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Tri-trophic effects of transgenic insect-resistant tobacco expressing a protease inhibitor or a biotin-binding protein on adults of the predatory carabid beetle *Ctenognathus novaezelandiae*

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Abstract

Tri-trophic impacts on adult predatory carabid beetles, *Ctenognathus novaezelandiae*, of insect-resistant transgenic tobacco plants expressing a serine protease inhibitor, bovine spleen trypsin inhibitor (BSTI), or a biotin-binding protein, avidin, were investigated. Both proteins could potentially affect this beetle, since avidin is known to be insecticidal to many beetle species and *C. novaezelandiae* midguts were shown to contain high levels of trypsin, a protease powerfully inhibited by bovine pancreatic trypsin inhibitor (a BSTI homologue) *in vitro*. Newly emerged field-collected adult *C. novaezelandiae* were fed exclusively for 280 days on *Spodoptera litura* larvae raised either on non-transgenic control, transgenic avidin (55 ppm) or transgenic BSTI (68 ppm) tobacco. Despite this long-term exclusive diet, there was no treatment effect on survival or fecundity and only minor and transient effects on beetles were observed. Data pooled across time and genders showed control-prey-fed beetles weighed 3% more than BSTI-prey-fed beetles and avidin-prey-fed beetles consumed 3–4% fewer prey than control- or BSTI-prey-fed individuals. Females in all treatments gained more mass and survived longer than males. Low exposure to the proteins because of dilution and deactivation within the prey is the most likely explanation for the lack of tri-trophic effects observed. Aditionally, the presence of a digestive chymotrypsin only partially inhibited by BSTI may provide an alternative path for proteolysis.

Keywords: Tri-trophic non-target impacts; Carabid beetle; Transgenic plant; Avidin; Bovine spleen trypsin inhibitor (BSTI); Aprotinin (BPTI)

1. Introduction

Transgenic insect-resistant crops expressing *Bacillus thuringiensis* (Bt) delta endotoxins are being used increasingly to control insect pests, either as a single trait, stacked together, or stacked with herbicide resistance genes (James, 2006). Alternatives to Bt-crops are also being developed. Protease inhibitors are now being expressed and field-tested in some crops (Graham et al., 2002; Wu et al., 2005), and biotin-binding proteins (BBPs) have been expressed experimentally in maize, tobacco, apple and rice (Kramer et al., 2000; Burgess et al., 2002a; Markwick et al., 2003; Yoza et al., 2005). Before approving field release of transgenic plants, regulatory authorities require data on their environmental safety. Insect-resistant plants may have the potential to harm beneficial non-target natural enemies such as predators and parasitoids. When pest control has negative impacts on natural enemies, this may result in disruption of biological or integrated control,

Abbreviations: BApNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; BTpNA, *N*-benzoyl-tyrosine *p*-nitroanilide; LpNA, L-leucine *p*-nitroanilide; SAAPLpNA, *N*-succinyl-L-alanyl-alanyl-propyl-leucine *p*-nitroanilide; γEpNA, γ-glutamic acid *p*-nitroanilide; DMF, dimethylformamide; DMSO, dimethyl sulphoxide; E64, trans-epoxysuccinyl-L-leucyl-amido (4-guanidino)butane; BSTI, bovine spleen trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor, α1AT, α_1-antitrypsin; SBTI; Kunitz soybean trypsin inhibitor; POT2, potato proteinase inhibitor 2; POT1, potato proteinase inhibitor 1; MBTI, mungbean trypsin inhibitor; CPTI, cowpea trypsin inhibitor; EDTA, ethylenediamine tetraacetic acid.

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leading to pest outbreaks (Cui and Xia, 2000; Sun and Zhang, 2003; Ito et al., 2005).

Carabid beetles are thought to be important components of both wild and cultivated ecosystems, contributing to the regulation of prey species and biological control of pests, and even having a role in the improvement of physical soil conditions (Thiele, 1977; Luff, 1987; Lovei and Sunderland, 1996; Symondson et al., 2002; Ostman et al., 2003; O'Neal et al., 2005). They are considered useful as an indicator species or as a key taxonomic group for assessing biodiversity and environmental impacts (Boscaini et al., 2000; Edwards et al., 1996; Ferris and Humphrey, 1999). Carabids have been used in forest ecosystems as indicators of ecosystem quality and functioning (Szyszko, 2002), stability and stress (Allegro and Sciaky, 2003), and productivity and ecological efficiency (Guido, 2000) and have been identified as good candidates for evaluating the potential unintended effects of transgenic crops (Lopez et al., 2005).

