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Effects of kiwifruit (*Actinidia deliciosa*) cysteine protease on growth and survival of *Spodoptera litura* larvae (Lepidoptera: Noctuidae) fed with control or transgenic avidin-expressing tobacco

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Effects of kiwifruit (*Actinidia deliciosa*) cysteine protease on growth and survival of *Spodoptera litura* larvae (Lepidoptera: Noctuidae) fed with control or transgenic avidin-expressing tobacco

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Abstract Kiwifruit (*Actinidia deliciosa*) produce a fruit cysteine protease, actinidain (synonym actinidin), of unknown function. Other plant cysteine proteases, such as Mir 1 from some varieties of maize and papain from papaya, confer protection against insect attack. To determine if actinidain could also reduce insect growth or survival, actinidain powder was painted on to tobacco leaves (at 0.1% of total leaf protein) and fed to neonate cotton leafworm larvae, *Spodoptera litura*. Larvae fed with actinidain were significantly smaller and took longer to pupate than those feeding on control, unpainted tobacco. To determine whether this growth inhibition could combine usefully with the effects of another insect control protein, *S. litura* larvae were also fed with transgenic tobacco leaves expressing (on average) 7.7 μM avidin, a biotin-binding protein, with or without actinidain painted on as described above. Larvae fed with avidin-expressing leaves painted with actinidain died slightly but significantly sooner than those on transgenic avidin-leaves without actinidain. We conclude that actinidain alone has

a small but significant negative impact on *S. litura* larval development and, when combined with avidin, can result in a slightly faster kill than transgenic avidin-expressing leaves without actinidain.

Keywords actinidain; avidin; biotin-binding protein; kiwifruit

INTRODUCTION

The first evidence of a plant cysteine protease having an insecticidal function was that of Pechan et al. (2000), who showed that when insect-resistant lines of maize were attacked by foliage-feeding larvae (Lepidoptera: Noctuidae), a 33-kDa cysteine protease, Mir 1, accumulated in the wounded whorls. They postulated that this protease was responsible for a 50% reduction in larval mass previously noted among *Spodoptera frugiperda* larvae fed with foliage or callus derived from resistant maize cultivars (Williams et al. 1985, 1987, 1990; Chang et al. 2000). *S. frugiperda* larvae were also significantly smaller than controls (70–80% reduction) when fed for 5–7 days on transgenic maize callus expressing the *mir1* gene (Pechan et al. 2000). When left to complete their development, these insects pupated and then emerged as adults about five days later than the control insects (Pechan et al. 2000). Scanning electron microscopy (SEM) also showed that affected larvae had severely damaged peritrophic matrices (Pechan et al. 2002), which could be the mode of action of the maize cysteine protease on *S. frugiperda*. Recently, Konno et al. (2004) showed that papaya leaves were highly toxic to Eri silkworms, *Samia ricini*, which had very reduced growth and high mortality after four days on this food. However, this toxicity was eliminated if latex exuded by the leaves was washed off or the leaves were painted with E-64 (a cysteine protease inhibitor). The cysteine protease papain was identified as the insecticidal component of the latex, with some activity against the noctuid pests *Mamestra brassicae* and *Spodoptera litura* as well (Konno et

al. 2004). Other plant cysteine proteases (ficin from wild fig and bromelain from pineapple) were also shown to inhibit growth and cause mortality of *S. ricini* larvae. Konno et al. (2004) suggested that plant latex, and its cysteine proteases in particular, may provide plants with a general defence against insect attack. The aim of the present study was to examine the possibility that kiwifruit may also produce a cysteine protease with anti-insect properties.

As the fruit of kiwifruit (*Actinidia deliciosa* (A. Chev.) C. F. Liang et A. R. Ferguson var. *deliciosa*) mature, they produce abundant quantities of actinidain (synonym: actinidin), a cysteine protease belonging to the papain family (Praekelt et al. 1988; Podivinsky et al. 1989; Keeling et al. 1990; Sugiyama et al. 1996). Actinidain is first detected in half-size kiwifruit and both its protease activity and mRNA levels increase until the fruit is fully formed (Lin et al. 1993). Actinidain's physiological function is unknown. It is encoded by a large gene family, and expression of closely-related mRNAs have been detected in most tissues of the kiwifruit plant (R. Atkinson pers. comm.). *A. deliciosa* fruit are attacked by several insect species. In New Zealand, two species of larval leafrollers (Lepidoptera: Tortricidae), *Ctenopseustis obliquana* and *Cnephasia jactatana*, feed on both fruit and foliage (Stevens et al. 1995). The possibility that actinidain expression may be induced in response to wounding or insect damage has not yet been investigated.

