulins of both AdorGV-E and *Epinotia aporema* granulovirus (EpapGV), another GV from a tortricid host species (Ferrelli *et al*., 2012). Preliminary amplification and sequencing with AdorGV-specific granulin, fp25k, p10 and pep primers yielded nucleotide sequences sharing 99.2–100% sequence identity with homologous regions in the genome of the AdorGV-E (data not shown; Wormleaton *et al*., 2003). These results indicate that the Miyazaki *A. honmai* GV sample is an isolate of AdorGV.

### Characteristics of the relative pathogenicity, UV inactivation, and viral yield of AdorGV-M and AdorGV-E

To determine if the unusual shape and size of the OBs produced by this virus were associated with altered pathogenicity and persistence, bioassays were carried out with the AdorGV-M and AdorGV-E isolates against larvae

| Table 1. Mortality of *Adoxophyes* spp. larvae collected in the field in Miyazaki Prefecture |
|-----------------|-----------------|-----------------|
| **Cause of death** | **No. larvae collected (%)** |
| GV (large cuboidal OBs) | 1 (1.0) |
| GV (ovo-cylindrical OBs) | 12 (11.8) |
| Entomopoxivirus | 1 (1.0) |
| Fungi | 12 (11.8) |
| Parasitoid | 15 (14.7) |
| Unknown | 27 (26.5) |
| Healthy | 34 (33.3) |
| Total | 102 (100) |

![Fig. 1. Morphology of OBs for GVs. (a) Phase-contrast microscopy of OBs for AdorGV-M. (b, c) Low-magnification (b) and high-magnification (c) transmission electron micrographs of OBs for AdorGV-M. (d) Transmission electron micrograph of OBs for AdorGV-To.](http://vir.sgmjournals.org)
of *A. honmai*. The two AdorGV isolates killed *A. honmai* larvae with similar LC$_{50}$ (50% lethal concentration) values and dose–response curves (Table 2) at both first instar and fourth instar. The LC$_{50}$ of AdorGV-M was 2.1- and 2.3-fold greater than AdorGV-E LC$_{50}$ against first-instar *A. honmai* larvae and fourth-instar larvae, respectively. These differences were significant at the $P<0.05$ level. No significant difference was observed for the concentration–response slope of AdorGV-E and -M with either instar ($P>0.05$) (Table 2).

In an experiment examining the loss of pathogenicity upon irradiation with UV light, the AdorGV-M retained significantly more pathogenicity towards *A. honmai* larvae during prolonged periods of UV irradiation (Fig. 2), as the relative ratio of inactivation ($r$) was greater for OBs of AdorGV-E than of AdorGV-M. The half-life ($t_{1/2}$) of the OBs for AdorGV-M was 3.1 ± 0.28 min, which was significantly longer than that for AdorGV-E (0.61 ± 0.04 min; Table S1, available in the online Supplementary Material). On the other hand, $r$ and $t_{1/2}$ values for ODVs from both strains were not significantly different ($P>0.05$). This suggests that the unusual OB morphology of AdorGV-M was associated with UV resistance.

Yield of infectious progeny virus generated by infection with AdorGV-E or AdorGV-M was estimated by bioassay of homogenates prepared from GV-killed larvae (Fig. 3). The yield of infectious OBs produced per larva inoculated with AdorGV-E and -M was estimated at 2.0 ± 0.30 × 10$^{11}$ and 5.5 ± 0.9 × 10$^{9}$ OBs per larva (mean ± SE), respectively, which was significantly different ($t=5.1$, df=4, $P<0.01$). These results also corresponded to a difference in OBs produced per unit body mass for AdorGV-E and -M, which was calculated to be 2.7 ± 0.30 × 10$^9$ and 8.9 ± 1.8 × 10$^7$ OBs mg$^{-1}$, respectively ($t=6.6$, df=4, $P<0.01$). The infectious viral yield of AdorGV-E per larva was 36 times higher than that of AdorGV-M, and that per unit body mass was 31 times higher.

