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Optical Brighteners Do Not Influence Covert Baculovirus Infection of *Spodoptera frugiperda*

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Covert infection with *Spodoptera frugiperda* multiple nucleopolyhedrovirus, detected by reverse transcription-PCR of virus gene transcripts (*ie-0* and *polh*), was not significantly affected by the presence of an optical brightener (Tinopal UNPA-GX), indicating no change in virus virulence. Detection of the covert infection was dependent on insect life stage and the viral mRNA used for diagnosis.

Baculoviruses are virulent pathogens of insects, especially Lepidoptera, that form the basis for several biological insecticides (16). Baculovirus infections usually kill the host but may also survive as sublethal covert infections (17) and transmit themselves vertically, from parent to offspring (3, 10, 11, 14). Stilbene optical brighteners increase the probability of virus infection by inhibiting chitin synthesis (1, 7), thus increasing the permeability of the midgut peritrophic membrane (21), and by reducing the rate of turnover of infected gut cells (22).

We hypothesized that the optical brightener Tinopal UNPA-GX (Sigma, St. Louis, Mo.), administered in mixtures with occlusion bodies (OBs) of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV; *Baculoviridae*), would result in a higher prevalence of covert infection in larvae and adults of the fall armyworm, *S. frugiperda* (Lepidoptera: Noctuidae). For this experiment, we employed reverse transcription (RT)-PCR with primers specific for two SfMNPV genes, *ie-0* and *polh*. The *ie-0* gene is a *trans*-activator capable of stimulating the transcription of genes required for the replication of viral DNA and is essential for initiating virus replication (9). The polyhedrin (*polh*) gene encodes a major structural protein that forms the matrix of the viral OB late in the infection cycle (15). *polh* is not essential for virus replication but is required for horizontal transmission (2).

OBs of SfMNPV, originally isolated in Nicaragua (8), were produced in *S. frugiperda* from a laboratory colony, purified by centrifugation, and quantified in a bacterial counting chamber (18). Groups of 500 to 600 newly molted second instars were fed OB suspensions (with or without Tinopal UNPA-GX) by the droplet feeding technique (13) that were estimated to cause ~90% mortality in experimental insects (6.2×10^5 OBs/ml of SfMNPV alone or 4.4×10^4 OBs/ml of SfMNPV and 0.1% [wt/vol] Tinopal UNPA-GX). The procedure was repeated with groups of 75 to 100 newly molted fifth instars treated with 1.3×10^9 OBs/ml of SfMNPV alone or 1.2×10^5 OBs/ml of SfMNPV and 0.1% Tinopal UNPA-GX. Inoculated insects were reared individually on diet plugs and weighed 2 days after pupation. Control larvae were treated identically with solutions not containing virus. The experiment was performed three times with second instars and four times with fifth instars. To confirm the suitability of target genes (*ie-0* and *polh*) as indicators of covert infection, an initial sample of 22 insects inoculated with SfMNPV alone in the fifth instar, which showed no signs of NPV disease, were randomly selected as sixth instars and subjected to RT-PCR. Subsequently, between 20 and 27 insects inoculated in the second or fifth instars were randomly selected in the fifth or adult stage, respectively, and used in RT-PCR analysis.

Total RNA was extracted from the posterior part of the larval body of fifth- and sixth-instar larvae and from the dissected abdomen of adult *S. frugiperda* as described elsewhere (19). RT was performed on total RNA (up to 1 μg) by using an ImProm-II kit (Promega) following the manufacturer's recommendations. PCR amplification of cDNA was performed by using 5 μl of the RT reaction in a volume of 50 μl. Primers were designed by using SfMNPV sequences of the immediately early gene *ie-0* (5′ TACGCTCGAGATGAGTATTAATCAT AGATT 3′ [forward] and 5′ CGTACTCGAGTCTGGCAAA TGTTACACT 3′ [reverse]) (O. Simón, unpublished data) and very-late polyhedrin gene *polh* (12) (5′ TCGAGGAGAGGA

TABLE 1. Prevalence of different life stages of *S. frugiperda* positive for SfMNPV-specific transcripts of the immediate-early *ie-0* gene and very late *polh* gene detected by RT-PCR[a]

Target gene	Life stage at:		No. of insects positive for transcript/No. tested (%)	
	Inoculation	Analysis	SfMNPV	SfMNPV and Tinopal UNPA-GX
ie-0	Second instar	Fifth instar	4/21 (19)	1/21 (5)
		Adult	0/21 (0)	0/20 (0)
polh	Second instar	Fifth instar	1/21 (5)	1/21 (5)
		Adult	7/27 (26)	3/27 (11)
	Fifth instar	Adult	1/21 (5)	1/21 (5)

[a] Insects were inoculated in the second and fifth instar with SfMNPV alone or in mixtures with Tinopal UPNA-GX. Virus-specific RNA was not detected in any of the untreated control insects, and no PCR products were amplified in any of the RNA controls. In all cases, Southern hybridization analysis confirmed the identity of all the amplification products (Fig. 1D to F).