If, like some other plant cysteine proteases, actinidain could inhibit insect growth or increase mortality, then further investigation into its potential utility either as a biologically-based pesticide (or adjuvant to other pesticides) or in transgenic plants (alone or in combination with other pest-resistance genes) would be warranted.

Transgenic apple, tobacco, and maize plants, expressing the biotin-binding protein avidin, have been shown to be protected against attack by a range of insect pests (Kramer et al. 2000; Burgess et al. 2002; Markwick et al. 2003). Avidin is thought to bind with dietary biotin and deprive the insect of this vitamin sufficiently to interfere with moulting and cause death. Plants expressing avidin have the potential to control a much wider range of insect pests than currently available transgenic crop cultivars, which express various toxins from *Bacillus thuringiensis* (Bt) with relatively narrow host specificities (James 2003). Transgenic crops expressing non-Bt pest-resistance genes, such as those encoding biotin-binding proteins, chitinases, lectins, protease inhibitors, and perhaps also cysteine proteases, are

likely to be commercialised in the future. Most of these modifications can target a wider range of pest insects than Bt and operate via completely different mechanisms, so that cross-resistance with Bt-resistant insects is very unlikely. These genes may well be deployed in multi-gene combinations to improve efficacy and durability. Simultaneous deployment of multiple Bt-derived transgenes in a single crop, as a strategy to broaden specificity and manage resistance, is already a commercial reality, e.g., Bollgard II cotton (Chitkowski et al. 2003).

The first aim of this study was to determine whether actinidain has anti-insect properties similar to those reported for other plant cysteine proteases. The second aim was to discover whether actinidain could increase the speed of kill of transgenic plants expressing the insecticidal biotin-binding protein, avidin (Burgess et al. 2002; Murray et al. 2002).

MATERIALS AND METHODS

Actinidain

Actinidain powder extracted from *A. deliciosa* fruit and with a proteolytic activity of 127 units/mg was supplied by New Zealand Pharmaceuticals Ltd (batch no. 5601036). Titration against E64 showed that the powder contained 62% (w/w) active actinidain. For painting on to tobacco leaves, a 0.03% (w/v) aqueous solution of the powder was made up in 0.1% (v/v) BondXtra® (a wetting, spreading, and sticking agent). BondXtra® at 0.1% had no effect on actinidain activity. This dissolved powder was applied so that each g of leaf (fresh weight) carried c. 0.03 mg of actinidain powder (i.e., 0.02 mg of active actinidain per g leaf). This concentration approximated 0.1% active actinidain (w/w) of total leaf protein, a value comparable to the avidin levels used and a realistic value for actinidain transgene expression. When the mixture was applied to a microplate well, allowed to dry at room temperature and maintained for up to 48 h, no effect on activity was observed on dissolution.

Insects and plants

Spodoptera litura were obtained from a laboratory colony originally established from moths field-collected in Queensland, Australia, and reared on an artificial diet as described in McManus & Burgess (1995). For experiments, neonate larvae (i.e., <12 h post-emergence) were used.

Tobacco (*Nicotiana tabacum* cv. Samsun) plants were originally transformed to express vacuole-

targeted avidin, driven by a CaMV 35S promoter, as described by Murray et al. (2002). For the present experiments, second generation plants were produced by self-fertilisation. Non-transformed tobacco plants were used as controls. All plants were grown in a containment glasshouse at c. 28°C and used in insect trials once they had formed 12 leaves.

At the start of the first experiment, levels of expression of avidin in samples excised from leaves of the same age as those used to feed the insects were determined for each transgenic plant using methods described previously (Murray et al. 2002). Levels of avidin in tobacco leaves remain constant following leaf maturation (Murray et al. 2002). Briefly, plant tissue was ground in four volumes of sodium carbonate/bicarbonate buffer, pH 9.8, centrifuged and samples incubated overnight in microtitre plates in the same buffer. Standard curves were constructed in equal volumes of control plant extracts using egg white avidin (Sigma Chemicals, St Louis, MO, United States). Plates were developed using rabbit anti-avidin antibodies and goat anti-rabbit alkaline phosphatase-linked antibodies and the appropriate blocking and washing procedures. The level of avidin was determined from initial enzyme rates using sodium p-nitrophenyl phosphate as substrate. As avidin monomers bind to biotin on an equimolar basis, to allow meaningful comparison of avidin expression with plant biotin levels, avidin expression levels have previously been expressed as μM ($\mu\text{moles/kg}$ of fresh leaf tissue) (Burgess et al. 2002; Murray et al. 2002; Markwick et al. 2003). To allow comparison with other studies using transgenic avidin-expressing tobacco, this convention has been followed in the present study. To enable comparison with other studies where expression levels are reported as a percentage of leaf protein, and if we assume tobacco leaves contain protein as 2% of fresh weight, then avidin expressed at 13 μM is equivalent to 1% of total leaf protein.

