Filamentous actin is required for lepidopteran nucleopolyhedrovirus progeny production

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Autographa californica M nucleopolyhedrovirus (AcMNPV) is the prototypical member of the Nucleopolyhedrosis genus of the Baculoviridae, a family of large, double-stranded DNA viruses that are highly diverse. Nucleocapsid morphogenesis of AcMNPV and others in the Nucleopolyhedrovirus genus takes place within the nuclei of infected host cells. Previously, we showed that filamentous actin (F-actin) is essential for this process to occur in AcMNPV-infected cells, an unprecedented finding for a DNA virus that replicates within the nucleus. Because of the fundamental importance of this requirement to our understanding of virus–host interactions, and because of the diversity of viruses included within the Nucleopolyhedrovirus genus, we were compelled to determine whether the replication of other nucleopolyhedroviruses was also F-actin dependent. We report here that progeny virus production of six other lepidopteran nucleopolyhedroviruses, representing both phylogenetic groups I and II within the genus, is also F-actin dependent. The six viruses studied (Spodoptera frugiperda MNPV, Bombyx mori NPV, Orgyia pseudotsugata MNPV, Lymantria dispar MNPV, Anticarsia gemmatalis MNPV and Helicoverpa zea SNPV) were unable to produce progeny in the presence of either cytochalasin D or latrunculin A, two actin-binding agents that interfere with F-actin-dependent processes but differ in their modes of action. F-actin-dependent progeny morphogenesis, therefore, appears to be a characteristic common among viruses in this genus that have lepidopteran hosts.

Introduction

Baculoviridae is a family of diverse double-stranded DNA viruses with arthropod hosts. Family members infect hundreds of insect species that cover a wide range of habitats, including forests, fields and streams (Miller, 1997). This diversity in hosts is echoed by diversity among the baculoviruses themselves, and is reflected not only by differences in the host range of individual viruses, but also in pathogenesis, variation in genome size and G+C content (Miller, 1997; Ahrens et al., 1997). Baculovirus genomes are large and contain over 100 open reading frames, but only a small fraction of these have been functionally characterized even for the best-studied baculovirus, Autographa californica M nucleopolyhedrovirus (AcMNPV) (Miller, 1997). AcMNPV, the type-species of the Nucleopolyhedrovirus genus, has served as the de facto model for all baculoviruses even though it may not be representative of the group as a whole, or even, for that matter, of the Nucleopolyhedrovirus genus. For example, AcMNPV produces two different forms to complete its infection cycle in larval lepidopteran hosts. One form, released from an occluding protein matrix following ingestion by a susceptible host, initiates infection in midgut cells while the other form, budded virus (BV), transmits infection systemically. The major envelope glycoprotein of AcMNPV BV, gp64, is encoded by the AcMNPV genome and is essential for functional entry of AcMNPV BV. The recent completion of the genomic sequences of two other nucleopolyhedroviruses, Lymantria dispar (Ld)MNPV (Kuzio et al., 1999) and Helicoverpa zea (Hz)SNPV (Albert Lu, DuPont Agricultural Products, personal communication), however, has revealed that homologues of this essential AcMNPV gene are missing in those genomes. This finding leaves a void in our understanding of how the budded forms of these two viruses functionally enter their host...
cells, and also serves to remind us that what may be true for AcMNPV may not be true for every baculovirus.

Given the uncertainties of AcMNPV as a representative of other nucleopolyhedroviruses, we were anxious to determine whether our recent finding, that filamentous actin (F-actin) is essential for nucleocapsid morphogenesis of AcMNPV (Ohkawa & Volkman, 1999), holds true for other nucleopolyhedroviruses. The involvement of F-actin is quite unexpected and unique for viruses that replicate and assemble within the nucleus; hence, an understanding of the breadth of the requirement among baculoviruses is fundamental to any understanding of baculovirus–host interactions as a whole. Further, the implications are that baculoviruses that are found to be F-actin dependent must encode gene products that specifically interact with, and manipulate, the actin cytoskeleton of their hosts in an (as yet) unprecedented way.

