

Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle

Susan D. Lawrence* & Nicole G. Novak

United States Department of Agriculture, Agricultural Research Service, Insect Biocontrol Laboratory, BARC-West, Bldg. 011A, Room 214, Beltsville, MD 20705, USA

*Author for correspondence (Fax: +1-301-504-5104; E-mail: LawrenceS@ba.ars.usda.gov)

Received 2 December 2005; Revisions requested 12 December 2005; Revisions received 18 January 2006; Accepted 20 January 2006

Key words: chitinase, herbivore, insect resistance, *Lycopersicon esculentum*, *Populus*

Abstract

The previously described poplar chitinase, WIN6, is induced during infestation by gypsy moth (*Lymantria dispar* L.) larvae, thus suggesting a role in defense against insect pests. To test this hypothesis, we produced tomato seedlings infected with a recombinant potato virus X (PVX), which produces WIN6, and tested its insecticidal properties on Colorado potato beetle [CPB; *Leptinotarsa decemlineata* (Say)], which is a serious pest of tomatoes and other crops. The advantage of PVX is that plant material is ready for insect bioassay within 3–4 weeks of constructing the recombinant virus. Considering that production of transgenic tomato seedlings using *Agrobacterium* takes at least 6 months, this hastens the rate at which genes can be examined. Upon insect bioassay, only 47% CPB neonates feeding on leaves containing >0.3% w/w WIN6 developed to 2nd instar while 93% of controls reached 2nd instar. To our knowledge this is the first plant chitinase that retards development of an insect pest.

Introduction

Colorado potato beetle [CPB; *Leptinotarsa decemlineata* (Say)] is a serious pest of many solanaceous crops. It has the ability to evolve resistance to virtually all insecticides used to control it (Bishop & Grafius 1996). Transgenic methods are available to tomato breeders but multiple forms of resistance will be required for such an adaptable insect pest. Rapid identification of genes conferring resistance to CPB would be helpful to these endeavors. Placing the gene of interest in the potato virus X (PVX) genome allows production of infectious recombinant transcripts. Upon spread of PVX in the growing seedling, leaf material containing the recombinant protein can be bioassayed to detect effects on CPB.

PVX is a plant potyvirus containing five genes (for reviews, see Chapman *et al.* 1992,

Baulcombe *et al.* 1995). The viral RNA-dependent RNA polymerase is used for production of infectious transcripts. The triple gene block consists of three viral proteins involved with movement of the virus within the plant. The coat protein encases the infectious RNA transcript. A vector containing the entire viral genome has been developed (Baulcombe *et al.* 1995) duplicating the coat protein promoter in order to drive expression of the recombinant gene. The recombinant virus moves through the phloem into newly forming leaves of the seedling and the coat protein promoter allows production of the recombinant protein (Baulcombe *et al.* 1995). PVX infects solanaceous crops, and tomato or tobacco is commonly selected for studies involving PVX. We have extended the use of PVX for testing the efficacy of insecticidal proteins (Lawrence & Novak 2001). After 4 days, tobacco budworm (*Heliothis virescens* F.) fed on tobacco leaves

expressing a trypsin inhibitor from poplar grew to 77% of the weight of insects feeding on leaves expressing PVX alone (Lawrence & Novak 2001).

In an attempt to discover genes that may be detrimental to CPB, we have identified a number of plant genes induced by wounding or insect herbivory (data in preparation). These genes may be defensive in nature and consequently good candidates for insect resistance genes. Wounding of poplar leads to the expression of genes presumed to be defensive against insect infestation (Parsons *et al.* 1989). The genes for a Kunitz type trypsin inhibitor and polyphenol oxidase are induced in poplar by infestation with forest tent caterpillar (Constabel *et al.* 2000, Haruta *et al.* 2001), and a role for polyphenol oxidase in inhibiting insect growth has been recently described (Wang & Constabel 2004). The systemically wound-induced (*win*) genes were initially isolated from RNA of upper leaves of poplar plants in which the lower leaves had been repeatedly wounded (Parsons *et al.* 1989). The gene we have chosen from this work is *win6*, in which expression of the gene in tobacco has demonstrated that it encodes a functional acidic four-domain chitinase (Clarke *et al.* 1998). Although *win6* is induced by wounding, both locally and in the systemic unwounded immature leaves of poplar (Parsons *et al.* 1989, Clarke *et al.* 1998), the effect of insect herbivory has not been tested.