### Characteristics of the AdorGV-M genome

The AdorGV-M genome was determined initially by Sanger dideoxy sequencing and subsequently by next-generation sequencing on an Illumina platform. Both sequencing methods yielded a consensus genome sequence of 99 507 bp, containing approximately 121 potentially protein-encoding ORFs (Tables S2 and S3). In the Illumina consensus sequence, positions 12 506 (within ORF 19/repeat region 2), 12 338 (within ORF 19/repeat region 2) and 77 258 (non-coding regions between ORFs 95 and 96/repeat region 8) were found to be highly polymorphic.

The genome sequences of the AdorGV-M and AdorGV-E isolates share 99.7% nucleotide sequence identity by Martinez/Needleman–Wunsch alignment, with 46 gaps inserted to optimize the alignment. The AdorGV-M sequence contains all of the 37 baculovirus core genes and all of the 26 additional genes found in all members of genus *Betabaculovirus* (Garavaglia et al., 2012). It also contains all of the repeat regions and all but one of the ORFs described for the AdorGV-E isolate, but is 150 bp shorter than the AdorGV-E isolate due mostly to deletions in the repeat region sequences of the AdorGV-M genome. AdorGV-M ORF 52, which is located within repeat region 6 in the AdorGV-E isolate, is truncated at 43 codons in the AdorGV-M isolate due to a C→A substitution which created a TAG stop codon. The AdorGV-M sequence has three ORFs not listed in the sequence of the AdorGV-E isolate, including ORF 65, ORF 104, and ORF 116. ORF 65 and ORF 104 are relatively small ORFs of 56 and 75 codons, respectively, and can also be found in the AdorGV-E isolate sequence. No homologues for these ORFs are present in other baculovirus genomes. ORF 116 is a homologue of the late expression factor-10 (*lef-10*) gene (Lu & Miller, 1994) and can also be found in the AdorGV-E isolate. ORF 116 is a homologue of the late expression factor-10 (*lef-10*) gene (Lu & Miller, 1994) and can also be found in the AdorGV-E isolate. Of the ORFs shared by AdorGV-M and AdorGV-E, only eight differed in the occurrence of upstream transcriptional motifs, including ORFs 22, 28 (*odv-c66*), 30, 31, 68 (*dbp*), 85 (*vp91*), 113 and 120 (*egt*) (Table S3).

### Comparison of selected genes among English and Japanese AdorGV isolates

Of the 121 ORFs that the AdorGV-M and AdorGV-E isolates have in common, 66 of them encode amino acid sequences that share 100% identity by BLAST. All putative homologues of AdorGV-M shared with that of AdorGV-E showed the same gene order (Table S3). Of the remaining ORFs, ORF 24 encodes the least conserved gene product (Tables S2 and S3). In the Illumina consensus sequence, positions 12 506 (within ORF 19/repeat region 2), 12 338 (within ORF 19/repeat region 2) and 77 258 (non-coding regions between ORFs 95 and 96/repeat region 8) were found to be highly polymorphic.

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### Table 2. Dose–response of AdorGV-M and -E on *A. honmai* larvae

<table>
<thead>
<tr>
<th>Instar</th>
<th>Virus</th>
<th>LC$_{50}$ (OBs ml$^{-1}$)</th>
<th>95% Confidence limit</th>
<th>Slope</th>
<th>$\chi^2$</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
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<tr>
<td>first</td>
<td>AdorGV-E</td>
<td>6.0 × 10$^5$</td>
<td>4.6 × 10$^5$</td>
<td>1.20</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>AdorGV-M</td>
<td>1.3 × 10$^5$</td>
<td>9.5 × 10$^5$</td>
<td>1.02</td>
<td>2.58</td>
</tr>
<tr>
<td>fourth</td>
<td>AdorGV-E</td>
<td>1.1 × 10$^7$</td>
<td>8.5 × 10$^6$</td>
<td>0.96</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>AdorGV-M</td>
<td>2.5 × 10$^7$</td>
<td>1.9 × 10$^7$</td>
<td>0.98</td>
<td>0.77</td>
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found in the genome of *Pieris rapae* granulovirus (PiraGV; Zhang et al., 2012), and other poorly conserved homologues of this ORF appear to be present in other GV genomes (Wormleaton et al., 2003).

