



A rapid method for the production and characterization of recombinant insecticidal proteins in plants

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Abstract

Development and evaluation of transgenic plants containing genes that confer insecticidal activity can be a labor-intensive and time-consuming process. Transformed plants require months of growth before they can be analyzed. Transient gene expression by plant viral vectors offers an alternative. Using this approach, infected plants can be screened after only two weeks. Genes coding for the enhanced green fluorescent protein (EGFP), the *Bacillus thuringiensis* (Bt) Cry1Ac toxin and a wound-induced (Kunitz-type) proteinase inhibitor from poplar (WIN3) were introduced into a potato virus X (PVX) expression vector, pPC2S. Infectious full-length RNA transcripts synthesized *in vitro* or infectious viral particles were used to inoculate plants. Western blots demonstrate expression of the proteins in *Lycopersicon esculentum* cv. Rutgers and *Nicotiana benthamiana* leaves. Fluorescence microscopy was also used to monitor EGFP expression. To demonstrate the effectiveness of the PVX expression system, *N. benthamiana* expressing Cry1Ac or WIN3 was used and growth of *Heliothis virescens*, tobacco budworm was assayed. Leaves that expressed Cry1Ac were almost completely resistant to larval feeding. Tobacco budworm fed on leaves expressing WIN3 grew to only 77% of the weight of insects fed with leaves expressing PVX alone. These results illustrate that the PVX expression system allows rapid and reliable production of recombinant plant material for characterization of insecticidal genes in insect bioassays.

Abbreviations: Bt – *Bacillus thuringiensis*, PVX – potato virus X, tsp – total soluble protein, WIN3 – wound-induced protein 3

Introduction

Assessing new insecticidal genes *in planta* is a tedious process, since it involves the time-intensive task of transforming plants with the test gene. In tomato, it takes months before transgenic plants can be tested for the presence of the newly inserted gene. Additional time is also required to propagate material before enough is available for insect bioassays. However, creating a plant containing a recombinant virus allows much earlier screening and assay of insecticidal genes.

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Indeed, only two weeks after plants are inoculated with a recombinant PVX, they are ready for use in an insect bioassay. In addition, by infecting the plant with a mutated gene, structure and function studies of the protein can be quickly carried out (Rommens et al. 1995).

PVX is a plant potexvirus containing 5 genes (for reviews, see Chapman et al. 1992; Baulcombe et al. 1995). The entire genome of the virus is present in the vector described below (Baulcombe et al. 1995). A duplicated coat protein (CP) promoter is used to drive expression of the foreign gene. The CP and the triple gene block (M1-3) are required for systemic viral movement. The RNA-dependent RNA polymerase (RdRp) is necessary for subsequent production of in-

fectious transcripts. Placement of a foreign gene after the duplicated CP promoter allows production and spread of the protein along with PVX through the phloem into the newly forming leaves (Baulcombe et al. 1995). PVX infects the solanaceous plants tobacco, tomato and potato.

Bacillus thuringiensis (Bt) is a well-described source of insecticidal proteins (Estruch et al. 1997). The Bt endotoxin Cry1Ac is toxic to lepidopteran insects and therefore provides a valuable test of PVX as a vectoring system for insecticidal genes. The bacterial gene has been modified to function in plant cells (accession number AF177675) and *cry1Ac* genes have been inserted into numerous plant species producing increased resistance to a number of insect pests (for reviews, see Schuler et al. 1998; de Maagd et al. 1999). However, the existence of Bt-tolerant pest species and increasing concern over the development of insects resistant to Bt (Tabashnik 1994; de Maagd et al. 1999) drive the continued search for novel insecticidal genes.

Wounding of poplar leads to the expression of genes presumed defensive against insect predation (Parsons et al. 1989). However, since none of these *win* (wound-induced) genes have been tested for insecticidal activity, they make excellent candidates for this assay. The translated sequence of one of these wound-responsive genes, *win3*, has homology to a Kunitz-type trypsin inhibitor from soybean (Bradshaw et al. 1989). Trypsin inhibitors have been found to deter insect feeding when tested in transgenic plants (for review, see Schuler et al. 1998). Therefore, we wondered whether WIN3 enhances resistance to a pest insect.

