Enhanced Resistance to Control Potato Tuberworm by Combining Engineered Resistance, Avidin, and Natural Resistance Derived from, *Solanum Chacoense*

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Abstract Potato tuberworm, *Phthorimaea operculella* (Zeller), is a destructive insect pest of potato, *Solanum tuberosum* (L.), found primarily in tropical and sub-tropical regions. It has recently become established in the northwestern United States. Avidin is a natural protein found in chicken (*Gallus gallus* L.) egg whites that has insecticidal properties against a number of lepidopteran and coleopteran pests. Biotin is a cofactor of carboxylases that are required for many important processes like lipogenesis, gluconeogensis, fatty acid and amino acid catabolism. Without biotin, an insect's growth is severely stunted, eventually leading to death. Avidin binds and sequesters biotin, limiting its availability during insect growth and development. Previous studies have demonstrated that avidin is effective against potato tuberworm. We sought to elevate resistance by combining avidin with natural host plant resistance factors from the wild species *Solanum chacoense* Bitter. We expressed avidin in two potato lines: MSE149-5Y, a susceptible potato line, and ND5873-15, a line with *S. chacoense*-derived resistance. The avidin expression was determined by ELISA to be 10.6 µM in MSE75.7 (avidin) and 12.5 µM in ND75.3 (avidin + *S. chacoense*-derived) in the tuber. Potato tuberworm bioassays were performed on MSE149-5Y, MSE75.7, ND5873-15, and ND75.3. Mortality was measured after 28 d. Mortality of larvae fed on MSE149-5Y (susceptible) did not differ significantly compared to the mortality of larvae fed on MSE75.7 (avidin) or ND5873-1 (*S. chacoense*-derived). Significantly higher mortality (98%) was observed for larvae fed on ND75.3 (avidin + *S. chacoense*-derived) tubers than for larvae fed on MSE149-5Y (susceptible). The present study is the first report of combining avidin with natural host plant resistance factors against potato tuberworm. Expressing avidin in combination with natural host plant resistance may be of value in managing potato tuberworm.

Resumen El gusano del tubérculo *Phthomaea operculella* (Zeller) es una plaga destructiva de papa *Solanum tuberosum* L., se encuentra principalmente en las regiones tropical y subtropical. Se le ha encontrado recientemente en los estados del noroeste de los EE.UU. La avidina es una proteína que se halla en la clara de huevo de las aves de corral (*Gallus gallus* L.) que tiene propiedades insecticidas contra ciertos lepidópteros y coleópteros. La biotina es un cofactor de las carboxilasas requerido para muchos procesos importantes como la lipogénesis, gluconogésis y el catabolismo de los ácidos grasos y aminoácidos. Sin la biotina, el crecimiento del insecto es severamente afectado conduciéndolo eventualmente a la muerte. La avidina une y secuestra a la biotina, limitando su disponibilidad durante el crecimiento y desarrollo del insecto. Estudios previos han demostrado que la avidina es efectiva contra el gusano del tubérculo de papa. Buscamos elevar la resistencia mediante la combinación de avidina con factores de resistencia natural de plantas hospedantes provenientes de *Solanum chacoense* Bitter. Expresamos la avidina en dos líneas de papa: MSE149-5Y, línea susceptible y ND5873-15 línea resistente derivada de *S. chacoense.* La presencia de avidina fue determinada por ELISA como 10.6 µM en MSE75.7 (avidina) y 12.5 µM en ND75.3 (avidina + derivado de *S. chacoense*) en los tubérculos. Se realizaron bioensayos del gusano del tubérculo en MSE149-5Y, MSE75.7, ND58.73-15 y ND75.3.. La mortalidad de larvas sobre

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MSE149-5Y (susceptible) no fue significativamente diferente comparado a la mortalidad de la larva alimentada con MSE75.7 (avidina) o ND5873-1 (derivado de *S. chacoense*). Se observó una mayor mortalidad (98%) significativa en larvas alimentadas sobre tubérculos de ND75.3 (avidina + derivado de *S. chacoense*) que en larvas alimentadas sobre tubérculos de MSE149-5Y (susceptible). El presente estudio da el primer informe de una combinación de avidina con factores de resistencia natural de la planta hospedante contra el gusano del tubérculo de papa. La expresión de la avidina en combinación con la resistencia natural de la planta hospedante puede ser una herramienta valiosa para el manejo del gusano del tubérculo de papa.