Among baculovirus genes that are known or suggested to be involved, directly or indirectly, in baculovirus OB morphogenesis, the AdorGV granulin (*gran*, ORF 1), *pep-1* (polyhedral envelope protein; ORF 16), and *fp25k* (ORF 101) genes share 100% sequence identity at the amino acid level between the AdorGV-M and AdorGV-E isolates (Table S3). The *p10* gene product (ORF 13) differs in sequence by one amino acid. Also, genes encoding a second *pep* homologue (*pep-2*; ORF 18) and a protein that appears to be a fusion of P10 and PEP sequences (ORF 17) also exhibit differences in amino acid sequence between the AdorGV-M and AdorGV-E isolates (Table S3).

In this study, AdorGV-M showed higher UV resistance than AdorGV. Like other baculoviruses, GVs are rapidly inactivated by exposure to UV light in sunlight (David et al., 1968). A capacity to resist inactivation by UV light could conceivably cause an increase in the fitness of a virus strain that possessed such a trait. GV genes that influence susceptibility to inactivation by UV light may therefore be under positive selection pressure (Yang, 2001). To identify genes in AdorGV-M that may have undergone positive selection during the divergence of the AdorGV-M and AdorGV-E isolates, the non-synonymous and synonymous

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**Fig. 2.** Persistence of AdorGV-E and AdorGV-M exposed to UV-B light. Percentage of original activity remaining (OAR) is shown for OBs and ODVs from AdorGV-E and -M exposed to UV-B light.

**Fig. 3.** Infectious viral yield of *A. honmai* larvae inoculated with AdorGV-E and AdorGV-M. Infectious viral yield per larva (a) or larval body mass (b) at 20 days p.i. Bars indicate SEM. Different lower-case letters indicate significant difference (*P*<0.05).
substitution rates ($d_N$ and $d_S$, respectively; Yang, 2001) between homologous ORF pairs bearing non-synonymous substitutions were estimated using a maximum-likelihood method. Of 30 genes examined, two ORFs, ORF 14 and ORF 68, were found to have $d_N/d_S$ ratios of 1.3 and 1.1, respectively, suggesting that these ORFs may have experienced positive selection pressure. Homologues of ORF 14 are present in GVs of *Clostera anachoreta* (Liang et al., 2011), *Clostera anastomosis* (Liang et al., 2013) and PirAGV (Zhang et al., 2012), but there are no clues about the function of this gene. ORF 68 encodes a homologue of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) ac25, or *dbp* (DNA-binding protein; Mikhailov et al., 1998).

To identify amino acid differences unique to the AdorGV-M that may account for this isolate’s unusual OB morphology, a selection of nine genes were amplified from the genomes of three additional AdorGV isolates from Tokyo, Kagoshima and Tokushima, Japan, and sequenced. These isolates produce OBs with typical GV granule size and morphology (data not shown). The nine genes selected included three genes, ORF 13 (p10), ORF 17 (p10-pep) and ORF 18 (pep-2), putatively involved in OB assembly that differed in sequence in AdorGV-M, and the two genes with relatively high $d_N/d_S$ ratios, ORF 14 and ORF 68 (*dbp*). In addition, the sequences for ORF 30 and ORF 112 of the English isolate also were amplified and sequenced. The AdorGV-M ORF 30 sequence contains a frameshift at codon 92 that alters the amino acid sequence relative to ORF 30 of AdorGV-E. Likewise, the predicted amino acid sequence of AdorGV-M genome ORF 113 aligns with positions 35–132 of ORF 112 of the English strain; an upstream ORF in a different reading frame encodes the first 17 amino acids of ORF 112 in AdorGV-E, but a frameshift at this position results in a different sequence in AdorGV-M. While ORF 30 is unique to the AdorGV genome, ORF 112/113 has homologues in the *A. orana* and *A. honmai* nucleopolyhedrovirus genome sequences (Hilton et al., 2016; Nakai et al., 2003), as well as a less conserved homologue in the *Agrotis segetum* granulovirus sequence (GenBank accession no. YP_006286). Finally, the p47 (ORF 57) and desmoplakin (ORF 95) genes also were amplified and sequenced. The p47 gene is a baculovirus core gene that encodes a subunit of the baculovirus RNA polymerase (Guarino et al., 1998). The p47 gene of AdorGV-E and AdorGV-M genes are distinguished by three non-synonymous substitutions, with an absence of synonymous substitutions. The *desmoplakin* gene, also a core gene, encodes a nucleocapsid-associated protein that is required for nucleocapsid egress from the nucleus of infected cells (Ke et al., 2008). Numerous non-synonymous and synonymous substitutions distinguish the AdorGV-M copy of this gene from that of the AdorGV-E.