We have examined the expression of recombinant genes by PVX in *Nicotiana benthamiana* and *Lycopersicon esculentum*. We have subsequently used these plants in insect bioassays to characterize the effectiveness of the test gene in limiting larval growth of *Heliothis virescens*, tobacco budworm.

Materials and methods

PVX plasmids

Standard molecular methods were used to construct plasmids. The PVX expression vector pPC2S in Figure 1 (Baulcombe et al. 1995) was used to construct the following plasmids. *win3* from pwin3.12 (Hollick and Gordon 1993) was amplified by means of primers PacI.*win3* (5'-CCTTAATTAATATGAAGATCACTA-AATTTCTAGGG-3') and SalI.*win3* (5'-CCGCGT-CGACTTCCCTCAATATCATTCTGACACC-3') and

cloned into the *EcoRV* site of pPC2S to create PVX-WIN3. The EGFP coding sequence from pEGFP (Clontech, Palo Alto, CA) was amplified with primers PacI.EGFP (5'-CCTTATTATCGCCACCATGGTGA-GCAAGGGCGAGGAGC-3') and XhoI.EGFP (5'-CCGCTCGAGCGCTTTACTTGTACAGCTCGTCC-3') and cloned into the *EcoRV* site of pPC2S to create PVX-EGFP. The plasmid pPC2S was modified in the multiple cloning site (data in preparation) and used to construct NPVX-CRY1Ac. The *cry1Ac* gene from pGL896 (J. Kemp, New Mexico State University) was amplified with primers BsiWI.Cry1Ac (5'-ATACGTACGCCGACCATGGCTATCGAGACCGG-3') and BstEII.Cry1Ac (5'-ATTCATGGTGACCTTC-GAGTGTTCAGTAACTGG-3') and cloned into NPVX at the BsiWI and BstEII sites to create NPVX-CRY1Ac. In this work we refer to NPVX-Cry1Ac as PVX-Cry1Ac.

Plant material

Nicotiana benthamiana and *Lycopersicon esculentum* cv. Rutgers (tomato) were transplanted to individual pots 4 weeks and 2 weeks after sowing, respectively. Plants were grown under 16 h day/8 h night cycles at room temperature for ca. 2 weeks before infection.

Transcript production, plant inoculation and viral purification

Plasmids were digested with *SpeI* and further purified by proteinase K (0.2 mg/ml) digestion for 1 h at 50 °C, followed by phenol/chloroform extraction and ethanol precipitation. Ca. 1 µg of linearized template was used in the transcription reaction conditions outlined in the mMessage mMachine T7 kit (Ambion, Austin, TX). The reaction was incubated for 15 min at 37 °C, GTP was added to a final concentration of 1.5 mM and reactions were continued for another 40 min.

To inoculate leaves with infectious transcript, the leaf was dusted with carborundum, 0.03 U of AntiRNAse (Ambion) was added to the transcription reaction and 2–10 µl of transcript was gently rubbed onto the entire leaf surface with a gloved finger. Inoculation of leaves with purified virus was performed the same way except that AntiRNAse was omitted and 5–20 µl of viral stock was used.

For recombinant virus isolation, symptomatic *N. benthamiana* were harvested 10–14 days post infection. Leaves were ground to a fine powder in liquid nitrogen and 50 ml 0.1 M sodium phosphate, 10 mM EDTA pH 7 was added for every 10 g of leaf tissue. The sample was mixed well and filtered through 8

