Genomic Sequence Analysis of Granulovirus Isolated from the Tobacco Cutworm, *Spodoptera litura*

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Abstract

**Background:** *Spodoptera litura* is a noctuid moth that is considered an agricultural pest. The larvae feed on a wide range of plants and have been recorded on plants from 40 plant families (mostly dicotyledons). It is a major pest of many crops. To better understand *Spodoptera litura* granulovirus (SpliGV), the nucleotide sequence of the SpliGV DNA genome was determined and analyzed.

**Methodology/Principal Findings:** The genome of the SpliGV was completely sequenced. The nucleotide sequence of the SpliGV genome was 124,121 bp long with 61.2% A+T content and contained 133 putative open reading frames (ORFs) of 150 or more nucleotides. The 133 putative ORFs covered 86.3% of the genome. Among these, 31 ORFs were conserved in most completely sequenced baculovirus genomes, 38 were granulovirus (GV)-specific, and 64 were present in some nucleopolyhedroviruses (NPVs) and/or GVs. We proved that 9 of the ORFs were SpliGV specific.

**Conclusions/Significance:** The genome of SpliGV is 124,121 bp in size. One hundred thirty-three ORFs that putatively encode proteins of 50 or more amino acid residues with minimal overlap were determined. No chitinase or cathepsin genes, which are involved in the liquefaction of the infected host, were found in the SpliGV genome, explaining why SpliGV-infected insects do not degrade in a typical manner. The DNA photolyase gene was first found in the genus Granulovirus. When phylogenic relationships were analyzed, the SpliGV was most closely related to *Trichoplusia ni* granulovirus (TnGV) and *Xestia c-nigrum* granulovirus (XecnGV), which belong to the Type I-granuloviruses (Type I-GV).

Introduction

The family Baculoviридae includes invertebrate-specific viruses with circular, coavely closed, double-stranded DNA genomes ranging in size from 80–180 kb [1]. To date, more than 600 baculoviruses have been described to infect species from the insect orders Lepidoptera, Diptera, and Hymenoptera, and it is likely that baculoviruses represent the largest and most diverse family of DNA viruses [2,3]. Previously, the family Baculoviридae was subdivided into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), mainly based on the morphology of their occlusion bodies (OBs) [1]. Recently, a proposed reclassification has expanded the family to include four genera: the viruses of Lepidoptera are divided into the Alpha- and Beta-baculoviридae, encompassing the NPVs and GVs, respectively, and those infecting Hymenoptera and Diptera are named the Gamma- and Delta-baculoviридae, respectively [5]. While NPVs have OBs with many virions and have been isolated from lepidopteran and non-lepidopteran hosts, the OBs of GVs each contain a single virion and have only been isolated from lepidopteran insects [6]. The lepidopteran-specific NPVs are further classified into two groups, I and II, based on the phylogenetic analysis of their polyhedrin (*polh*) genes [7,8]. GVs cause three distinct types of pathology in infected hosts [9]: Type I-GVs only infect the fat body, usually resulting in a relatively slow speed of killing; Type II-GVs infect most of the insect host’s tissues, resulting in a faster speed of killing; and Type III-GVs infect only the midgut epithelium, resulting in the rapid death of the host. At present, Type III-GVs contain only one member, *Harrissia brillians* granulovirus (HabrGV). Phylogenetic analysis of GV sequences suggests that these different types of GV pathogenesis do not have monophyletic origins [10].

As a novel and steady pesticide, baculoviruses have been used as agents for the biological control of certain insect pest species. Baculoviридae possess several suitable properties, including high efficacy in controlling insect pests and a less negative impact on the environment and non-target species than chemical pesticides [11,12]. However, their use has been limited due to their slow speed of killing and narrow host specificity. Recent studies have
shown that baculoviruses expressing foreign genes, such as a *Bacillus thuringiensis* crystal protein gene and an insect-specific neurotoxin gene have accelerated killing speeds and promise to be effective biological insecticides [13]. To date, GVs have been isolated only from lepidopteran larvae. In particular, GVs infect both agricultural and forest insect pests, making them potentially important as biological insecticides [14].