After the first experiment, the plants were cut back, re-potted, fertilised, and newly-emerged buds grown to the 12-leaf stage before being used in the second and third experiments. Leaves from plants used in the first experiment had a mean avidin expression level of $7.15 \pm 0.91 \mu\text{M}$ ($n = 11$), those in the second experiment had a mean level of $9.55 \pm 2.22 \mu\text{M}$ ($n = 3$), and those in the third experiment had a mean avidin expression level of $6.41 \pm 1.32 \mu\text{M}$ ($n = 10$). There were no significant differences in the expression levels of these three groups of plants (ANOVA).

For each experiment, the uppermost young, fully expanded leaves (1–3 per plant) were excised from

control and avidin-plants. At each harvest time, plants of each type were selected at random and sufficient leaves of similar age taken to feed the larvae in the experiment. The midribs were dissected out and the leaf blades weighed. These were then painted evenly with either actinidain/BondXtra® solution as described above, or with a control solution of 0.1% (v/v) BondXtra® at a rate of 100 μl solution/g of fresh leaf. Once dry, the leaves were cut into c. $3 \times 3 \text{ cm}$ squares and each placed on moist filter paper in a 60 ml clear plastic pottle. A single neonate *S. litura* larva was placed in each pottle, and they were incubated at $24.5 \pm 1^\circ\text{C}$, 60% relative humidity, under a 16:8 h light:dark regime. Thirty-five larvae were set up for each of the four treatments (control tobacco $\pm$ actinidain, avidin-tobacco $\pm$ actinidain). On day 10 (after first being placed on tobacco), all larvae were moved to 250 ml clear plastic tissue culture pottles and fed with larger painted leaf portions (c. $6 \times 6 \text{ cm}$). Each pottle was checked daily, and dead larvae, signs of pre-pupation (shredded leaf or paper, cessation of feeding), pupation, and adult emergence recorded. Leaves were replaced with new ones of the same type if necessary. All surviving larvae were weighed 3, 6, 9, and 12 days from the beginning of the experiment. In the first experiment, insects were also weighed on day 14. Each experiment was terminated after all insects had either died or become adults. The entire experiment was repeated three times.

Statistical analysis

All data were analysed to determine the influence of actinidain on the growth and survival of all insects (whether on control or transgenic tobacco) and of avidin on all insects (regardless of whether they received actinidain as well). In addition to this, the effects of each of the four treatments (control, actinidain only, avidin only, actinidain and avidin) were analysed separately. To assess growth, the masses of larvae on each weighing occasion, and the mean time elapsed until pre-pupation, pupation, and adult emergence (if it occurred) were compared using ANOVA. To investigate effects on survival, three tests were performed. For treatments in which some insects were alive at the end of the experiment, analysis of mean and median times until death required the assumption that each surviving insect died the next day. ANOVA was used (as described above) to compare the mean time elapsed until death for each treatment and a chi-squared test was used to compare median survival times. Survival curves were plotted and Mantel-Haenzel (log-rank) tests

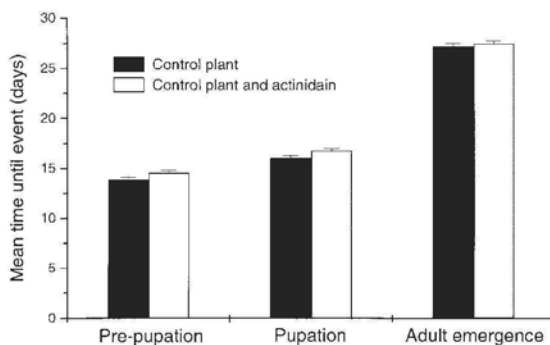
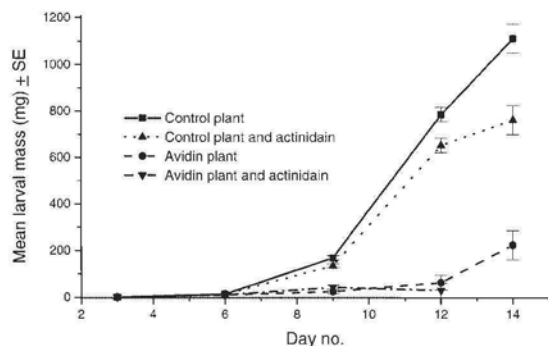