Chitinases are often induced in the pathogen resistance pathway associated with defense against plant disease. Since chitinase degrades chitin found in the wall of fungal pathogens, plants expressing a recombinant chitinase have been produced to inhibit fungal disease organisms (for a review see Schnickler & Chet 1997). The peritrophic membrane surrounding the insect midgut is also made of chitin. A bean (*Phaseolus vulgaris*) chitinase has been shown to be induced by wounding, fungal elicitor and pathogen infection (Hedrick *et al.* 1988), so a role in insect defense could be assumed. Studies using a complimentary bean chitinase as a potential insect resistance gene (Gatehouse *et al.* 1996, 1997) however, showed little promise. A chitinase present in larval molting fluid from the late stages of the fifth instar of the insect pest tobacco hornworm, *Manduca sexta*, when expressed in tobacco caused significant reduction in growth of

tobacco budworm (Ding *et al.* 1998). We examined the effect of the poplar chitinase, *Win6*, on CPB neonate development. In order to rapidly assay *win6*, we used PVX to transiently express *win6* in tomato plants.

Materials and methods

Plant materials

An F₁ hybrid poplar genotype (*Populus trichocarpa* Torr. and Gray × *P. deltoides* Bartr. ex Marsh. hybrid H11-11) was used for infestation and grown in 3.8 l pots at 23 °C under 16 h light/8 h dark cycles. Plants were vegetatively propagated and used in experiments after achieving a height of ~80 cm as described previously (Lawrence *et al.* 1997). *Nicotiana benthamiana* were grown until the first true leaf measured approximately 3 cm. *Lycopersicon esculentum* cv. Rutgers (tomato) were grown until the first true leaf appeared. *N. benthamiana* and *L. esculentum* were grown under 16 h days/8 h night cycles at 24 °C.

Gypsy moth rearing and feeding experiments

Gypsy moth (*Lymantria dispar* L.) eggs were obtained from USDA-Aphis (Pest Survey Detection and Exclusion Laboratory, Otis, MA) and maintained at 25 °C in the dark on artificial diet. When larvae reached 2nd instar they were placed in empty diet cups to starve for 24 h. Ten larvae were placed in a fabric bag and placed on the seventh leaf down from the index leaf, which measures approximately 3.5 cm. Larvae fed on poplar leaves for 24 h; the infested leaf was harvested and flash frozen in liquid N₂. Two plants were pooled and two biological reps were performed.

RNA extraction and Northern blot analysis

Tissue was ground with a mortar and pestle to a fine powder in liquid nitrogen. RNA was isolated using a procedure illustrated in Chang *et al.* (1993). Northern blots were performed as described by Lawrence *et al.* (1997).

Cloning of win6 and production of tomato leaves expressing Win6 and enhanced green fluorescent protein (eGFP)

The *win6* (Parsons *et al.* 1989) cDNA was produced using 1 μ g of total poplar RNA and the Titanium One-Step RT-PCR kit (Clontech). To amplify *win6*, primers for PCR were designed to include *NotI* 5'-ACAGCGGCCGCACATGGT-GTCCTTCAATCC-3' and *SphI* 5'-GAACTCG-AGCATGCTAACAAGAAATGA-3' restriction sites. The *win6 NotI/SphI*-digested PCR product was ligated to NPVX (containing the AcV5

epitope tag, see Monsma & Blissard 1995, Lawrence *et al.* 2003) at the *NotI* and *SphI* sites. NPVX.*win6* was partially digested with *SpeI*. Cloning of NPVX.*egfp* and preparation of transcript, plant inoculation and viral purification for NPVX.*win6* and NPVX.*egfp* were as described in Lawrence & Novak (2001) except that 2 μ g of linearized DNA was used to make infectious RNA transcript using the T7 MEGAscriptTM High Yield Transcription kit (Ambion). Symptomatic *Nicotiana benthamiana* leaves were harvested for viral isolation 10–14 days post-infection. Five to twenty microlitre of a viral stock was rubbed onto tomato leaves. Figure 1 is a flow chart showing the steps required for making infected tomato leaves expressing recombinant protein.

Colorado potato beetle rearing and bioassay

CPB were reared on potato plants (*Solanum tuberosum*, var. Kennebec) in the Insect Biocontrol Laboratory, Beltsville, MD. Egg masses were harvested and maintained at 28 °C until neonates hatched. Tomato leaf material was infected with recombinant PVX virions. After 14 days, ten 12 mm discs were cut from a uniformly symptomatic leaf containing EGFP or WIN6 and placed in wet filter paper-lined Petri dishes with 10 neonate CPB larvae. The remaining portion of the leaf was assayed for protein concentration as described below. Larvae were allowed to feed for 3 days and then scored as first or 2nd instars. For each of 3 experiments, a minimum of 21 *win6* and 5 *egfp*-expressing plants were tested. A Mann-Whitney rank sum test was run from the program SigmaPlot v.9.0, since the data was non-parametric, to determine differences between CPB bioassays comparing *win6*- and *egfp*-expressing leaf discs.

