

## Epitope tagging: a monoclonal antibody specific for recombinant fusion proteins in plants

Susan D. Lawrence, Nicole G. Novak, and Jeffrey M. Slack  
United States Department of Agriculture, Beltsville, MD, USA

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*The easy identification of a recombinant protein in plant material becomes increasingly relevant as more transgenic plants are used for research and commercial applications. Tagging recombinant proteins with a small peptide (epitope) can perform such a task. However, available epitope antibodies will also cross-react with endogenous plant proteins at a level that may be unacceptable. Here we describe the new epitope antibody AcV5. Whether it is attached to the carboxyl terminal end of enhanced green fluorescent protein (EGFP) or the Bacillus thuringiensis endotoxin Cry3A, these proteins remain functional. In addition, using less than 250 pg AcV5-tagged EGFP produces a strong signal on Western blots with no cross-reactivity of proteins from a broad range of plants of agronomic importance.*

### INTRODUCTION

Epitope tagging has been successful for protein identification using a variety of immunological methods. The DNA sequence specific for the epitope tag is added to a gene of interest, and the resulting fusion protein is detected with an antibody that is specific for the tag. The advantage of using this foreign peptide is that it allows for the unequivocal identification of recombinant proteins from any closely related endogenous proteins. Additionally, cloning transgenes into a vector already containing an epitope tag cassette saves the time and expense of isolating proteins and producing antibodies for each recombinant protein. Epitope tagging was first described in 1987 (1) and may be extremely useful in applications such as the high-throughput screening of recombinant proteins.

To date, the commercially available epitopes c-myc (human c-myc oncogene) HA (influenza virus hemagglutinin), and FLAG<sup>®</sup> have been periodically used to tag transgenes in plants. However, all of these products have the disadvantage of cross-reacting with plant proteins. For example, *Arabidopsis* proteins react with c-myc antibody (2), anti-HA with tobacco (3), and anti-FLAG with potato (4). In addition, c-myc and HA have the perceived risk of being human disease markers.

AcV5 is one of a series of monoclo-

nal antibodies (MAbs) that were generated by Hohmann and Faulkner (5). It recognizes the GP64 envelope fusion protein (efp) of *Autographa californica* multiple nucleopolyhedrosis virus, AcMNPV (5). AcV5 MAb immunoprecipitates GP64 efp, and immunofluorescent microscopy localizes GP64 to the cell membrane of transfected Sf9 cells (5,6). Monsma and Blissard (7) identified the AcV5 epitope as a short 9-amino acid peptide. We have constructed two vectors that contain the AcV5 epitope for the production of tagged recombinant proteins in plants. The AcV5 MAb is highly specific in plants and produces a strong signal on Western blots without the presence of cross-reacting bands.

### MATERIALS AND METHODS

#### PVX Plasmids

A 69-nucleotide primer was designed to code for a 9-amino acid AcV5 epitope of the baculovirus gp64 protein (7) and seven restriction sites. This oligonucleotide and its complement were annealed by heating at 95°C for 5 min and cooled to room temperature. Reaction conditions were 150 ng/μL primers, 100 mM Tris, pH 7.5, and 50 mM MgCl<sub>2</sub>. The annealed fragment was ligated to the *EcoRV* site in the PVX vector pPC2S (8) to produce NPVX (Figure 1). The

AcV5 epitope and additional restriction sites were confirmed by sequencing.

The *win3* sequence from pwin3.6 (9) that encodes a wound-induced proteinase inhibitor was amplified and cloned into the *BstEII/BsiWI* sites of NPVX to create NPVX.WIN3. The enhanced green fluorescent protein (EGFP) coding sequence from pEGFP (BD Biosciences Clontech, Palo Alto, CA, USA) was amplified and cloned into the *BstEII/BsiWI* sites of NPVX to create NPVX.EGFP. The *cryIAC* gene from pGL896 (provided by J. Kemp, New Mexico State University, Las Cruces, NM, USA) was amplified and cloned into NPVX at the *BsiWI/BstEII* sites to create NPVX.CRY1Ac. The *cry3A* sequence from pGC689 (J. Kemp; GenBank<sup>®</sup> accession no. M84650) was amplified and cloned into NPVX at the *SphI/NotI* sites. The genes *cryIAC* and *cry3A* encode insecticidal endotoxins from *Bacillus thuringiensis*. Figure 1 outlines the position of the introduced genes in NPVX. The sequence of the chimeric gene EGFP-AcV5 was amplified from NPVX.EGFP, and the product was digested with *SacI* and *XbaI* and ligated to *XbaI/SacI*-digested pBI121 (BD Biosciences Clontech) to create pBI121.EGFP/AcV5.