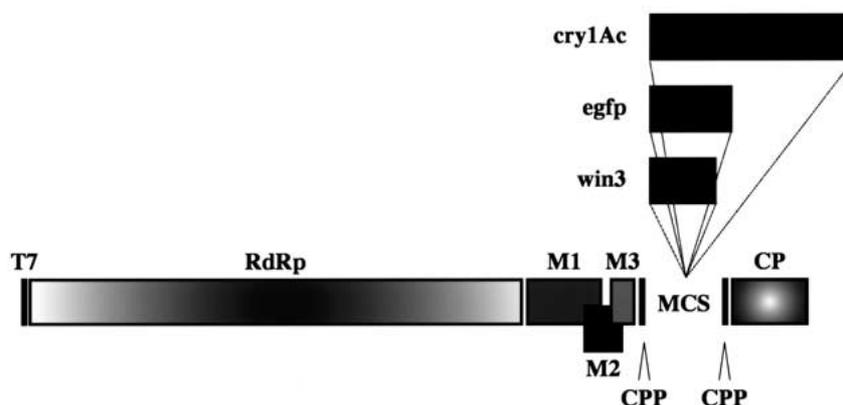


Figure 1. Foreign genes were cloned into the PVX vector, pPC2S. Viral genes and the MCS, multiple cloning site are shown. RdRp, RNA-dependent RNA polymerase; M1–M3, movement proteins; CPP, coat protein promoter; CP, coat protein.

layers of cheesecloth. The extract was centrifuged at $3400 \times g$ for 10 min and the supernatant was spun for 90 min at $44\,000 \times g$. The pellet was resuspended in 4 ml water and centrifuged at $4900 \times g$. The supernatant was layered over 4 ml of 20% sucrose and centrifuged at $160\,000 \times g$ for 1 h. The resuspended pellet was centrifuged at $3400 \times g$ for 10 min and the supernatant containing purified virus was stored at 4°C .

Fluorescent photography

N. benthamiana leaves were viewed with a mineral light at 366 nm (UVP, Upland, CA). Photographs of *L. esculentum* leaves were taken on a Fujifilm Intelligent Dark Box (Fuji Medical Systems, Stamford, CT) equipped with a Dia illuminator and FI filter Y515-Di and captured by a Fujifilm LAS-1000 digital camera.

Escherichia coli expression of WIN3

The pET System (Novagen, Madison, WI) was used for the expression of WIN3. A 589 bp fragment of the *win3* ORF from pwin3.6 (Hollick and Gordon 1993) was amplified with Elongase (Life Technologies) with primers EcoRI.*win3* (5'-GGAATTCCTTCTCTTTGACTTTGCCTTCGCAGC-3') and XhoI.*win3* (5'-CCGCYCGAGATTTTATGTGCTCTCAATGCGCGC-3'). The product was digested with EcoRI, filled in with T4 DNA polymerase (Life Technologies) and digested with XhoI. The fragment was ligated to the *SalI* (also treated with T4 DNA polymerase) and XhoI sites of pET24-d (Novagen). DH5 α cells were transformed to isolate pET24-d.*win3*.6.7, which was subsequently established in the BL21(DE3) inducible cell line. Cells were induced

as described by the manufacturer (Novagen). The pET24-d.*win3*.6.7 plasmid was verified by sequencing in both directions and the molecular mass of WIN3 was verified by SDS-PAGE at 22.6 kDa. Inclusion bodies were isolated according to the method outlined by Lin and Cheng (1991). Inclusion body-purified protein was separated by SDS-PAGE and bands were excised. Protein was recovered by electroelution with 50 mM ammonium hydrogen carbonate, 0.1% SDS volatile buffer in the BioRad Electro-eluter model 422 according to the manufacturer's instructions. Protein was sent to Cocalico Biologicals (Reamstown, PA) for polyclonal WIN3 antiserum production.

Western blots and protein quantification

Leaves were extracted in a buffer containing 50 mM Tris pH 8, 10 mM KCl, 5 mM MgCl₂, 10 mM BME, 0.4 M sucrose and 20% v/v glycerol. Total protein was determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Extracts were fractionated by SDS-PAGE, transferred onto nitrocellulose and processed with the appropriate antibody at the following dilutions: 1:1000 GFP Monoclonal Antibody (Clontech, Palo Alto, CA), 1:10 000 polyclonal WIN3 antiserum (Cocalico Biologicals), and 1:1000 Cry1Ac antibody donated by D. Dean (Ohio State University). Horseradish peroxidase (HRP)-conjugated secondary antibodies were used at 1:5000. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate by Pierce and Hyperfilm ECL by Amersham Pharmacia Biotech (Piscataway, NJ).