Protein extraction and western blotting

Proteins were extracted and separated by SDS-PAGE as described in Lawrence & Novak (2001). Total protein was determined with the coomassie plus protein assay reagent (Pierce). After transfer to nitrocellulose, WIN6 was detected using a 1:500 dilution of MAb AcV5 (Hohmann & Faulkner 1983). Blots were developed using the ECF Western Blotting Kit (Amersham Pharmacia Biotech) and fluorescence

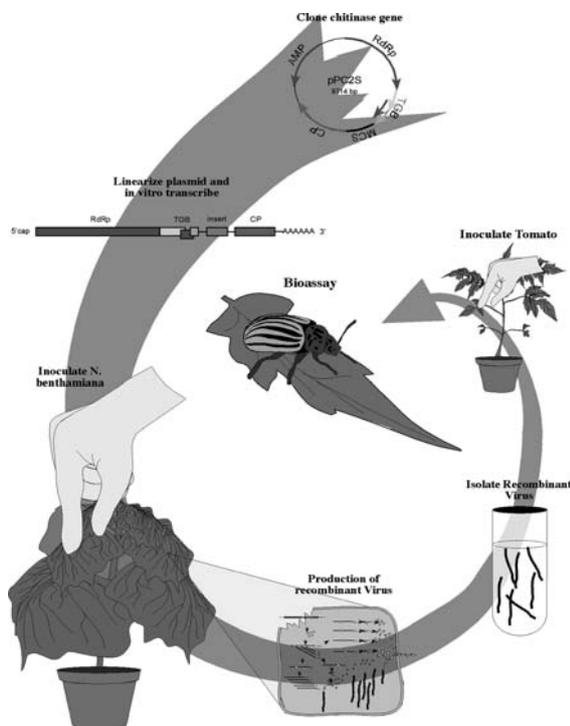


Fig. 1. Production of recombinant leaf material for insect bioassay. Step (1) The gene of interest is ligated at the multiple cloning site to the PVX vector. RdRp=RNA dependent RNA polymerase, TGB=triple gene block, MCS= multiple cloning site, CP=coat protein and AMP=ampicillin resistance gene. Step (2) The plasmid is linearized and *in vitro* transcribed to produce infectious transcript. Step (3) *N. benthamiana* is inoculated with infectious RNA transcript by rubbing onto the leaves. Viral RNA is transcribed, protein is produced and virus is packaged. Newly made virus moves through the phloem into developing leaves. Step (4) Recombinant virus is isolated. Step (5) Tomato plants are inoculated by rubbing recombinant virus onto the leaves. Virus infects the plant and newly formed leaves express the recombinant protein. Step (6) PVX infected leaves are used in assays measuring effects on Colorado potato beetle development.

was detected using a Typhoon 8600 phosphorimager (Amersham Pharmacia Biotech). WIN6 bands were quantified by volume integration using ImageQuant software. Protein concentrations were calculated using sucrose cushion-purified baculovirus-budded virions (O'Reilly *et al.* 1992), which have native GP64 protein (the original source of the AcV5 epitope). GP64 was separated by SDS-PAGE, coomassie stained to estimate protein amount, and then used as a standard in ECF western blots performed as described above.

Results

win6 transcript was measured to determine whether it was induced by insect infestation. Since *win6* has been cloned as a wound induced gene from poplar, and wounding is used to mimic infestation, poplar seedlings were subjected to gypsy moth feeding for 24 h. Infestation resulted in abundant expression of the *win6* transcript (Figure 2). Therefore, we reasoned that *win6* may be detrimental to insects.