The tobacco cutworm, *Spodoptera litura*, has totally polyphagous noctuid larvae as well as high reproductive potential and the ability to migrate long distances as adults. The host range of *S. litura* covers over 40 families [15]. Among the main crop species attacked by *S. litura* in the tropics are *Colocasia esculenta*, cotton, flux, groundnuts, jute, alfalfa, maize, rice, soybeans, tea, tobacco, and vegetables (including eggplants, brassica, capsicum, cucurbit vegetables, wild beans, and sweet potatoes). Other hosts include ornamentals, wild plants, weeds, and shade trees (e.g., *Leucaena leucocephala*, the shade tree of cocoa plantations in Indonesia). These factors contribute to the role of *S. litura* as a major pest of many agricultural crops throughout its geographical range, and as a result, many insecticide treatments target this pest [16].

Several properties of baculoviruses, such as their relatively slow killing speed, narrow host spectrum, and high production costs, are disadvantageous. To overcome these disadvantages, it is necessary to develop a better understanding of the biology and pathology of baculoviruses. One approach is to conduct extensive research into diverse viruses that possess distinct biological and pathological characteristics. Detailed information about a wide range of isolates will provide a more comprehensive overview of baculoviruses and help to overcome their shortcomings as biological pest control agents. In this study, to add our knowledge of granulovirus molecular genetics, the complete genome of SpliGV was sequenced and analyzed.

**Results and Discussion**

**Characteristics of the SpliGV genome sequence**

So far, 49 baculovirus genomes have been sequenced (Table 1). Thirty-eight NPV genome sequences have been reported, but to date, complete genome sequences have only been reported for seven GVs [1, 17–22], with genome sequences for another four GVs (from *Plutella xylostella* opercularia, *Agris segetum*, *Pseudalatta unipuncta* and *Peris rapae*) on file in GenBank. A 6 x sequence of the SpliGV genome was compiled from all sequence data generated. The size of the final draft sequence was 124,121 nt. The SpliGV genome has a A+T content of 61.2%, which is closest to that of *Pseudalatta unipuncta GV* (60.2%) (PsunGV, GenBank accession no. EU678671). Among GV genomes, that of Cryptophlebia leucotreta GV (CrleGV) has the highest A+T content, 67.6%, and that of Cydia pomonella granulovirus (CpGV) has the lowest A+T content, 54.3%. Coding sequences represent 86.3% of the genome of SpliGV.

One hundred thirty-three ORFs of at least 50 codons in length that had minimal overlap with larger ORFs or shared significant sequence identity with previously characterized baculovirus ORFs were identified (Fig. 1). Among these, 31 ORFs were conserved in most completely sequenced baculovirus genomes, 38 were GV-specific, and 64 were present in some NPVs and/or GVs. By convention, the first nucleotide of the methionine start codon of the *granulin* gene was defined as nucleotide 1 of the genome, and the sequence was numbered in the direction of transcription of the *granulin* gene. As with other baculovirus genomes, the ORFs were randomly distributed with 77 ORFs in the *granulin*-sense orientation and 56 in the opposite orientation. Canonical baculovirus early and late gene promoter sequences were associated with 119 ORFs of the SpliGV (Table S1). As we have known, there is little overlap in baculovirus genomic DNAs. Minimal overlaps (less than 16 codons) were observed between 57 adjacent ORFs. More notably, greater levels of overlap were found between Spli22 and Spli23 (62 bp), Spli23 and Spli24 (110 bp), Spli35 and Spli36 (100 bp), Spli50 and Spli59 (110 bp), Spli74 and Spli75 (57 bp), Spli107 and Spli108 (113 bp), and Spli128 and Spli129 (152 bp) (Fig. 1).

BLAST comparisons of the nucleotide sequences and the deduced amino acid sequences of 133 ORFs of SpliGV indicated that 9 ORFs have no similarity to any reported baculovirus genes. We proved that these 9 SpliGV-specific ORFs could be transcribed from the SpliGV genome by RT-PCR (Fig. 2). These 9 ORFs were named Spli30, Spli40, Spli51, Spli61, Spli63, Spli75, Spli88, Spli121, and Spli133. Except for Spli51, the other SpliGV-specific ORFs had some similarities to genes from other microbes.

