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INSECTICIDAL ACTIVITY OF TRANSGENIC POTATO (*Solanum tuberosum* L.) EXPRESSING AVIDIN FROM CHICKEN (*Gallus gallus* L.)

presented by

Susannah G. Cooper

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Doctoral degree in Plant Breeding and Genetics

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INSECTICIDAL ACTIVITY OF TRANSGENIC POTATO, (*Solanum tuberosum* L.) EXPRESSING AVIDIN FROM CHICKEN (*Gallus gallus* L.)

By

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ABSTRACT

INSECTICIDAL ACTIVITY OF TRANSGENIC POTATO, (*Solanum tuberosum* L.) EXPRESSING AVIDIN FROM CHICKEN (*Gallus gallus* L.)

By

Susannah Greene Cooper

Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and potato tuberworm, *Phthorimaea operculella* (Zeller), are destructive pests of potato, *Solanum tuberosum* L. Avidin is derived from chicken (*Gallus gallus* L.) that has demonstrated insecticidal activity to a broad spectrum of pests.

The potential for avidin as an insecticidal transgene was evaluated against Colorado potato beetle. The LC_{50} for avidin was determined to be 136 µg /ml (108-188). We sought to enhance resistance by combining avidin with natural host plant resistance derived *S. chacoense.*

Avidin was expressed in two potato lines: MSE149-5Y, a susceptible line, and ND5873-15, a *S. chacoense*-derived resistant line. The putative transformants were screened with PCR to validate insertion, Southern analysis to determine copy number, and ELISA to quantify avidin expression. The copy number ranged from 1 – 3. Avidin expression ranged from 0.0 – 64.5 µM ± 0.3 S.E. Fourteen transgenic MSE149-5Y lines and 7 transgenic ND5873-15 lines were screened for Colorado potato beetle resistance. In general, larvae fed on transgenic avidin plants were significantly smaller and had significantly less survivors than the non-transgenic parental line at 3 d.

Insect resistance was further analyzed for four lines: MSE149-5Y (susceptible line), MSE75.7 (avidin line), ND5873-15 (*S. chacosense*-derived resistance), and ND75.3

(avidin + *S. chacoense*). Survival and was significantly less for Colorado potato beetle larvae fed on MSE75.7 or ND75.3 compared to MSE149-5Y or ND5873-15.

The development for Colorado potato beetle larvae was monitored over 56 d. Development from first to pre-pupal stage was significantly prolonged for larvae fed on MSE75.7 compared to larvae fed on MSE149-5Y. Significantly fewer larvae survived to adults fed on MSE75.7 or ND75.3 compared to larvae fed on MSE149-5Y or ND5873-15.

The development for potato tuberworm larvae was monitored over 28 d. Mortality of potato tuberworm larvae fed on the MSE149-5Y did not differ significantly from the mortality of larvae fed on the MSE75.7 or ND5873-15. Mortality (98% ± 9 S.E.) of larvae fed on ND75.3 was significantly higher than larvae fed on MSE149-5Y.

Avidin-based resistance, alone or in combination with other natural or engineered host plant resistance factors, may be a useful in managing insect pests.

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GENERAL INTRODUCTION

Potato, *Solanum tuberosum* L.

Potato, *Solanum tuberosum* L., is an important crop worldwide, ranking fourth in production and cash value following wheat (*Triticum aesitvum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.). It is cultivated in three-fourths of the world with 18.7 million hectares grown annually (Anon 1984, Anon 2006a). In 2005, 440,000 hectares were harvested in the US alone, with a farm gate value of $2.3 billion dollars (Anon 2006b). The importance of potatoes is increasing due to the rising world population and the capability of potato to grow well in adverse conditions (Anon 1984). Potatoes offer great nutritional value with high caloric content, high quality protein, many critical vitamins, such as vitamin C, minerals, such as potassium, and trace elements necessary for the human diet. Potatoes are rich in antioxidants that are associated with many health benefits, including lower incidences of heart-disease, and reductions in some types of cancers, macular degeneration, and cataracts (Brown 2005).

Morphology

The cultivated potato, *S. tuberosum,* is in the subgenus *Pachystemonum* and section *Tuberium*. It is a perennial plant that has pinnately compound leaves, with 7-9 ovate leaflets (Howard 1970) (Figure 1.1). The flowers are complete and about 4 cm diam.; the flower color varies from white to pink or blue (Cutter 1992). The anthers are typically bright yellow. Potato is principally self-pollinated, but cross-pollination can occur in nature. Bumblebees, and to a lesser extent the wind, are the chief means of

cross-pollination (Sanford and Hanneman 1981). A number of potato cultivars are not able to produce fruit due to failure to flower, male sterility, or other factors. The fruit is a small green berry. Sexual reproduction is mostly used for crop improvement rather than commercial production of potatoes (Dean 1994). "Seed" potatoes (small tubers or cut tubers) are used for commercial production

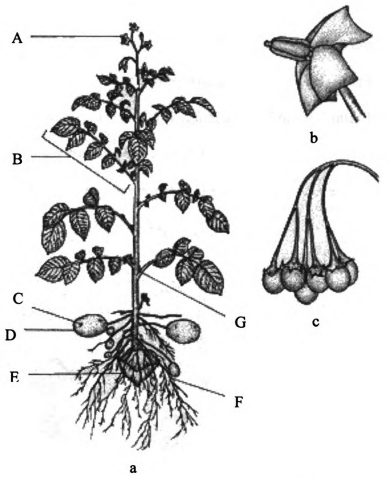

Figure 1.1: An illustration of a potato plant: (a) entire potato plant (A) flower (B) compound leaf (C) "eye" (D) tuber (E) seed potato (F) roots (b) flower (c) fruit (Schumann 1991)

Although many lay people consider the tuber a root, it is actually a swollen stem, called a stolon, which grows underground. The tuber is formed due to the translocation and storage of carbohydrates (Artschwager 1924). Typically, seed potatoes (whole or cut

tubers) rather than true seed from fruit are planted for commercial production (Dean 1994). True seed is heterozygous and extremely variable, while seed potatoes are clonally propagated and therefore, genetically identical. Disease transmission (fungi, bacterium and viruses) is the major disadvantage of the use of seed potatoes compared with the use of true seed; disease transmission is a huge concern for commercial growers (Struik and Wiersema 1999, Schumann 1991).

Potato has five growth stages (Fig. 1.2). In stage I, the tubers break dormancy and sprouts grow from the eyes of the seed potato toward the surface of the soil. All the energy required for this stage is contained within the seed piece (Cutter 1992). At stage II, the potato plant undergoes vegetative growth that includes leaves, vines, roots and stolons. The plant is no longer dependent on the seed piece for energy and acquires energy from photosynthesis (Dean 1994).

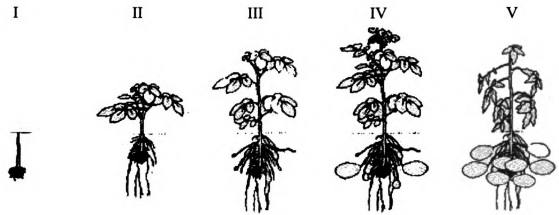

Figure 1.2: The five developmental stages of a potato plant. Stage (I) Sprout development (II) Vegetative growth (III) Tuber initiation (IV) Tuber Bulking (V) Tuber maturation (Anon 2004)

The duration of stage I – II is generally between 30-70 days depending on planting date, variety, environment, and climate (Anon 2004). At stage III, the stolons begin to swell,

marking the initiation of tuber formation. At stage IV, the tuber cells continue to swell and accumulate water, nutrients, and carbohydrates (Dean 1994). The tuber bulking stage is the longest, lasting up to 3 months (Cutter 1992). At stage V, the potato plant begins to senesce, evident by yellowing leaves. At this stage, the tubers are mature with thickened and hardened skin and can be harvested with little damage (Anon 2004).

Origin

The potato belongs to the family *Solanaceae* and genus *Solanum*. The genus *Solanum* contains over 2,000 species, including 199 tuber-bearing species with only of these seven species cultivated (Spooner and Hijmans 2001). Wild potato species are dispersed throughout the Americas, including the southwestern United States, Mexico, Central America, and South America (Hijmans and Spooner 2001) (Fig. 1.3). Due to the extensive natural distribution, potato has also adapted to a wide range of climates. The two major epicenters of potato diversity are located in (1) the southwestern United States and central highlands of Mexico and (2) the Andean highlands of South America, including Bolivia, Ecuador and Peru (Fig. 1.3) (Spooner et al. 2005a). Primitive landraces are distributed throughout the Andes from Venezuela to Chile. Chilean landraces, *S. tuberosum* subsp. *tuberosum,* are derived from Andean landraces, *S. tuberosum* subsp. *andigenum* (Hawkes 1990). Based on chloroplast DNA and archeological evidence, it is believed that Andean potatoes were initially domesticated near Lake Titicaca at the borders of Peru and Bolivia around 7000 B.C.E. (Hosaka 2003, Hawkes 1990). Spaniards introduced potatoes to Europe around 1570. *Solanum* species vary in ploidy level, from diploids ($2n = 2x = 24$) to hexaploids ($2n = 6x = 72$), with

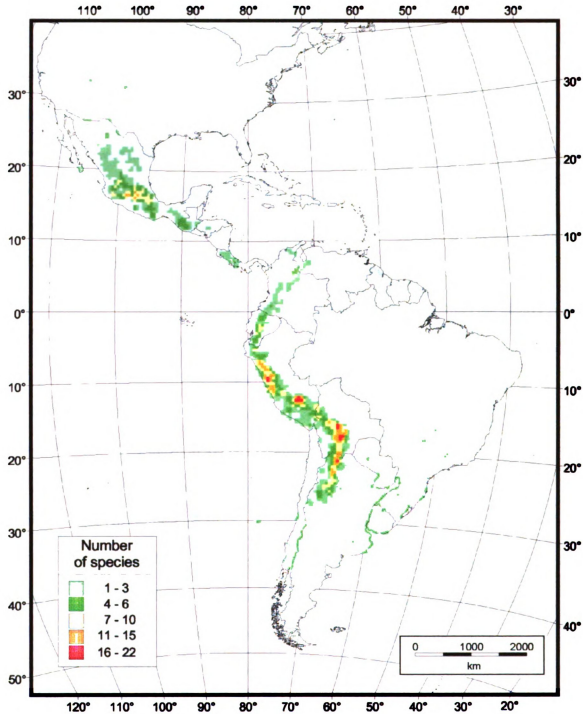

Figure 1.3: Distribution of wild potatoes (Image is presented in color) (Hijmans and Spooner 2001)

about 75% of the species are diploids (Hawkes 1994). The cultivated potato *Solanum tuberosum* is an autotetraploid ($2n = 4x = 48$).

The origin of early introductions of potato, either the Andes or Chile, has been debated for the last century. The Russian botanists, Juzepczuk and Bukasov, believed European potatoes were derived from Chilean landraces (*S. tuberosum* subsp. *tuberosum*) because they were adapted to long day conditions similar to the day length conditions in Europe (Hawkes 1990). Historical and scientific evidence contradicts Juzepczuk and Bukasov's conclusion. First, there was no direct route from Chile to Spain until 1579; seed potatoes would not likely survive the time-consuming and arduous journey (Salaman 1946). Second, early herbarium specimens of European potato are clearly from Andean landraces (*S. tuberosum* subsp. *andigenum*); in addition, descriptions suggest the first introductions were short-day adapted, tuberizing in November and December (Salaman and Hawkes 1949). Simmonds (1968) demonstrated that short day South America source could adapt to long day conditions after two generations of selection; with further selection, the leaf size increased from Andean landraces to large leaves similar to leaves of Chilean landraces.

The initial introductions, in part, may have been Andean landraces originating from Peru or Columbia that subsequently adapted to long-day conditions, converting to types similar to Chilean landraces (Glendinning 1968). The chloroplast DNA of relic cultivars related to the first European potatoes is similar to that of chloroplast DNA of Andean landraces, further indicating that the first European potatoes originated from Peru (Hosaka and Hanneman 1988). The most recent molecular evidence, however, supports Juzepczuk and Bukasov's original hypothesis that European potatoes were

derived from Chilean landraces (Spooner et al. 2005b). Potato was introduced to India from Europe and Indian varieties still include remnants of the germplasm from the early introduction to Europe (Swaminathan 1958). From microsatellite DNA analysis, the Indian landraces are more closely related to the Chilean landraces than to Andean landraces (Spooner et al. 2005b). However, a number of Indian landraces lacked chloroplast DNA similar to Chilean landraces, suggesting early introductions of potatoes maybe from both the Andes and Chile (Spooner et al 2005b). Moreover, Chilean landraces were likely the predominant cultivar in Europe prior to 1840 (Spooner et al. 2005b).

Endosperm Balance Number Hypothesis

Germplasm diversity is one of the greatest attributes of potatoes. Breeders typically access new genetic resources through traditional crossing of plants. Incompatibility between species can limit access to a number of important species with important traits such as disease and insect resistance. The endosperm balance number hypothesis was developed to predict successful interspecific and interploidy crosses of *Solanum* species (Peloquin et al. 1989). The endosperm balance number represents a species effective ploidy level and is not indicative of the actual ploidy level. The assignment of an endosperm balance number value is based on the *Solanum* species crossability to standard tester species (Johnston and Hanneman 1980, Carputo et al. 2003). Endosperm balance number values range from 1 to 4 depending on ploidy level. Diploid species have an endosperm balance number value of either 1 or 2; triploid species have an endosperm balance number value of 2; tetraploids have an endosperm balance

number value of either 2 or 4; pentaploids have an endosperm balance number value of 4. Doubling the ploidy level of a diploid species also doubles the endosperm balance number value. Ploidy levels are manipulated by producing 2*n* gametes that be attained by crossing the species with *S. phureja* (Ross 1986). Species with the same endosperm balance number value cross freely (Fig. 1.4). For viable seed development, the ratio of maternal to paternal endosperm balance number must be 2 to 1 (Hawkes 1994).

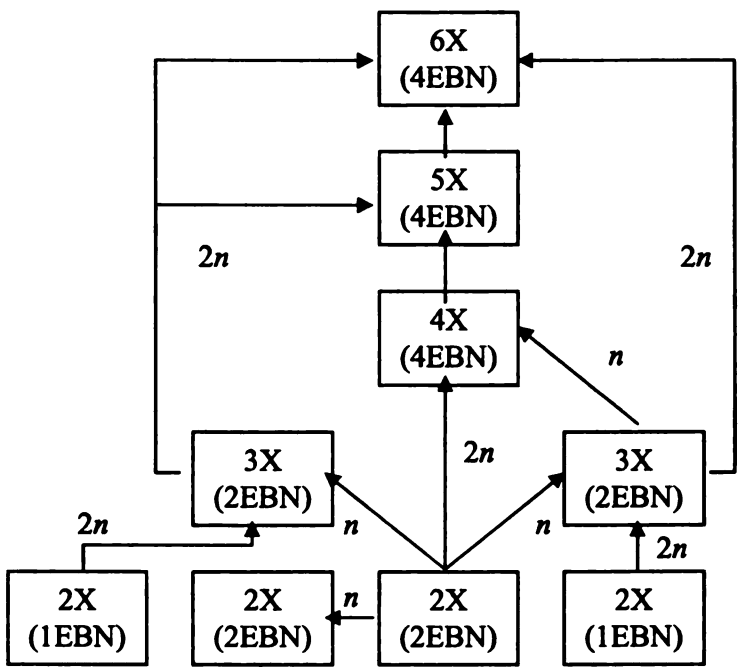

Figure 1.4: Endosperm balance number (EBN) hypothesis: Intraploid crosses are likely to produce viable seed when the EBN ratio is 2EBN female: 1EBN male adapted from Carputo et al. 2003)

Endosperm balance number and ploidy barriers are primarily overcome by using 2*n* gametes. A 2*n* gamete results from genes alter normal meiosis, causing the chromosome number to double in the gamete. The mechanisms for 2*n* gamete formation are: premeiotic doubling, first-division restitution, chromosome replication during the meiotic interphase, second-division restitution, postmeiotic doubling, and apospory; the most common mechanisms in potato are first-division restitution and second-division

restitution (Fig. 1.5) (Peloquin et al. 1989). First-division restitution and second-division restitution are the most common methods of 2n gamete formation in potato. First-division restitution is a result of the failure of spindle formation leading to the nuclear membrane reforming around the chromosomes without movement to opposite poles during meiosis I. Second-division restitution is a result of failure of the cell plate to form during meiosis II.