#### Plant Material

*Nicotiana benthamiana* was transplanted to individual pots two weeks after sowing. The plants were grown another two weeks under a 16/8 light/dark cycle at room temperature before infection. *Zea mays* L. cv. 76007 corn, *Brassica napus* L. cv. Westar canola, *Glycine max* (L) Merrill soybean, *Gossypium hirsutum* cotton, *Solanum tuberosum* cv. superior potato, and *Arabidopsis thaliana* ecotype Columbia were grown for three weeks at 25°C on a 12/12 light/dark cycle. Transcript production, virus production, plant inoculation, and protein extraction were as previously described (Figure 2) (10).

#### Protein Quantification and Western Blot Analysis

Total leaf protein (Figure 2B) was extracted at a 1:1 ratio in a buffer containing 100 mM HEPES, pH 7.4, 5 mM EDTA, 10 mM dithiothreitol (DTT), 5 μg/mL each protease inhibitor [an-

tipain, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and pepstatin], and 10% glycerol. The samples were vortex mixed and sonicated for 2–3 short bursts of 10 s each. The tube was microcentrifuged at 1400× *g* at 4°C for 30 min. The supernatant was collected and recentrifuged for 30 min. Cotton, canola, soybean, maize, potato, and *Arabidopsis* samples were collected. Extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with AcV5 MAb at a ratio of 1:500. Horseradish peroxidase (HRP)-conjugated secondary antibodies were used at a ratio of 1:5000. Proteins were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL, USA). Maize cells were transiently transformed via microprojectile bombardment (11,12).

### Insect Bioassays

A colony of Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Say) is maintained at the Insect Biocontrol Laboratory (USDA-ARS, Beltsville, MD, USA). Newly hatched larvae were used in experiments that measured the inhibition of larval development and mortality. Ten neonate CPB larvae were placed in wet filter paper-lined Petri dishes with leaves that expressed NPVX.CRY3A or NPVX.EGFP. Addi-

tional leaves were added as the feeding progressed. Larval stage and survival of CPB were noted after 5 days.

## RESULTS

### Genes Containing the AcV5 Sequence-Produced Proteins

Figure 1 illustrates that four different genes were separately cloned into the modified potato virus X vector NPVX that contained the epitope tag AcV5. A duplicate copy of the coat protein promoter of the NPVX vector drives the expression of the engineered protein. *N. benthamiana* was infected with the four different viruses, NPVX.EGFP, NPVX.Cry1Ac, NPVX.Cry3A, or NPVX.WIN3, and the presence of the proteins in the leaf extracts was monitored using AcV5 MAb on Western blots. All four proteins migrated at slightly higher than expected molecular weight (Figure 2A) with AcV5 attached because the computer-aided calculation was 69.5, 67.5, 21.7, and 23.5 kDa for Cry3A, Cry1Ac, EGFP, and WIN3, respectively.

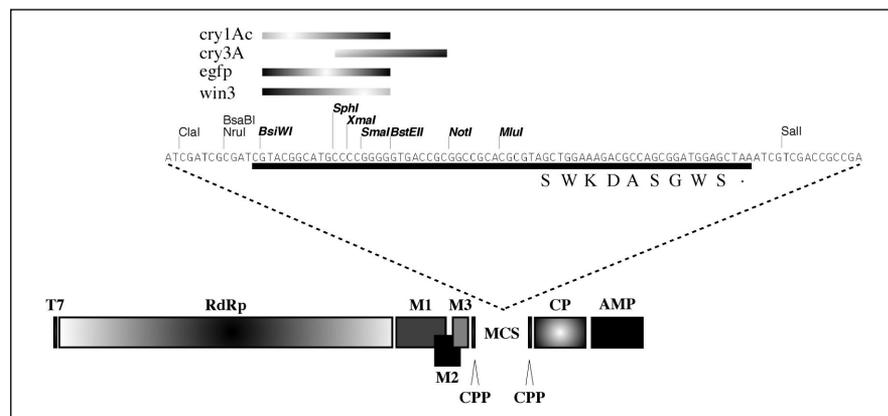
To analyze the specificity of the AcV5 MAb against cross-reacting protein from a variety of plant species, seedlings were grown from crop plants of agronomic importance (i.e., the most widely grown crops in agriculture). First, the quantity of a tobacco leaf extract with