Nucleotide sequence alignments and phylogenetic inference with the *pep*-p10, p47, *dbp* and *desmoplakin* coding sequences indicated that all five AdorGV isolates examined are likely variants of the same GV species (Fig. 4a). The sequences of the AdorGV-K, AdorGV-To and AdorGV-E isolates were almost identical, and these taxa were placed together in a clade with strong bootstrap support (Fig. 4b). The AdorGV-Tks and AdorGV-M sequences are more divergent, though AdorGV-Tks was still placed in a larger group with AdorGV-K, AdorGV-To and AdorGV-E. Phylogenetic inference with all the nucleotide sequence data generated for all nine loci produced a tree with very similar topology, except that AdorGV-Tks was placed into a group with AdorGV-M with low bootstrap support (data not shown).

Alignment of the encoded amino acid sequences from the nine loci revealed that the sequences for the predicted polypeptides of AdorGV-K, AdorGV-To and AdorGV-E were identical, while amino acid differences were found in the AdorGV-M and AdorGV-Tks sequences (Fig. 4). These two isolates had the same amino acid differences in the p10, ORF 14 and pep-2 sequences relative to the AdorGV-K/To/ E consensus sequence. The AdorGV-M sequences for *pep*-p10, p47, *dbp* and desmoplakin contained amino acid differences that were unique to AdorGV-M. Likewise, the AdorGV-Tks sequences also contained unique amino acid differences in its *pep*-p10, p47 and desmoplakin sequences (Table 3). While both the AdorGV-M and AdorGV-Tks isolates bear frameshifts in the same approximate area of ORF 30 (Fig. 5), only the AdorGV-M contains a frameshift in ORF 112/113. Since electron microscopy analysis shows that AdorGV-Tks does not produce OBs that are cuboidal or unusually large (data not shown), the amino acid substitutions occurring in *pep*-p10, p47, *dbp*, desmoplakin and ORF 112/113 correlate with the distinctive OB morphology of AdorGV-M and may contribute to the occurrence of their large size and cuboidal shape.

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**Fig. 4.** Phylogenetic analysis of nucleotide sequences for ORFs of the genes *pep*-p10, p47, *dbp* and desmoplakin, showing relationships of the AdorGV isolates together with PirAGV (Zhang et al., 2012) as an outgroup (a) and details of the AdorGV group (b). Minimum evolution (ME) phylogenies inferred from the concatenated alignment are shown with bootstrap values ≥50% for ME and maximum-parsimony (MP) trees at each node where available (ME/MP). Bars, nucleotide substitutions per site.
Aberrant OB morphology in other baculoviruses

Watanabe & Imanishi (1972) described OBs of an abnormal shape in GV-infected larvae of *A. honmai* (previously named *Adoxophyes fasciata*), alongside OBs with the standard GV ovo-cylindrical shape. These GVs were not cubic but exhibited an elongate or irregular shape, and some appeared to be ‘compound’ granules consisting of multiple granules fused together. Cuboidal OBs of an abnormally large size have been reported for other GVs isolated from *Plodia interpunctella*, *Choristoneura fumiferana* and *Cydia pomonella* (Bird, 1976; Arnott & Smith, 1968; Stairs, 1964; Stairs et al., 1966). The cubic granules reported by Arnott & Smith (1968) occurred alongside elongate and compound granules in *Pl. interpunctella*. Both Arnott & Smith (1968) and Stairs (1964) reported that the cubic granule phenotype persisted through consecutive passages in healthy insects, suggesting that this phenotype was genetically encoded by the GVs in these studies.