Our study was conducted by testing the ability of six divergent members of the Nucleopolyhedrovirus genus to produce progeny in the presence of two actin-binding drugs, cytochalasin D (CD) and latrunculin A (LA), which differ in their modes of action. The viruses we used were selected because cloned isolates and cell lines for their propagation were available, and because phylogenetic studies based on two conserved, virus-encoded proteins (polyhedrin and DNA polymerase) indicated that this set of lepidopteran viruses spanned the Nucleopolyhedrovirus genus with regard to evolutionary relatedness (Zanotto et al., 1993; Hu, 1998; Bulach et al., 1999). Notably, we included the two nucleopolyhedroviruses known not to encode gp64. Here we present results demonstrating that for all six, progeny production was sensitive to both CD and LA, suggesting that F-actin is a genus-wide requirement for the replication of those nucleopolyhedroviruses with lepidopteran hosts.

Methods

**Cell lines and media.** Spodoptera frugiperda SF-9 and SF-21 cells were grown in Grace's medium with 10% foetal calf serum. Trichoplusia ni TN-368 and Lymantria dispar L652Y cells (the latter provided by G. Rohrmann, Oregon State University, Corvallis, OR, USA) were carried in TNMFH medium with 10% foetal calf serum. Heliotis virescens HvE1a cells (provided by D. E. Lynn, Insect Biocontrol Laboratory, USDA/ARS, Beltsville, MD, USA), were maintained in modified TC-100 medium and passaged as described (Lynn & Shapiro, 1998). Bombyx mori BmN cells (provided by S. George Kamita at the University of California, Davis, CA, USA) were grown in TC-100 medium and maintained as described (Maeda, 1989). Heliotis virescens-derived BCIRL-HV-AM1 cells (McIntosh et al., 1981) were provided by James Wong (DuPont Agricultural Products, Newark, DE, USA) and grown in TNMFH medium with 10% foetal bovine serum. SF-9, SF-21, TN-368 and BCIRL-HV-AM1 cells were sometimes grown in suspension cultures, at which time their media were supplemented with Pluronic acid F-68 (Sigma) to 0.1%.

**Viruses.** All viruses used were cloned isolates and included AcMNPV-hsp70/lacZ (Engelhard et al., 1994), Spodoptera frugiperda (Sf)MNPV, Bombyx mori (Bm)NPV, Orgyia pseudotsugata (Op)MNPV, LdMNPV, Anticarsia gemmatalis (Ag)MNPV and HzSNPV-hsp70/lacZ. AcMNPV-hsp70/lacZ was a first-passage stock amplified in SF-9 cells after inoculation with infectious H. virescens haemolymph. BmNPV budded virus stock was the gift of S. George Kamita. OpMNPV budded virus was generously provided by George Rohrmann. Susan Thiem (Michigan State University, East Lansing, MI, USA) and James Slavicek (USDA Forest Service, Delaware, OH, USA) both provided budded virus stocks of LdMNPV A21 (Slavicek et al., 1996). AgMNPV-ag3 and SfMNPV-clone 1 were obtained from D. E. Lynn. HzSNPV-hsp70/lacZ was provided by James Wong.

**Drug treatments.** CD (Sigma) and LA (Molecular Probes) were dissolved in dimethylsulfoxide at a minimum concentration of 500 μM and frozen in aliquots at −80°C until needed. Cells from exponential phase cultures (8 × 10^5 cells/ml for cells grown in flasks and 1 × 10^6 cells/ml for suspension cultures) were seeded at 10^6 per well in six-well plates (Falcon) and allowed to settle for 1–2 h. The exception was HvE1a cells, which were passaged by trypsinization. These cells were seeded at one-half density and allowed to settle for 24 h in order to recover. The media were aspirated from the wells, and equal amounts of inocula were added at an m.o.i. of 10–20. The m.o.i.s used with LdMNPV and SfMNPV were lower because of lower titres (2 and 0.1, respectively). The six-well plates were rocked slowly at room temperature for a 1 h adsorption period, after which the inocula were removed and the cells washed gently twice with fresh medium. Each well then received 2 ml of fresh medium, with or without CD or LA. This medium was immediately sampled (30 μl or 100 μl for SfMNPV and LdMNPV) for baseline (residual) BV titres. Additional samples were taken at 24, 48 and sometimes 72 h post-infection (p.i.). All samples were frozen at −80°C immediately after they were taken, and kept frozen until they were titred by immunoplaque assay. Each experiment was done independently twice.