EGFP/AcV5 was determined to be less than 250 pg by comparing it on Western blot to a dilution series of nickel column-purified spectrophotometer-quantified histidine (His)-tagged EGFP. The EGFP/AcV5 was added to each sample of total leaf extract, and then total protein samples from different species were equally loaded onto an SDS-polyacrylamide gel. The equal loading of the different plant samples was verified by the quantification of each lane of a Coomassie® Blue (Pierce Chemical)-stained protein blot using ImageQuant™ software (Molecular Dynamics, Sunnyvale CA, USA). A strong signal to the AcV5 MAb was found on immunoblots with no cross-reactivity observed to host proteins from cotton, canola, soybean, or maize (Figure 2B). *Arabidopsis* and potato leaf samples were also tested, and no additional cross-reactivity was noted (data not shown). Therefore, the AcV5 antibody specifically identifies the recombinant protein in a variety of plant species.

### AcV5-Tagged Fusion Proteins Are Functional in Dicots and Monocots

*N. benthamiana* was infected with NPVX.EGFP. Fourteen days following infection, the illumination of leaves with ultraviolet light revealed patches of green fluorescence caused by the activity of the EGFP tagged with AcV5 (Figure 3A).

To test whether AcV5 worked with a different vector and in a monocot species, the EGFP/AcV5 fusion protein gene replaced the *uidA* (*gus*) gene from the vector pBI121, bringing the EGFP chimeric gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Maize leaf pieces were biolistically and transiently transformed with the EGFP/AcV5-modified pBI121. The cells that were positively transformed with pBI121.EGFP/AcV5 contained bright green fluorescence in the nucleus and along the cytoplasmic strands of single cells (Figure 3B). Bombarding leaf pieces with unmodified pBI121 containing the  $\beta$ -glucuronidase (*gus*) gene produced no such fluorescence; instead, upon staining the tissue with X-glucuronide, the expected blue-stained cells were evident (data not shown). This confirms that the transient transformation of tissue led to gene expression and that the



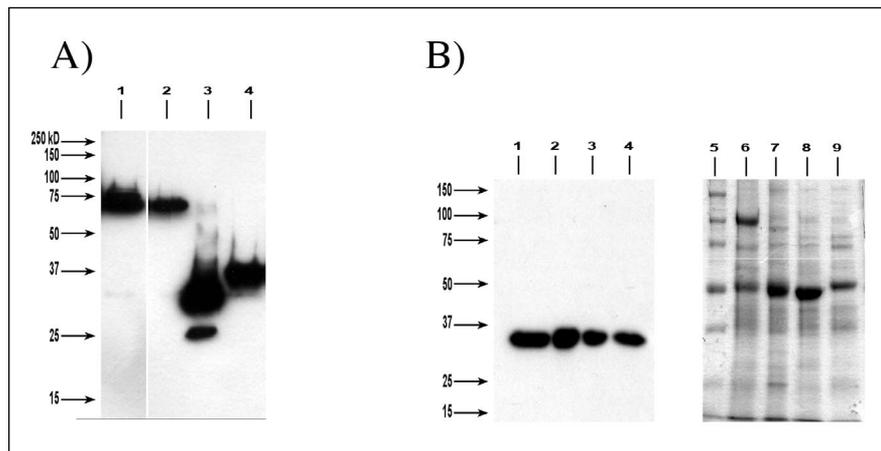
**Figure 1. A map of the major coding regions of NPVX and sequence modifications.** The multiple cloning site (MCS) has been expanded to illustrate the addition of the AcV5 epitope and seven restriction sites. The added DNA sequence is underscored, and restriction sites are bold and italicized. The AcV5 protein sequence is shown beneath the DNA sequence. There are bars above this sequence that depict how the construct was opened for the insertion of the genes. *Cry1Ac*, *egfp*, and *win3* were all inserted into the *BsiWI* and *BstEII* sites. *Cry3A* was inserted into the *SphI* and *NotI* sites. T7, T7 promoter; RdRp, RNA-dependent RNA polymerase; M1–M3, movement proteins; CPP, coat protein promoter; CP, coat protein; AMP, ampicillin resistance gene.