**Relationships with other baculoviruses**

Previously, when the nucleotide and the deduced amino acid sequences of the SpliGV granulin gene were aligned with those of granulin and polyhedrin genes from other baculoviruses, SpliGV was most closely related to TuGV and XecnGV, which belong to the Type I-GVs [23]. To further investigate to the relationship between SpliGV and other baculoviruses, phylogenetic trees were inferred from a set of concatenated, aligned, partial amino acid sequences of 24 genes from SpliGV and 40 other completely sequenced lepidopteran baculoviruses (Fig. 3). This result did not confirm a clear relationship between SpliGV and XecnGV, as shown in Figure 3. However, we can place SpliGV and XecnGV in a clade of closely related GV's isolated from Lepidoptera of the family Noctuidae, including AgerGV, *Plutella xylostella* GV (PhyGV), PsunGV and Helicoverpa armigera GV (HearGV). These viruses, along with XecnGV, are considered to be isolates of the same virus species [2].

Dot plot sequence comparisons revealed a strong degree of colinearity between the genome of SpliGV and those of other GVs (Fig. 4). SpliGV lacked many of the ORFs found in XecnGV, which is expected given the significantly smaller size of the genome of SpliGV. Comparison of the SpliGV genome with that of *Spodoptera littoralis* multicapsid NPV (SpliMNPV) revealed that the order of some ORFs was conserved between the two viruses, but the orientation of a large proportion of these ORFs was inverted relative to the polyhedron gene (Fig. 4).

Comparative analysis of the gene organization of the SpliGV genome with those of 44 other baculoviruses was carried out using a gene order diagram (GOD) (Fig. 5). The GOD analysis was performed using the 24 genes of the SpliGV genome along with those of 44 other baculoviruses. Homologs of the 24 genes of the SpliGV genome are found in all 40 of the lepidopteran baculovirus genomes. However, some of these homologs have been lost from the three hymenopterous baculovirus genomes and the one dipteran baculovirus genome (Fig. 5). The homologs of Spli73, Spli77, Spli78, Spli83, Spli84, Spli89, Spli92, Spli94, and Spli98 occur in the same order in all 44 lepidopteran baculovirus genomes in either the forward direction or reverse direction relative to the SpliGV genome. In all of the lepidopteran GV genomes, a total of 13 homologs of genes of the SpliGV genome, including Spli55, Spli104, Spli115, and Spli119 as well as the homologs found in all of the lepidopteran baculovirus genomes, are organize in the same order and direction as in the SpliGV genome (Fig. 5). We also found that the order of the SpliGV gene homologs was generally more conserved in the group I NPVs than in the group II NPVs (Fig. 5). The GOD analysis technique may be able to supplement other baculovirus classification methods.
### Table 1. Characteristics of baculovirus genomes (February 2010).