Volume integration values were calculated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). WIN3 was quantified on the immunoblots by comparison of experimental samples to the linear

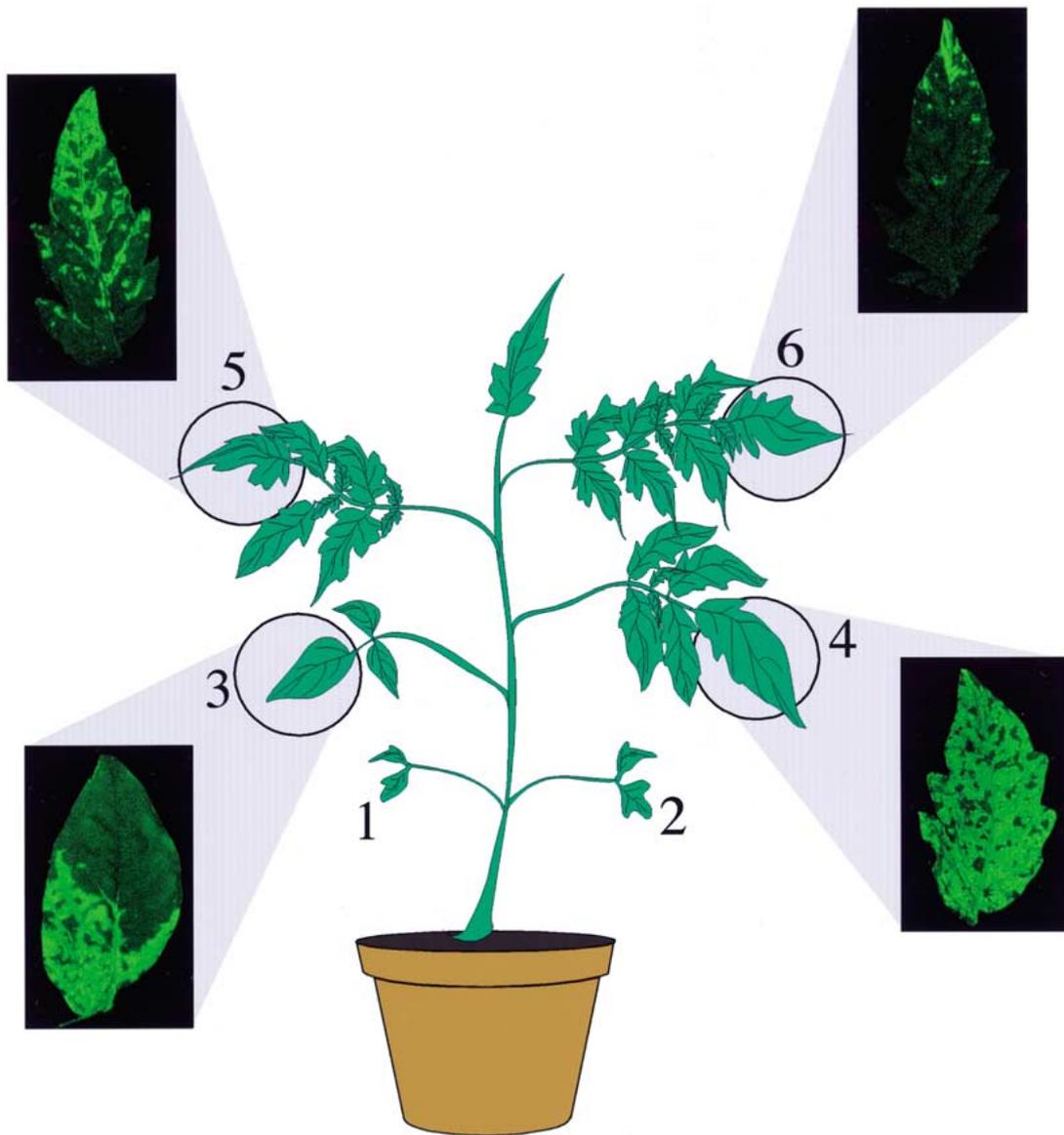


Figure 2. EGFP accumulation in a PVX-EGFP infected tomato plant at 14 days after infection. The plant was inoculated on the first true leaf as soon as it unfolded (leaf 1).

portion of a dilution series of WIN3 purified from *E. coli*.