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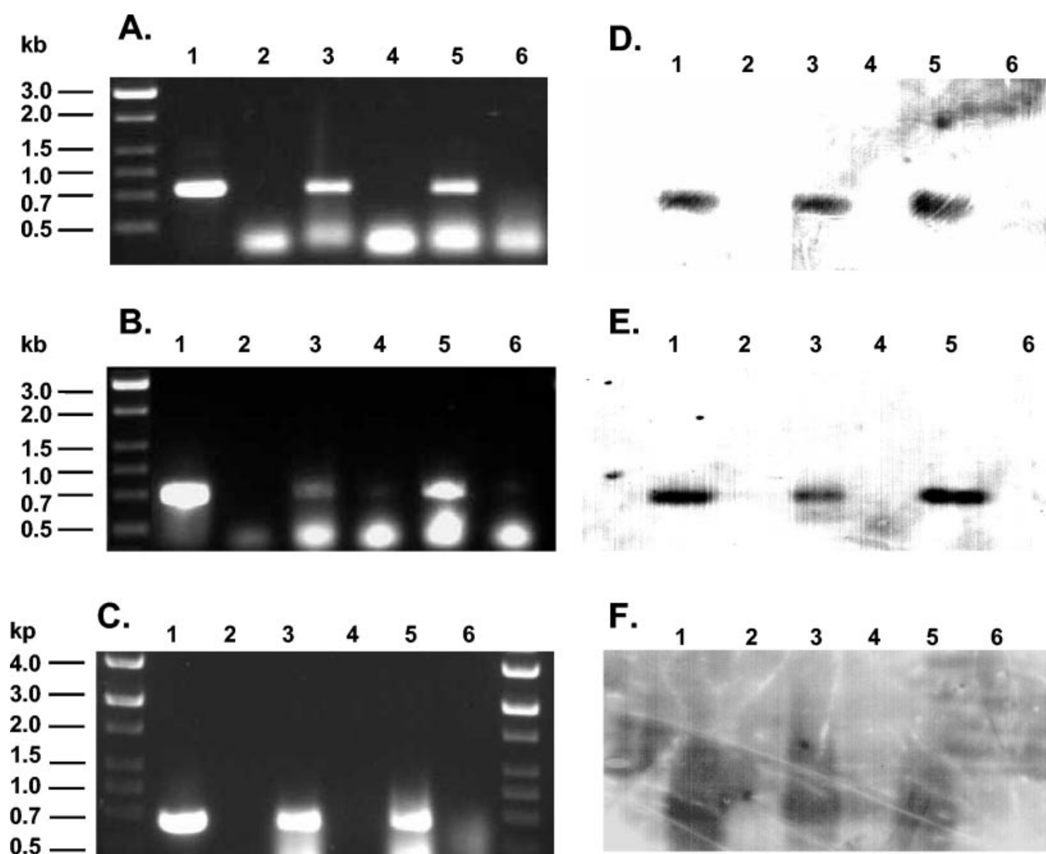

FIG. 1. (A) RT-PCR fragments amplified with SfMNPV-specific primers for *ie-0* from fifth-instar *S. frugiperda* that survived inoculation with SfMNPV alone (lane 3) or with SfMNPV and Tinopal UNPA-GX (lane 5) in the second instar. (B) RT-PCR fragments amplified with SfMNPV *polh* primers from fifth-instar *S. frugiperda* that survived inoculation with SfMNPV alone (lane 3) or with SfMNPV and Tinopal UNPA-GX (lane 5) in the second instar. (C) RT-PCR fragments amplified with SfMNPV *polh* primers from adults of *S. frugiperda* that survived inoculation with SfMNPV alone (lane 3) or with SfMNPV and Tinopal UNPA-GX (lane 5) in the second instar. Positive controls (lethally infected larvae) for *ie-0* (A, lane 1) and *polh* (B and C, lanes 1), negative controls (uninfected larvae) for *ie-0* (A, lane 2) and *polh* (B and C, lanes 2), and RNA controls (PCR on total RNA) (A to C, lanes 4 and 6) are shown. Southern blot analyses of the agarose gels shown in panels A to C used labeled SfMNPV *ie-0* (D) or *polh* (E and F) genes as probes. The molecular marker used was 12-kb DNA ladder (Stratagene); fragment sizes are given on the left.