First-division restitution via parallel or fused spindles is most useful for potato breeders because the progeny contains about 80% of the heterozygosity of the parent (Hermsen 1984). The endosperm balance number value of a 2*n* gamete will be the same value as the parent, allowing for intro-endosperm balance number crosses (Ehlenfeldt and Hanneman 1984). The uses of 2*n* gametes have allowed potato breeders to access wild diploid species.

Natural Resistance Factors

The genus *Solanum* has tremendous natural diversity, including a large number of natural host plant resistance factors to insect pests (Tables 1.1 – 1.5). The two most commonly exploited host plant resistance factors in *Solanum* are glandular trichomes and glycoalkaloids.

Glandular Trichomes

The wild species, *Solanum berthaultii* Hawkes, *Solanum polyadenium* Greenm, and *Solanum tarijense* Hawkes, are resistant to aphids and leaf hoppers, largely due to glandular trichomes on the surface of the plant (Fig. 1.6) (Gibson 1971). Glandular trichomes are hair-like structures and there are two types of trichomes: A and B. Type A

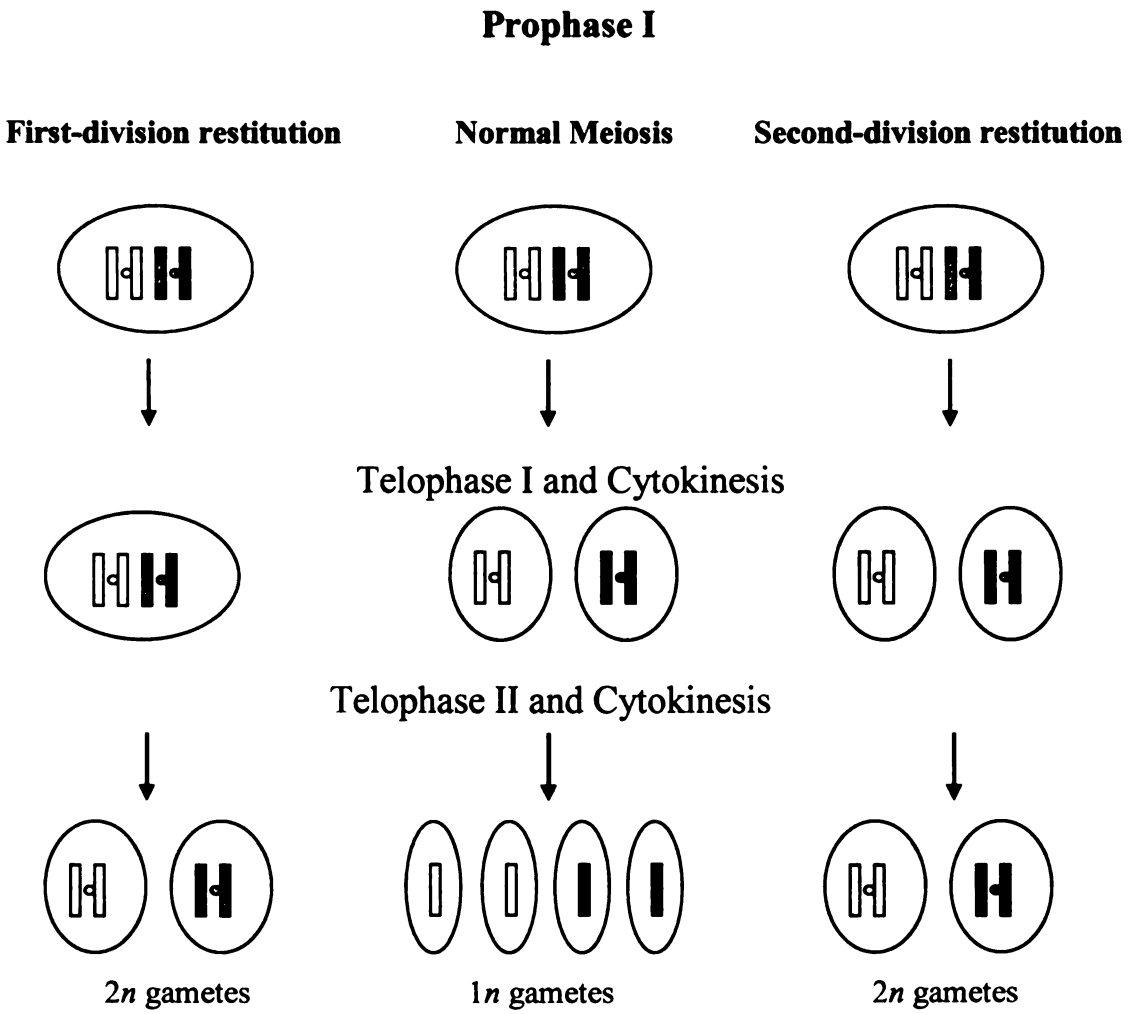

Figure 1.5: Development of 2n gametes.

provides greater insect resistance than type B. Type A trichomes have a four-lobed gland at the apex (Kowalski et al. 1992). As small insects traverse the leaf, the glands rupture releasing an exudate, which oxidizes in air and hardens, trapping the insects (Dimock and Tingey 1987). Type B trichomes have an ovoid gland that continuously discharges clear, more viscous exudates, which act as repellent against aphids and other small insects (King et al. 1987). Trichomes also release sequiterpenes that inhibit insect feeding (King et al. 1988).

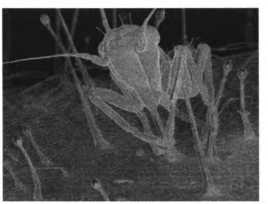

Figure 1.6: Entrapment of a first instar potato leafhopper by exudate from type B glandular trichomes vied by scanning electron microscopy. The glands adhere to various parts of the nymphs (Ranger and Hower 2001)

Glycoalkaloids

The cultivated potato naturally produces glycoalkaloid compounds, which can deter insect feeding (Sinden et al. 1986; Sinden et al. 1980). High glycoalkaloid levels are useful host plant resistance factors, but they impart a bitter taste in the tuber and induce nausea and vomiting in mammals at high concentrations (Sinden and Webb 1972; Van Gelder 1990). Most glycoalkaloids are distributed throughout the potato plant, in tubers and foliage. However, *Solanum chacoense* Bitter, a wild relative of potato, produces novel glycoalkaloids called leptines that are expressed only in the foliage (Lorenzen et al.

Table 1.1 Natural insect resistance sources in *Solanum* to green peach aphid *Myzus persicae* (Sulzer), and potato aphid, *Macrosiphum euphorbiae* (Thomas) (continued on p. 13)

Name	2*n* =	Endosperm Balance Number	Reference
S. berthaultii Hawkes	24	2	Casagrande 1982
S. brachistotrichum Bitter	24	1	Mndolwa et al. 1984
S. bukasovii Juz.	24	2	Mndolwa et al. 1984
S. bulbocastanum Dunal	24	1	Mndolwa et al. 1984
S. canasense Hawkes	24	2	Mndolwa et al. 1984
S. chancayense Ochoa	24	1	Mndolwa et al. 1984
S. chomatophilum Bitter	24	2	Mndolwa et al. 1984
S. etuberosum Lindl.	24	1	Mndolwa et al. 1984
S. hjertingii Hawkes	48	2	Mndolwa et al. 1984
S. hougasii Correll Madroño	72	4	Mndolwa et al. 1984
S. infundibuliforme Phil.	24	2	Mndolwa et al. 1984
S. jamesii Torr.	24/36	1	Mndolwa et al. 1984
S. lignicaule Vargas	24	1	Mndolwa et al. 1984
S. marinasense Vargas	24	2	Mndolwa et al. 1984
S. medians Bitter	24	2	Mndolwa et al. 1984
S. multidissectum Hawkes	24	2	Mndolwa et al. 1984
S. phureja Juk. and Buk.	24	2	Mndolwa et al. 1984
S. sanctae-rosae Hawkes	24	2	Mndolwa et al. 1984
S. stoloniferum Schlecht. and Bouche	48	2	Mndolwa et al. 1984

Table 1.1 Natural insect resistance sources in *Solanum* to green peach aphid *Myzus persicae* (Sulzer), and potato aphid, *Macrosiphum euphorbiae* (Thomas) (continued from p. 12)

Name	2*n* =	Endosperm Balance Number	Reference
S. tarijense Hawkes	24	2	Mndolwa et al. 1984
S. toralapanum Cárdenas and Hawkes	24	2	Mndolwa et al. 1984
S. trifidum Correll	24	1	Mndolwa et al. 1984
S. tuberosum L. *ssp. andigena*	48	4	Mndolwa et al. 1984
S. tuberosum L. *ssp. stenotomum*	48	2	Mndolwa et al. 1984
S. verrucosum Schltdl.	24	2	Mndolwa et al. 1984

Table 1.2 Natural insect resistance sources in *Solanum* to potato tuberworm, *Phthorimaea operculella* Zeller

Name	2*n* =	Endosperm Balance Number	Reference
S. berthaultii Hawkes	24	2	Chavez et al. 1988, Malakar and Tingey 1999
S. commersonii Dunal	24/36	1	Chavez et al. 1988
S. sparsipilum Bitter	24	2	Chavez et al. 1988
S. sucrense Hawkes	48	2	Chavez et al. 1988
S. tarijense Hawkes	24	2	Chavez et al. 1988

Table 1.3 Natural insect resistance sources in *Solanum* to Colorado potato beetle, *Leptinotarsa decemlineata* (Say) **(continued on p. 15)**

Name	2*n* =	Endosperm Balance Number	Reference
S. acroglossum Juz.	24	2	Carter et al. 1987, Flanders et al. 1992
S. berthaultii Hawkes	24	2	Pelletier et al. 2001
S. caldasii Humb. and Bonpl.	24	?	Torka et al. 1950
S. canasense Hawkes	24	2	Carter et al. 1987, Flanders et al. 1992
S. capsicibaccatum Cárdenas	24	1	Pelletier et al. 2001
S. cartarthrum Bitter	?	?	Torka et al. 1950
S. chacoense Bitter	24	2	Sikinyi et al. 1997
S. chomatophilum Bitter	24	2	Carter et al. 1987
S. circaeifolium Bitter	24	1	Carter et al. 1987
S. commersonii Dunal	24/36	1	Torka et al. 1950
S. demissum Lindl.	72	4	Carter et al. 1987, Flanders et al. 1992
S. immite Dunal	?	?	Carter et al. 1987, Flanders et al. 1992
S. jalcae Ochoa	?	?	Carter et al. 1987, Flanders et al. 1992
S. jamesii Torr.	24/36	1	Pelletier et al. 2001
S. lycopersicoides Dunal	24	1	Carter et al. 1987, Flanders et al. 1992
S. macolae Buk.	24		Torka et al. 1950
S. megistacrolobum Bitter	24	2	Carter et al. 1987, Flanders et al. 1992
S. milanii Pet.	?	?	Torka et al. 1950

Table 1.3 Natural insect resistance sources in *Solanum* to Colorado potato beetle, *Leptinotarsa decemlineata* (Say) **(continued from p. 14)**

Name	2*n* =	Endosperm Balance Number	Reference
S. neocardenasii Hawkes	24	?	Dimock et al. 1986
S. okadae Hawkes	24	2	Pelletier et al. 2001
S. oplocense Hawkes	48, 72	4	Pelletier et al. 1999
S. spegazzinii Bitter	24	2	Carter et al. 1987, Flanders et al. 1992
S. tarijense Hawkes	24	2	Pelletier et al. 2001
S. trifidum Correll	24	1	Sikinyi et al. 1997

Table 1.4 Natural insect resistance sources in *Solanum* to potato leaf hopper, *Empoasca fabae* (Harris)

Name	2*n* =	Endosperm Balance Number	Reference
S. agrimonifolium Rydb.	48	2	Tingey and Plaisted 1976, Flanders et al. 1992
S. berthaultii Hawkes	24	2	Flanders et al. 1992, Tingey and Yencho 1994
S. brachycarpum Correll	72	4	Flanders et al. 1992, Tingey and Yencho 1994
S. demissum Lindl.	72	4	Flanders et al. 1992, Tingey and Yencho 1994
S. etuberosum Lindl.	24	1	Flanders et al. 1992, Tingey and Yencho 1994
S. polyadenium Greenm	24	1	Flanders et al. 1992, Tingey and Yencho 1994

Table 1.5 Natural insect resistance sources in *Solanum* to potato flea beetle, *Epitrix cucumeris* (Harris)

Name	2*n* =	Endosperm Balance Number	Reference
S. alandiae Cárdenas	24	2	Tingey and Yencho 1994
S. berthaultii Hawkes	24	2	Tingey and Sinden 1982, Tingey and Yencho 1994
S. bulbocastanum Dunal	24	1	Tingey and Yencho 1994
S. lignicaule Vargas	24	1	Tingey and Yencho 1994
S. marinasense Vargas	24	2	Tingey and Yencho 1994
S. megistacrolobum Bitter	24	2	Tingey and Yencho 1994
S. microdontum Bitter	24	2	Tingey and Yencho 1994
S. mochicense Ochoa	24	1	Tingey and Yencho 1994
S. pampasense Hawkes	24	2	Tingey and Yencho 1994
S. polyadenium Greenm	24	1	Tingey and Yencho 1994
S. polytrichon Rydb.	48	2	Tingey and Yencho 1994
S. sanctae-rosae Hawkes	24	2	Tingey and Yencho 1994
S. stoloniferum Schlecht. and Bouche	48	2	Tingey and Yencho 1994
S. toralapanum Cárdenas and Hawkes	24	2	Tingey and Yencho 1994

2001). Although leptines have not been introgressed into any current commercial cultivars, leptines could provide protection from foliar pests and alleviate the human health concern associated with high glycoalkaloid content in the tuber (Sinden et al. 1986). Due to human health issues associated with glycoalkaloids, the industry has limited tuber glycoalkaloids levels to 20mg/100g of fresh tissue for newly released cultivars (Van Gelder 1990). North Dakota State University recently released a cultivar, Dakota Diamond (ND5822C-7), with reported insect resistance attributed to glycoalkaloids.