Insect bioassays

Heliothis virescens eggs were obtained from the USDA Western Cotton Research Facility, Phoenix, AZ, and maintained at 28 °C on an artificial diet with 16 h day/8 h night cycle until they reached their second or third instar. Uniformly symptomatic leaves containing EGFP or Cry1Ac were cut into 3 cm × 3 cm pieces and placed in a petri dish with five larvae. Lar-

vae fed for 16 h. Newly hatched larvae were used in experiments measuring larval growth inhibition. Ten neonate *H. virescens* larvae were placed in wet filter paper-lined petri dishes with leaves expressing WIN3 or PVX alone. Additional leaves were added as feeding progressed. The GLM procedure of SAS Institute (1992) was used to perform analysis of vari-

ance (ANOVA) to determine any significant difference in weight or survival between larvae fed the two diets.

Results

Recombinant gene expression

Fourteen days after infection with PVX-EGFP transcripts, illumination of tomato leaves with UV light revealed patches of green fluorescence. Tomato was used to monitor movement and expression of EGFP because leaf age is easily correlated to position on the stem. When different leaves of the same plant were monitored it was clear that the pattern of EGFP expression was not uniform (Figure 2). Although the first leaf was initially infected, the most extensive signal was found in the fourth leaf, distal to the infection site. Fluorescence was not observed in uninfected plants or plants infected with PVX alone. Classic mosaic symptoms were also present on the leaves synthesizing EGFP, indicating a productive viral infection.

The PVX vector was used to produce WIN3, EGFP and Cry1Ac (Figure 3a). All three proteins were produced at the expected molecular mass (Cry1Ac, 67.5 kDa; EGFP, 27 kDa; WIN3, 21.7 kDa). Because a purified supply of *E. coli*-synthesized WIN3 was available, an accurate quantification of the WIN3 produced in plants could be made. Examination of four plants inoculated with PVX-WIN3 virus showed the amount of WIN3 synthesized varied from 0.1–0.2% of total soluble protein (TSP) (Figure 3b).

Insect bioassays

N. benthamiana, was infected with PVX-EGFP or PVX-Cry1Ac. Symptomatic leaf pieces, systemic to the infection site were fed to second and third instar tobacco budworm. After 16 h, leaf pieces producing Cry1Ac had incurred little damage (Figure 4). In contrast, plant material that synthesized EGFP was decimated. Therefore, although the pattern of signal for one gene, namely EGFP, was patchy, feeding deterrence was obvious in leaves producing the Cry1Ac toxin and seemed to occur throughout the leaf tissue. No effect on feeding deterrence was noted with insects fed on leaves producing WIN3 (data not shown).

Insect bioassays were also performed with newly hatched tobacco budworm larvae. Ten larvae per dish were placed on excised leaves and allowed to feed. Each dish was supplied with leaf material from a single infected plant. After 4 days, 80% of the larvae feeding on leaves producing WIN3 had survived,