CTTTGGAC 3′ [forward] and 5′ CACGGTTGATGAACTC TTCG 3′ [reverse]). The primers were designed to give amplifications of 783 bp for *ie-0* and 770 bp for *polh*. All positive RT-PCR amplifications were checked for the presence of DNA contamination by PCR using the original samples. Positive and negative controls were also included in each RT-PCR amplification. PCR products were observed by electrophoresis in 0.7% agarose gels stained with ethidium bromide and photographed. Each gel was transferred to nylon membrane (Hybond-N+; Amersham), hybridized with digoxigenin-labeled probes (Boehringer Mannheim), washed twice, and subjected to autoradiography.

The genes *ie-0* and *polh* proved suitable as indicators of covert infection. RT-PCR analysis from sixth instars that survived treatment with SfMNPV alone in the fifth instar showed that 9 of 22 (41%) and 2 of 22 (9%) samples tested positive for *ie-0* and *polh* transcripts, respectively. No virus mortality was observed with the mock-infected control insects.

Larvae of *S. frugiperda* suffered 91 or 89% mortality when inoculated in the second instar with SfMNPV alone or with SfMNPV and Tinopal UNPA-GX, respectively. When inoculated in the fifth instar, insects suffered 83 or 85% mortality, respectively.

The transcriptional activity of early and late genes differed markedly between the larval and adult life stages. Of the fifth instar insects that survived treatment with SfMNPV alone in the second instar, 4 of 21 (19%) gave a positive result for *ie-0* transcripts whereas a single positive individual was detected among insects treated with SfMNPV and Tinopal UNPA-GX (Table 1; Fig. 1A and D). In the case of very late *polh* gene transcripts, the number of positive insect larvae was extremely low (1 of 21) for both treatments (Fig. 1B and E).

No *ie-0* transcripts were detected in any of the adult insects derived from larvae inoculated in the second instar. In contrast, transcription of the *polh* gene was detected in 7 of 27 (26%) or 3 of 27 (11%) of adult insects treated with SfMNPV or with SfMNPV and Tinopal UNPA-GX in the second instar, respectively (Table 1; Fig. 1C and F). No difference in the prevalence of *polh* transcripts was detected between treatments with virus alone and virus with optical brightener for adult insects that had been inoculated in the fifth instar (1 of 21 insects tested positive for each treatment).

The mean weights (± standard errors) of pupae derived from larvae inoculated in the second or fifth instar did not differ significantly in the treatments involving SfMNPV alone (second instar, 186.2 ± 3.5 mg [n = 124]; fifth instar, 157.7 ± 3.5 mg [n = 46]) or SfMNPV and Tinopal UNPA-GX (second instar, 187.2 ± 3.7 mg [n = 64]; fifth instar, 179.8 ± 4.0 mg [n = 67]) compared to their respective controls (second instar, 195.2 ± 3.0 mg [n = 74]; fifth instar, 175.1 ± 4.4 mg [n = 43]) (t test or Kruskal-Wallis test, $\alpha = 0.05$).

Contrary to our initial hypothesis, the presence of an optical brightener in inocula did not greatly affect the prevalence of covert NPV infections in *S. frugiperda* larvae or adults. We conclude that the optical brightener does not alter the virulence of the virus, i.e., the severity of disease, in infected hosts (20). Infected cells may struggle to control viral replication or limit the spread of the disease by apoptosis (6). Indeed, baculoviruses contain a number of apoptosis-inhibiting genes designed to reduce the probability of cell suicide (5). The interaction between optical brighteners and baculovirus apoptosis suppressors appears to merit further study.

The persistence of baculoviruses in covertly infected hosts is an effective mechanism that ensures virus survival when the density of the host population is low and opportunities for horizontal transmission are limited (4). The ability to determine accurately the prevalence of covert infection in insect populations depends on the life stages at sampling and the nature of the viral gene mRNA used to detect baculovirus infection.