Introduction

Potato tuberworm, *Phthorimaea operculella* (Zeller) is distributed throughout the world, including the Americas, Europe, Africa, Asia, and Australia (Trivedi and Rajagopal 1991). Potato tuberworm established itself in California as early as 1855 and in Texas as early as 1917 (Berthon 1855; Graf 1917). Potato tuberworm was chiefly considered a tropical pest until an outbreak of potato tuberworm in a field near Hermiston, Oregon caused severe damage to potato crops (Alvarez et al. 2005). Mild winters and dry summer conditions have expanded the potato tuberworm's geographic range to the Columbia Basin (Alvarez et al. 2005). Due to the devastation, potato growers in the Pacific Northwest are evaluating strategies to control potato tuberworm.

Potato tuberworm feeds on solanaceous plants, including potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melogena* L.), and tobacco (*Nicotiana tabacum* L.) (Das and Raman 1994). Larvae damage potato crops by mining leaves and boring into tubers in the field. The greatest damage occurs in storage where the larval mining of tubers causes rotting and renders the tubers unmarketable (Kroschel and Koch 1994). Potato tuberworm typically has two generations in the summer and a third generation in storage in the United States (Chittenden 1912). The potato tuberworm does not have discrete generations in tropical climates; multiple generations occur in the field and in storage, and all stages of larvae and adults may be present at any one time (Trivedi and Rajagopal 1991).

Potato tuberworm can cause significant economic damage. Potato tuberworm infestations accounted for losses of 42% of the stored crop in Ethiopia and 86% of the stored crop in Tunisia (Roux et al. 1992; Sileshi and Teriessa 2001). In 2003, economic losses due to potato tuberworm damage were approximately US$2 million in Oregon, and have increased significantly in 2004 and 2005 (Rondon 2007). Insecticides remain the chief means of control for potato tuberworm (Alvarez et al. 2005). Cultural practices, such as irrigation regimens and planting depth, can reduce infestation in the field (Shelton and Wyman 1979; Coll et al. 2000). Host plant resistance factors, both natural and engineered, would augment current pest management practices.

Natural host plant resistance factors from the *Solanum* species can be introgressed into the cultivated potato through traditional breeding. *Solanum berthaultii* 'Hawkes', *S. commersonii* 'Dunal', *S. sparsipilum* 'Bitter', *S. sucrense* 'Hawkes' and *S. tarijense* 'Hawkes' have reported resistance to potato tuberworm (Chavez et al. 1998; Malakar and Tingey 1999). Additionally, potatoes naturally produce compounds such as glycoalkaloids that are associated with resistance to a number of insect pests (Maga 1994). The wild species, *S. chacoense*, produces a number of compounds, including leptine glycoalkaloids, which confer resistance to Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Sinden et al. 1986). ND5873-15 is a breeding line from North Dakota State University derived from *S. chacoense* with uncharacterized Colorado potato beetle resistance partially attributed to glycoalkaloids; ND5873-15 has not been previously screened for potato tuberworm resistance.

Genetic engineering is a powerful tool that allows plant breeders to introgress resistance genes from any organism into potato. Crystal (Cry) proteins derived from the soil-borne bacterium *Bacillus thuringiensis (Bt)* are the most well studied class of insecticidal proteins and are commonly engineered into plants to confer insect resistance (Whalon and Wingerd 2003). Several Bt-Cry1 proteins have been engineered into potato to provide protection against potato tuberworm (Beuning et al. 2001; Naimov et al. 2003; Davidson et al. 2004; Douches et al. 2004).