Genetic Engineering

Genetic engineering is a relatively new tool to plant breeding that may lead to improvements to potato. Individual genes or a cassette of genes can be inserted into the genome of a plant via genetic engineering. Plant breeders have potential access to genes from any organism in any kingdom. Additionally, genetic engineering also allows for the reintroduction of individual potato genes. *Solanum* has immense diversity with many beneficial traits, including natural resistance to pests. Unfortunately, many resistant species are not readily accessible to breeders using traditional breeding techniques due to issues with endosperm balance numbers and incompatibility (Tables 1.1 – 1.5). Furthermore, wild weedy potato relatives possess beneficial genes, but these genes are often masked and/or difficult to remove from the wild background. Even if crosses can be performed between domesticated potato and wild relatives, multiple generations of backcrossing are usually required to remove undesirable traits. Although a great deal of work is required, important genes can be identified and cloned from a wild *Solanum*

species. If an important gene is cloned, it can be inserted and expressed into a number of elite potato lines relatively easily via genetic engineering compared to traditional breeding methods.

Potato late blight, *Phytophthora infestans* L., is among the most important pests of potato. Natural resistance factors exist within wild *Solanum* species. For example, *S. bulbocastanum* is highly resistant to late blight. Recently, a late blight resistant gene from *S. bulbocastanum* was cloned (Ballvora et al. 2003, Song et al. 2003). The resistant gene, *RB,* was inserted into a susceptible cultivar, cv. Katahdin, conferring resistance to late blight in the transformed plants (Song et al. 2003). This example demonstrates the great potential of genetic engineering. Prior to the use of genetic engineering techniques, plant breeders did not readily have access to this resistance source. *S. bulbocastanum* is a diploid (2x = 24) species with a endosperm balance number of 1; therefore *S. bulbocastanum* must be crossed with a bridging species before the genome can be introgressed into *S. tuberosum* (Hawkes 1994).

Late blight resistance from *S. bulbocastanum* was transferred to cultivated potato through the use of somatic hybrids (Helgeson et al. 1998). Leaf cells of the diploid *S. bulbocastanum* and the tetraploid *S. tuberosum* were fused via PEG-mediated fusion, producing a hexaploid somatic hybrid (Helgeson et al. 1998). The somatic hybrids were crossed with susceptible potato cultivars and retained a high level of late blight resistance (Helgeson et al. 1998). Through genetic engineering, the resistant gene can be incorporated into many different potato cultivars relatively easily avoiding undesirable traits. Even in cases where sexual incompatibility is not an issue, genetic engineering can shorten breeding time because extensive backcrossing is not needed to remove unwanted

traits of the wild parent or to pyramid and combine traits for durable host plant resistances or for resistance to multiple pests.

Transformation

Potatoes were among the first successful transgenic crop plants (An et al. 1986). Potato transformation is achieved by a number of methods and can be directed to either the nuclear or plastid genome. Electroporation and *Agrobacterium tumefaciens* - mediated techniques are used to incorporate genes into the nuclear genome, while biolistic methods are used for the incorporation of genes into both the nuclear and plastid genome (Daniell et al. 1998, Huang et al. 2002, Maliga 2004, Nguyen et al. 2005). *A. tumefaciens* -mediated transformation is the predominant method currently used in potato.

Agrobacterium tumefaciens

Crown gall disease affects dicotyledonous plants, particularly members of the Rosae family. The disease is characterized by large tumors forming at the crown of the plant just above the soil surface. The causative agent is a soil-borne bacterium, *Agrobacterium tumefaciens* Smith and Townsend (Smith and Townsend 1907). In the mid-1970s, researchers determined that a large plasmid, named tumor-inducing or Ti plasmid, is essential for *A. tumefaciens* to be virulent (Van Larebeke et al. 1974). Moreover, a segment of DNA from the Ti plasmid is transferred into the nuclear DNA of the tumor plant cells (Chilton et al. 1980). The segment of DNA transferred from the Ti plasmid is named Transfer DNA or T-DNA.

The Ti plasmid contains genes that assist in integrating T-DNA into the nuclear DNA of the plant host; the set of genes are called Virulence (*vir*) genes. Wounded plants release phenolic compounds, like acetosyringone, that stimulate the *vir* genes (Hoekema et al. 1983). VirA and VirG recognize phenolic compounds, such as acetosyringone; VirA autophosphorylates in the presence of sugar and phenolic compounds (Pan et al. 1993). VirA then transfers the phosphate group to VirG (Jin et al. 1993). After VirG is activated, it increases the transcription of the *vir* genes (Fullner et al. 1996). VirD1/D2 is expressed and nicks the dsDNA in the right border of the T-DNA on the Ti plasmid (Dürrenberger et al. 1989). VirD1 and VirE bind to the single stranded T-DNA to stop it from annealing to itself. In addition, VirD1 and VirE protect the single stranded T-DNA from degradation (Zupan et al. 1996). VirB complex form a pilus of the bacterium to infect the plant cell (Zupan et al. 1998). VirD1 and VirE guide the single stranded T-DNA into the plant genome. VirD2 integrates the T-DNA into the plant genome (Tinland et al. 1995).

The T-DNA contains two types of genes: oncogenic and opine synthesis. Oncogenic genes cause the production of auxins and cytokinins, resulting in tumor formation (Guadin et al. 1994). Opines are critical carbon and nitrogen sources for survival of the bacterium, but cannot be synthesized by the bacterium itself. By inserting the genes for opine synthesis into the plant genome, the bacterium uses the plant machinery to manufacture opines (Hooykass and Beijersbergen 1994). Outside the T-DNA region, the Ti plasmid contains genes that code for proteins that allow the bacterium to break down opines (Hooykass and Shilperoot 1992).

A. tumefaciens -mediated transformation exploits this natural phenomenon to insert foreign genes from various organisms into plants. To use this system for genetic engineering, the tumor-causing genes within the Ti plasmid are removed so that infected cells can produce fertile plants (Bottino et al. 1989). The two essential elements for gene insertion are: border repeats of the T-DNA region and the *vir* genes. Co-integrated and binary vectors incorporate these two elements using slightly different approaches. The co-integrated vector system contains the *vir* genes, the border repeats of the T-DNA, and the gene of interest on one plasmid; often the co-integrated vector is large and inefficient (Hoekema et al. 1983). The binary vector system contains two plasmids; one plasmid contains the T-DNA with the gene of interest; the second plasmid contains the *vir* genes for insertion (Hoekema et al. 1983). The binary system increases efficiency and allows for easier manipulation of the plasmids compared to the co-integrated vector system.

Genes of Interest: Insect resistance

Altering natural resistance

Potatoes naturally produce many compounds, such as glycoalkaloids, that are associated with insect resistance (Sinden et al. 1980). Pathways can be manipulated with genetic engineering by altering the expression of enzymes within the pathways. For example, high glycoalkaloid levels inhibit insect feeding, but also impart a bitter taste in the tuber and can cause nausea and vomiting in mammals (Sinden and Webb 1972, Van Gelder 1990). Glycoalkaloids are distributed throughout the entire potato plant, both tuber and foliage. The predominant glycoalkaloids in potatoes are α–solanine and α-chaconine, constituting 95% of total glycoalkaloids and also causing the majority of

toxicity of potatoes (Potus and Adrain 1995). α–solanine and α-chaconine have the same aglycone precursor, solanidine, but differ in addition of glycosyl residues, galactose (α–solanine) or glucose (α-chaconine). The enzyme UDP-galactose solanidine glycosyltransferase (SGT) transfers a galactose to the solandine forming the intermediate γ-solanine; UDP-glucose solanidine glycosyltransferase transfers a glucose forming the intermediate γ-chaconine (Zimowski 1991). The *sgt1* gene has been cloned and developed into an anti-sense RNA transgene (McCue et al. 2005). The anti-sense *sgt1* gene stops the conversion of solandine to γ-solanine, thereby reducing the levels of α–solanine. While the integration of *sgt1* into potatoes inhibited the production of α-solanine accumulation, the levels of α-chaconine were elevated, compensating for the reduction of α-solanine, resulting in similar level of total glycoalkaloids in the wild type and transgenic potato lines (McCue et al. 2005).

Bacillus thuringiensis derived – Crystal proteins

Bacillus thuringiensis-Crystal (Cry) proteins are the most well studied class of insecticidal proteins. They are derived from the soil-borne bacterium *Bacillus thuringiensis* Berliner; transgenic plants that contain *Bt*-Cry genes are commonly referred to as *Bt* plants in popular publications (Slaney et al. 1992). During sporulation, *B. thuringiensis* produces Cry protein inclusions that only dissolve at a specific pH inside the insect's midgut, converting the inclusion into a δ-endotoxin by proteolytic cleavage (Kaur 2000). The δ-endotoxin binds to receptors in the brush-border membranes of the gut epithelium cells creating a pore in the membrane. This pore disrupts the osmotic balance and eventually causes the cell to swell and lyses leading death (Whalon and

Wingerd 2003). Due to specificity of gut pH and membrane receptors, *Bt*-Cry proteins are highly specific to individual insect orders; additionally, *Bt*-Cry proteins are non-toxic to mammals (Ferre and Van Rie 2002).

Strains of the *B. thuringiensis* have been formulated for use as a foliar spray for many decades, but *Bt* sprays provide limited protection because it is photosensitive and degrades quickly compared to most insecticides (Whalon and Wingerd 2003). To increase efficiency, genes coding for *Bt*-Cry proteins have been inserted into many agricultural crops; the resulting plants express *Bt*-Cry proteins constantly in their tissue alleviating problems associated with foliar applications. The specificity of *Bt*-Cry proteins allows plant breeders to target a single insect pest and not kill most beneficial insects; on the other hand the specificity does not provide a wide range of protection (Ferre and Van Rie 2002). *Bt*-Cry3A, from *B. thuringiensis* subsp. *tenebrionis*, targets coleopteran pests and is effective against Colorado potato beetle; *Bt*-Cry1Ia1, from *B. thuringiensis* subsp. *kurstaki,* targets lepidopteran pests and is effective against potato tuberworm (Perlak et al. 1993; Douches et al. 2004) Recently, chimeric Bt genes have been engineered to broaden the range of pests affected (Naimov et al. 2003, Singh et al. 2004, Chen et al. 2006).

Vegetative insecticidal proteins (Vips)

Vegetative insecticidal proteins (Vip) are less well known than Cry proteins, but may be active against a wider range of insects (Estruch et al. 1996). Vip proteins are derived from *B. thuringiensis* or *Bacillus cereus* Frankland and Frankland (Sharma et al. 2002). As previously mentioned, *Bt*-Cry proteins are produced during sporulation, while

Vip proteins are produced in the secreted supernatant fluids collected during the vegetative growth stage prior to sporulation, making them distinctly different proteins (Estruch et al. 1996). Vip3A, isolated from *B. thuringiensis*, is active against a variety of Lepidoptera (Estruch et al. 1996). Vip1 and Vip2, isolated from *B. cereus*, are toxic to Coleoptera (Estruch et al. 1996; Moellenbeck et al. 2001). Unlike *Bt*-Cry proteins, the solubility of Vip3A is not highly affected by the insect gut pH allowing the toxin to have a broader range of insecticidal activities (Yu et al. 1997). The exact mechanism of Vips is not well understood, but Vips result in the lysing of the gut epithelium cells (Yu et al. 1997, Lee et al. 2005). Although both *Bt*-Cry and Vip toxins lyse the cells, the membrane receptors and pH requirement are different; therefore the development of cross-resistance between the two classes of toxins is unlikely (Lee et al. 2005).

Novel fusion proteins

Arachnid venom is selective for its prey, which are often insects. Spider toxins are excellent candidate genes for transformation because they are broad spectrum and insect specific, have a unique mode of action, are likely to degrade in the environment, and cannot easily be used as foliar applications because they are not readily absorbed through insect cuticle (Fitches et al. 2004). Three polypeptide toxins from the SFI1 family were recently isolated from venom glands of the spider *Segestria florentina* (Rossi). Moreover, SFI1-SFI8 have recently been cloned and expressed in yeast (Lipkin et al. 2002). Tobacco budworm, *Heliothis virescens* (Fabricus), is paralyzed when injected with SFI1 toxins; adult mice are not negatively affected when injected with the same toxin (Lipkin et al. 2002). The SFI1 family of toxins is a selective

agonist/antagonist of different voltage-dependent Ca^{2+} channels causing flaccid paralysis (Lipkin et al. 2002).

SFI1 is not currently transformed into any crops, but recombinant SFI1 and a fusion protein SFI1/GNA are only expressed in the yeast (Fitches et al. 2004). GNA is a plant lectin; its function as a fusion protein is to deliver SFI1 toxin to the hemolymph of the insects. GNA, SFI1, and SFI1/GNA were isolated and fed to tomato moth, *Lacanobia oleracea* (L.), green-peach aphid, *Myzus persicae* (Sulzer) and rice brown planthopper, *Nilaparvata lugens* (Stål) in artificial diets. GNA or SFI1 alone did not exhibit toxic effects to any larvae, while SFI1/GNA killed 100% of tomato moth and rice brown planthopper and 51% of green peach aphid (Down et al. 2006, Fitches et al. 2004). SFI1 toxins are excellent candidate genes to combine with *Bt*-Cry proteins due to differences in mode of action.

Avidin

Avidin is derived from chicken eggs (*Gallus gallus* L.) and belongs to class of proteins termed biotin-binding (Green 1990, Stevens 1991). Avidin is produced in egg whites; it is a glycoprotein tetramer (67 kDa) with four nearly identical subunits approximately 17kDa. Each subunit of avidin tightly binds with a single molecule of biotin. The avidin-biotin complex has one the strongest bonds found in nature (K_d=10^{-15} M) (Stevens 1991). Biotin, also called vitamin H or B_8, is an essential vitamin for all organisms. It is a cofactor that covalently binds to several carboxylases that serve in many important biosynthetic pathways such as the citric acid cycle, lipogenesis, gluconeogensis, fatty acid and amino acid catabolism (Knowles 1989, Alban et al. 2000).

Although a requirement for all life, biotin synthesis is restricted to plants, many bacteria, and a number of fungi. Therefore, animals, along with many fungi and bacterium, must acquire biotin their diet or environment. Avidin protects the chicken embryo by sequestering the essential biotin from disease causing organisms. Without accessible biotin, the harmful microorganisms cannot perform many essential processes needed for growth and survival (Stevens 1991).

The insecticidal activities of avidin were first discovered in 1959 when it was added to the artificial diet of the housefly (*Musca domestica* L.) (Levinson and Bergmann 1959). A molar excess of avidin in an insect diet causes a deficiency in accessible biotin, resulting in abnormal larval development and even death in a range of insect orders (Morgan et al. 1993, Marwick et al. 2001, Malone et al. 2002). Avidin is an excellent candidate for plant transformation due to its insecticidal properties; in addition it is a single gene product. The gene coding for avidin production has been inserted into a few crops, including maize, tobacco (*Nicotiana tabacum* L.), potato, and rice (*Oryza sativa* L. var. Nipponbare) and confers resistance to a wide spectrum of insect pests (Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Malone et al. 2005, Yoza et al. 2005).

Although all insects require biotin, transgenic plants expressing avidin do not appear to negatively affect non-target insects. The development of newly emerged honeybees, *Apis mellifera* L., was not negatively affected by the addition of avidin to pollen-food (Malone et al. 2002, Malone et al. 2004). Moreover, only 10 – 28% of the avidin was recovered from tobacco cutworms, *Spodoptera litura* Fabricius, feeding on transgenic tobacco plants expressing avidin was able to bind to biotin, suggesting

predators feeding on tobacco cutworms would not be negatively impacted by the avidin within the insect. (Christeller et al. 2005).