Figure 3 shows the results of three CPB bioassays using tomato leaves expressing *win6*. In two experiments, only 41% ± 8% and 54% ± 5% of the CPB feeding on WIN6-expressing tomato leaves reached 2nd instar, compared to 96% ± 3% and 90% ± 4% feeding on eGFP-expressing tomato leaves, suggesting that WIN6 has a detrimental effect on CPB development. Using a Mann-Whitney rank sum test, bioassays 1 and 2 were both significantly different from the eGFP controls ($p < 0.001$ and $p < 0.005$, respectively). A third bioassay, however, had no difference between CPB fed on tomato leaves expressing WIN6 compared with larvae eating leaves infected with PVX-expressing EGFP. We found that leaf discs that produced a high inhibition of CPB development did not necessarily have a high level of WIN6 protein (as determined by western blot). Figure 4 shows the amount of Win6 protein produced for the three experiments. WIN6 protein present in the leaves used in the third experiment averaged 0.1% w/w ± 0.1% of WIN6 to total protein. More than twice as much WIN6 protein was present (0.3% w/w ± 0.1%) in the first experiment and even higher WIN6 levels (0.6% w/w ± 0.2%) were

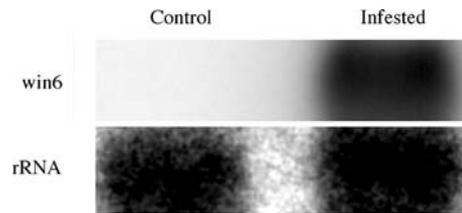


Fig. 2. Abundant *win6* transcript is produced upon infestation of poplar leaves with 2nd instar gypsy moth larvae for 24 h. 7.5 µg RNA was added per lane. C=control and I=infested poplar leaves.

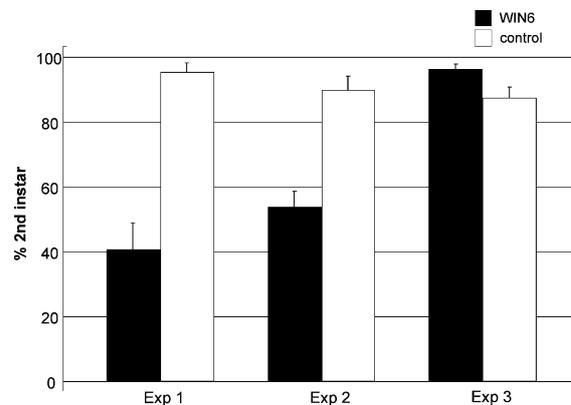


Fig. 3. Development of Colorado potato beetle larvae are retarded when insects are fed tomato leaves expressing the recombinant poplar chitinase WIN6. The Y-axis is the average percent of insects that had reached 2nd instar after 3 days. The X-axis represents each bioassay in which CPB larvae were fed either control leaf discs or WIN6-expressing leaf discs. Control leaves were infected with PVX-expressing EGFP, the enhanced green fluorescent protein. In experiment 1, 210 CPB were exposed to WIN6-expressing leaf discs and 50 CPB were exposed to control leaf discs. In experiment 2, 240 CPB were exposed to WIN6-expressing leaf discs and 80 CPB were exposed to control leaf discs. In experiment 3, 220 CPB were exposed to WIN6-expressing leaf discs and 80 CPB were exposed to control leaf discs. Error bars represent standard error.

obtained in the second experiment. Thus, it appears that protein expression levels varied from experiment to experiment. This also suggests that a threshold level of WIN6 protein (perhaps > 0.3% w/w) is required for an effect on insect development to be observed.

Discussion

We have shown that *win6* is induced by gypsy moth infestation. Proving that *win6* plays a role

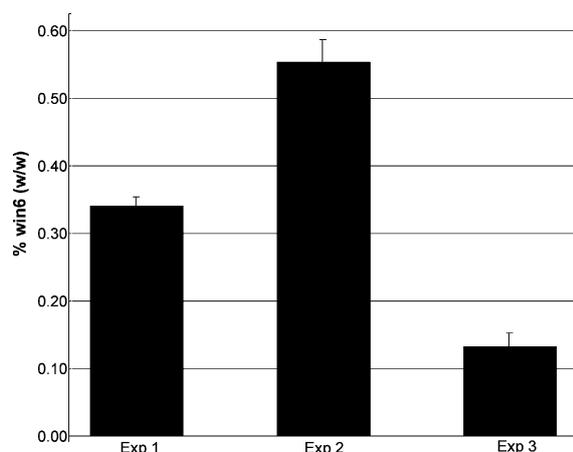


Fig. 4. Amount of Win6 protein produced in tomato plants for each experiment. Four nanogram of total protein was loaded for each plant onto SDS-PAGE gels. The Y-axis is the average percent of Win6 protein of the total plant proteins as measured by western blot. The X-axis is the three individual experiment in which leaves were expressing WIN6. Experiment 1 represents the average of 21 plants. Experiment 2 represents the average of 24 plants and experiment 3 represents the average of 22 plants. Error bars denote standard error.

on the inhibition of gypsy moth infestation would require transgenic experiments in poplar. It would be interesting to inhibit chitinase activity by antisense or overexpress chitinase and see if the recombinant plants become more or less susceptible to gypsy moth feeding. Expression of *win* genes could have greater deleterious effects on non-host insects since they have not evolved with these genes.