Recently, Markwick et al. (2003) obtained a high level of resistance against potato tuberworm by engineering tobacco to express avidin. Avidin is a naturally produced protein in the white of chicken eggs (*Gallus gallus* L.) (Stevens 1991). It has a strong affinity for biotin, (vitamin H or B_8) an essential vitamin for all organisms (Trager 1948; Green 1990). Biotin is a required cofactor for a number of important carboxylases involved in such pathways as the citric acid cycle, lipogenesis, and fatty acid and amino acid catabolism (Mistry and Dakshinamurti 1964). Insects require biotin-dependent carboxylases to store and use fat (Miura et al. 1967). When avidin is added to insect diets, it causes a deficiency of biotin that delays slow growth, causes abnormal development, and eventually kills the insect (Levinson and Bergmann 1959; Morgan et al. 1993;

Marwick et al. 2001; Malone et al. 2002). Transgenic maize (*Zea mays* L.), tobacco, and apple (*Malus domestica* Borkh.) expressing avidin are resistant to a broad spectrum of insect pests, including Lepidoptera and Coleoptera (Kramer et al. 2000; Markwick et al. 2001, 2003; Burgess et al. 2002).

Insect mortality, as a result of biotin depletion, is slow (Levinson et al. 1992; Kramer et al. 2000; Markwick et al. 2001; 2003; Burgess et al. 2002). Combining avidin with other resistance strategies, such as natural host plant resistance, may enhance the level of plant protection. Moreover, combining resistance mechanisms into a plant may also delay adaptation of the pest (Gould 1998; Roush 1998; Zhao et al. 2005).

The objective of this study was to evaluate the performance of potato tuberworm first stage larvae on MSE149-5Y (a susceptible potato) and ND5873-15 (*S. chacoense*-derived) and also on transgenic tubers from these lines that are expressing avidin.

Materials and Methods

Plant Material

The potato lines selected were MSE149-5Y, MSE75.7, ND5873-15 and ND75.3. MSE149-5Y is a breeding line from Michigan State University that is susceptible to insects. ND5873-15 is a breeding line from North Dakota State University with *S. chacoense*-derived resistance to Colorado potato beetle (Coombs et al. 2002). Tuber total glycoalkaloid levels of ND5873-15 and MSE149-5Y were previously measured and determined to be below the accepted limit of 20 mg% (B. Perkins, unpublished data). Fourteen transgenic MSE149-5Y lines constitutively expressing the 472 bp avidin gene using the 35S CaMV promoter and seven transgenic ND5873-15 lines were developed in our lab from the two parental lines (Cooper et al. 2007). *Agrobacterium tumefaciens*-mediated transformation was used according to Douches et al. (1998), and putative transgenic lines were confirmed by PCR and Southern analysis. Avidin expression in the plants was confirmed via ELISA. MSE75.7 and ND75.3 were selected for this study because of high insecticidal activity to Colorado potato beetle larvae, high avidin expression in the leaf tissue in both lines, and they did not significantly differ from each other (Cooper et al. 2006). Tuber total glycoalkaloid levels of MSE75.7 and ND75.3 are below the accepted level of 20 mg% (B. Perkins, unpublished data).

The potato lines, MSE149-5Y, MSE75.7, ND5873-15 and ND75.3 were maintained in tissue culture as previously described (Coombs et al. 2002). Rooted tissue culture plants were transferred to soil in seedling trays (50 cells per tray, 3 cm diam.) in the greenhouse. After a month, the seedlings were transferred into plastic pots (3.78 L). When the plants senesced, tubers were harvested. The tubers were kept in cold storage (4°C, 90% relative humidity) for approximately 2 months prior to the potato tuberworm assay.