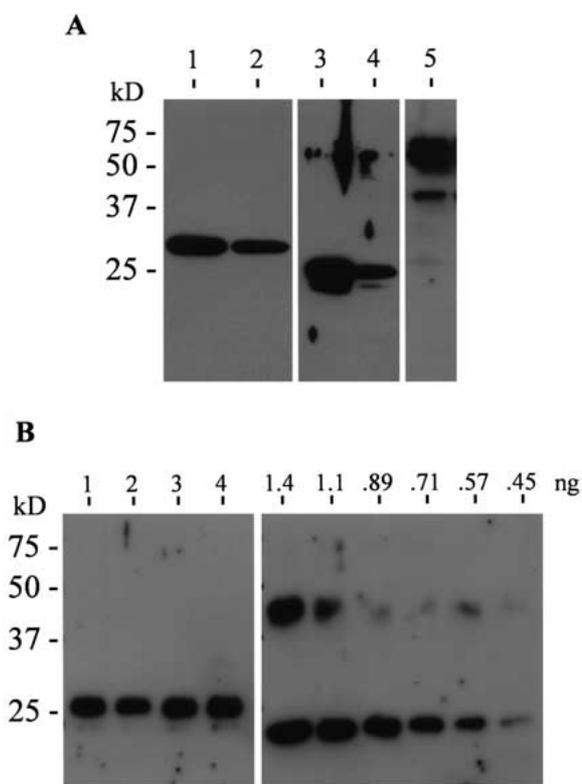


Figure 3. Protein produced in infected plants. A. Immunoblot illustrating synthesis of different genes in *N. benthamiana* and tomato. 1 and 2, EGFP in *N. benthamiana* and tomato, respectively; 3 and 4, WIN3 in *N. benthamiana* and tomato, respectively; 5, Cry1Ac in *N. benthamiana*. B. Immunoblot illustrating WIN3 accumulation in individually infected *N. benthamiana* plants. 1–4, total soluble leaf protein (500 ng/lane); purified *E. coli* expressed WIN3 standard (1.4, 1.1, 0.89, 0.71, 0.57, 0.45 ng).

compared with 91% of those eating leaves synthesizing PVX alone. This difference, however, was not significant ($F=4.92$; $df=1,16$; $p=0.0724$). Measurement of insect biomass after four days demonstrated that larvae feeding on leaves infected with PVX-WIN3 grew to only 77% of the weight of larvae eating leaves producing PVX alone (Figure 5). The difference between larval weight was significant ($F=3.70$; $df=1,16$; $p=0.0414$). Consequently, synthesis of WIN3 using the PVX vector and insect bioassay revealed insecticidal properties of this poplar gene.

For plant inoculation, the use of purified viral stocks rather than *in vitro* transcripts offers several advantages. Viral stocks are cheaper, produce more reliable infections, are more consistent (the integrity of *in vitro* transcripts varies from experiment to experiment), cut plant inoculation time significantly,



Figure 4. *N. benthamiana* synthesizing Cry1Ac deters *Heliothis virescens* feeding. The leaves from 3 individually infected plants producing Cry1Ac or EGFP were fed to 2nd- or 3rd-instar larvae for 16 h. Bar = 1 cm.

store well, and are extremely stable. Inoculation with purified virus often resulted in more rapid systemic infection, allowing plants to be used as early as one week after viral infection.

Discussion

When interpreting bioassay results it is important to know if appropriate levels of the test gene have been expressed. Production of GUS in PVX-infected *N. clevelandii* and *N. tabacum* plants resulted in comparable levels to those obtained with transgenes driven

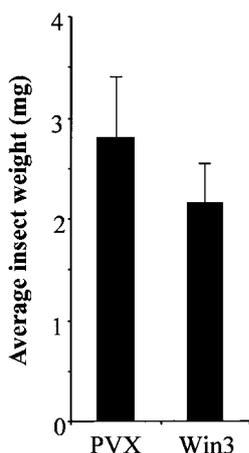


Figure 5. *N. benthamiana* leaves synthesizing WIN3 retard *Heliothis virescens* growth. The leaves from individually infected plants producing WIN3 or wildtype PVX were used to feed 10 neonate larvae per dish for 4 days. Data represent the average of 90 neonates from 9 infected plants for each sample.

by the cauliflower mosaic virus (CaMV) 35S promoter (Chapman et al. 1992). Transformation of *N. tabacum* with cowpea trypsin inhibitor linked to the CaMV 35S promoter produced inhibitor at 1% of TSP (Hilder et al. 1987). This is a typical level of protein for genes driven by the 35S promoter (Burgess and Gatehouse 1997). However, slightly lower levels (0.16–0.4%) were reported when proteinase inhibitors linked to 35S were transformed into tobacco and *Arabidopsis* (Jongsma et al. 1995; Urwin et al. 1998). In *N. benthamiana* we found that only 0.1–0.2% of the total protein in leaf tissue was WIN3. Perhaps different plant species or particular genes effect the level of protein produced. Our results and those of Chapman et al. (1992) suggest that the CP promoter of PVX expresses foreign genes at a similar level to the CaMV 35S promoter.