| Virus                  | No. ORFs | Genome size (bp) | Accession No. | AT content (%) | Sequenced |
|------------------------|----------|------------------|---------------|----------------|-----------|
| Autographa californica MNPV | 155      | 133,894          | NC_001623     | 59.3           | 1994–07–16 |
| Bombyx mori NPV        | 143      | 128,413          | NC_001962     | 59.6           | 1996–01–18 |
| Orgia pseudotsugata MNPV | 152      | 131,995          | NC_003529     | 44.9           | 1997–03–27 |
| Mamestra configurata NPV–A | 169      | 155,060          | NC_002169     | 62.1           | 1999–05–29 |
| Lymantria dispar MNPV  | 166      | 161,046          | NC_002654     | 60.0           | 2001–01–25 |
| Spodoptera exigua MNPV | 139      | 131,403          | NC_003084     | 49.1           | 2001–10–02 |
| Helicoverpa armigera NPV (G4) | 135      | 130,759          | NC_003905     | 54.2           | 2002–05–09 |
| Leucania separata NPV  | 169      | 168,041          | NC_004117     | 51.5           | 2002–10–02 |
| Orgyia leucostigma NPV | 135      | 156,179          | NC_004778     | 51.5           | 2003–11–02 |
| Helicoverpa armigera NPV | 147      | 172,544          | NC_005721     | 49.0           | 2003–12–02 |
| Spodoptera littura NPV | 141      | 139,342          | NC_006776     | 51.4           | 2004–01–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_007124     | 51.4           | 2004–03–01 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_007749     | 51.4           | 2004–04–12 |
| Mamestra configurata NPV–B | 168      | 158,482          | NC_008725     | 61.2           | 2004–05–01 |
| Rachiplusia ou MNPV    | 146      | 130,869          | NC_009001     | 61.1           | 2004–06–12 |
| Helicoverpa armigera NPV | 147      | 132,125          | NC_009434     | 61.1           | 2004–07–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_009749     | 61.1           | 2004–08–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_009839     | 61.1           | 2004–09–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_010276     | 61.1           | 2004–10–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_010439     | 61.1           | 2004–11–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_011354     | 61.1           | 2004–12–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_011749     | 61.1           | 2005–01–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_012149     | 61.1           | 2005–02–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_012549     | 61.1           | 2005–03–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_012949     | 61.1           | 2005–04–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_013349     | 61.1           | 2005–05–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_013749     | 61.1           | 2005–06–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_014149     | 61.1           | 2005–07–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_014549     | 61.1           | 2005–08–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_014949     | 61.1           | 2005–09–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_015349     | 61.1           | 2005–10–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_015749     | 61.1           | 2005–11–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_016149     | 61.1           | 2005–12–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_016549     | 61.1           | 2006–01–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_016949     | 61.1           | 2006–02–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_017349     | 61.1           | 2006–03–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_017749     | 61.1           | 2006–04–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_018149     | 61.1           | 2006–05–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_018549     | 61.1           | 2006–06–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_018949     | 61.1           | 2006–07–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_019349     | 61.1           | 2006–08–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_019749     | 61.1           | 2006–09–12 |
| Helicoverpa armigera NPV | 134      | 130,759          | NC_020149     | 61.1           | 2006–10–12 |
| Orgyia pseudotsugata MNPV | 152      | 131,995          | NC_020549     | 61.1           | 2006–11–12 |
| Spodoptera litura NPV | 141      | 139,342          | NC_020949     | 61.1           | 2006–12–12 |

References:
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SpliGV genes involved in DNA replication and transcription

There are 19 lef genes in Autographa californica multicapsid NPV (AcMNPV) that have been implicated in DNA replication and transcription [24]. Early baculovirus genes are transcribed by the host cell RNA polymerase II, but these genes are often transactivated by genes such as ie-0, ie-1, ie-2, and pe38 [25]. Among these genes, only ie-1 is present in the SpliGV genome (Table 2). Both ie-2 and pe38 are also absent from all group II NPVs and GVs with the exception of CpGV and PhopGV, which have a pe38 gene [22]. Six genes have been reported to be essential for baculovirus DNA replication: lef-1, lef-2, lef-3, dnapol, helicase, and ie-1 [26]. Homologs of all of these essential genes are present in SpliGV (Table 2). They are moderately well conserved, with the exception of lef-3 and ie-1 (Table 2). SpliGV does not have the lef-7 or lef-12 typically found in group I NPVs. SpliGV encodes a DNA ligase (Spli107) and a second helicase (Spli116) (Table 2), as do Lymantria dispar multicapsid NPV (LdMNPV) and other GVs. In LdMNPV, neither the helicase-2 nor the dna ligase gene stimulates DNA replication in transient assays. As their homologs are involved in DNA repair and recombination, these genes could also be involved in DNA repair [27].

SpliGV lacks genes for enzymatic functions in nucleotide metabolism, such as the large (rr1) and small (rr2) subunits of ribonucleotide reductase, but it does have the deoxyuridyl-triphosphate (dUTPase) gene (Table 2). These enzymes are found in several baculoviruses and are involved in nucleotide metabolism. They catalyze the reduction of host cell rNTPs to dNTPs [1].

Many genes required for late gene transcription have been described, including lef-4, lef-6, lef-8, lef-9, lef-10, lef-11, 39k, p47, and vlf-1 [28]. All of these genes are found in SpliGV (Table 2). Generally, these genes are more conserved than the early transcription activators [29]. The lef-6 genes of GVs are smaller than the lef-6 genes of NPVs (86–102 amino acids vs. 138–187 amino acids).