Insect Pests

Over 170 arthropods co-exist within the potato ecosystem, but Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and potato tuberworm, *Phthorimaea operculella* Zeller, are among the most destructive. Colorado potato beetle attacks potato crops in North America, Europe and Asia (Radcliffe 1982). The potato tuberworm is typically found in tropical and subtropical climates, but has recently been established in the northwestern United States (Trivedi and Rajagopal 1991, Alvarez et al. 2005).

Colorado potato beetle

Colorado potato beetle's name is deceiving; the beetle neither originates from Colorado nor is the native host plant potato. Colorado potato beetles are native to southern Mexico; early settlers unknowingly imported the beetle into the western United States. In the early 1800s, Colorado potato beetle was noted in the western United States as a colorful beetle that feeds on such noxious weeds as nightshade, *Solanum datura* L. and remained an entomological novelty until the gold rush (Jacques 1988). With the gold rush and westward expansion, farms populated the landscapes and potato fields became abundant (Salaman 1985). The beetles shifted from feeding on nightshade to more succulent and abundant potato leaves. The obscure insect quickly multiplied and became a major pest.

In 1859, Colorado potato beetle destroyed potato fields outside of Omaha, Nebraska (Forgash 1981). By 1865, it was observed east of the Mississippi, it reached Ohio by 1869, and Maine by 1872. Colorado potato beetle led to the first large-scale use of arsenical insecticides, Paris green, in 1864 (Gauthier et al. 1981). Since the onset of arsenical insecticides, pesticides have been primary means to control the pest (Casagrande 1987). Colorado potato beetle is highly adaptable and has consistently adapted to insecticides; it has developed resistance to every chemical insecticide used to control it and is presently resistant to over 40 insecticides (Bishop and Grafius 1996, Whalon et al. 2006). Without control, it will consume all of the plant's foliage and begin feeding on the stem and exposed tubers, as well. As little as 12.5% to 25% defoliation can significantly decrease potato yields (Mailloux et al. 1996). Complete defoliation can reduce potato yields as much as two-thirds (Hare 1980).

Both larvae and adult Colorado potato beetles feed on solancecous plants, including potato, tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melogena* L.), nightshade (*Solanum nigrum* L.), and tobacco (*Nicotiana tabacum* L.) (Jacques 1988). A single adult female can lay as many 300 eggs in four to five weeks (Mailloux et al. 1996). Egg masses generally hatch within 4 to 7 d (Figure 1.7) (Walgenbach and Wyman 1984). Larvae are dark orange with a black head capsule. The larval stage consists of four instars. Depending on the climate, development from first to fourth instar takes about 9 – 34 d (Walgenbach and Wyman 1984). Fourth stage larvae cause the greatest damage due to the need to accumulate energy for pupation. During later stages, toxins and insecticide treatments are less effective (Wierenga et al. 1996). The fourth instar digs 5-10 cm in the

soil and undergoes pupation. After about 10 d, the adult beetles emerge from the soil; within 5 d, adults mate and are able to produce viable eggs (Hare 1990).

Figure 1.7: Stages of the Colorado potato beetle: (A) adult (B) larva (C) pupa (Carter et al. 1996)

The entire life cycle generally lasts between 16 –37 d (Mailloux et al. 1996). In Michigan, Colorado potato beetles normally have one or two generations in a season, but in warmer climates three generations per year can occur.

Potato tuberworm

Potato tuberworm is found worldwide, but is chiefly considered a tropical pest of potato. Recently, it has established itself in the Pacific Northwest. In 2002, potato tuberworm was first reported to cause severe damage to a field near Hermiston, Oregon (Alvarez et al. 2005). Potato tuberworm is not a recently imported pest; it was documented in California in 1855 and Texas in 1917 (Berthon 1855, Graf 1917). Due to mild winters and dry summer conditions, potato tuberworm has expanded its geographic

range to the Columbia Basin (Alvarez et al. 2005). Unfortunately, more farms in the region have suffered from potato tuberworm infestations since 2002.

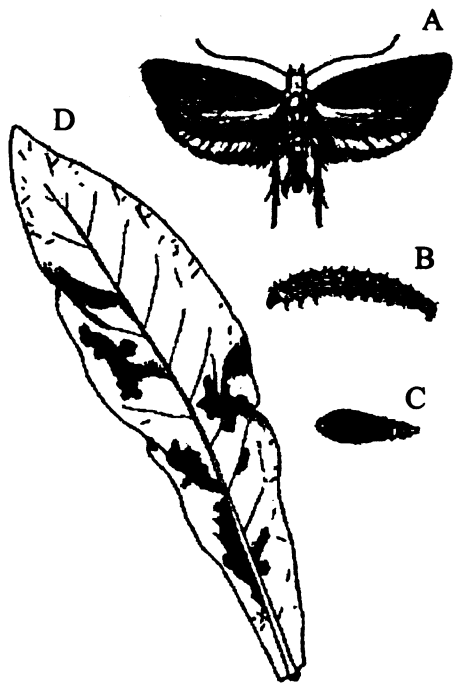

Figure 1.8: Stages of potato tuberworm (A) Adult (B) larva (C) pupa (D) leaf mining damage caused by potato tuberworm larvae (Carter et al. 1996)

Potato tuberworm feeds on solanaceous plants, including potato, tomato, eggplant, nightshade and tobacco (Das and Raman 1994). The larvae mine leaves or bore into potato tubers or tomato fruits; in potatoes, the greatest damage is due to larval mining in storage causing the tubers to rot (Kroschel and Koch 1994). Potato tuberworm typically has two generations in the summer and a third generation in storage in the United States, while potato tuberworm can have multiple generations per year with all stages of larvae and adults found throughout the year in tropical and subtropical climates (Chittenden 1912, Trivedi and Rajagopal 1991).

A single female lays between 60 and 300 eggs (Graf 1917). In warmer temperatures, eggs hatch within 4-6 d. The eggs are white and turn dark brown just prior to hatching. Neonates have a white body with a brown head capsule and are less than 2 mm in length (Fig. 1.8). In warmer climates, the development from first to fourth stage can take between 15 – 17 d; in storage, the larval period can last up to seven months (Graf 1917). Potato tuberworm pupates below the soil in the field. In storage, potato tuberworm makes a cocoon in crevices or near the tuber eye and pupates (Trivedi and Rajagopal 1991). In the field, the pupal stage last 6-9 d (Moregan and Crumb 1914). The adults are weak flyers and predominately active at dawn and dusk (Coll and Yuval 2004).

Potato tuberworm causes significant economic damage. Crop losses due to potato tuberworm have been reported to be 42% of the stored crop in Ethiopia, 70% in India, and 86% in Tunisia (Saxema and Rizvi 1974, Roux et al. 1992, Sileshi and Teriessa 2001). While insecticides remain the chief means to control potato tuberworm, cultural practices can reduce infestation (Coll et al. 2000). Potato tuberworm damage can be abated by storing tubers in moth-free environments at temperatures below 10°C (Sporleder et al. 2004). In addition, planting tubers at a depth of 10 cm significantly reduced damage and infestation compared with 6 cm (Akahde et al. 1970). Moreover, sprinkler irrigation reduces damage compared to the use of furrow irrigation (Shelton and Wyman 1979).

Resistance Management

Deployment of genetically engineered insect resistant crop varieties is a critical issue for the implementation of biotechnology in crop pest management (Gould 1998). The major biological concerns surrounding crops engineered to produce *Bacillus thuringiensis* (Bt) toxins are sustainability and management. Continual exposure to Bt toxins from bio-pesticides, transgenic crops, and laboratory selection has led to the development of resistance in several species (Tabashnik 1994, Ferre and Van Rie 2002). The rate of a pest's resistance development is positively correlated with increasing selection pressure (Tabashnik et al. 1990). Transgenic crops can increase selection pressure compared to foliar sprays of the pesticide toxin by (1) increasing the level of toxin exposure to the pest; (2) producing toxin over a long period in all plant parts (3) increasing acreage of crops expressing Bt (Gould 1998; Hilder and Boulter 1999; Whalon and Norris 1999).

The higher dose of transgenic plants along with the rising acreage of Bt plants using the same or similar toxin can increase selection pressure on an insect pest (Hilder and Boulter 1999). From 1996-2003, Bt cotton acreage has increased from 12% to 75 % in the US and Bt corn acreage has increased from 1% to 40% in the US (Carpenter and Gianessi 2001, NASS Prospective Plantings 2002, Anon. 2003). With acreage of Bt crops increasing, it is important to evaluate methods to preserve Bt and other host plant resistance mechanisms, both natural and novel insecticidal toxins, like avidin.

Mostly, single host plant resistance factors are available commercially (*Bacillus thuringiensis* toxins), so current discussion emphasizes a high dose/refugia model for managing the adaptation of insect pests to resistant crop varieties (Whalon and Norris

1999). According to Shelton et al. (2002), the high dose/refugia model is the "only strategy currently available". Regulations in place for deployment of Bt transgenic crops in the US are based on this model (Anon 2001), but there are serious concerns about the level of compliance (Jaffe 2003). A model combining genetically engineered and traditionally bred host plant resistance has the potential to be more widely adopted and more durable.

Host plant resistance management methods typically fall into one of three categories: (1) maintaining a susceptible insect population through seed mixtures, refuges, and crop rotation; (2) using trap crops to attract pests away from more economically important crops; and (3) combining different toxins assuming the insect is less likely to develop resistance to more than one toxin simultaneously (Neppl 2000). Combined toxins can be employed by combining insecticides with host plant resistance factors or by stacking host plant resistance factors into plants (Mani 1985, Roush 1998, Zhao et al. 2005). Combining multiple resistance factors can delay resistance development exponentially (Roush 1998, Zhao et al. 2005). *Solanum* has immense potential genetic diversity for host plant resistance. In addition to insect resistance through traditional breeding, potato is also amenable to genetic engineering. Potato breeders are in unique position with ability to readily access both natural and engineered host plant resistance for plant protection.

Research Objectives

This research evaluates avidin as a potential host plant resistance factor. The objectives were:

1) Evaluate the efficacy of avidin against Colorado potato beetle larvae.
2) Genetically engineering MSE149-5Y, a susceptible cultivar, and ND5873-15, a naturally resistant cultivar, to express the avidin protein.
3) Evaluate the efficacy of transgenic potato plants expressing avidin against Colorado potato beetle larvae.
4) Monitor the development of Colorado potato beetle larvae exclusively feeding on transgenic potato plants expressing avidin.
5) Evaluate effects of combining avidin and natural host plant resistance to enhance resistance against Colorado potato beetle larvae.
6) Evaluate effects of combining avidin and natural host plant resistance to enhance resistance against potato tuberworm larvae.

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CHAPTER II:

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Insecticidal Activity of Avidin Combined with Genetically Engineered and Traditional Host Plant Resistance Against Colorado Potato Beetle (Coleoptera: Chrysomelidae) Larvae

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ABSTRACT Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a destructive pest of potato, *Solanum tuberosum* (L.), in North America. It is renowned for adapting to insecticides. With the arsenal of effective insecticides decreasing, it is important to consider alternative forms of control. Biotin is an essential coenzyme for insect growth and development. Avidin is a protein found in chicken egg that sequesters biotin and has shown insecticidal properties against a range of insect. We assessed the effectiveness of avidin against the Colorado potato beetle neonates in a no-choice detached leaf bioassay at 0, 17, 34, 51, 102, and 204 μg avidin/ml over 12 d. The LC_{50} was 136 μg avidin/ml (108-188 95% CL). The combined effects of avidin (136 μg avidin/ml) with Bt-Cry3A or leptines were evaluated with neonates and third instars over 12 and 6 d, respectively. Three potato lines were used: susceptible line, a line engineered to express Cry3A from *Bacillus thuringiensis*, and a line expressing the natural resistance factor leptines. The addition of avidin at the LC_{50} concentration significantly reduced consumption by neonates, but it did not affect consumption by third instars feeding on the susceptible line and the leptine line. Survival of neonates feeding on the susceptible line with avidin was significantly reduced compared with the susceptible line. Survival of third instars on the Bt-Cry3A with avidin was significantly reduced after 3 d compared with survival on the Bt-Cry3A, suggesting the addition of avidin may increase susceptibility to Bt-Cry3A.

KEY WORDS avidin, Bt-Cry3A, host plant resistance, leptine, *S. chacoense*

Biotin, also called vitamin H or B_8, is an essential vitamin for all organisms. It is a cofactor that covalently binds to several carboxylases that serve in many important biosynthetic pathways such as the citric acid cycle, lipogenesis, gluconeogensis, and fatty acid and amino acid catabolism (Knowles 1989, Alban et al. 2000). Although biotin is a requirement for all life, biotin synthesis is restricted to plants, many bacteria, and a number of fungi (Alban et al. 2000). Animals, along with many fungi and bacteria, must acquire biotin from outside sources such as diet or environment.

Biotin binding proteins have a strong affinity for biotin, with the strongest noncovalent bond found in nature ($K_d = 10^{-15}$ M) (Izrailev et al. 1997). One of the most well-known biotin binding proteins is avidin from chicken, *Gallus gallus* L. (Green 1990, Stevens 1991). Avidin is produced in egg whites. It is a glycoprotein tetramer (66 kDa) comprised of four nearly identical subunits ≈17 kDa. Each subunit of avidin tightly binds to a single molecule of biotin. Avidin protects the chicken embryo from disease-causing organisms by sequestering the essential biotin. Without accessible biotin, harmful microorganisms cannot perform essential processes needed for growth and survival (Stevens 1991).

The insecticidal activities of avidin were first discovered in 1959 when it was added to the artificial diet of the housefly, *Musca domestica* L. (Levinson and Bergmann 1959). A molar excess of avidin in an insect diet causes a deficiency in accessible biotin, resulting in abnormal larval development and even death in a range of insect orders (Morgan et al. 1993, Malone et al. 2002, Markwick et al. 2003). Avidin is an excellent candidate for plant transformation because it is a single gene product with insecticidal activity. The gene coding for avidin production has been cloned and has been inserted into a few crops, including maize, tobacco, and potato, providing resistance to a wide spectrum of insect pests (Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002). Avidin is safe for consumers because cooking denatures it.

Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is among the most economically significant pests of potatoes, *Solanum tuberosum* spp. *tuberosum* L., in North America, Europe, and western Asia. As little as 12.5% defoliation significantly reduces potato yields; complete defoliation can lead to crop failure (Hare 1980, Mailloux and Bostanian 1989). Colorado

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potato beetle is renowned for development of insecticide resistance with resistance reported to >40 insecticides (Whalon et al. 2005). Therefore, examining novel control strategies may be of great consequence for the management of the Colorado potato beetle.

Compared with many other crops, *Solanum* has immense potential genetic diversity for host plant resistance. Many wild *Solanum* species, including *Solanum berthaultii* Hawkes, *Solanum chacoense* Bitter, *Solanum polyadenium* subsp. *orizabae* Bitter, and *Solanum tarijense* Hawkes, are thought to have genetic traits causing insect resistance (Pelletier et al. 1999). Glycoalkaloids have long been associated with resistance to insects and plant pathogens (Maga 1994). *S. chacoense* produces compounds called leptine glycoalkaloids that confer resistance to Colorado potato beetle resistance (Sinden et al. 1986, Lorenzen et al. 2001). Most glycoalkaloids are distributed throughout the plant, including the tuber. However, high levels of glycoalkaloids in the tuber impart a bitter taste and also may be toxic to humans (Van Gelder 1990). Leptine glycoalkaloids are only expressed in the foliage, conferring insect resistance and also alleviating human health concerns associated with high levels in the tuber (Sinden et al. 1986).