EGFP/AcV5 fusion protein is functional in a monocot.

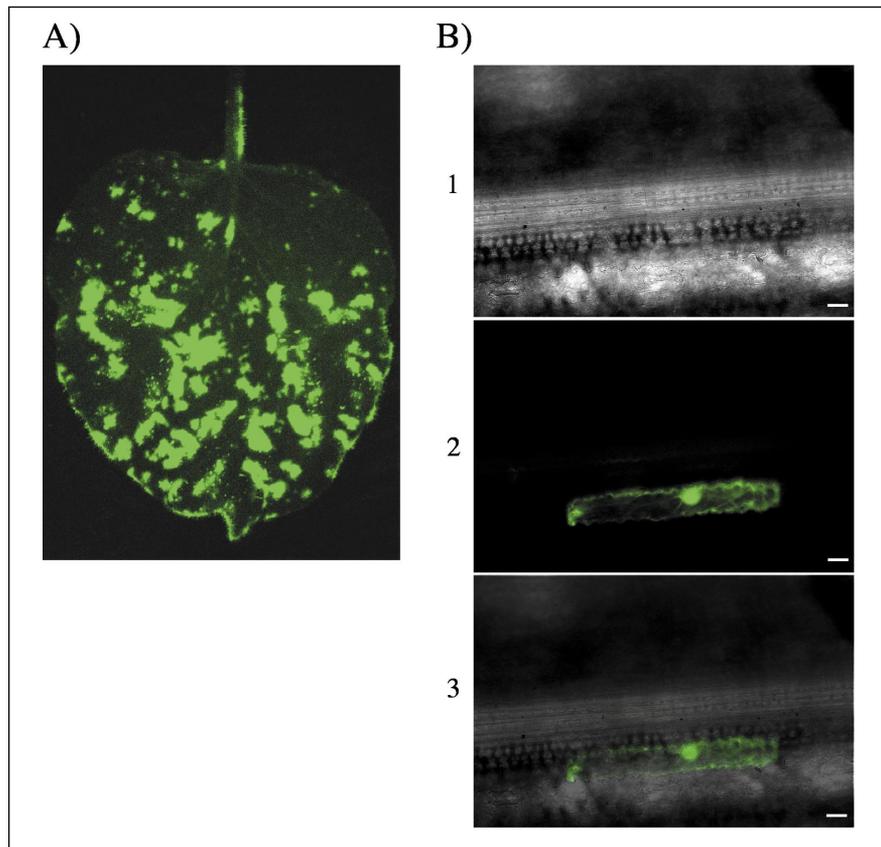
The EGFP protein is functional with AcV5 attached to the carboxyl terminal end, but we wanted to determine if another gene would function under these circumstances. The *B. thuringiensis* endotoxin gene was ligated into NPVX, and the NPVX.Cry3A virus was used to infect tomato plants. The presence of the AcV5 epitope fused to the Cry3A protein should result in a functional insect toxin. After 14 days, the material was used to feed first instar larvae of CPB. We measured the developmental and mortality differences of CPB that were fed tomato leaves either infected with NPVX.Cry3A or NPVX.EGFP. Fifteen Petri dishes were prepared with 10 CPB each. Each dish received leaves from a single inoculated plant. Five plants were infected with the control NPVX.EGFP, and 10 plants were infected with NPVX.Cry3A. After five days, 93.8% of the CPB that were fed the control were live second instar larvae (data not shown). For the NPVX.Cry3A-inoculated leaves, individual plants exhibited different outcomes, but two groups of plants could be identified. The consumption of one group of five plants resulted in all CPB remaining as first instars, with the majority of them dead (8–10 dead per Petri dish). The second group of five plants infected with NPVX.Cry3A resulted in 0–4 dead per dish and 3–9 live second instar CPB. The intermediate group was different than the five control NPVX.EGFP-infected plants because the control leaves only resulted in a single CPB death. Different levels of the Cry3A protein are probably responsible for this result because a subsequent experiment suggested that high levels of Cry3A protein correlated with low amounts of leaf material eaten. Clearly, the presence of the Cry3A/AcV5 gene in the plant material is required for the inhibition of larval development and an increase in mortality. The presence of the AcV5 epitope fused to the Cry3A protein resulted in a functional insect toxin.