ELISA for Quantification of Avidin

Indirect sandwich ELISA was conducted on the tubers of greenhouse grown potato plants. Microtiter plates (Nunc, West Chester, PA) were coated with mouse anti-avidin antibody (Sigma Chemical, St. Louis, MO) overnight at 4°C. Protein was extracted from the tuber eyes (by grinding 1 g of tissue in 1 ml of 50 mM PBS pH 7.0 containing 0.05% Tween (Sigma Chemical) before being adjusted to a final dilution of 1:10 (w/v). The avidin protein from the leaf extracts was captured overnight at 4°C. The avidin protein reacted with rabbit anti-avidin antibody (Sigma Chemical) (1.25 h, 37°C). Finally, the plates were incubated with an anti-rabbit conjugated to alkaline phosphatase (Sigma Chemical) (1.25 h, 37°C). The alkaline phosphatase was detected with para-nitro-phenyl phosphate (pNPP) at 1 mg/ml at 37°C. Absorbance was detected at 405 nm after 60 min incubation with the PNP substrate using an automated microplate reader (Wallac Victor[2] V 1420 multi-label counter, Perkin Elmer, Wellesley, MA). The ELISA analysis was replicated three times for each line. The means of protein expression level were compared using Fisher's LSD (SAS Inst. 2006).

Potato Tuberworm

The potato tuberworm colony was initiated from insects collected in South Africa in 2004 by Dr. Walter Pett (Michigan State University, Department of Entomology). The colony of potato tuberworm is maintained at Michigan State University, Department of Entomology on potato tubers as previously described in Mohammed et al. (2000).

Potato Tuberworm Bioassay

A Magenta box (77 mm×77 mm×97 mm) (Chicago, Illinois) was filled with 2 cm of vermiculite (Therm-o-Rock, New Eagle, PA). A single tuber was placed inside each Magenta box. Ten first stage potato tuberworm larvae were placed on the tuber. The Magenta box was sealed with a vented lid to allow for gas exchange. The number of emerged adults was counted after 28 d. Avidin is anti-nutritional and delays growth and development in insects (Levinson et al. 1992; Morgan et al. 1993). Therefore, the tubers were cut to locate larvae within the tubers that had not emerged as adults. Missing individuals were considered

Fig. 1 Examples of damage caused by potato tuberworm larvae fed potatoes after 28 d. **a** MSE149-5Y (susceptible), **b** MSE75.7 (avidin) **c** ND5873-15 (*S. chacoense*-derived), and **d** ND75.3 (avidin + *S. chacoense*-derived)
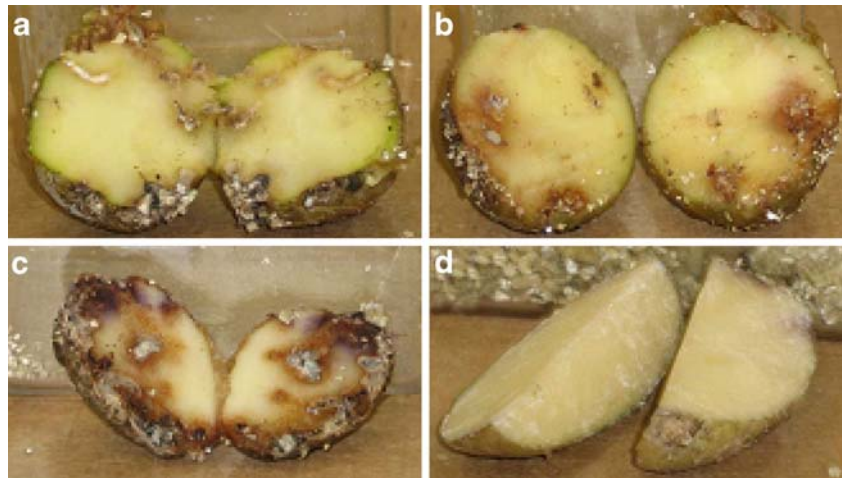
dead. Percentage mortality was transformed with the arcsine of the square root to homogenize variance and these data were analyzed using Fisher's protected least significant difference test (LSD, $\alpha=0.05$) in the general linear models procedure of SAS (SAS Inst. 2006). Mean arcsine values were retransformed into percentages for presentation. The potato tuberworm bioassay was replicated five times (50 individuals per plant line).