Figure 1. Representation of the SpliGV genome. ORFs and transcriptional direction are indicated by arrows. The ORFs present in most completely sequenced baculovirus genomes are colored gray; GV-specific ORFs are in blue; SpliGV unique ORFs are in red; and ORFs present in some NPVs and/or some GVs are in black. doi:10.1371/journal.pone.0028163.g001

Figure 2. RT-PCR of 9 SpliGV-specific ORFs using mRNAs from S. littura larvae infected with SpliGV as a template. M1, 100-bp DNA ladder (Fermentas, USA); 1, Spli30; 2, Spli40; 3, Spli51; 4, Spli61; 5, Spli63; 6, Spli75; 7, Spli88; 8, Spli121; 9, Spli133; M2, 1-kb DNA ladder (Fermentas, USA). doi:10.1371/journal.pone.0028163.g002
Figure 3. Phylogenetic relationship between 41 complete baculovirus genomes based on the nucleotide sequences of 24 genes. The numbers on the branches represent bootstrap values for 1,000 replicates.

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SpliGV structural genes

The most conserved baculovirus structural protein is polyhedrin/granulin (93% maximal amino acid identity), the major component of OBs [30]. SpliGV lacked homologs of two structural genes, the p80/87-capsid gene and ORF 1629 (p78/73). The p80/87-capsid gene is also absent from the other sequenced GV. A putative ORF 1629 (Xecn2) has been identified in XecnGV (Xecn2), although it is less than half the size of the NPV ORFs and...
Table 2. SpliGV genes grouped according to functional comparison with other baculoviruses.

| Genes present in SpliGV | Genes missing in SpliGV |
|-------------------------|-------------------------|
| Transcription           |                         |
| 39 K (Spli44), lef-11 (Spli45), p47 (54), lef-6 (Spli65), lef-5 (Spli73), lef-4 (Spli83), vlf (Spli94), lef-9 (Spli104), lef-8 (Spli119) | p26 (Ac136), pe38 (Ac153) |
| Replication             |                         |
| ie (Spli7), lef-2 (Spli28), dUTPase (Spli47), lef-1 (Spli58), dbp (Spli66), 38 k (Spli74), hel-1 (Spli77), dna pol (Spli98), lef-3 (Spli100), pkn/pnl (Spli105), dna ligase (Spli107), hel-2 (Spli118), me33 (Spli132) | ptp (Ac1), lef-7 (Ac125), ie0 (Ac147-0), nudix (Ac38), ie2 (Ac151), pkn polyomaviridae kinase (Ac33), r1 (Pci127), r2 (Pci128) |
| Structural              |                         |
| granulin (Spli1), pk (Spli3), p10-1 (Spli4), adv-e18 (Spli10), adv-e56 (Spli13), pep (Spli18), p10-2 (Spli19), efp (Spli22), pif-2 (Spli24), p1 (Spli34), pif-2 (Spli35), cg3-10 (Spli48), p74 (Spli52), vp24capsid (Spli56), p10-3 (Spli59), pif-1 (Spli60), bvod-e42 (Spli71), p6.9 (Spli72), pif-4 (Spli74), adv-e25 (Spli78), p33 (Spli80), vp39 capsid (Spli84), adv-e27 (Spli85), vp91capsid (Spli89), tlp20 (Spli90), gp1 (Spli92), desmop (Spli99), fp (Spli106), cg30-2 (Spli122), adv-e65 (Spli123), vp1054 (Spli129) | bvod-e26 (Ac16), pkl (Ac24), gp64 (Ac128), vp80,vp87 (Ac104), exo-n (Ac141) |
| Auxiliary               |                         |
| p49 (Spli11), iap-3 (Spli16), bro-1 (Spli17), lef-10 (Spli128), mp-nase (Spli33), ubi (Spli41), dna photolyase (Spli46), bro-2 (Spli49), bro-3 (Spli50), fyg-1 (Spli62), iap-5 (Spli103), bro-4 (Spli109), bro-5 (Spli112), bro-6 (Spli113), fyg-2 (Spli114), alk-exo (Spli115), fyg-3 (Spli131) | p94 (Xecn21), cathespin (Xecn58), sod (Xecn68), chinatine (Xecn103), gp37 (Xecn107), cta (Xecn127), enhancin-1 (Xecn150), enhancin-2 (Xecn152), enhancin-3 (Xecn154), enhancin-4 (Xecn166), lef-12 (Ac41) |
| Unknown                 |                         |
| Spli2, Spli5, Spli6, Spli9, Spli12, Spli14, Spli15, Spli20, Spli21, Spli23, Spli25, Spli26, Spli27, Spli29, Spli31, Spli32, Spli35, Spli38, Spli39, Spli42, Spli43, Spli53, Spli55, Spli57, Spli64, Spli67, Spli68, Spli69, Spli70, Spli79, Spli81, Spli82, Spli86, Spli87, Spli91, Spli93, Spli95, Spli96, Spli97, Spli101, Spli102, Spli108, Spli110, Spli111, Spli117, Spli118, Spli120, Spli124, Spli125, Spli126, Spli127, Spli130 |                         |
| SpliGV unique           |                         |
| Spli30, Spli40, Spli51, Spli61, Spli63, Spli75, Spli88, Spli121, Spli133 |                         |