Several studies with alphabaculoviruses (NPVs) indicate that the sequence of the polyhedrin (*polh*) gene is a primary determinant of OB morphology in infected cells (Carstens *et al.*, 1986; Cheng *et al.*, 1998; Eason *et al.*, 1998; Jarvis *et al.*, 1991; Katsuma *et al.*, 1999; Lin *et al.*, 2000; López *et al.*, 2011). In a number of cases, mutation of the polyhedrin gene resulted in the formation of cuboidal OBs (Carstens *et al.*, 1986; Jarvis *et al.*, 1991; Lin *et al.*, 2000; Katsuma *et al.*, 1999).

In this study, the AdorGV-M and -E sequences of the *gran* genes (the betabaculovirus orthologues of alphabaculovirus *polh* genes) were identical, indicating that the mutant morphology of the AdorGV-M OBs cannot be attributed to mutations in the *gran* gene. This observation is consistent with results from other alphabaculovirus studies showing that the *polh* sequence is not the sole determinant of OB morphology (Hu *et al.*, 1999; Woo *et al.*, 1998). Eason *et al.* (1998) reported that recombinant clones of AcMNPV in which the native *polh* gene has been replaced by the *gran* gene of *Trichoplusia ni* granulovirus produced large, cuboidal OBs in cell culture, further indicating that a WT granulin sequence can generate OBs of the sort observed for AdorGV-M. In contrast with the OBs formed by AdorGV-M, the granulin...
OBs formed by these recombinant AcMNPV clones contained few or no virions, as was also the case for the cuboidal OBs generated by NPV polh mutants. Mutation of the gene encoding FP25K is also known to reduce virion occlusion of AcMNPV (Harrison & Summers, 1995); however, no mutations in the fp25k gene were discovered in AdorGV-M.

The surface of baculovirus OBs is covered by a layer referred to as the calyx, or polyhedral envelope, which consists mostly of carbohydrate (Minion et al., 1979). A phosphorylated protein encoded by the pep (polyhedral envelope protein) gene is a structural component of this envelope (Whitt & Manning, 1988). Also, the p10 gene appears to be involved in the formation of the calyx, though it is not itself a component of the calyx (Van Oers & Vlak, 1997). The calyx appears to lend physical stability to the OBs, but it has also been speculated that the calyx serves to delimit the boundaries of OBs and define their shape (Eason et al., 1998). Recombinant clones of the Orgyia pseudotsugata multiple nucleopolyhedrovirus in which either the pep gene or both the pep and p10 genes had been disrupted by insertion of a marker gene produced cuboidal OBs during infection of cells in culture, similar to the OBs of AdorGV-M (Gross et al., 1994). The AdorGV-M ORF 17 (pep-p10) gene, which encodes a PEP–P10 fusion protein, contains a serine to phenylalanine mutation (relative to the AdorGV consensus sequence for this gene; Table 3) that is unique to AdorGV-M. This mutation may account for the large size and cuboidal morphology of AdorGV-M OBs.

Finally, few differences exist between AdorGV-E and AdorGV-M in terms of the occurrence and distribution of transcriptional motifs (Table S3), and no differences were found in the transcriptional motifs upstream of the gran, pep-1, pep-2, pep-p10 or p10 genes. Nevertheless, we cannot formally exclude the possibility that differences in the expression of one or more of these genes may contribute to the OB morphology phenotype exhibited by AdorGV-M.