Results and Discussion

ELISA for Quantification of Avidin

The level of avidin expression in tubers of MSE75.7 (10.6 μM) did not differ significantly compared to the level of avidin expression in tubers of ND75.3 (12.5 μM) (data not shown). The level of avidin expression was much lower in the tubers than previously observed in the leaf tissue of the same potato lines (63.8 μM for MSE75.7 line and 61.6 μM for ND75.3) (Cooper et al. 2006).
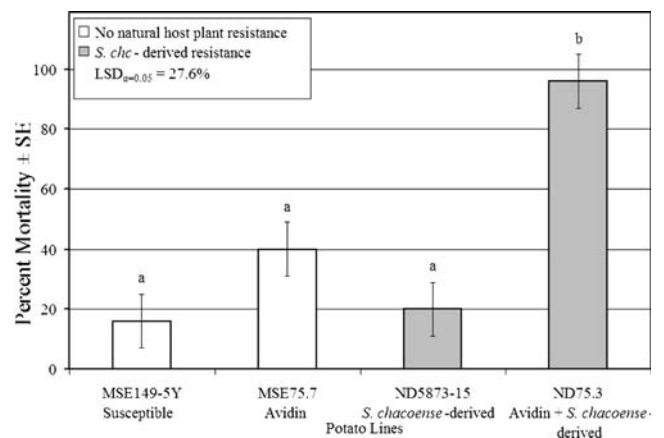
Potato Tuberworm Bioassays

Mining damage was apparent on the *S. chacoense*-derived resistance line (ND5873-15) tubers (Fig. 1). The susceptible, avidin and *S. chacoense*-derived line tubers were rotting with extensive mining damage after 28 d (Fig. 1). The avidin + *S. chacoense* (ND75.3) tubers were relatively free of damage.

Larvae fed on the susceptible line had the lowest mortality (16.0%) (Fig. 2). The mortality for larvae fed on the avidin line was higher (40.0%), but did not differ significantly compared to the mortality for larvae fed on the susceptible line. Markwick et al. (2003) observed much higher mortality (~97%) in a shorter time (9 d) with transgenic avidin tobacco leaves. Avidin expression was

much lower in transgenic tobacco (3.1–4.6 μM) compared to transgenic potato (10.6 μM) (Markwick et al. 2003). Additionally, potatoes produce low levels of biotin (0.14 μg/100 g) compared to leafy vegetable crops such as lettuce (0.62 μg/100 g) or cabbage (0.83 μg/100 g), therefore, it is unlikely the natural biotin content within potato counteracted the effects of avidin (James 1952).

The *S. chacoense*-derived resistance in ND5873-15 tubers did not confer appreciable resistance against potato tuberworm. The mortality for larvae fed on ND5873-15 was low (20.0%) and did not differ significantly from the mortality for larvae fed on MSE149-5Y (Fig. 2). The low tuber total glycoalkaloid levels of MSE149-5Y and ND5873-15 concur with the observed tuberworm mortality, with neither line demonstrating resistance.

Fig. 2 Mean percentage mortality of potato tuberworm larvae fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND75.3 (avidin + *S. chacoense*-derived) at 28 d in a no-choice tuber bioassay. Means followed by different letters are significantly different ($P<0.05$) based on analysis of arcsine square-root transformed data. Means were separated using Fischer's least squared differences test. Untransformed data are presented

Although avidin or *S. chacoense*-derived resistance alone did not appear to provide plant protection, the expression of avidin in ND5873-15 (ND75.3) provided a high level of protection (Fig. 1–2). The mortality (98.0%) for larvae fed on ND75.3 was significantly higher than the mortality for larvae fed on the susceptible line (MSE149-5Y), the MSE75.7 avidin line, or ND5873-15. The sole survivor on the avidin+*S. chacoense*-derived resistance line was a fourth instar larva found in one of the tubers; the remaining four tubers were clean with no evidence of potato tuberworm damage (Fig. 1).