In addition to insect resistance through traditional breeding, potato is also amenable to genetic engineering. Cry toxin genes have been inserted into potato to impart resistance to several insects (Adang et al. 1993, Perlak et al. 1993, Douches et al. 1998, Coombs et al. 2002). Cry toxins are a class of insecticidal proteins from the soil-borne bacterium *Bacillus thuringiensis* Berliner (Bt) (Sharma et al. 2000). Cry proteins are highly specific in activity. Specificity is often limited to individual insect orders and frequently only a few species within an order are affected. The Bt-Cry3A toxin is active against Colorado potato beetle larvae (Adang et al. 1993, Perlak et al. 1993, Coombs et al. 2002).

The objectives of this study were to 1) assess the potential for using avidin in potato for control of the Colorado potato beetle and 2) examine the combined effects of avidin with the natural host plant resistance, leptines, or the engineered resistance, Bt-Cry3A.

Materials and Methods

Determination of LC_{50}. Colorado potato beetle egg masses were obtained from the New Jersey Department of Agriculture's Phillip Alampi Beneficial Insect Rearing Laboratory, West Trenton, NJ. This strain was originally collected in 1983 from potato and eggplant fields in New Jersey and has been continuously reared without exposure to insecticides. Potatoes ('Yukon Gold') were grown under greenhouse conditions. Fully expanded leaves were collected, and then each petiole was immersed in a water-filled vial sealed with Parafilm. Aqueous solutions of avidin (0, 17, 34, 51, 102, and 204 μg avidin/ml) (Sigma, St. Louis MO) were prepared using distilled water and 0.01% Tween 20 (Sigma). Leaves were dipped and air-dried and then individually placed in petri dishes (125 mm in diameter) lined with filter paper. Ten neonates were placed on each leaf for 12 d in a no-choice test. Leaves were replaced with fresh leaves dipped in the same avidin solution as needed. Mortality was assessed every 4 d. Larvae were considered dead if no movement was observed after being lightly touched with a paintbrush. This procedure was replicated four times (40 larvae per avidin concentration). Percentage of mortality was adjusted with Abbott's formula to correct for mortality on untreated foliage (Abbott 1925). The avidin concentrations were log transformed and analyzed with Probit analysis (PROC PROBIT, SAS Institute 2002). The 50% lethal concentration (LC_{50}) along with 95% fiducial limits (FL) was obtained for avidin.

An identical experiment was conducted (same avidin concentrations and methods) with biotin added to counteract the effects of avidin. Aqueous solutions of avidin (0, 17, 34, 51, 102, and 204 μg avidin/ml) (Sigma) with biotin (0, 0.98, 1.96, 2.94, 5.88, and 11.76 μg biotin/ml) (Sigma) were prepared using distilled water and 0.01% Tween 20 (Sigma). Leaves were dipped and air-dried and then individually placed in petri dishes (125 mm in diameter) lined with filter paper. Percentage of mortality was transformed with the arcsine of the square root to homogenize variance. Data were analyzed design using SAS general linear model procedure for analysis of variance (ANOVA) (SAS Institute 2002). Mean comparisons were conducted using Fisher's least significant difference (LSD) test ($\alpha = 0.05$).

Combined Effects of Avidin. The LC_{50} concentration of avidin was used to determine the combined effects of avidin on a natural and engineered resistant host plants. Three potato clones—Yukon Gold, USDA8380-1 (leptine line), and YGc3.12 (Yukon Gold with Bt-Cry3A)—were evaluated in this study. USDA8380-1 was derived from the wild potato *S. chacoense*, which expresses leptines as a natural host plant resistance factor in the foliar tissue of the plant (Lorenzen et al. 2001). The codon-modified-*cry3A* (coleopteran specific) gene used for transformation of Yukon Gold in this study was obtained from John Kemp (New Mexico State University, Las Cruces, NM) (Sutton et al. 1992). The constitutive *ocs*$_3$*mas* promoter (Ni et al. 1995) was used to promote expression of the *Bt-cry3A* gene. The *Bt-cry3A* transgenic potato line was generated using *Agrobacterium tumefaciens*-mediated transformation (Coombs et al. 2002).

The three potato lines were grown under greenhouse conditions. Leaves were collected, petioles were placed in vials of water, as described above, and dipped into: 0.01% Tween 20 (wt:vol) (Sigma), or 136 μg/ml avidin (Sigma) in 0.01% Tween 20. Leaves were air-dried and then individually placed in petri dishes (125 mm in diameter) lined with filter paper. Neonates (10 per leaf) were placed on the detached leaves in a no-choice test. If leaf quality had degraded significantly or a large area of leaf was consumed, the leaf was replaced. Consumption was visually estimated with square millimeter grid paper and recorded for each group of larvae (Coombs et al. 2002). Consump-

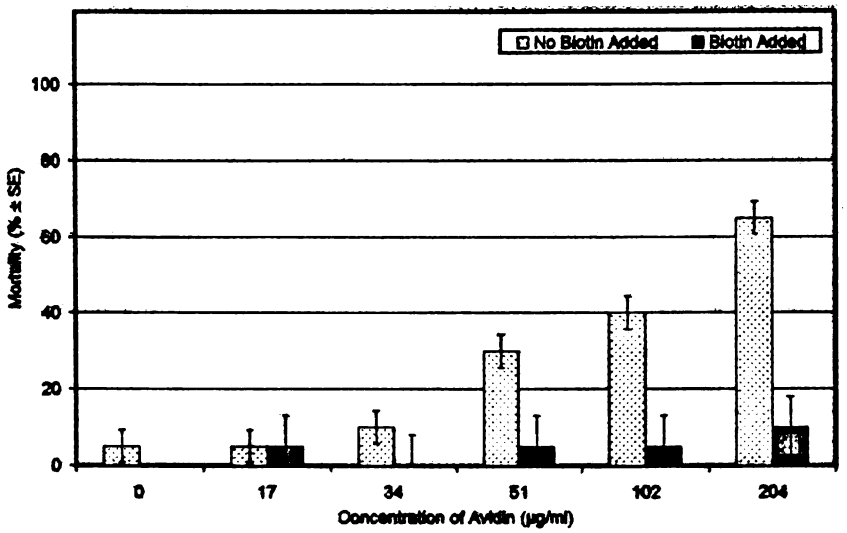

Fig. 1. Mean percentage of dead Colorado potato beetle neonates fed on Yukon Gold dipped in 0, 17, 34, 51, 102, and 204 µg avidin/ml alone or combined with biotin at 4 times the molar concentration of avidin (0, 1.0, 2.0, 2.9, 5.9, and 11.8 µg biotin/ml). The LC_{50} was determined to be 136 µg avidin/ml (108–188 95% CL). There was no significant difference between avidin combined with biotin at any concentration compared with at 0 µg of avidin/ml ($LSD_{\alpha = 0.05}$ = 18.6%).

tion, survival, and biomass of the survivors were measured every 2 d for 12 d. Six replications were performed (60 individuals per potato line × avidin treatment). Percentage of survival was transformed with the arcsine of the square root to homogenize variance. The data sets (consumption, final survivor biomass, arcsine and square root mortality) were analyzed using SAS least-squared means model procedure for a two-factorial design ANOVA, with the factors of potato line and avidin treatment, used to analyze consumption. The means were separated using a pairwise comparison (SAS Institute 2002).

Previous studies have suggested that neonate larvae may be so sensitive to individual resistance factors that combined effects may not be evident; effects of combined resistance strategies may be apparent at the third or fourth instar (Cooper et al. 2004). Therefore, leaf dip bioassays also were performed on third instars to further differentiate resistance strategies. Leaf dip assays were performed similarly to the neonate assays described above. Egg masses were obtained from New Jersey Department of Agriculture and reared in the laboratory on Yukon Gold until the third instar. Five newly molted third instars (within 48 h of molting) were placed on dipped leaves. Leaf tissue was replaced daily. Consumption, survivorship, and biomass of survivors were measured daily for 6 d. Twelve replications were performed (60 individuals per potato line × avidin treatment). The data were analyzed as in the neonate assay (SAS Institute 2002).

Results and Discussion

Determination of LC_{50}. Avidin was toxic to Colorado potato beetle larvae. Colorado potato beetle exhibited a dose–response to avidin in the LC_{50} assay (Fig. 1). At concentrations higher than 102 µg/ml, larvae did not develop past the third instar. Larvae consuming leaves dipped in concentrations of 51 µg/ml or higher of avidin had significantly higher mortality at 12 d than larvae consuming leaves dipped in 0 µg/ml ($F = 18.26$, df = 5, $P < 0.0001$) (Fig. 1). The LC_{50} values was determined to be 136 µg avidin/ml (n = 40, slope = 2.3 ± 0.3, 136 µg avidin/ml (108–188 95% CL), $Pr < \chi^2 = 4.3$) at 12 d. The addition of biotin to the solutions counteracted the negative effects of avidin (Fig. 1). There was no significant difference between mortality at any concentration and mortality at 0 µg avidin/ml + 0 µg biotin/ml ($F = 0.45$, df = 5, $P = 0.8078$). Fisher's LSD value was determined to be 18% mortality, and the highest mean percentage of mortality (10%) was observed for larvae feeding on leaves dipped in 204 µg avidin/ml + 11.76 µg biotin/ml.

The LC_{50} value for avidin and Colorado potato beetle neonates was higher than that previously observed with potato tuberworm, *Phthorimaea operculella* (Zeller) (LC_{50} of 2.3 µg/ml at 9 d); light brown apple moth, *Epiphyas postvittana* (Walker) (LC_{50} of 43.4 µg/ml at 21 d); and *Ctenopseustis obliquana* (Walker) (LC_{50} of 45.7 µg/ml at 21 d) (Markwick et al. 2001). The reported expression levels of transgenic tobacco, *Nicotiana tabacum* L., and transgenic apple, *Malus domestica* Borkh, range from 0 to 24.5 µM (0–416.5 µg/ml) and 1.9–11.2 µM (32.3–190.4 µg/ml), respectively (Murray et al. 2002, Markwick et al. 2003). Although the concentration of the dip solution is comparable with avidin expression in transgenic plants, the actual dose in the dip assay is much lower. The dip was a topical application, whereas the transgenic plants express avidin in each cell of the plant. If transgenic potato plants have comparable expression

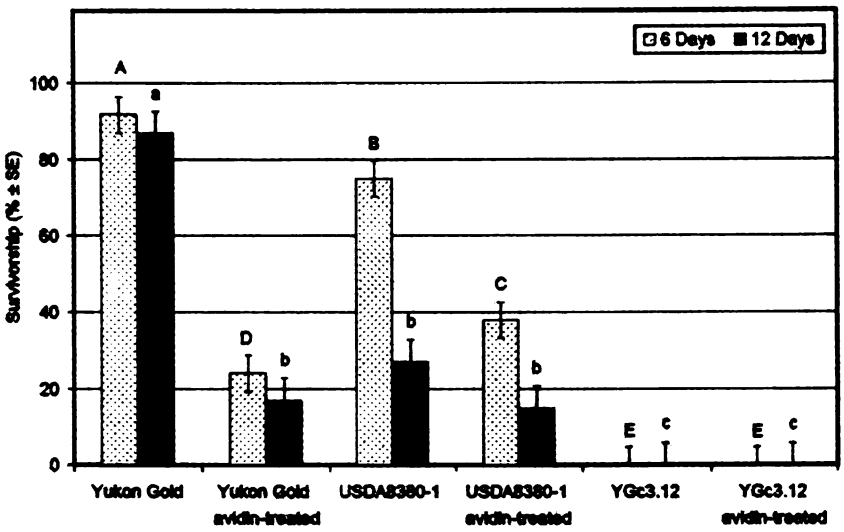

Fig. 2. Mean percentage of Colorado potato beetle neonate survivors fed on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 6 and 12 d in a no-choice detached leaf bioassay. Means followed by different letters within a date are significantly different at 0.05 level based on analysis of arcsine square-root transformed data; means were separated using a pairwise comparison.

levels to that of transgenic tobacco or apple, mortality of Colorado potato beetle would likely be higher than observed in the leaf dip assay because of higher avidin exposure and continuous expression.

Combined Effects of Avidin. *Neonate Assay.* Both potato line ($F_{6\ d}$ = 77.23, df = 2, $P < 0.0001$; and $F_{12\ d}$ = 30.80, df = 2, $P < 0.0001$) and avidin treatment ($F_{6\ d}$ = 45.49, df = 1, $P < 0.0001$; and $F_{12\ d}$ = 18.64, df = 1, $P = 0.0002$) significantly affected survival at 6 and 12 d. There was a significant interaction between potato line and avidin treatment on larval survival at 6 d (F = 17.17, df = 2, $P < 0.0001$) and 12 d (F = 14.15, df = 2, $P < 0.0001$). Larvae consuming avidin-treated Yukon Gold had significantly lower survival compared with larvae feeding on untreated Yukon Gold at 6 and 12 d, suggesting avidin is detrimental to the survival of neonates (Fig. 2). Colorado potato beetle neonates are more sensitive to toxins than later instars because they lack the nutritional and metabolic resources to cope with toxins, and they also are receiving a higher dose per larval mass (Wierenga et al. 1996, Hilton et al. 1998). Consumption rates (milligrams of food per milligram of body weight per day) are highest for neonates. Young larvae also have limited detoxification ability compared with larger larvae or adults contributing to their sensitivity of neonates to toxins (Zhao et al. 2000). Survival of larvae feeding on avidin-treated USDA8380-1 was significantly reduced compared with untreated USDA8380-1 at 6 d. Survival did not significantly differ for larvae feeding on avidin-treated USDA8380-1 compared with untreated USDA8380-1 at 12 d. USD8380-1 produces leptines, which are strong feeding deterrents for Colorado potato beetle (Tingey 1984). The larvae consuming avidin-treated USDA8380-1 may have not received a large enough dose to have detrimental effects on survival. Survival of larvae did not significantly differ between larvae feeding on avidin-treated Yukon Gold, avidin-treated USDA8380-1, or untreated USDA8380-1 at 12 d, suggesting comparable susceptibility to avidin and leptines in early instars. Survival did not significantly differ between larvae feeding on avidin-treated YGc3.12 compared with larvae feeding on untreated Ygc3.12 at 6 and 12 d. Regardless of the addition of avidin, nearly a 100% of larvae consuming YGc3.12 were dead by 4 d. This was expected because of the strong effect of Bt-Cry3A (Cooper et al. 2004)

Both potato line ($F_{6\ d}$ = 56.49, df = 2, $P < 0.0001$; and $F_{12\ d}$ = 61.98, df = 2, $P < 0.0001$) and avidin treatment ($F_{6\ d}$ = 61.87, df = 1, $P < 0.0001$; and $F_{12\ d}$ = 54.17, df = 1, $P < 0.0001$) had a significant effect on the amount of feeding at 6 and 12 d. There was a significant interaction between the effects of potato line and avidin treatment on consumption at 6 d (F = 23.70, df = 2, $P < 0.0001$) and 12 d (F = 25.72, df = 2, $P < 0.0001$), suggesting the addition of avidin to a host plant resistance factor may decrease larval feeding. Consumption was significantly less on avidin-treated Yukon Gold than on untreated Yukon Gold at 6 and 12 d (Fig. 3). Avidin is antinutritional; it retards the development of larvae, eventually leading to death (Levinson et al. 1992). Smaller larvae consume less foliage than large larvae. The retarded growth of larvae feeding on avidin-treated Yukon Gold likely accounts for the reduced consumption rather than the avidin possessing deterrent properties. The health of larvae feeding on Yukon Gold treated with avidin was severely compromised. The larvae feeding on avidin were often slower than larvae of a similar size.