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has similarity concentrated only around a conserved proline-rich region [20]. Except for HearGV, all of the GVs have a Xcen2 homolog with similarity in the first 65 aa of the amino-terminal region of the protein but show no similarity to ORF 1629 and do not contain a proline-rich region. Pif is found on the surface of the OBs and is important for the formation of polyhedra, as it stabilizes them and prevents them from fusing [31]. SpliGV does have a pif (Spli13) that shares 55% amino acid identity with its homolog from XecnGV, Xecn18 (Table 2).

Occlusion-derived viruses (ODVs) contain more than 10 different envelope proteins. Five of these, denoted pif-1, pif-2, pif-3, pif-4, and pif-7, have been identified as essential for per os infection of insect larvae [32–36]. All five proteins are highly conserved in Baculoviridae and are encoded by the so-called core genes [32, 37–39]. These PIF proteins function in the early stage of virus infection, and are deleted or inactivated at any of these pif genes leads to a block in infection prior to viral gene expression in midgut epithelial cells [33, 35, 40]. All five genes involved in per os infectivity were present in SpliGV (Table 2). In the SpliGV genome, p74 encodes a small, truncated protein (144 aa) containing the conserved C-terminal region of the XecnGV p74 (Xecn77) gene (43% identity over 140 aa), but the p74 of SpliGV is substantially shorter than that of XecnGV (144 aa vs. 710 aa).

In NPV-infected cells, P10 forms fibrous structures in the nucleus and cytoplasm [21]. This protein is implicated in OB morphogenesis and disintegration of the nuclear matrix, resulting in the dissemination of OBs [41]. P10 proteins from different baculoviruses are characterized by the size differences in their shared domains, and their sequences are generally poorly conserved [17]. Three XecnGV ORFs (Xecn3, Xecn19, and Xecn83) present similarities to p10. Homologs of these three ORFs are present in PtxyGV (Ptxy2, Ptxy21, and Ptxy50), and Hashimoto et al. [19] suggested that they are all p10 homologs. SpliGV contains homologs of the three putative p10 homologs of XecnGV, which are Spli4, Spli19, and Spli59 (Table 2). They showed maximal similarity to Xecn5, Xecn19, and Xecn83, respectively. Whereas Spli4 (p10–1) and Spli19 (p10–2) have a proline-rich domain and a heptad sequence, Spli59 (p10–3) has only a heptad sequence significantly smaller than that of Xecn83 (97 vs. 182 amino acids). The close association of P10 and the polyhedron envelope has been well documented in many NPVs, and it has been known that the presence of P10 is essential for the formation of the polyhedron envelope [17]. In some GVs, the functional association of PEP and P10 might have been conserved in a single protein [1]. Further work must be done to fully understand the role of the different p10 homologs in SpliGV.