**Plaque assays.** Immunoplaque assays were performed essentially as previously described (Volkman & Goldsmith, 1982), with modifications specific to each virus (Table 1). Briefly, all assays were done with microscope slides that were embossed with twelve 5 mm circular wells per slide (Carlson Scientific no. 101205). The slides were prepared for cell culture by immersing them overnight in glass staining-dishes containing a solution of 70% ethanol and 1% HCl, followed by 10 rinses in tap-water and then glass-distilled water. Dishes containing rinsed slides were covered and baked overnight at 75°C and used without further sterilization. Prepared slides were placed in sterile, covered, plastic dishes (Falcon no. 1012) and seeded with a subconfluent monolayer of actively growing cells. After the cells had settled for 30 min to 1 h, the medium was removed and replaced with 10 μl of a serial dilution of sample cell supernatant, and the slide incubated at 27°C. After 1 h, the inoculum was aspirated and quickly replaced with 0.1% hydroxypropylmethylcellulose (Dow Chemical) dissolved in the appropriate medium. Incubation at 27°C was then continued for 40–90 h, depending on the virus. After incubation, the cells were fixed for 5 min in formyl-buffered acetone, or 3.7% formaldehyde for the lacZ-expressing viruses (AcMNPV-hsp70/lacZ and HzSNPV-hsp70/lacZ), and then washed with PBS until the methylicholesterol was removed. In order to elucidate plaques for the lacZ-expressing viruses, the cells were overlaid with X-Gal in enhancement buffer [0.8 mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside in 5 mM KFe(CN)₆, 5 mM K₂Fe(CN)₆, 2 mM MgCl₂ in distilled water] and incubated for 1–2 h to develop the signal. For the remaining viruses, cells were blocked with dilute normal goat serum for 5 min after fixing and rinsing, and then incubated with the appropriate primary antibody for 25 min (Table 1). Antibodies used for this purpose were rabbit antibodies to AcMNPV polyhedrin (Volkman, 1983), OpMNPV MBP–ORF2 (Russell et al., 1997; generously provided by G. F. Rohrmann) and mouse monoclonal antibody to AcMNPV gp64.
(Keddie et al., 1989). After several PBS rinses, a secondary horseradish peroxidase-conjugated antibody was incubated with the cells for 25 min. After a final rinse. True Blue Peroxidase substrate (Kirkegaard and Perry Laboratories) was overlaid to produce a blue stain at the locations of plaques. Each slide was then air-dried, mounted in D. P. X. (Aldrich), and the plaques counted using a compound microscope equipped with a 40 x objective. Each serial dilution was plated in quadruplicate, and each relevant dilution was assayed twice.

Western blots. Suspension cultures of SF-9, SF-21 and TN-368 cells in exponential phase growth, all growing in TNMFH + 10% FBS + 0.1% Pluronic acid, were seeded into duplicate 35 mm plates at 10^6 cells per plate. Cells were allowed to settle and spread for 12 h, and then they were rinsed twice quickly with PBS, drained, and lysed in 150 µl cell lysis buffer (PBS + 0.5% NP-40). Plates were scraped with a cell scraper, and the lysate was placed in microfuge tubes (one per plate) on ice. Fifty µl of 4 x SDS–PAGE sample buffer containing E64 proteinase inhibitor (final concentration 30 µg/ml) was added to each tube (Hom & Volkman, 1998), and the contents were then immediately boiled for 2 min, chilled on ice briefly, and stored at –80 °C. Thawed samples were diluted 1:2 in series up to 1:64 in sample buffer (1 x), boiled again for 1 min, and then loaded onto 10% acrylamide SDS–PAGE gels. After electrophoresis, the proteins were transferred to Immobilon-P membrane (Millipore) and probed with C4 anti-actin antibody (Lessard, 1988) diluted 1:2000 in TBS containing 0.2% Tween 20, followed by goat anti-mouse horseradish peroxidase-conjugated antibody diluted 1:3000 in the same diluent (Sigma). Reactions were elucidated using the Supersignal Chemiluminescent Substrate Luminol/Enhancer (Pierce).