Consumption was significantly reduced on untreated USDA8380-1 compared with untreated Yukon Gold, likely because of the deterrent properties asso-

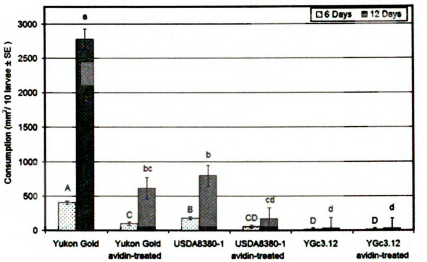

Fig. 3. Mean consumption by Colorado potato beetle neonates on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 6 and 12 d in a no-choice detached leaf bioassay. Means followed by different letters within a date are significantly different at 0.05 level; means were separated using a pairwise comparison.

ciated with leptines (Fig. 3) (Tingey 1984). Consumption significantly decreased for larvae feeding on avidin-treated USDA8380-1 compared the USDA8380-1 in at 6 and 12 d, but the biomass did not significantly differ (Fig. 3 and 4). The lower consumption is likely the result of fewer surviving larvae eating the avidin-treated USDA8380-1 compared with untreated USDA8380-1 (Fig. 2). Larvae feeding on avidin-treated USDA8380-1 seemed much weaker and had slower movement than larvae feeding on USDA8380-1. Feeding on untreated USDA8380-1 did not significantly differ from avidin-treated Yukon Gold at 12 d, but the combined resistance of the avidin-treated USDA8380-1 did have significantly less consumption than untreated USDA8380-1 at 12 d.

Potato line significantly affected the biomass of survivors at 6 d ($F = 16.33$, df = 2, $P < 0.0001$) and 12 d ($F = 16.94$, df = 2, $P < 0.0001$), but the addition of avidin did not significantly affect biomass at 6 d ($F = 0.07$, df = 1, $P = 0.7999$) or 12 d ($F = 3.39$, df = 2, $P < 0.0755$) (Fig. 4). Biomass of survivors was significantly reduced for larvae fed on avidin-treated Yukon Gold compared with larvae fed on untreated Yukon Gold at 12 d, demonstrating the negative effect of avidin on

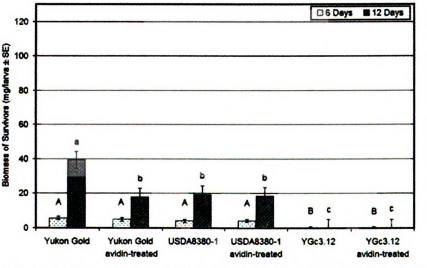

Fig. 4. Mean biomass of surviving Colorado potato beetle neonates fed on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 6 and 12 d in a no-choice detached leaf bioassay. Means followed by different letters within a date are significantly different at 0.05 level; means were separated using a pairwise comparison.

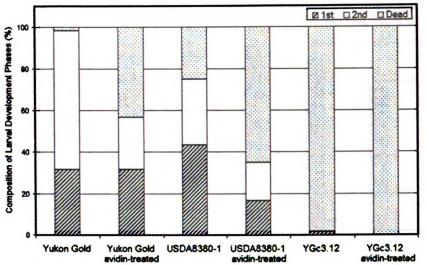

Fig. 5. Developmental stages of Colorado potato beetle neonates fed on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 6 d in a no-choice detached leaf bioassay.

larval growth. Biomass did not significantly differ between the untreated and avidin-treated USDA8380-1; perhaps neonates did not receive a sufficient dose to retard growth (Fig. 4). USDA8380-1 produces leptines that deters Colorado potato beetle feeding (Tingey 1984). At 12 d, larvae fed on the untreated Yukon Gold consumed almost 3 times as much as larvae fed on the untreated USDA8380-1. The leaves were dipped in avidin solutions; therefore, larvae consuming more tissue ingested more avidin. Larvae feeding on avidin-treated USDA8380-1 likely consumed far less leaf tissue and less avidin than larvae feeding on avidin-treated Yukon Gold. Because of the rapid mortality on YGc3.12, biomass data were only collected at 2 d. Larvae fed on Ygc3.12 died within 4 d and did not develop past first instar (Fig. 5).

Although the biomass of survivors did not significantly differ for larvae feeding on avidin-treated USDA8380-1 compared with larvae fed on untreated USDA8380-1, the addition of avidin did retard larval development. At 8 d, >40% of the surviving larvae that fed on untreated USDA8380-1 were third instars, whereas <6% of surviving larvae that fed on avidin-treated USDA8380-1 treated were third instars. No larvae survived to second instar feeding on Ygc3.12 regardless of avidin treatment (Figs. 5 and 6).

Colorado potato beetles spend ≈12–17 d as larvae, with ≈7–11 d to reach fourth instar (Walgenbach and

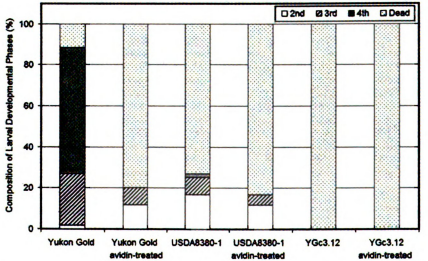

Fig. 6. Developmental stages of Colorado potato beetle neonates fed on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 12 d in a no-choice detached leaf bioassay.

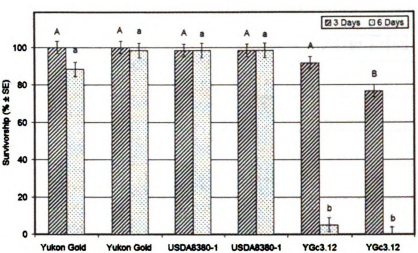

Fig. 7. Mean percentage of Colorado potato beetle third instar survivors fed on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 3 and 6 d in a no-choice detached leaf bioassay. Means followed by different letters within a date are significantly different at 0.05 level based on analysis of arcsine square-root transformed data; means were separated using a pair wise comparison.

Wyman 1984). In our 12-d neonate assay, no larvae feeding on avidin-treated leaves progressed to fourth instar, whereas 61.7% of larvae feeding on untreated Yukon Gold progressed to the fourth instar (Fig. 6). The addition of avidin to artificial diets delays insect and mite growth and also compromises reproduction of mites (Levinson et al. 1992, Markwick et al. 2001). With transgenic plants expressing high levels of avidin, Colorado potato beetle larvae may not be able to develop to fourth instar, survive pupation, or efficiently reproduce. Further studies need to be performed to more closely examine effects of avidin on rate of development of neonates to adulthood and on the fecundity of surviving adults.

Third Instar Assay. Potato line significantly affected survival at 3 d ($F = 14.24$, df = 2, $P < 0.0001$) and 6 d ($F = 314.33$, df = 2, $P < 0.0001$), but the avidin treatment did not significantly affect larval survival at 3 d ($F = 3.44$, df = 1, $P = 0.0681$) and 6 d ($F = 0.11$, df = 1, $P = 0.7413$). There were significant interactions between effects of potato line and avidin treatment on third instar survival at 3 d ($F = 3.44$, df = 2, $P < 0.0379$) but not at 6 d ($F = 2.36$, df = 2, $P < 0.1019$). Survival of third instars was not significantly affected by the avidin treatment on Yukon Gold or USDA8380-1 at 3 or 6 d (Fig. 7). The avidin LC_{50} was determined with neonates, which are typically much more susceptible to toxins than later stages such as third instars. Also, the dose/larval mass would be less for the third instars compared with neonates consume at a higher rate (milligrams of food per milligram of body size) than larger larvae.

Third instars fed on avidin-treated YGc3.12 had significantly lower survival compared with third instars fed on untreated Ygc3.12 after 3 d. However, nearly 100% of larvae consuming Ygc3.12 were dead by 6 d regardless of the avidin treatment because of the high toxicity of Bt-Cry3A to Colorado potato beetle larvae (Fig. 7) (Perlak et al. 1993). When combining resistance factors with Bt-Cry3A, the added effects are often masked, especially in early instars (Cooper et al. 2004). Avidin did not seem to reduce survivorship of larvae on Bt-Cry3A in the neonate assay. The addition of avidin may increase the susceptibility of larger Colorado potato beetle larvae to the Bt-Cry3A toxin. *Helicoverpa armigera* (Hübner) larvae had a significantly higher mortality when fed on a transgenic avidin plant painted with Bt-Cry1Ba compared with the transgenic avidin plant or Bt-Cry1Ba-painted plant alone (Burgess et al. 2002). Therefore, insects feeding on plants expressing high levels of a Bt toxin and avidin may have a higher mortality than insects feeding on a plant expressing either resistance factor alone.

Both potato line ($F_{3\ d} = 90.11$, df = 2, $P < 0.0001$; and $F_{6\ d} = 399.71$, df = 2, $P < 0.0001$) and avidin treatment ($F_{3\ d} = 12.61$, df = 1, $P < 0.0001$; and $F_{6\ d} = 4.97$, df = 1, $P = 0.0292$) significantly affected the area consumed by third instars at 3 and 6 d. There was a significant interaction between potato line and avidin treatment on consumption by third instars at 3 d ($F = 4.6$, df = 2, $P = 0.0135$) but not at 6 d ($F = 0.99$, df = 2, $P = 0.3777$). Larvae consumed significantly less avidin-treated Yukon Gold than untreated Yukon Gold at 3 d (Fig. 8). After 6 d, consumption did not significantly differ between untreated Yukon Gold and avidin-treated Yukon Gold. A higher dose of avidin may increase the length of development of third instars. Larvae consumed significantly less of untreated and avidin-treated USDA8380-1 than untreated Yukon Gold; consumption did not significantly differ between untreated and avidin-treated USD8380-1 at 3 or 6 d. Larvae consumed significantly

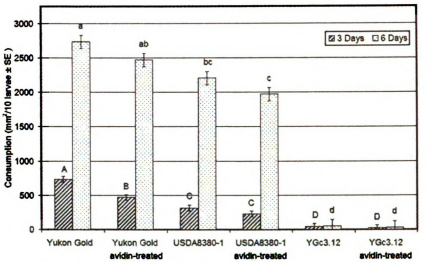

Fig. 8. Mean consumption of Colorado potato beetle third instars on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 μg avidin/ml or 136 μg avidin/ml solution at 3 and 6 d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different at 0.05 level; means were separated using a pairwise comparison.

less YGc3.12 than Yukon Gold or USDA8380-1 regardless of avidin treatment. Consumption did not significantly differ between untreated and avidin-treated YGc3.12 at 3 or 6 d.

Potato line significantly affected the biomass of survivors at 3 d ($F = 74.47$, df = 2, $P < 0.0001$) and 6 d ($F = 190.82$, df = 2, $P < 0.0001$), but the avidin treatment did not significantly affect the biomass of survivors at 3 d ($F = 0.21$, df = 1, $P = 0.6481$) or 6d ($F = 0.17$, df = 1, $P < 0.6853$). The LC_{50} was determined using neonates. Neonates are more susceptible to toxins than third instars. If a higher concentration of avidin was used, avidin may have demonstrated negative effects on third instars such as retarded growth. Surviving larvae fed on avidin-treated and untreated Yukon Gold had the highest biomass, but the two groups did not differ significantly from each other (Fig. 9). The biomass of surviving larvae feeding on either avidin-treated or untreated USDA8380-1 was significantly lower than biomass of larvae fed on Yukon Gold, but similarly it did not significantly differ from each other. The biomass of survivors fed on the avidin-treated and untreated YGc3.12 was significantly lower than the biomass of larvae fed on Yukon

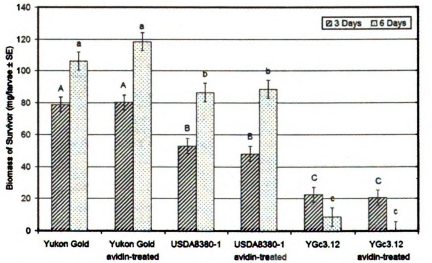

Fig. 9. Mean biomass of surviving Colorado potato beetle third instars fed on three potato lines (Yukon Gold, USDA8380-1, and YGc3.18) dipped in either 0 or 136 μg avidin/ml solution at 3 and 6 d no-choice detached leaf bioassay. Means followed by different letters are significantly different at 0.05 level; means were separated using a pairwise comparison.

Gold or USDA8380-1, but it did not significantly differ from each other.

The current study suggests avidin may be an effective resistance factor against Colorado potato beetle larvae. Avidin is an excellent candidate gene for plant transformation because of its insecticidal properties. Similar to Bt-cry toxins, avidin is a single gene product. If avidin is expressed throughout the plant, it may disturb the biosynthetic pathways of plants, which use carboxylase that requires biotin (Alban et al. 2000). Biotin is located throughout much of the plant cell, including the cytoplasm, mitochondria, and chloroplast, but it is not present in the vacuole of the cell (Baldet et al. 1992). Previous studies have attached a vacuolar targeting leader sequence to the avidin, diverting the produced avidin into vacuole of the cell and reducing or eliminating interference with plant biochemical pathways (Murray et al. 2002).

Currently, most commercial insecticidal transgenic plants rely on *Bt-cry* genes that are designed to combat select groups of pests. With the universal dependence of biotin, avidin may confer broad-spectrum resistance. A broad-spectrum insecticide can kill beneficial insects such as predators or pollinators along with the targeted pest. Using transgenic plants to deploy avidin could decrease the negative effects on beneficial insects. It may be effective against a number of potato insect pests such as wireworms, *Conoderus falli* (Lane), and variegated cutworms, *Peridroma saucia* (Hübner). In addition, avidin may even provide some protection against other noninsect potato pests such as scab, *Streptomyces scabies* (Thaxter), or late blight, *Phytophthora infestans* (Mont.) de Bary.

Combining resistance factors with distinctly different modes of action has been shown to increase insecticidal activity and also may increase the durability of individual toxins (Roush 1998, Zhao et al. 2005). Avidin has antinutritional activity and may not have the "quick kill" effect like Cry toxins. Combining avidin with stronger toxins such as Bt-Cry or natural host plant resistance, may increase the both the effectiveness and longevity of the resistance factors.