**Increased UV resistance and its possible impact on fitness**

AdorGV-M exhibited an LC50 that was significantly higher than that of AdorGV-E in bioassays at the P<0.05 level, but the magnitude of difference was only approximately twofold. In contrast, AdorGV-M OBs were significantly more resistant to inactivation by UV irradiation, with a t½ that is fivefold longer than that of AdorGV-E OBs. The larger OBs of AdorGV-M imply that the occluded virions of this strain are surrounded by a thicker layer of crystalline granulin matrix than those of AdorGV-E. Studies examining the capacity of the OB crystalline protein matrix to protect occluded virions against UV inactivation have provided contradictory results. Ignoffo et al. (1992) found...
that OBs of *Helicoverpa zea* single nucleopolyhedrovirus actually possessed a slightly shorter $t_{1/2}$ than alkali-liberated, non-occluded virion (ODV) when both were exposed to a simulated sunlight UV source, indicating that the crystalline protein matrix of OBs provides no protection against UV inactivation. Behle et al. (2000) found that OBs of an NPV from *Anaglyphra falcifera* lost a significantly smaller proportion of activity upon exposure to simulated sunlight than did non-occluded ODV of the same virus, which indicated that the OB matrix does provide ODV with some protection against UV inactivation. If resistance to inactivation by sunlight makes a significant contribution to fitness of a baculovirus in the field, then one would expect that the large OB phenotype may provide a fitness advantage compared with the smaller, typically ovo-cylindrical OB phenotype. On the other hand, yield of infectious OBs was approximately 30 times less in AdorGV-M than in AdorGV-E. If infectious viral yield reflects number of OBs, it is consistent with the observation that the numbers of aberrant cuboidal OBs reported for other baculoviruses tend to be low when they occur in infected cells. Although the larger OB phenotype has been reported from time to time among GVs, the standard ovo-cylindrical shape of GV OBs appears to be far more prevalent, as was the case with OBs of the GV-killed larvae harvested in Miyazaki (Table 1).

In conclusion, we identified a strain of *Adoxophyes orana* granulovirus with an unusual cuboidal morphology that exhibited an increased degree of resistance to inactivation by UV irradiation. Genomic sequencing of this virus and comparison with sequences from other strains of the same species have revealed the possible genetic basis for the unusual properties of this virus. Further studies comparing AdorGV-M and other AdorGV isolates may provide more insight into the occurrence of aberrant OB morphology and, more generally, into the molecular basis of baculovirus OB morphogenesis and its evolution.

**METHODS**

**Viruses and insects.** The AdorGV-M isolate was obtained from an infected *Adoxophyes* spp. larva collected from a tea field in Kawaminami-cho, Miyazaki Prefecture on 1 June 1999. OBs from the cadaver were propagated in healthy *A. honmai* larvae after removing midguts, because the sample was initially contaminated with cypovirus. To produce a clonal isolate of AdorGV-M, OBs were subjected to three passages through larvae after killing larvae harvested in Miyazaki (Table 1).

**Transmission electron microscopy.** *A. honmai* neonate larvae were infected by allowing them to consume droplets containing $1.0 \times 10^6$ OBs ml$^{-1}$ of AdorGV-M or AdorGV-To. Infected fifth-instar larvae showing typical granulosis gross pathology were dissected and the fat body was fixed, dehydrated, occluded in resin, ultrasectioned and stained for analysis by electron microscopy (H-7100, Hitachi) at 100 kV as described by Nakai & Kunimi (1997).

**Bioassays.** Susceptibility of neonate and fourth-instar *A. honmai* larvae to OBs of AdhoGV-M and AdhoGV-E was examined by the food disc and droplet-inoculation methods, respectively. For the food disc method, neonate larvae in 30 mm diameter plastic dishes were exposed to a disc (6 mm diameter, 6 mm thickness) of Insecta LF diet (Nihon Nosan-Kogyo) onto which $10 \mu$l OB suspension ($10^4.5$, $10^5$, $10^5$, $10^5$, $10^5$ and $10^6$ OBs ml$^{-1}$) had been pipetted. The control larvae were fed a diet disc treated with $10 \mu$l sterilized distilled water. After 24 h of feeding at 25 °C, each larva was individually transferred into a piece of fresh artificial diet in a half-ounce cup (3 cm diameter × 2.5 cm height). For the droplet-inoculation method, newly moulted fourth-instar *A. honmai* larvae were exposed to droplets containing OB suspension ($10^4.5$, $10^5$, $10^5$, $10^5$, $10^5$ and $10^6$ OBs ml$^{-1}$), 1% red food dye and 10% sucrose. Inoculated larvae were reared on artificial diet at 25 °C, with 16L:8D photoperiod, until either pupation or larval death. Development and mortality were observed daily. More than 35 larvae were used for each treatment with three replicates. The dose–response data were analysed by probit analysis using POLO-PC (Lebra Software).