The genus *Solanum* has immense natural diversity and natural host plant resistance. Although the *S. chacoense*-derived ND5873-15 was not highly resistant against potato tuberworm, it provided strong protection in combination with avidin. The insect resistance associated with the wild species *S. chacoense* is partially attributed to leptine glycoalkaloids (Sinden et al. 1986). The breeding line ND5873-15 does not have leptine glycoalkaloids (D. Douches unpublished data) and the tuber total glycoalkaloids levels (solanine and chaconine) are not outside the range found for released varieties. The current literature is limited regarding potato tuberworm resistance in Solanum (Malakar and Tingey 1999). The enhanced resistance observed in this study may be due to an interaction of avidin and other undefined tuber glycoalkaloids. Avidin combined with stronger natural host plant resistance factors may confer a more robust and broad-spectrum resistance. Furthermore, avidin is detrimental to other potato pests, such as Colorado potato beetle (Cooper et al. 2006). It is also effective against number of lepidopteran and coleopteran pests in other crops; therefore, it may negatively impact other pests of the potato like wireworms, Elateridae spp., Andean potato weevil, *Premnotrypes suturicallus* Kuschel, and variegated cutworms, *Peridroma saucia* (Hübner) (Kramer et al. 2000, Markwick et al. 2001, 2003; Burgess et al. 2002; Yoza et al. 2005).

At present, avidin does not appear to negatively impact such non-target organisms as young honeybees, *Apis mellifera* L. (Malone et al. 2002; Malone et al. 2004). Additionally, avidin will not likely accumulate in the food chain or affect natural enemies of pests. Only 10–28% avidin recovered from the gut of the tobacco cutworm fed on transgenic avidin tobacco was active and able to bind to biotin (Christeller et al. 2005).

Impacts of avidin from transgenic potatoes on consumers would be negligible. Humans regularly consume avidin in the form of egg whites. Avidin denatures during cooking and loses its ability to bind to biotin (Durance 1991). Less than 3% of the avidin in transgenic rice was able to bind to biotin after cooking (Yoza et al. 2005). Similarly, potato is typically cooked before being consumed. Moreover, avidin is not highly allergenic; the major allergens in egg are ovomucoid, ovalbumin, ovotransfferin, and lysozyme (Subramanian and Adiga 1997). Finally, humans have a diverse diet and do not rely solely on potatoes for nutrition or biotin. The average person consumes 35–70 μg of biotin daily from varied food sources, including vegetables and nuts (Hardinge 1961).

Transgenic avidin plants may also delay the growth and development of pathogens. For instance, biotin deficiencies stunt the growth of a number of Fusarium species (Robbins and Ma 1941); transgenic avidin potatoes may delay the development of Fusarium dry rot, *Fusarium sambucinum*. Biotin also stimulates sporulation in some fungi (Yoshida and Shirata 2000). Similarly, transgenic avidin potatoes may inhibit proliferation of pathogenic fungi by sequestering available biotin.

The present study is the first report of combining avidin with natural host plant resistance factors against potato tuberworm. Combining avidin with other resistances factors, including natural host plant resistance factors, such as leptines or glandular trichomes, or other transgenes, such as Bt-Cry proteins, may provide strong and broad-spectrum plant protection. For example, combining avidin with a stronger natural host plant resistance factor from *S. berthaultii*, *S. commersonii*, *S. sparsipilum*, *S. sucrense* or *S. tarijense* may provide superior protection against insect pests.

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