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CHAPTER III:

TRANSGENIC POTATOES, *Solanum tuberosum* L., EXPRESSING AVIDIN CONFERS RESISTANCE TO COLORADO POTATO BEETLE LARVAE, *Leptinotarsa decemlineata* (Say)

ABSTRACT

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most destructive insect pest of potato, *Solanum tuberosum* (L.), in North America. Avidin is a protein found in chicken egg whites that has demonstrated insecticidal properties against a number of lepidopteran and coleopteran pests. Biotin is an essential co-enzyme required for all organisms, including insects; it is a cofactor for a number of carboxylases involved in such processes as citric acid cycle. Avidin binds to biotin, thereby limiting availability of biotin during insect growth and development. Without biotin, an insect's growth is severely stunted, eventually leading to death. We sought to elevate resistance by combining avidin with natural host plant resistance derived from the wild relative of potato, *S. chacoense* Bitter. We expressed avidin in two potato lines: MSE149-5Y, a susceptible potato line, and ND5873-15, *S. chacoense*-derived resistant potato line. The avidin expression ranged from 0 - 63.8 μM ± 0.25 S.E. Detached leaf bioassays were performed on transgenic and non-transgenic clones of the susceptible and *S. chacoense* lines using first stage Colorado potato beetle larvae. Survival, and survivor growth were measured after 3 d. Twenty-one transgenic lines were screened. In general, survival was significantly less for larvae fed on transgenic avidin lines compared to the non-transgenic lines. In addition, the mass of survivors was often significantly reduced for larvae fed on

the transgenic avidin lines compared to the non-transgenic lines. Avidin, either alone or in conjunction with other engineered or natural host plant resistance factors, may suppress Colorado potato beetle larvae.

INTRODUCTION

Biotin, also called vitamin H or B_8, is critical for all organisms, including insects (Trager 1948). A number of important carboxylases require biotin as a cofactor, including carboxylases involved in such important biosynthetic pathways such as the citric acid cycle, lipogenesis, gluconeogensis, and fatty acid and amino acid catabolism (Mistry and Dakshinamurti 1964). All organisms require biotin, but only a number of plants, bacteria and fungi synthesize biotin. Hence, most organisms, including insects, must acquire biotin from their diet or environment (Trager 1948).

A class of proteins termed biotin-binding proteins sequesters biotin (Izrailev et al. 1997). The most well known biotin-binding proteins are avidin from chicken (*Gallus gallus* L.) and streptavidin from *Streptomyces avidinii* (Bayer et al. 1990. Green 1990, Stevens 1991). These proteins have a strong affinity for biotin, with the strongest non-covalent bond found in nature (K_d=10^{-15} M) (Izrailev et al. 1997). Insects require biotin-dependent carboxylases to store and use fat (Miura et al. 1967). Insects with little biotin available die due to their inability to store or access stored fat. Insects are particularly sensitive to biotin depletion during molting because of the high-energy requirement of this process (Miura et al. 1967). The addition of avidin to the diet of an insect causes a deficiency in accessible biotin resulting in abnormal larval growth and development leading to death (Levinson and Bergmann 1959). Due to the universal dependence of biotin, avidin is effective against a broad range of plant pests such as Diptera, Lepidoptera and Coleoptera (Levinson and Bergmann 1959; Morgan et al. 1993, Kramer et al. 2000, Marwick et al. 2001, Burgess et al. 2002, Malone et al. 2002).

Colorado potato beetle, *Solanum tuberosum* (L.), is one of the most destructive insect pests of potatoes, *Solanum tuberosum* spp. *tuberosum* L., in North America, Europe, and western Asia. Infestations by this insect significantly impact potato yield and may even lead to crop failure (Hare 1980, Mailloux and Bostanian 1989). Potato growers rely on insecticides to control Colorado potato beetle. Unfortunately, this pest readily adapts to insecticides and is currently resistant to over 40 insecticides (Whalon et al. 2006). Therefore, it is important to examine novel control strategies, including genetic engineering, to manage this pest.

Avidin in high concentrations kills Colorado potato beetle larvae (Cooper et al. 2006). The LC_{50} for avidin value is much higher (136 μg /ml (108-188)) than the LC_{50} value for insecticidal proteins such as Bt-Cry3A (1.84 μg /ml (1.35-2.54)) (Namiov et al. 2001, Cooper et al. 2006). Therefore, combining avidin with other resistance strategies, such as insecticides, other protein toxins, or natural host plant resistance factors, may elevate the level of control. Furthermore, combining multiple resistance factors into a plant, may delay resistance development (Gould 1986, Roush 1998, Zhao et al. 2005).

Potatoes include a large number of closely related species with natural insect resistance factors that can be introgessed into cultivated potato through traditional breeding. In particular, potatoes produce natural compounds called glycoalkaloids that are associated with insect and disease resistance (Maga 1994). The wild species *Solanum chacoense* produces a number of compounds, including leptine glycoalkaloids, which confer resistance to Colorado potato beetle (Sinden et al. 1986, Lorenzen et al. 2001). ND5873-15 is an elite breeding line from North Dakota State University derived from *S. chacoense* Bitter with insect resistance partially attributed to glycoalkaloids (Lorenzen et

al. 2001). Engineering ND5873-15 to expresses avidin may enhance resistance to Colorado potato beetle.

The objectives of this study were: (1) transform potato with PPI/avidin fusion gene (2) to evaluate the performance of Colorado potato beetle larvae feeding on transgenic potato plants expressing avidin in detached leaf tests and (3) examine the combined effects of avidin with natural host plant resistance derived from *S. chacoense*.

MATERIALS AND METHODS

Plant material

Plant lines used for transformation were MSE149-5Y ($2n = 4x = 48$) and ND5873-15 ($2n = 4x = 48$). 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 resistance to Colorado potato beetle derived from *S. chacoense.* The potato lines were maintained in tissue culture by nodal propagation in 25 x 150 mm culture tubes or GA-7 Magenta vessels (Magenta Corp, Chicago, IL) in modified Murashige and Skoog (MS) (1962) medium (MS salts at 4.3 gL^{-1}, 3% sucrose, 1.4 mM sodium phosphate, 1.1 μM thiamine, 0.55 mM myo-inositol, pH 6.0, and Bactoagar at 8 gL^{-1} (Difco, Detroit, MI). All culture tubes, Magenta vessels, and Petri dishes were sealed with Micropore surgical tape (3M Co., St. Paul, MN). Cultures were maintained at 25 ± 2 °C with a 16:8 photoperiod of light: dark.

Construction of plasmid for transformation

The avidin cDNA carried on plasmid, pgn1cpk008.d3, was obtained from Delaware Biotechnology Institute (Newark, DE). Previous studies demonstrated that avidin could be safely stored in the vacuole of the plant by adding the signal sequence tag, potato protease inhibitor-I (PPI-I), to the avidin gene (Genbank Accession L06606, Beuning et al. 1994, Murray et al. 2002). If avidin is expressed throughout the plant cell, it can interfere with biotin-dependent carboxylases and cell function (Murray et al. 2002). A 111 bp olgio-nucleotide was synthesized at Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University; the sequence included the PPI-I

sequence (93 bp) with 11 bp of 5' end of the avidin gene. Primers were designed to amplify the remaining avidin gene from the pgn1cpk008.d3, resulting in a 370 bp avidin fragment. The primer sequence complimentary to the transcribed strand was 5'-CCA GAA AGT GCT CGC TGA CTG G -3'. The second primer was complimentary to the non-transcribed strand and had a sequence of 5'-CGC GGA TCC TCA CCT GTG TGC GCA G -3'. The amplified avidin fragment (370 bp) and the synthesized PPI-I- avidin fragment were cut with BsiHKA I and ligated, resulting in the fusion gene PPI-I/avidin (Fig. 3.1). The resulting PPI-I/avidin fusion protein has a total of 158 amino acids:

MESKFAHIIV FFLLATPFET LLARKESDGP EIPARKCSLT GKWTNDLGSN MTIGAVNSRG EFTGTYITAV TATSNEIKES PLHGTQNTIN KRTQPTFGFT VNWKFSESTT VFTGQCFIDR NGKEVLKTMW LLRSSVNDIG DDWKATGINI FTRLRTQV.

The PPI-I/avidin gene was sub-cloned into vector pE1120 resulting in a plasmid (pSPUD75) that included the constitutive CaMV35S promoter and the selectable marker neomycin phosphotransferase (*nptII*) under the control of its own nopaline synthase promoter (Ni et al. 1995) (Fig. 3.1). The plasmid pSPUD75 was introduced into *Agrobacterium tumefaciens* Smith and Townsend strain LBA4404 (Clontech, Palo Alto, Calif.) by triparental mating (Bevan 1984).

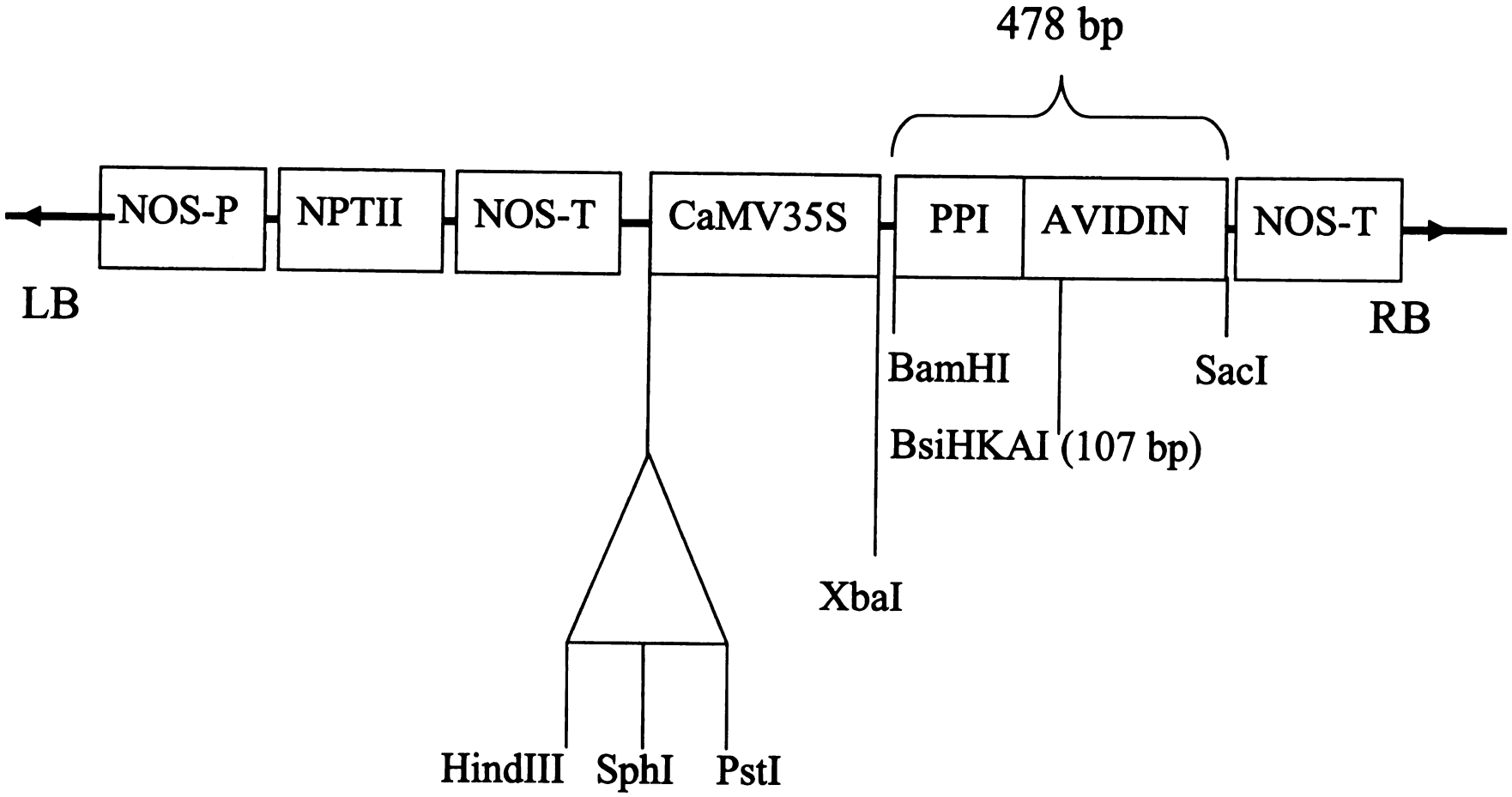

Figure 3.1: Schematic of gene construct pSpud75.

Transformation

Transgenic PPI-I/avidin potato lines were generated using *A. tumefaciens*-mediated transformation (Li et al. 1999). The explants were prepared for transformation by cutting internodes of the stem from tissue culture plantlets. When callus nodules produced shoots 5 - 7 mm in length, the shoots were excised and placed in rooting medium (modified MS medium with the addition of kanamycin at 50 mgL^{-1}) in 25 x 150 mm culture tubes. A single shoot was removed from each callus to ensure selection from independent transformation events. Rooted transformants expressing resistance to kanamycin were maintained by micropropagation and were transplanted to trays in the greenhouse for further analyses.

Molecular characterization

Polymerase Chain Reaction (PCR)

DNA was isolated by the quick DNA method from one 8-mm diameter leaf disc of a young (4-5 weeks old), greenhouse-grown, tissue culture transplant (Hosaka 2004). For PCR, 10 µL of the resulting DNA solution was used directly. PCR components for 50 µL reactions were used following RedTaq instructions (Sigma St. Louis, MO) (1X PCR buffer, 0.2 mM dNTP mixture, 1.0 µM of each primer, 100 ng template DNA, and 1 U Taq DNA polymerase).

The length of the synthetic PPI-I/avidin gene is 483 base pair (bp). A 25-base primer and a 26-base primer were chosen to amplify the 383 base pair length DNA fragment between bases 84 and 467 of the synthetic PPI-I/avidin gene. The primer sequence complimentary to the transcribed strand was 5'-GGA CCA GAA GCC AGA

AAG TGC TCG G-3'. The second primer was complimentary to the non-transcribed strand and had a sequence of 5'-GTG TGC GCA GGC GAG TGA AGA TG -3'. PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and primer extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The reactions were held at 4 °C before being analyzed. Reaction products were electrophoresed on a 0.9% (w/v) agarose gel containing ethidium bromide at 0.5 μgmL^{-1} in 1X Tris-acetate/EDTA (pH 8.0) buffer at 100 mV for 1 h (Sambrook et al. 1989) and viewed under ultraviolet light (254 nm).

Enzyme-linked immunosorbent assay for quantification of avidin

Indirect sandwich Enzyme linked immunosorbent assay (ELISA) was conducted on the leaves of PCR-positive 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 leaf by grinding 1g of tissue in 1 ml of 50mM 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 determined with para-nitrophenyl phosphate at 1mg/ml at 37°C. Absorbance was measured at 405 nm after 60 min incubation 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. Mean protein expression levels were compared using Fisher's least significant difference test ($P = 0.05$) (SAS Inst. 2005). Transgenic lines were selected from each parent line (MSE149-5Y and ND5873-15) to be further characterized by Southern analysis and detached leaf bioassays with Colorado potato beetle larvae.

Southern analysis

Total plant genomic DNA was extracted from the fresh leaf tissue (2 g) of greenhouse-grown tissue culture transplants using the CTAB (cetyltrimethylammonium bromide) extraction protocol (Saghai-Maroof et al. 1984), modified by adding 2% beta-mercaptoethanol to the extraction buffer. DNA was quantified using a UV-VIS spectrometer (Genesys 10 series spectrophotometers, ThermoSpectronic, Rochester, NY).

To determine the number of PPI-I/avidin gene insertion events, the DNA was digested with *XbaI*. Agarose-gel electrophoresis, Southern blotting, membrane hybridization, and detection were performed as per Li et al. (1999), with the exception of the PPI-I/avidin RNA probe, which was made by in vitro SP6 RNA polymerase transcription of the PPI-I/avidin gene cut from pSP73 with *BamHI* as per manufacturer's instructions (Roche, Indianapolis, IN).