Testing putative insect resistance *win* genes using PVX to produce recombinant material quickly distinguishes candidates for further study. Although it lacks the refinement of an insect bioassay performed with purified protein, it more readily resembles the result expected from a transgenic plant, since the protein is produced within the plant. We found that 87–95% of the CPB reached 2nd instar when fed eGFP-expressing PVX infected plant material. Although PVX infection itself would result in expression of pathogen resistance genes, tomatoes expressing PVXeGFP serve as appropriate controls for those effects. Experiments were performed for 3 days because control leaf discs had been eaten by this time and leaf material was limiting. Neonates were used for bioassay because they readily eat tomato leaves. Since the CPB larvae are reared on potato plants, habituated older larvae

do not immediately feed on tomato leaves. PVX infection of potato should allow testing of later instar. The PVX vector allows testing of a number of agronomic pests such as tobacco budworm *Heliothis virescens*, corn earworm *Helicoverpa zea*, beet armyworm *Spodoptera exigua* and CPB. Since these include both coleopteran and lepidopteran pests this assay covers a broad taxonomic spectrum.

PVX expression of the green fluorescent protein can be observed microscopically in a tomato leaf and this expression appears to be patchy (Lawrence & Novak 2001). After 14 days post-infection of the first true leaf the fourth newly formed leaf contains the most uniform eGFP expression and this leaf was used for bioassay. However, even in this leaf, eGFP expression was not always uniform throughout. The patchiness does not seem to be cell type specific. However it is not too surprising that protein expression levels and bioassay results within the same leaf do not correspond. The amount of Win6 protein produced per experiment also varied. This may be due to differences in susceptibility of plants to infection from experiment to experiment.

We have found a developmental delay of CPB larvae when fed a diet containing more than 0.3% w/w WIN6. It is difficult to say why the protein levels of Win6 were so much lower in the third experiment compared to the first two. Perhaps the plants produced a thicker epidermis so that viral penetration was inhibited. The observed delay in CPB development suggests that WIN6 has a significant effect on larval development, but it is less dramatic than CPB feeding on plants infected with *Bacillus thuringiensis* (Bt) *cry* toxin expressed via PVX (Lawrence & Novak, unpublished). Using the *cry* toxin CPB neonates are killed within 3 days. Chitinases have been shown to damage the peritrophic membrane of the insect *in vivo* and *in vitro* thus causing the midgut to be compromised (Kramer & Muthukrishnan 1997). This may be a mechanism by which WIN6 influenced insect development in our study. To date, only transgenic insect molting chitinase from *Manduca sexta* or the chitinase from the insect virus AcMNPV have been shown to either inhibit insect growth or kill outright (Ding *et al.* 1998, Rao *et al.* 2004). Both of these chitinases are directed at insect larvae and may be mechanistically different than plant

chitinases. In addition, these chitinases belong to the family 18 chitinases, which contain mainly non-plant chitinases. Chitinases belong to either family 18 or 19, due to their amino acid sequence similarities (Henrissat 1999). WIN6, on the other hand, is in family 19 showing similarity to other plant class Ib chitinases (Clarke *et al.* 1998, Henrissat 1999), so both families contain a chitinase that effects insect pests. Currently, we have the chitinase from AcNMPV cloned into PVX and we are testing whether it affects CPB larvae.

Initially we hypothesized that WIN6 was a wound-induced chitinase responsible for inhibiting opportunistic fungal infections at the wound site (Clarke *et al.* 1998). Although we have not tested Win6 on fungal pathogens, clearly this assumption should be modified in consideration of these results. Since WIN6 does not kill larvae within the 3 days that we have used in our assay, it may contribute additively to insect resistance in concert with other host defenses. Perhaps WIN6 could be used in conjunction with lower levels of Bt (Kramer & Muthukrishnan 1997) or with parasitoids or predators of CPB in an IPM program resulting in a reduction in the use of insecticides. Win6 may be advantageous if a plant derived gene is more acceptable in a transgenic plant than a gene from outside the plant kingdom. It will be interesting to determine whether this chitinase inhibits other insect pests so that it could be used as a broad spectrum resistance gene.