Immunofluorescence microscopy. Approximately 10^5 actively growing TN-368 cells in 200 µl medium were seeded onto 22 mm square coverslips which had been prepared in the same way as the microscope slides described under Plaque assays. Cells were allowed to settle for 30 min and were then inoculated (where indicated) with AcMNPV-hsp70/lacZ at an m.o.i. of 50. After 1 h, the inocula were removed and replaced with 1 ml fresh medium containing either no drug, 1 µg/ml (2 mM) CD or 0.4 µg/ml (1 mM) LA. After incubation for 25 h at 27 °C, these cells were fixed and treated with monoclonal antibody 39P10 (to AcMNPV p39) for confirmation of infection, TRITC–phalloidin for F-actin staining and DAPI for DNA staining to locate the nucleus as described (Charlton & Volkman, 1991). The cells were viewed using a Zeiss Axiophot photomicroscope equipped for fluorescence microscopy.

Results

Treatment of cells with CD or LA induces changes in F-actin

CD and LA are actin-binding drugs with different modes of action. LA forms a 1:1 molar complex with soluble G-actin thereby sequestering it and preventing it from polymerizing (Coué et al., 1987; Spector et al., 1989); CD binds F-actin at the barbed ends and prevents association and dissociation of G-actin monomers from that end (Brown & Spudich, 1981). Both drugs interfere with F-actin-dependent processes, however. The effects of these drugs on F-actin in AcMNPV-infected and uninfected TN-368 cells are shown in Fig. 1. In the absence of drug treatment, the redistribution of F-actin from micospikes and the cortical regions of the cells to the nuclei was clearly evident 25 h p.i. (compare Fig. 1 A to 1D). Addition of CD (1 µg/ml) to uninfected cells also resulted in a change in the distribution of F-actin from surface structures (Fig. 1A) to aggregates or foci primarily within the cytoplasm at 25 h p.i. (Fig. 1B). In comparison, addition of CD to infected cells did not appear to affect the distribution so much as the content: CD diminished the amount of F-actin apparent within the nucleus, but some small cytoplasmic aggregates were also evident (compare Fig. 1 D to 1E). Incubation of cells for 25 h with LA (0.4 µg/ml), whether uninfected or infected, resulted in the disappearance of F-actin.

Sensitivity of AcMNPV-hsp70/lacZ BV production to LA is inversely correlated with cellular actin content

Because the dependence of AcMNPV replication on F-actin had been determined previously using a different method (Ohkawa & Volkman, 1999), we initiated our study by testing the sensitivity of AcMNPV-hsp70/lacZ BV production to CD and LA in three permissive cell lines, TN-368, SF-21 and SF-9 (Fig. 2A). BV production was sensitive to both drugs individually in all three cell lines, but the amount of drug required for a similar level of inhibition differed for each. Among the three cell lines tested, the greatest sensitivity of BV production to CD was observed in TN-368 cells and the least sensitivity in SF-21 cells, even though Western blot analysis indicated that the actin content of TN-368 and SF-21 cells, uninfected, was equivalent (Fig. 2B). These differences in sensitivity to CD suggested that AcMNPV-infected SF-21 cells contained more F-actin barbed ends than AcMNPV-infected TN-368 cells. This could occur, even if the total F-actin content of both cell types was identical, if the filaments were shorter but more numerous in infected SF-21 cells. Whatever the cause, the results indicate (not unexpectedly) that cellular factors play a role in the number and character of the actin filaments in AcMNPV-infected cells.

The greatest sensitivity of AcMNPV BV production to LA was evident in SF-9 cells with a less severe but roughly equivalent level of inhibition in infected TN-368 and SF-21 cells (Fig. 2). These results were consistent with, and inversely correlated with, total actin content per cell for the three cell types tested (Fig. 2B). Because the activity of LA is to sequester monomeric actin, the degree of sensitivity of AcMNPV to LA should be an indication of the size of the pools of G-actin available to the virus. The results, therefore, indicated that the size of the pools of G-actin was roughly equivalent within AcMNPV-infected TN-368 and SF-21 cells, but smaller in SF-9 cells.