High levels of proteinase inhibitors are needed to see an effect on larval growth. Cowpea trypsin inhibitor at 0.25% TSP was needed to cause death of tobacco budworm (Hilder et al. 1987), while a trypsin inhibitor from *Manduca* in transgenic cotton at 0.1% of TSP was able to reduce whitefly emergence (Thomas et al. 1995). In rice transformed with the soybean Kunitz trypsin inhibitor a range of 0.3–0.5% of TSP was sufficient to cause 40% mortality of the brown planthopper (Lee et al. 1999). The level of WIN3 in our experiments was such that it resulted in a reduction in larval growth, but did not affect survival of the insect. It is also possible that WIN3 is not as active a proteinase inhibitor or is not as effective

against proteinases of the *H. virescens* gut. Christeller and Shaw (1989) demonstrated that Kunitz trypsin inhibitors from different plant species varied in their potency against a single insect gut enzyme. Our results demonstrated that production of WIN3 was able to retard the growth of tobacco budworm.

Bt δ -endotoxins are more effective insecticidal proteins than proteinase inhibitors. Cry1Ac at 0.04% of TSP completely controlled tobacco budworm in transgenic soybean (Stewart et al. 1996). In *Arabidopsis*, Cry1Ac present at 0.005% of TSP increased mortality of tobacco budworm (Santos et al. 1997). Therefore, less *Bt* toxin protein is required to deter larvae. Although we cannot estimate the amount of Cry1Ac present in our PVX-infected plants, less should be required to prevent defoliation and limit larval growth.

The stability of expression of the foreign gene has been an issue with PVX vectors (Chapman et al. 1992). Placing the β -glucuronidase (GUS) gene in a PVX vector and examining GUS and PVX transcript expression revealed that plants might contain populations of deleted forms of PVX-GUS that Chapman et al. (1992) attributed to loss of GUS by homologous recombination. This explained their observation that symptomatic leaf areas did not always coincide with GUS expression. Deleted forms of the recombinants were more common in *N. tabacum* than *N. clevelandii*. They concluded that the large size of the GUS gene might be responsible for the instability. However, in our experiments, symptoms of viral infection were always indicative of production of the test protein in both *L. esculentum* and *N. benthamiana*. However, since our largest gene is *cry1Ac* (1.8 kb) and only small amounts of the protein are needed for insecticidal effects, it is possible that any instability of this construct was not detectable in our assay. If instability is a concern in the insect bioassay, leaves from different plants can be pooled to circumvent this problem.

We considered the effect of PVX infection on the wound response in the infected plants. In fact, Preston et al. (1999) had shown that tobacco mosaic virus (TMV) inoculation inhibits wound responses and causes *Manduca sexta* larvae to eat 1.7–2.7 times more TMV-infected leaf tissue. Using infected plant material for comparison in bioassays should allow unambiguous analysis of the insecticidal gene. This assay is meant as a first *in planta* test of a gene's insecticidal activity. Once a promising candidate gene is selected, transformation by more traditional means

should allow production of a clone that expresses the transgenic protein in a heritable manner.

PVX infects many solanaceous plants such as tobacco, tomato and potato. We are currently testing the use of this vector in these additional plant species. Many important agronomic pests such as tobacco budworm *Heliothis virescens*, corn earworm *Helicoverpa zea*, beet armyworm *Spodoptera exigua* and Colorado potato beetle *Leptinotarsa decemlineata* will feed on these species (Kota et al. 1999). Although not all insect pests can be assayed using this system, both lepidopteran and coleopteran species are represented in the aforementioned list.

This assay allows rapid testing of an insecticidal protein *in planta*. Unlike testing proteins produced in *E. coli*, these PVX vectored proteins should be correctly modified post-translationally. Although bacterial genes, such as *Bt* endotoxins, require modification before they can function with nuclear or viral-based plant expression systems, PVX could be used to rapidly assess the effectiveness of any necessary gene modifications. Furthermore, unlike feeding assays, which can use tediously purified protein, this system allows the testing of a single gene product so that effects from other isozymes can be factored out. Expression of a candidate gene through PVX infection should add to the arsenal of high-throughput approaches in the search for new insecticidal genes.

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