Acknowledgements

We would like to acknowledge Mike Blackburn, John Davis and Kevin Thorpe for advice on statistical analysis and insightful discussion. We would also like to thank Kevin Thorpe and Fernando Vega for critical reading of the manuscript. 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.

References

- Baulcombe DC, Chapman S, Santa Cruz S (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* **7**: 1045–1053.
- Bishop BA, Grafius EJ (1996) Insecticide resistance in the Colorado potato beetle. In: Jolivet PHA, Cox ML, eds. *Chrysomelidae Biology*, Vol. 1. Amsterdam: Academic Publishing, pp. 355–377.
- Chang S, Puryear J, Caimey J (1993) A simple method for isolating RNA from pine trees. *Plant Mol. Biol. Reporter* **11**: 113–116.
- Chapman S, Kavanagh T, Baulcombe D (1992) Potato virus X as a vector for gene expression in plants. *Plant J.* **2**: 549–557.
- Clarke HRG, Lawrence SD, Flaskerud J, Korhnak TE, Gordon MP, Davis JM (1998) Chitinase accumulates systemically in wounded poplar trees. *Physiol. Plantarum* **103**: 154–160.
- Constabel CP, Yip L, Patton JJ, Christopher ME (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.* **124**: 285–295.
- Ding X, Gopalkrishnan B, Johnson LB, White FF, Wang X, Morgan TD, Kramer KJ, Mutukrishnan S (1998) Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Res.* **7**: 77–84.
- Gatehouse AMR, Down RE, Pwell KS, Sauvion N, Rahbe Y, Newell CA, Merryweather A, Hamilton WDO, Gatehouse JA (1996) Transgenic potato plants with enhanced resistance to the peach-potato aphid *Myzus persicae*. *Entomol. Exp. Appl.* **79**: 295–307.
- Gatehouse AM, Davison GM, Newell CA, Merryweather A, Hamilton WDO, Burgess EPJ, Gilbert RJC, Gatehouse JA (1997) Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: growth room trials. *Mol. Breeding* **3**: 49–63.
- Haruta M, Major IT, Christopher ME, Patton JJ, Constabel CP (2001) A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. *Plant Mol. Biol.* **46**: 347–59.
- Hedrick S, Bell JN, Boller T, Lamb CJ (1988) Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *Plant Physiol.* **86**: 182–186.
- Henrissat B (1999) Classification of chitinase modules. *EXS* **87**: 137–156.
- Hohmann AW, Faulkner P (1983) Monoclonal antibodies to baculovirus structural proteins: determination of specificities by western blot analysis. *Virology* **125**: 432–444.
- Kramer KJ, Muthukrishnan S (1997) Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem. Mol. Biol.* **27**: 887–900.
- Lawrence SD, Greenwood JS, Korhnak TE, Davis JM (1997) A vegetative storage protein homolog is expressed in the growing shoot apex of hybrid poplar. *Planta* **203**: 237–244.
- Lawrence SD, Novak NG (2001) A rapid method for the production and characterization of recombinant insecticidal proteins in plants. *Mol. Breeding* **8**: 139–146.
- Lawrence SD, Novak NG, Slack JM (2003) Epitope tagging: a monoclonal antibody specific for recombinant fusion proteins in plants. *Biotechniques* **35**: 488–492.
- Monsma SA, Blissard GW (1995) Identification of a membrane fusion domain and an oligomerization domain in the baculovirus GP64 envelope fusion protein. *J. Virol.* **69**: 2583–2595.
- O'Reilly D, Miller LK, Luckow VA (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. New York: W.H. Freeman and Co.

- Parsons TJ, Bradshaw HD, Gordon MP (1989) Systemic accumulation of specific mRNAs in response to wounding in poplar trees. *Proc. Natl. Acad. Sci. USA* **86**: 7895–7899.
- Rao R, Findra L, Giordana B, de Eguileor M, Congiu T, Burlini N, Arciello S, Corrado G, Pennacchio F (2004) AcMNPV ChiA protein disrupts the peritrophic membrane and alters midgut physiology of *Bombyx mori* larvae. *Insect Biochem. Mol. Biol.* **34**: 1205–1213.
- Schnickler H, Chet I (1997) Heterologous chitinase gene expression to improve plant defense against phytopathogenic fungi. *J. Indus. Micro. Tech.* **19**: 196–201.
- Wang J, Constabel CP (2004) Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*). *Planta* **220**: 87–96.