Fig. 1 Effects of four treatments on the growth of *Spodoptera litura* larvae. "Control" insects were fed with non-transgenic tobacco leaves ($n = 104$ on day 3, $n = 103$ on days 6 and 9, $n = 99$ on day 12, and $n = 33$ on day 14). "Actinidain" insects were fed with non-transgenic tobacco leaves painted with a solution of actinidain ($n = 101$ on day 3, $n = 99$ on days 6 and 9, $n = 95$ on day 12, and $n = 30$ on day 14). "Avidin" insects were fed with transgenic avidin-expressing tobacco leaves ($n = 104$ on day 3, $n = 101$ on day 6, $n = 73$ on day 9, $n = 22$ on day 12, and $n = 2$ on day 14). "Avidin and actinidain" insects were fed with transgenic avidin-expressing tobacco leaves painted with a solution of actinidain ($n = 101$ on day 3, $n = 97$ on day 6, $n = 61$ on day 9, and $n = 13$ on day 12). Only one replicate was weighed on day 14 and there were no "avidin and actinidain" survivors on that day.

Fig. 2 Effects of an actinidain solution painted onto non-transgenic tobacco leaves on the timing of pre-pupation, pupation, and adult emergence of *Spodoptera litura* fed as larvae on those leaves.

given the combined treatment were dead, the few still alive in the avidin treatment were much smaller than the controls, and the actinidain-fed larvae were of intermediate size. Larvae fed with actinidain-painted non-transgenic tobacco were significantly smaller than those on non-transgenic tobacco on days 9, 12, and 14 ($P < 0.05$).

All avidin-fed larvae failed to pupate, but analysis of the other insects showed that actinidain-fed larvae, when compared with control-fed larvae were, very slightly, but significantly and consistently, delayed in reaching pre-pupation and pupation ($P < 0.05$) but not adulthood (Fig. 2). The mean number of days to pre-pupation of actinidain-fed larvae was 14.55 days and that of control-fed larvae was 13.85 days (LSD (0.05) = 0.502). Actinidain-fed larvae reached pupation after 16.78 days and control-fed larvae pupated after 16.05 days (LSD (0.05) = 0.482).

As expected, avidin had a very marked effect on *S. litura* survival (Fig. 3). Most insects fed with non-transgenic tobacco, with or without actinidain, were still alive at the end of the experiment. The mean time until death was significantly shorter for insects fed with control tobacco painted with actinidain (30.2 days) than for those fed with control tobacco (31.4 days) ($P < 0.05$). When painted onto avidin-expressing tobacco, actinidain did not significantly reduce the mean time until insect death. However, analysis of other measures of longevity suggested a slight negative impact of actinidain on *S. litura* survival. Comparisons of median survival times for these insects revealed a significant but slight reduction in the actinidain-plus-avidin-treated insects (10 days) compared with avidin-only treated insects (10.5 days) ($P < 0.01$). Comparisons of the survival

(Kalbfleisch & Prentice 1980) were carried out to compare Kayden-Meir estimates of survival distribution, $S(t)$, of the insects in each treatment.

RESULTS

The masses of insects in the first experiment were significantly less than those in the second and third experiments. However, this was consistent across all treatments and all days. Insects in the first experiment also had significantly greater longevity than the others. Once again this was consistent across all treatments. There were no significant differences in mean avidin expression levels of the transgenic plants used in each experiment that could explain this, and the results of the three experiments were combined for further analysis.

Both avidin and actinidain had significant effects on the growth of *S. litura* larvae, although avidin's effects were much greater than those of actinidain (Fig. 1). Larvae on avidin-expressing tobacco with or without actinidain were significantly smaller than those on the two non-transgenic tobacco treatments on days 9 and 12 ($P < 0.05$). On day 14, all larvae