CD and LA inhibit BV production by six divergent nucleopolyhedroviruses

Table 1 lists the viruses used in this study, their phylogenetic groups, the host cell lines used to support their replication and the antibody or reagent used to determine their titre. The sensitivities of six of these viruses to CD and LA are
Fig. 1. Rhodamine–phalloidin staining of F-actin. TN-368 cells were either mock infected (A, B, C) or infected with AcMNPV-hsp70/lacZ at an m.o.i. of 50 (D, E, F), and then either mock-treated (A, D) or treated with CD at 1 µg/ml (B, E) or LA at 0-4 µg/ml (C, F) and incubated for 25 h. Cells were subsequently fixed and stained with TRITC–phalloidin.
F-actin is required for NPV production

Fig. 2. Drug sensitivity of AcMNPV (A) and actin content (B) in three permissive cell lines. (A) Parallel cultures of TN-368, Sf-21 and Sf-9 cells were infected with AcMNPV-hsp70/lacZ and then treated with CD or LA as indicated. Samples were taken at 0, 24 and 48 h p.i. and titred by plaque assay. Each point is the mean of duplicates run in parallel, each titred in quadruplicate. Error bars indicate ±1 standard deviation. (B) Western blots of serially diluted lysates made from equal numbers of uninfected TN-368, Sf-21 and Sf-9 cells and then probed for actin. Shown is a 1 s exposure after development with chemiluminescent substrate. Dilutions 1:8, 1:16, 1:32 and 1:64 are shown. Duplicate experiments were done and gave identical results.

shown in Figs 3 and 4, respectively. BV production for all six was sensitive to both drugs individually suggesting that they all were dependent upon F-actin for progeny virus production. Infections by all six baculoviruses were completely inhibited by the lowest concentration of CD used, 0.5 µg/ml (Fig. 3). Replication of one of the six, SfMNPV, took place in Sf-21 cells, the same cells wherein this concentration of CD had very little effect on AcMNPV replication (Fig. 2). This difference in CD sensitivity indicated that AcMNPV-infected Sf-21 cells contained more F-actin barbed ends than SfMNPV-infected Sf-21 cells, which, in turn, suggested that virus-encoded factors also had an effect on the number and character of actin filaments within infected cells.

Sf-21 cells generated a 56-fold higher level of BV progeny by 48 h.p.i. when infected with AcMNPV than by SfMNPV in the absence of CD or LA (compare Fig. 2 with Fig. 4). Similarly, by 72 h p.i., L652Y cells had generated a 112-fold higher level of BV when infected by OpMNPV than by LdMNPV (Fig. 4). Further, AcMNPV progeny production in Sf-21 cells was not significantly affected by the addition 0.08 µg/ml of LA at either 24 or 48 h.p.i. while SfMNPV progeny production, by comparison, was reduced by 80% at 24 h.p.i. and by 70% at 48 h.p.i. (compare Fig. 2 with Fig. 4). Likewise, in the presence of 0.08 µg/ml of LA, OpMNPV progeny production was depressed 16-fold by 72 h.p.i. compared with 157-fold for LdMNPV at that time (Fig. 4). Together, these results demonstrated that for each pair of viruses infecting a given cell line, the one that replicated the best was the least sensitive to LA. The implications were that the G-actin pool size was affected by virus replication, and the degree of virus en-
hancement of the pool size may have been a factor in the relative productivity of infection in these cell lines. Because these NPVs do not encode actin (Ahrens et al., 1997) and there is no increase in actin synthesis upon infection (Talhouk & Volkman, 1991), the source of G-actin is likely to be depolymerized cytoplasmic F-actin.

Discussion

Many different viruses appropriate the actin cytoskeleton of their host cells for a specific function (reviewed in Cudmore et al., 1997), but the functions in question all take place within the cytoplasm with the exception of AcMNPV nucleocapsid morphogenesis (Ohkawa & Volkman, 1999). The very existence of nuclear actin has been controversial until recently, but although widely accepted now, unequivocal functions for nuclear actin have been difficult to demonstrate (Gonsior et al., 1999). In this study we have shown that like AcMNPV, the type species, progeny BV production by six divergent nucleopolyhedroviruses is inhibited by two drugs that interfere with F-actin function. While specificity is always a concern when drawing conclusions from studies involving drugs, the fact that the activities of the two drugs differ (LA sequesters monomeric actin while CD binds the barbed end of F-actin) supports the conclusion that actin is the functional target of the drugs. Further evidence for this is the inverse correlation of the cellular actin content and the concentration of LA needed to interfere with AcMNPV-hsp70/lacZ BV production (Fig. 2). The fact that a similar correlation could not be drawn for CD could be among the factors positively influencing the number of barbed ends available. They also could explain the diminished staining of F-actin observed in infected TN-368 cells treated with CD (Fig. 1E) compared with control infected cells (Fig. 1D).