SpliGV auxiliary genes

Auxiliary genes are not essential for viral replication, but they do provide some selective advantages [42]. SpliGV does not contain either a chinatine or a cathepsin gene. It appears that baculoviruses encode these enzymes to aid breakdown of insect tissues at the end of infection to release OBs into the environment and thereby aid their horizontal spread. SpliGV-infected larvae do not lyse at the end of infection. The cadavers of S. litura larvae infected by SpliGV appeared smaller than normal larvae, as if they had lost much water, and they were very soft.

Enhancin is a metalloproteinase that disrupts the insect peritrophic membrane, facilitating the initiation of infection [43, 44]. These genes were first found in GV OBs, which can enhance the infection of some NPVs. Also referred to as viral enhancing or synergistic factors, enhancins were first identified and isolated by Tanada and colleagues [45]. Enhancin genes have been found in several GVs, including HearGV [46], Psun GV [46], TnGV [47], XecnGV [20], AgeGV (GenBank: AY322332), and ChfuGV (GenBank: AAG33872). The first GV genome to be completely sequenced, that of XecnGV, was found to have four different enhancin genes [20]. In contrast, no enhancin homolog is present in SpliGV.

Superoxide dismutase (sod) is a well-conserved gene of baculoviruses. This gene is presumed involved in the removal of free radicals but is non-essential, and its role in the virus life cycle is not known [48]. Of all of the GVs that have been sequenced to date, SpliGV is not the only one to lack sod; sod is also not found in a few NPV genomes.
Ubiquitin is the most conserved auxiliary gene and is present in all sequenced baculovirus genomes. The main function of cellular ubiquitin is to signal protein degradation [49]. Viral ubiquitin is nonessential, and its role is unclear [50]. SpliGV contains a homolog of ubiquitin, SpliH1 (Table 2).

Another SpliGV ORF of interest is Spli46, a homolog of the DNA photolyase gene (Table 2). DNA photolyase genes encode photolyase enzymes that are involved in the repair of UV-damaged DNA [51]. To date, in the sequenced baculoviruses other than SpliGV, photolyase genes are found only in ChichNPV and Trichoplusia ni NPV (TuNPV).

**Inhibitors of apoptosis (IAP)**

Apoptosis represents an important virus-host interaction process that probably influences viral pathogenesis. As an antiviral response in multicellular organisms, apoptosis can limit viruses in the suicide cells, thereby reducing the yield of progeny viruses, which results in abortive infection [52,53]. Although many viruses, including baculoviruses, can trigger apoptosis in infected cells, they can synthesize proteins that prevent apoptosis [54–57]. Baculoviruses possess two families of genes that suppress apoptosis, the p35/p49 family and the inhibitor of apoptosis (IAP) family. P35 was the first antiapoptotic baculovirus protein discovered, and it has only been identified in AcMNPV, Bombyx mori NPV (BmNPV), SpliMNPV, and Maruca vitrata MNPV (MavMNPV) [58–60]. A larger p35 homolog, p49, has been found in some baculoviruses [57,60]. P49 has a similar three-dimensional structure and the same mode of action as P35. However, P49 is able to inhibit initiator caspases that P35 is unable to inhibit [57,61,62]. All baculoviruses have been found to contain IAP homologs. The IAP-3 protein of CpGV was the first member of the baculovirus IAP family to be identified [56]. IAP homologs generally contain two baculovirus IAP repeats (BIR), which are associated with binding to apoptosis-inducing proteins, and a C-terminal zinc finger-like motif (RING) Cys/His motif [54,56,63]. According to amino acid sequence similarity, baculovirus IAPs can be divided into five types, named iap-1 through iap-5 [21]. There are two IAP genes, iap-3 (Spli16) and iap-5 (Spli103), and one p49 (Spli11) gene in the genome of SpliGV (Table 2).