**Resistance of OBs and ODVs to inactivation by UV exposure.** UV tolerance of AdorGV-E and -M OBs was compared by measuring the mortality of larvae inoculated with OBs exposed to UV light. Drops of OB suspension ($5 \mu$l of each drop containing $8.0 \times 10^7$ OBs ml$^{-1}$ for AdorGV-E and $1.3 \times 10^7$ OBs ml$^{-1}$ for AdorGV-M) were placed on a plastic film (Parafilm) and irradiated with a UV-B lamp (15 W, G15T8E; Sankyo Denki) in a black box (50 × 16 × 9.51 cm) in a 4 °C cold room. The distance between the UV light source and the drops was 9 cm, which corresponded to a light intensity of 390–410 μW cm$^{-2}$ as measured by a UV meter (UV-340; Custom). After 0, 10, 60, 120, 180 and 300 s of UV exposure, the drops were transferred to light-shaded plastic tubes and kept at 4 °C until use. Because of the shorter persistence of AdorGV-E OBs, two additional exposure times, 30 and 90 s, were used with trials involving AdorGV-E. All the drops examined were placed on the plastic film for a total of 300 s, including the time spent exposed to UV light.

Bioassay using the UV-exposed OB was done by the droplet-inoculation method as described above. Newly moulted fourth-instar larvae were exposed to sucrose/red dye drops containing OB suspensions on Parafilm that had been exposed to UV light. Larvae showing red colour in the midgut area were transferred to half-ounce cups supplied with artificial diet (Insecta LF) and were reared at 25 °C, 16L:8D. The percentage of larvae exhibiting symptoms of granulosis was determined by light microscopy. Three biological replicates were performed.

To measure the resistance of ODVs to UV inactivation, OB suspensions of both strains were dissolved with DAS buffer (0.1 M Na$_2$CO$_3$, 0.17 M NaCl, 0.01 M EDTA, pH 10.5) for 30 min, then neutralized with 0.18 vol 1 M Tris/HCl (pH 7.4) and centrifuged at 5000 g for 10 min at 4 °C to remove undissolved debris. The ODV
particles were purified by 30–60% sucrose gradient ultracentrifugation at 120,000 g for 30 min at 4 °C. The visible ODV band was collected and washed twice with TE solution (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and suspended in TE solution. The ODV solution was diluted to an LC_{70} concentration (as determined by bioassay), exposed to UV light as described above and kept at 4 °C until use. Bioassays were set up with 35 newly moulted fourth-instar larvae per exposure time as described above. Three biological replicates were performed.

**Measuring yield of infectious virus.** Neonate larvae were inoculated with an inoculum corresponding to an LC_{50} of AdorGV-E or AdorGV-M (1.4 x10^5 and 5.0 x10^7 OBs ml^-1, respectively) by the food disc method as described previously (Nakai et al., 2004). After 20 days, three larvae were collected randomly, Nakai et al. (2004) observed that the viral yield of Adho GV (closely related to AdorGV-E, because same strain as AdorGV-T) reached a maximum on 14 days post inoculation (p.i.) without significant further increase. Some larvae infected with AdorGV-M were already dead at 20 days p.i., and the yield at this harvest time was considered equivalent to the maximum yield of AdorGV-E and -M. Serial dilutions of each homogenate (AdorGV-E, ×10^{-3}, ×10^{-3}, ×10^{-4}, ×10^{-5}, ×10^{-6}, ×10^{-6}, ×10^{-7}, AdorGV-M; ×10^{-22}, ×10^{-3}, ×10^{-1}, ×10^{-2}, ×10^{-3}, ×10^{-5}, ×10^{-7}) were administered to 35 neonate larvae by the food disc method as described above. The dilution points at LC_{50} were estimated by probit analysis. Infectious viral yield was determined by comparison with previously obtained linear regressions for known amounts of AdorGV-E and -M OBs. Data were analysed by t-test with JMP software (SAS).