**DISCUSSION**

The addition of an epitope tag allows for the immunological analysis of recom-



**Figure 2. Western blots of recombinant proteins and Coomassie-stained gel of total plant extracts from different plant species.** For Western blots, total plant extracts from virally infected plants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Transgenic proteins were detected using monoclonal antibody (MAb) AcV5 at a ratio of 1:500. (A) *Nicotiana benthamiana* was infected with (i) PVX.Cry3A/AcV5, (ii) PVX.Cry1Ac/AcV5, (iii) PVX.eGFP/AcV5, and (iv) PVX.WIN3/AcV5. (B) Lanes 1–4, Western blot with less than 250 pg enhanced green fluorescent protein (EGFP)/AcV5 loaded per lane; lanes 5–9, Coomassie-stained gel of equally loaded total plant extracts; lane 5, molecular weight standards (Precision Plus Protein™ standards; Bio-Rad Laboratories, Hercules, CA, USA); lanes 1 and 6, cotton; lanes 2 and 7, canola; lanes 3 and 8, soybean; and lanes 4 and 9, corn.



**Figure 3. AcV5-tagged fusion proteins are functional.** (A) *Nicotiana benthamiana* leaf transformed with PVX.EGFP/AcV5 expressing the enhanced green fluorescent protein (EGFP). (B) EGFP/AcV5 is produced in maize epidermal cells 48 h after particle bombardment. The adaxial surface of a mature maize leaf was viewed with (lane 1) visible light and (lane 2) blue light. Note the presence of EGFP/AcV5 in the cytoplasm and nucleus of the epidermal cell. (Lane 3) Overlay of lanes 1 and 2 with 50% opacity. Size bar = 10  $\mu$ m.

binant protein without producing specific antibody to each protein of interest. It has been noted in the literature that the detection of some proteins on Western blots with the c-myc epitope may result in either quantitatively less protein than expected or none at all (13,14). However Fan et al. (14) noted that the proteins not present on Western blots are observable with immunofluorescence or immunoprecipitation, which shows that other methods could be utilized if a protein is not initially discernable. Recently, a T7 epitope was introduced at either the amino or carboxyl terminal end of avidin and phosphinothricin-N-acetyl transferase (PAT). Although the T7 epitope was stable on both ends of PAT, it tended to be cleaved from the carboxyl terminal end of avidin (15). It appears that the tagged protein plays a more pivotal role in determining the stability of the protein. Although we have not tested AcV5 at the amino terminal end, we expect that in general the epitope would be stable at either end of a protein. Clearly, the placement of the tag should be a consideration if initially no protein is found.

Low levels of AcV5-tagged protein (<250 pg) were easily identified with no cross-reactivity on a Western blot loaded with protein samples from several different species. This means that cross-reacting endogenous proteins are minimally present, which suggests that the AcV5 epitope-tagging system may be a useful tool to identify the presence of small amounts of recombinant protein in plant extracts. If multiple copies of AcV5 are used as an epitope tag, then it is possible that this sensitivity may be further enhanced.

Epitope-tagged EGFP and Cry3A are functional proteins. Different plants seemed to produce different levels of Cry3A toxicity. Because the same DNA construct was used for each of the plants, it seems most likely that the differences in the toxicity observed resulted from different levels in the expression of the cry3A/AcV5 gene. The levels of expression could vary if the gene were unstable, as observed for *gus* gene expression in PVX by Chapman et al. (16). Exactly half of the plants infected with NPVX.Cry3A that were fed to CPB resulted in dead or developmentally delayed larvae. In plants, c-myc (2,17,18), HA (19,20),

and FLAG (4)-tagged proteins have been functional. However, because the non-AcV5 epitope antibodies strongly cross-react with plant proteins, their use in the unequivocal identification of recombinant proteins is compromised.

Epitope-tagged proteins in transformed plants can be a useful way to differentiate between recombinant and endogenous proteins when only small immunological differences exist. The AcV5 MAb has the added advantage of high specificity, which allows for the unambiguous identification of the recombinant protein. This specificity may provide a broadly applicable tag for the identification of recombinant proteins in plant material.

#### ACKNOWLEDGMENTS

*The mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.*

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*Address correspondence to Susan D. Lawrence, United States Department of Agriculture, Agricultural Research Service, Insect Biocontrol Laboratory, BARC-West, Bldg. 011A, Rm 214, Beltsville, MD 20705, USA. e-mail: lawrencs@ba.ars.usda.gov*