**Baculovirus repeated ORFs (bro genes)**

One to sixteen copies of bro are present in all lepidopteran and dipteran NPV sequences to date and in some of GV's. They comprise a highly repetitive and conserved gene family that is widespread among insect DNA viruses [64]. Gene expression, nucleic acid binding activity, nucleosome association, protein localization and protein trafficking have been characterized for some NPV bro genes and proteins, but the functions of bro gene products in the baculovirus life cycle are still unclear [64–68]. SpliGV has 6 bro genes, named bro-1 to bro-6 based on their order in the genome, including homologs of XecnGV bro-a (Xecn60), bro-b (Xecn76), bro-c (Xecn130), and bro-l (Xecn131) (Table 2). In SpliGV, there are two adjacent pairs of bro ORFs (bro-2 and bro-3; bro-5 and bro-6). Interestingly, the bro-1, bro-4, and bro-6 genes are all homologs of Xecn60 (Table 2). The bro-3 gene of SpliGV encodes a small, truncated protein (67 aa) containing the conserved N-terminal region of the Xecn76 homolog.

**Materials and Methods**

**Viral DNA extraction**

The granules produced in larval cadavers were purified by a standard method [69]. To extract virus DNA, the purified granules were resuspended in 0.1 M sodium carbonate solution [0.1 M Na₂CO₃, 0.17 M NaCl, 0.01 M EDTA (pH 10.9)] and incubated at 37°C overnight with 0.5 mg/ml proteinase K (Sigma) and 1% SDS. A further extraction with phenol and chloroform-isomyl alcohol (24:1) was performed, and the DNA was ethanol precipitated. The DNA was resuspended in TE buffer [10 mM Tris-HCl, pH 8; 1 mM EDTA].

**Sequencing and sequence analysis of SpliGV genomic DNA**

The complete nucleotide sequence of SpliGV genomic DNA was determined using a shotgun strategy on an ABI Model 3700 sequencer (PE-Applied Biosystems, USA). The SpliGV DNA sequence was determined at least six times, and additional assessments were carried out for ambiguous sequences using gene-specific primers. Putative coding regions of the SpliGV genome were predicted using FGENESV0 [http://www.softberry.com/berry.pl?flhtml] [70] and the NCBI ORF finder [http://www.ncbi.nlm.nih.gov/gorf/gorf.html] by locating translation start and stop codons of ORFs of 50 or more amino acids. Dot plot sequence comparisons were generated with Advanced PipMaker [http://pipmaker.bx.psu.edu/cgi-bin/pipmaker/advanced]. Predicted amino acid sequence identities were obtained from the results of protein database searches using the standard protein-protein BLAST algorithm [http://blast.ncbi.nlm.nih.gov/Blast.cgi]. The genomic DNA sequence was deposited in GenBank under the accession number NC990503.

**Phylogenetic analysis**

For phylogenetic analysis, 24 genes from 30 NPVs and 10 GV's, which all have homologs in the SpliGV genome, were obtained from GenBank. Phylogenetic analysis was carried out using the maximum-parsimony (MP) method [71] incorporated in the parsimony program PAUP [Phylogenetic Analysis Using Parsimony and Other Methods] version 4.0 b10 program [72]. The reliability of the trees was tested with bootstrap re-sampling using 1,000 replicates.

**Total RNA extraction and RT-PCR**

Total RNA was isolated from infected S. litura larvae with TRIZOL Reagent (Invitrogen, USA) according to the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using AccuPower® RT/PCR PreMix (Bioneer, Korea) with SpliGV unique gene primer sets (Table S2). One microgram of total RNA and 20 pmol reverse primers were mixed, incubated at 70°C for 5 min and placed on ice. The incubated mixtures and 20 pmol forward primers were transferred to an AccuPower® RT/PCR PreMix tube, and the reaction volumes were brought up to 20 microliters with DEPC-DW. cDNA synthesis reactions and DNA PCR reactions were performed under the following temperature cycles: one cycle at 42°C for 60 min; 94°C for 5 min; one cycle at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec; 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec; and 1 cycle at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 7 min.

**Supporting Information**

Table S1 Predicted SpliGV ORFs by BLAST Search.

Table S2 Primers used to confirm new SpliGV genes.

**Author Contributions**

Conceived and designed the experiments: JYC, JYR, YHJ. Performed the experiments: YW, QE, JBP. Analyzed the data: YW, XYT, JSK. Wrote the paper: YW, JYC, YHJ.
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