**Viral DNA isolation and sequencing.** Viral DNA was isolated from viral OBs purified as described elsewhere (Nakai & Kunimi, 1997). The OB suspension was solubilized with 0.1 M Na_2CO_3 at 37 °C for 30 min and neutralized with 0.04 vol 1 M HCl. Viral DNA was extracted with phenol/chloroform as described elsewhere (Ishi et al., 2003). The DNA solution of the AdorGV-M isolate was sequenced both by primer walking with Sanger dyeoxy DNA sequencing and by next-generation sequencing on an Illumina platform. For primer walking, the first round of sequence reactions was carried out with a set of 226 primer pairs designed from the genome sequence of AdorGV-E to amplify regions of approximately 1200 bp. Fragments amplified by PCR were sequenced from both ends. The ampicons generated with these primers overlapped each other by 200 bp. Fragments were assembled into contigs, and a second round of ampicons overlapping the first set was generated and sequenced using an additional set of 246 primer pairs. The contigs from this second round were assembled with those of the first round to produce a draft of the genome sequence.

To determine the AdorGV-M genome sequence with next-generation sequencing technology, a library was prepared from fragmented AdorGV-M DNA and used to generate 7710 053 sequencing reads (76 cycles, single-end) with the Genome Analyzer Ix (Illumina). Sequencing reads were mapped on the genome DNA sequence of AdorGV-E with Bowtie2 v.2.2.3 (Langmead & Salzberg, 2012), and automated correction of genome sequence errors was performed by SAMtools (Li et al., 2009) and in-house Perl (v. 5.10.1) scripts. The final sequence draft was confirmed with the IGV (v. 2.3) genome browser (Robinson et al., 2011).

In addition to sequencing of the AdorGV-M genome, individual loci from AdorGV isolates collected in Tokushima, Kagoshima and Tokyo were PCR amplified and sequenced. To obtain template for PCR, granules were solubilized as previously described (Rowley et al., 2010), and viral DNA was extracted from occluded virus by phenol/chloroform extraction and ethanol precipitation. Two microtubes of a 100-fold dilution of the DNA templates was used in 50 μL PCRs as described by Rowley et al. (2010) using primers designed to amplify the AdorGV ORFs p10, ORF 14, pep-p10, pep-2, ORF 30, p47, dbp, desmoplakin and AdorGV-E ORF 112 (Table S4). Amplimers were precipitated away from excess primers and nucleotides using 20% PEG/2.5 M NaCl and subsequently sequenced with gene-specific primers as described by Harrison & Lynn (2007).

**Genome annotation, selection pressure analysis, and phylogenetic inference.** The GeneQuest program of the Lasergene DNASTAR suite (version 10) was used to identify ORFs of at least 50 codons in the AdorGV-M sequence. Those ORFs that did not overlap adjacent ORFs by more than 75 bp were selected for annotation, BLASTP queries and further characterization. If two ORFs overlapped by more than 75 bp, the larger ORF was selected for annotation, as were ORFs for which annotated homologues in other baculovirus genomes were identified.

The ORFs in AdorGV-M were compared with homologous ORFs in the genome of the AdorGV-E isolate. For those ORFs distinguished by non-synonymous substitutions, the rates of non-synonymous substitution per non-synonymous site (d_{NS}) and of synonymous substitution per synonymous site (d_{S}) between the AdorGV-M and -E sequences were calculated using the CODEML program of PAML v. 4.4c (Yang, 2007) with the individual frequency of each codon as a free parameter.

Alignments of AdorGV nucleotide and conceptual amino acid sequences were performed by CLUSTAL W using the MEGALIGN program of Lasergene v. 10 set at default values for gap and gap-length penalties. Pairwise alignments of nucleotide sequences were carried out using the Marti nez/Wunsch–Needleman algorithm of the same program. For phylogenetic inference, alignments of individual genes were concatenated with BioEdit (Hall, 1999). Phylogenetic relationships were inferred by minimum evolution and maximum-parsimony using MEGA4 (Tamura et al., 2007) with parameters as described previously (Harrison et al., 2008; Rowley et al., 2010).

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**REFERENCES**