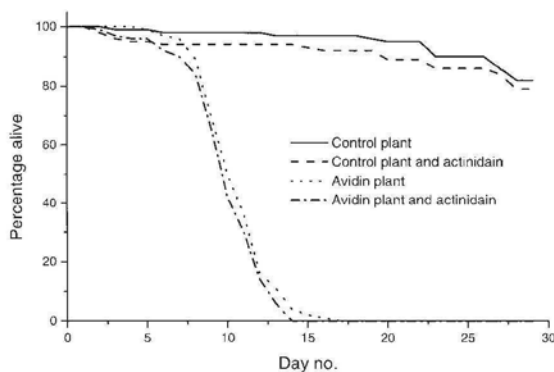


Fig. 3 Impacts of actinidain (solution painted onto tobacco leaves) and avidin (expressed by transgenic leaves) on the survival of *Spodoptera litura* larvae. Avidin plants, with or without actinidain, significantly reduced insect survival compared to control plants. The addition of actinidain to control leaves had no effect on the survival curves for insects on those types of plants, but when added to avidin-expressing plants, actinidain treatment resulted in a slight but significant reduction in survival in addition to the effects of avidin alone. The log-rank tests used to compare these curves do not generate error bars, as the entire curves are compared, not just fixed points.

curves for these insects also showed a slight but significant reduction in survival of insects given actinidain in addition to avidin ($P < 0.01$) (Fig. 3).

DISCUSSION

Spodoptera litura larvae fed with actinidain applied to control or avidin-expressing transgenic leaves at a rate approximating 0.1% (w/w) of total protein were smaller than those not fed with this protease. Avidin-fed larvae all died before pupation, slightly more quickly when actinidain was administered as well. When added to control tobacco, actinidain delayed the onset of pre-pupation and pupation of *S. litura* larvae, but did not reduce their survival or slow the emergence of adult moths.

These slight but significant impacts suggest that, like cysteine proteases from maize, papaya, wild fig, and pineapple (Pechan et al. 2000, 2002; Konno et al. 2004), actinidain from kiwifruit has a debilitating effect on insect larvae. However, the concentration of actinidain used in the present study was low compared with others. For example, maize callus transformed with *mir1* and expressing cysteine protease at 1% of total soluble protein reduced insect growth (Pechan et al. 2000). Actinidain alone at the concentration tested here would not be useful in

controlling this species, although the presence of low levels of endogenous tobacco cystatins, inhibiting actinidain following ingestion, may have influenced the results of this study. Furthermore, the possibility of impurities in the actinidain powder being responsible for the negative impacts observed cannot be discounted. It is possible that higher concentrations of actinidain may have more marked effects on insect growth.

Another variable that could affect activity is the redox potential of the larval midgut, which can vary between different species and is inconsistent in herbivores (Appel & Martin 1990). Unless reducing conditions are present in the midgut, the enzyme, that has been exposed to an oxidising atmosphere on the leaf surface and will be inactive on ingestion, will not activate. It is likely that only insects with a reducing midgut environment will be susceptible to cysteine proteases.

Electron micrographs have shown that ingestion of Mir1 cysteine protease from maize disrupts the peritrophic matrix of *S. frugiperda* (Pechan et al. 2002), although its mode of action is not yet certain. Pechan et al. (2002) pointed out the discrepancy between the acidic pH optimum of Mir1 (Jiang et al. 1995) and the highly alkaline pH of the lepidopteran larval midgut (Dow 1992) and suggested that a microenvironment at the peritrophic matrix might account for the observed effects. Peritrophic matrix disruption has also been demonstrated with other proteins. For example, marked mortality has been demonstrated in grain beetles fed with an insect chitinase engineered into and then extracted from transgenic tobacco (Wang et al. 1996). Like Mir1, actinidain may affect the peritrophic matrix of *S. litura*, but confirmation of this must await electron microscopic examination. Actinidain differs from Mir1 in having a catalytic efficiency profile that extends well into the alkaline region (Topham et al. 1991), which could facilitate its action in lepidopteran midguts. Even so, actinidain may have greater effects on insects with less alkaline midguts.

Peritrophic matrix damage may not always cause significant insect mortality, but it may make insects more susceptible to other stressors, such as anti-nutritional compounds. Avidin is thought to act as an insecticide by binding with dietary biotin and creating a lethal deficiency of this vitamin in some insects (e.g., Markwick et al. 2001). When applied to transgenic leaves expressing avidin, actinidain caused a slight increase in the speed at which *S. litura* larvae died. Avidin is known to be very resistant to proteases (Green 1975), is apparently not degraded

by insect digestive proteases (J. Christeller unpubl. data), and is unlikely to be adversely affected by the presence of an additional protease, such as actinidain, in the insect's diet. Whether outdoor conditions could exacerbate the effects of actinidain and make it a useful adjunct to insecticidal compounds like avidin must await further experimentation.

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