The viruses chosen for this study have all been subjected to phylogenetic analysis using amino acid sequence data of conserved proteins inferred from the nucleotide sequences of the genes that encode them (Zanotto et al., 1993; Hu, 1998; Bulach et al., 1999). While the computer programs used to analyse the data differ, and the resulting phylogenetic trees are not identical, there is agreement that there are two major clades or groups of NPVs, I and II. AcMNPV, BmNPV, OpMNPV and AgMNPV all fall within group I, while LdMNPV, HzSNPV and SiMNPV fall within group II (Bulach et al., 1999). So far, only MNPVs (viruses having one to many nucleocapsids per virion) have been placed in group I, while both SNPVs (viruses with only a single nucleocapsid per virion) and MNPVs fall in group II. Interestingly, those NPVs lacking gp64 fall in group II. Our evidence suggests that the lepidopteran nucleopolyhedrovirus dependence on F-actin evolved prior to the establishment of the two groups as well as before the development of nucleocapsid packaging differences.

All evidence to date indicates that F-actin plays a role in nucleocapsid assembly of nucleopolyhedroviruses within the nucleus (Volkman et al., 1987; Volkman, 1988; Hess et al., 1989). The ability to commandeer and direct the transport of actin to the nucleus is a feature not shared by any other group of viruses. Whether this sort of manipulation of the actin cytoskeleton is more related to the unique capabilities of nucleopolyhedroviruses or to an unexplored vulnerability of

### Table 1. Viruses, their phylogenetic groups, host cell lines and methods of detection in plaque assays

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<th>Virus</th>
<th>Phylogenetic group</th>
<th>Host cell line</th>
<th>Method of detection</th>
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<tr>
<td>AcMNPV-hsp70/lacZ</td>
<td>I</td>
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<td>X-Gal</td>
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<td>I</td>
<td>L652Y</td>
<td>Anti-OpMNPV p01 (ORF2)</td>
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<td>I</td>
<td>HvE1A</td>
<td>Anti-Ac-polyhedrin</td>
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Fig. 3. Inhibition of BV production by CD. Infected cells were treated with medium containing either no drug, 0.5 µg/ml or 1 µg/ml CD. Samples were taken at 0, 24, 48, and in some cases 72 h p.i. Virus titres for each time-point were determined by plaque assay. Each graph point is the mean of two independent experiments, each titred in quadruplicate. Error bars represent ± 1 standard deviation.

Fig. 4. Inhibition of BV production by LA. Infected cells were treated with medium containing either no drug, 0.08 µg/ml or 0.4 µg/ml LA. Samples were taken at 0, 24, 48, 72, and in some cases 96 h p.i. Virus titres for each time-point were determined by plaque assay. Each graph point is the mean of two independent experiments, each titred in quadruplicate. Error bars represent ± 1 standard deviation.
lepidopteran cells remains an open question. It will be interesting in this regard to determine whether the granuloviruses which are restricted to lepidopteran hosts, or those nucleopolyhedroviruses that have non-lepidopteran hosts, also depend on F-actin for progeny production.

The authors are greatly indebted to George Rohrmann, Dwight Lynn, George Kamita, Suzanne Thiem, James Wong, James Lessard and James Slavicek for generously supplying virus, cells and/or antibodies. We thank Taro Ohkawa for assistance in preparing the figures and Steve Ruzin of the CNR Facility for Biological Imaging for use of the Zeiss microscope and imaging equipment. Financial support was provided by USDA NRRCG 97-35302-4340, by Novartis Agricultural Discovery Institute, Inc., and by federal Regional Research and HATCH funds.

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Received 12 January 2000; Accepted 31 March 2000