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REFERENCES

1. **Bartnicki, G. S., J. Persson, and H. Chanzy.** 1994. An electron microscope and electron diffraction study of the effect of calcofluor and Congo red on the biosynthesis of chitin *in vitro*. Arch. Biochem. Biophys. **310:**6–15.
2. **Blissard, G. W., and G. F. Rohrmann.** 1990. Baculovirus diversity and molecular biology. Annu. Rev. Entomol. **35:**127–155.
3. **Burden, J. P., C. M. Griffiths, J. S. Cory, P. Smith, and S. M. Sait.** 2002. Vertical transmission of sublethal granulovirus infection in the Indian meal moth, *Plodia interpunctella*. Mol. Ecol. **11:**547–555.
4. **Burden, J. P., C. P. Nixon, A. E. Hodgkinson, R. D. Possee, S. M. Sait, L. A. King, and R. S. Hails.** 2003. Covert infections as a mechanism for long-term persistence of baculoviruses. Ecol. Lett. **6:**524–531.
5. **Clem, R. J.** 2001. Baculoviruses and apoptosis: the good, the bad, and the ugly. Cell Death Differ. **8:**137–143.
6. **Clem, R. J., and L. K. Miller.** 1993. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. J. Virol. **67:**3730–3738.
7. **Elorza, M. V., H. Rico, and R. Sentandreu.** 1983. Calcofluor white alters the assembly of chitin fibrils in *Saccharomyces cerevisiae* and *Candida albicans* cells. J. Gen. Microbiol. **129:**1577–1582.
8. **Escribano, A., T. Williams, D. Goulson, R. D. Cave, J. W. Chapman, and P. Caballero.** 1999. Selection of a nucleopolyhedrovirus for control of *Spodoptera frugiperda* (*Lepidoptera*: *Noctuidae*): structural, genetic, and biological comparison of four isolates from the Americas. J. Econ. Entomol. **92:**1079–1085.
9. **Friesen, P. D.** 1997. Regulation of baculovirus early gene expression, p. 141–170. *In* L. K. Miller (ed.), The baculoviruses. Plenum Press, New York, N.Y.
10. **Fuxa, J. R.** 1991. Insect control with baculoviruses. Biotech. Adv. **9:**425–442.
11. **Fuxa, J. R., E. H. Weidner, and A. R. Richter.** 1992. Polyhedra without virions in a vertically transmitted nuclear polyhedrosis virus. J. Invertebr. Pathol. **60:**53–58.
12. **Gonzalez, M. A., G. E. Smith, and M. D. Summers.** 1989. Insertion of the SfMNPV *polyhedrin* gene into an AcMNPV *polyhedrin* deletion mutant during viral infection. Virology **170:**160–175.
13. **Hughes, P. R., N. A. M. van Beek, and H. A. Wood.** 1986. A modified droplet feeding method for rapid assay of *Bacillus thuringiensis* and baculoviruses in noctuid larvae. J. Invertebr. Pathol. **48:**187–192.
14. **Kukan, B.** 1999. Vertical transmission of nucleopolyhedrovirus in insects. J. Invertebr. Pathol. **74:**103–111.
15. **Lu, A., and L. K. Miller.** 1997. Regulation of baculoviruses late and very late gene expression, p. 193–216. *In* L. K. Miller (ed.), The baculoviruses. Plenum Press, New York, N.Y.
16. **Moscardi, F.** 1999. Assessment of the application of baculoviruses for control of Lepidoptera. Annu. Rev. Entomol. **44:**257–289.
17. **Rothman, L. D., and J. M. Myers.** 1996. Debilitating effects of viral diseases on host Lepidoptera. J. Invertebr. Pathol. **67:**1–10.
18. **Simón, O., T. Williams, M. López Ferber, and P. Caballero.** 2004. Genetic structure of a *Spodoptera frugiperda* nucleopolyhedrovirus population: high prevalence of deletion genotypes. Appl. Environ. Microbiol. **70:**5579–5588.
19. **Simón, O., T. Williams, M. López-Ferber, and P. Caballero.** 2004. Virus entry or the primary infection cycle are not the principal determinants of host specificity of *Spodoptera* spp. nucleopolyhedroviruses. J. Gen. Virol. **45:**2845–2855.
20. **Thomas, S. R., and J. S. Elkington.** 2004. Pathogenicity and virulence. J. Invertebr. Pathol. **85:**146–151.
21. **Wang, P., and R. R. Granados.** 2000. Calcofluor disrupts the midgut defense system in insects. Insect Biochem. Mol. Biol. **30:**135–143.
22. **Washburn, J. O., B. A. Kirkpatrick, E. J. Haas-Stapleton, and L. E. Volkman.** 1998. Evidence that the stilbene-derived optical brightener M2R enhances *Autographa californica* M nucleopolyhedrovirus infection in *Trichoplusia ni* and *Heliothis virescens* by preventing sloughing of infected epithelial cells. Biol. Control **11:**58–69.