The present study investigated the potential for prey-mediated effects of transgenic tobacco plants expressing insecticidal levels of either a protease inhibitor or a BBP on the adults of *Ctenognathus novaezelandiae* (Fairmaire), a univoltine forest-dwelling predatory carabid beetle endemic to the North Island of New Zealand. This beetle occurs in *Pinus radiata* (Monterey pine) forests, which are grown in intensively managed plantations in New Zealand. *P. radiata* has been transformed with Bt and other genes and long-term field trials using transgenic pines are now under way in New Zealand (Walter et al., 1998; Bishop-Hurley et al., 2001; Grace et al., 2005; Moller et al., 2005; Hofig et al., 2006; http://www.ermanz.govt.nz). Bovine spleen trypsin inhibitor (BSTI) and avidin have been expressed separately in the model plant tobacco and both shown to be insecticidal to larvae of the noctuid pest, *Helicoverpa armigera* (Christeller et al., 2002; Burgess et al., 2002a). Avidin-expressing tobacco was also insecticidal to *Spodoptera litura* (Fabricius) (Burgess et al., 2002a).

As *C. novaezelandiae* has a long generation time it provides an example of those species which are generally under-represented in laboratory-based biosafety risk assessments, where short-lived species are often favoured for convenience (Stark et al., 2004). In addition to its role as a provider of ecosystem services, this species also represents New Zealand's native beetle fauna. Thus, as a test subject it has relevance to two of the management goals identified in New Zealand's environmental protection legislation (Anon., 1996) as applied to transgenic plants.

This paper describes the impacts of exclusive diets of two types of insect-resistant tobacco-fed prey on the feeding, growth, survival and fecundity of newly emerged field collected *C. novaezelandiae* throughout the 280 days of the adult stage. This represents the first investigation of the effects of long-term dietary exposure of a carabid beetle to prey fed on transgenic plants expressing BSTI or avidin for insect control.

2. Methods

2.1. Plants

Tobacco plants (*Nicotiana tabacum* L. "Samsun") were transformed to express BSTI using a standard *Agrobacterium tumefaciens*-mediated system (Horsch et al., 1985), following mobilisation of the plant expression plasmid, pAK (Christeller et al., 2002) into *A. tumefaciens* (strain LBA4404) by standard tri-parental mating techniques (Ditta et al., 1980). Plants used in the present experiments were from generation T_3, i.e. the third generation resulting from self-fertilisation of the originally transformed plants (T_0) and their progeny. Levels of expression of BSTI were measured using enzyme-linked immunosorbent assay (ELISA) (Christeller et al., 2002).

Transformation of plants to express avidin, a BBP, was achieved as above using the plant expression plasmid, pLA2, that incorporates an *N*-terminal vacuolar targeting sequence derived from potato proteinase inhibitor I (POT1) fused to an avidin sequence (Murray et al., 2002). T_2 plants were used in these experiments (i.e. they were grown from seeds collected from self-fertilised plants that were the progeny of a selfed original transformant). Leaf samples were taken from expanding green leaves and avidin expression levels were determined using ELISA (Murray et al., 2002) and adjusted for recovery using spiked control leaf samples (Christeller et al., 2005).

Plants were grown in a containment greenhouse at 26 ± 4 °C with a 16:8 h light:dark cycle. To feed prey larvae, expanding green leaves ranging between 20 and 30 cm in length from leaf base to leaf tip were cut cleanly from the plants where the petiole joined the stem, and the petioles inserted into set 0.4% (w:v) agar in individual 40 ml plastic cups to keep the leaves fresh. Leaves were then placed in sealed plastic storage boxes lined with paper towels, infested with several hundred neonate *S. litura* larvae and incubated at 24.5 ± 1 °C and 60% relative humidity, 16:8 h light:dark. To ensure a continuous supply of fresh leaves for *S. litura* larvae, plants were cut back as they approached maturity and allowed to grow back once for leaf harvesting before being discarded. New batches of seeds were sown to provide fresh plants every 3 or 4 months.

2.2. Prey

Neonate *S. litura* larvae were obtained from a laboratory colony established from moths collected in Queensland, Australia, and sprinkled onto freshly harvested tobacco leaves. Larvae were randomly assigned to control, BSTI or avidin treatments. Leaves were replaced initially after 4 days and then every 2 or 3 days as necessary, and larvae were transferred to additional boxes as they grew. At the third instar, larvae were removed from leaves and frozen for later use as prey.