Colorado potato beetle

Colorado potato beetle egg masses were obtained from the New Jersey Department of Agriculture's Phillip Alampi Beneficial Insect Rearing Laboratory, West

Trenton, NJ. This strain was originally collected in 1983 from potato and eggplant fields in New Jersey and has been continuously reared without exposure to insecticides.

Detached-leaf bioassays

No-choice detached-leaf bioassays were performed using MSE149-5Y, 14 transgenic avidin lines in parental background MSE149-5Y, ND5873-15, and 7 transgenic avidin lines the parental background ND5873-15. The potato lines were maintained in tissue culture as previously described (Coombs et al. 2002). When tissue culture plants reached about 60 mm ht, they were transferred to soil in seedling trays (50 cells per tray, 3 cm diam.) in the greenhouse. After a month, seedlings were transferred into a single plastic pot (2.5 L). Young, fully expanded leaves of similar age and size were removed from greenhouse transplants. The petiole was immersed in a water filled vial (3.5 ml), sealed with Parafilm and placed into a Petri dish (125mm diam.) lined with filter paper. Ten first instars were gently transferred from egg masses to each leaf. The first instars had not fed on the foliage before the detached leaf bioassay. Detached-leaf bioassays were maintained at 25 ± 2 °C with constant light of 25 μmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps. The detached-leaf bioassays were conducted as a completely randomized design with five replications (50 individuals per potato line).

Percent survival, and mass of survivors were recorded after 3 d. Consumption was visually estimated with mm^2 grid paper and recorded for each leaf (Coombs et al. 2002). Larvae were considered dead if missing or if no movement was observed after being gently touched with a fine-tipped paintbrush. Percentage survival was transformed with the arcsine of the square root to homogenize variance. The data sets (arcsine square

root percent survival and survivor mass) were analyzed using Fisher's protected least significant difference test ($P = 0.05$) in the general linear models procedure of SAS (SAS Inst. 2005). Reported mean arcsine survival values were retransformed into percentages from presentation. The data sets (arcsine square root percent survival and survivor mass) were also analyzed using Pearson's correlation to compare insect bioactivity with avidin expression level.

RESULTS AND DISCUSSION

Transformation

Agrobacterium-mediated transformation was effective in producing avidin-transgenic potato lines. The number of shoots emerging from any one callus ranged from 0 to 15, however; only one shoot was removed from each callus to represent independent events. Thirty-four shoots were removed from approximately 75 MSE149-5Y explants. Of the 34 shoots, 92% rooted in MS medium with kanamycin at 50 mg/L. Rooted putative transgenic plants in the MSE149-5Y background number were denoted as MSE75. followed by the shoot number. Twenty-six shoots were removed from approximately 75 ND5873-15 explants of which 100% rooted in MS medium with kanamycin at 50 mg/L. Rooted putative transgenic plants in the ND5873-15 background number were denoted as ND75. followed by the shoot number. All of the putative transgenic plants appeared phenotypically normal in test tubes.

Molecular characterization

Eighty percent of the rooted MSE149-5Y avidin lines and 88% of the rooted ND5873-15 avidin lines were PCR positive following DNA amplification of the 383 bp *avidin* fragment. PCR was not possible on nine of the putative transgenic ND5873-15 due to fungal contamination of the tissue culture tubes. The copy number ranged from one – three copies for transgenic plants expressing avidin (Fig. 3.2). All of the transgenic avidin plants appeared phenotypically normal in test tubes. Hood et al. (1997) generated transgenic maize, *Zea mays* L., with three to five copies of avidin that appeared

phenotypically normal. There was no relationship between copy number and avidin expression ($r = -0.07$, $p = 0.5879$).

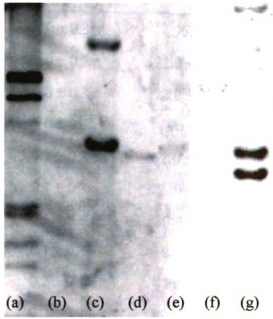

Figure 3.2: Southern analysis of total plant DNA from avidin transgenic lines digested with XbaI and hybridized with avidin RNA probe. The avidin plasmid, pSPUD75, was also digested. (a) Roche DIG-molecular weight marker III (b) MSE149-5Y (c) MSE75.21 (d) MSE75.7 (e) MSE75.25 (f) MSE75.27 (g) ND75.3

Avidin expression of the PCR positive lines ranged from 0.0 – 63.8 ± 0.3 μM ($F = 203.35$, $df = 49$, $P < 0.0001$) . The level of avidin expression was undetectable (0.0 ± 0.3 μM) in the transgenic lines MSE75.18, MSE75.28, and MSE75.30 (Table 3.1). This was generally comparable or higher than previous studies in other plants. Avidin expression ranged from 3.1 – 4.6 μM in transformed tobacco, 1.9 –11.2 μM in apple; ~ 100 μM in transformed maize and ~115 μM in transformed rice (Kramer et al. 2000; Burgess et al. 2002; Markwick et al. 2003; Yoza et al. 2005).

Table 3.1: Bioactivity of Colorado potato beetle first stage larvae after 3 d

Line	Avidin Expression (μM± SE)[†]	Percent Survival (± SE)[††]	Survivor Mass (mg/larva ± SE)[†††]
MSE149-5Y	0.0 ± 0.3 a	96.0 ± 8.0 a	50.5 ± 3.9 a
MSE75.18	0.0 ± 0.3 a	72.0 ± 8.0 bcdefg	20.6 ± 3.9 chdefghi
MSE75.28	0.0 ± 0.3 a	72.0 ± 8.0 bcdefg	23 ± 3.9 cdefgh
MSE75.30	0.0 ± 0.3 a	90.0 ± 8.0 ab	36 ± 3.9 b
MSE75.13	0.1 ± 0.3 a	72.0 ± 8.0 bcdefg	23 ± 3.9 cdefgh
MSE75.15	0.1 ± 0.3 a	72 .0 ± 8.0 bcdefg	24.5 ± 3.9 cdefg
MSE75.2	0.1 ± 0.3 a	80.0 ± 8.0 abcde	29 ± 3.9 bc
MSE75.25	0.1 ± 0.3 a	86.0 ± 8.0 abc	20.1 ± 3.9 cdefghij
MSE75.27	0.2 ± 0.3 a	82.0 ± 8.0 abcde	26.5 ± 3.9 bcde
MSE75.31	0.2 ± 0.3 a	80.0 ± 8.0 abcde	24.6 ± 3.9 cdef
MSE75.32	0.2 ± 0.3 a	72.0 ± 8.0 abcdef	28.5 ± 3.9 bcd
MSE75.19	0.5 ± 0.3 a	80.0 ± 8.0 abcde	21.8 ± 3.9 cdefgh
MSE75.24	0.6 ± 0.3 a	84.0 ± 8.0 abcd	16.5 ± 3.9 efghijk
MSE75.21	8 ± 0.3 b	72.0 ± 8.0 bcdefg	18 ± 3.9 defghijk
MSE75.7	64.9 ± 0.3 c	68.0 ± 8.0 bcdefg	13.5 ± 3.9 ghijk
ND7583-15	0 ± 0.3 a	88.0 ± 8.0 abc	20.8 ± 3.9 cdefghij
ND75.5	0.2 ± 0.3 a	72.0 ± 8.0 bcdefg	9.1 ± 3.9 jk
ND75.6	0.4 ± 0.3 a	62.0 ± 8.0 defg	12.2 ± 3.9 hijk
ND75.3	63.4 ± 0.3 c	50.0 ± 8.0 g	8.2 ± 3.9 k
ND75.2	63.5 ± 0.3 c	88.0 ± 8.0 abc	8.0 ± 3.9 k
ND75.10	64.0 ± 0.3 c	60.0 ± 8.0 defg	12.1 ± 3.9 hijk
ND75.7	64.1 ± 0.3 c	56.0 ± 8.0 efg	9.0 ± 3.9 k
ND75.16	65.3 ± 0.3 c	62.0 ± 8.0 defg	10 ± 3.9 jki

[†] Fisher Least Significant Difference$_{\alpha=0.05}$ = 4.8 μM
[††] Fisher Least Significant Difference$_{\alpha=0.05}$ = 23%
[†††] Fisher Least Significant Difference$_{\alpha=0.05}$ = 11.0 mg/larva

Detached leaf bioassays

First stage larvae fed on the susceptible line MSE149-5Y had significantly higher survival (96.0% ± 8.0 S.E.) than first stage larvae fed all the avidin lines, except MSE75.30 (90.0% ± 8.0 S.E.) that did not have detectable avidin expression (Table 3.1) ($F = 1.70$, $n = 22$, $P = 0.0291$). First stage larvae fed on MSE75.18 (72.0% ± 8.0 S.E.) or MSE75.2 (72.0% ± 8.0 S.E.), with undetectable avidin levels, had significantly lower survival than larvae fed on the susceptible line. There was not a significant correlation between avidin expression and mortality for larvae fed on the transgenic avidin lines ($r = -0.11988$, $P = 0.3056$). Avidin is an anti-nutritional protein that acts slowly on insects, therefore high mortality was not expected in 3 d (Markwick et al. 2001). Previous studies observed increasing mortality with increasing dose in Colorado potato beetle larvae, but the assay length was longer (12 d) (Cooper et al. 2003).

The survival (88.0% ± 8.0 S.E.) for first stage larvae fed on the *S. chacosense*-derived resistance line ND5873-15 did not differ significantly from first stage larvae fed the susceptible line (Table 3.1). First stage larvae fed on the *S. chacosense*-derived resistance line had significantly higher survival than first stage larvae fed all avidin + lines *Derived*-derived resistance lines except ND75.5 (72.0% ± 8.0 S.E.). The avidin expression in ND75.5 was low (0.2 μM ± 0.3 S.E.) and did not differ significantly from non-transgenic *S. chacosense*-derived resistance line ND5873-15. There was not a significant correlation between avidin expression and mortality for larvae fed on avidin + *S. chacoense*-derived resistance lines ($r = -0.20347$, $P = 0.2079$).

In general, the survival for first stage larvae fed on transgenic avidin lines did not differ significantly from the survival for first stage larvae fed on transgenic avidin + *S.*

chacoense-derived resistance lines of similar avidin expression (Table 3.1). The duration of the present assay (3 d) is too brief to determine efficacy of combining *S. chacoense*-derived resistance with avidin.

First stage larvae fed on any of the transgenic avidin lines were significantly smaller than first stage larvae fed on the non-transgenic susceptible line MSE149-5Y (Table 3.1). Larvae fed on the transgenic lines MSE75.18, MSE75.28, and MSE75.30 were significantly smaller than MSE149-5Y even though avidin expression was undetectable in the transgenic lines. There was small significant negative correlation between avidin expression and the mass of the surviving larvae fed on the transgenic avidin lines ($r = -0.44847$, $P < 0.0001$).

First stage larvae fed on the transgenic avidin + *S. chacoense*-derived resistance lines, ND75.2, ND75.3, ND75.7 were significantly smaller than first stage larvae fed on the non-transgenic *S. chacoense*-derived resistance line ND5873-15 (Table 3.1). There no correlation between avidin expression and the mass of the surviving larvae fed on avidin + *S. chacoense*-derived resistance lines ($r = -0.1802226$, $P = 0.2657$).

The mass of surviving first stage larvae fed on the transgenic avidin + *S. chacoense*-derived resistance lines did not differ significantly from mass of surviving first stage larvae fed on the transgenic avidin lines (Table 3.1).

In general, Colorado potato beetle larvae fed on transgenic avidin plants had higher insect mortality and lower larval mass in this brief 3 d assay. The present study suggests avidin may be useful in controlling Colorado potato beetle populations when first stage larvae are exposed to the plants. Combining multiple host plant resistance factors can increase the efficacy and effective life of individual host plant resistance

factors (Gould 1986, Roush 1998, Zhao et al. 2005). Combining *S. chacoense*-derived resistance with avidin did not confer elevated resistance in present brief 3 d bioassay. Longer bioassays are needed to elucidate the potential impacts of avidin alone and in combination with other resistance factors.

Avidin, especially in combination with other host plant resistance factors, is a promising insecticidal protein. Currently, commercial transgenic crops largely rely on crystal toxins (Cry) from the bacterium *Bacillus thuringiensis* for insect control (Ferry et al. 2006). Bt-Cry toxins are highly specific and generally only effective against a particular insect order and often only a few species within the order (Ferré and Van Rie 2002). Due to the universal dependence on biotin, avidin is broad-spectrum and effective against a variety of insect pests (Kramer et al. (2000), Morgan et al. 1993, Burgess et al. 2002, Markwick et al. 2003, Yoza et al. 2005, Cooper et al. 2006). Transgenic plants expressing avidin do not appear to negatively impact non-target insects even though avidin is broad spectrum. The development of newly emerged honeybees, *Apis mellifera* L., was not negatively affected by the addition of avidin to pollen-food (Malone et al. 2002, Malone et al. 2004). Additionally, only 10 – 28% of the avidin was recovered and active from tobacco cutworms, *Spodoptera litura* Fabricius, fed on transgenic tobacco plants expressing avidin, suggesting avidin will not accumulate in the food web (Christeller et al. 2005).

Combining host plant resistance factors with different modes of action can increase insecticidal activity and effective life of individual toxins (Gould 1986, Roush 1998, Zhao et al. 2005). Avidin has unique activity and is distinctly different from Bt-Cry toxins or natural host plant resistant factors in potato. Combining avidin with

stronger toxins like Bt-Cry or natural host plant resistance like leptines may increase both the effectiveness and longevity of the resistance factors. Additionally, avidin confers broad-spectrum resistance. Avidin is active against a number of lepidopteran and coleopteran pest and may be effective against other potato insect pests like wireworms, Elateridae spp. and variegated cutworms [*Peridroma saucia* (Hübner)], and potato tuberworm [*Phthorimaea operculella* (Zeller] (Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Markwick et al. 2003, Malone et al. 2005, Yoza et al. 2005).

Avidin is unlikely to negatively impact human health. Humans consume avidin regularly in the form of egg whites at concentrations >400 ppm (~26 μM) (Kramer et al. 2000). In addition, avidin is not highly allergenic (Subramanian and Adiga 1997). Also, Kramer et al. (2000) fed mice transgenic maize expressing avidin as their sole diet for 3 wks with no toxic effects. Moreover, avidin denatures after cooking and loses its ability to bind to biotin (Durance 1991). Avidin in transgenic rice loses most of its biotin-binding activity after 5 min at 95°C (Yoza et al. 2005). Potato is typically cooked before being consumed which will likely denature the protein. Finally, humans have a diverse diet and do not rely on potatoes solely for nutrition. On average, humans obtain 35-70 μg of biotin daily from varied food sources (Hardinge 1961).

This is the first report of combining avidin with natural host plant resistance to control an insect pest.

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CHAPTER IV:

COMBINING ENGINEERED RESISTANCE, AVIDIN, AND NATURAL RESISTANCE DERIVED FROM *Solanum chacoense* Bitter TO CONTROL COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* (Say)

ABSTRACT

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is the most destructive insect pest of potato, *Solanum tuberosum* (L.), in North America. Avidin sequesters available biotin, thereby causing abnormal growth and development of insects. We expressed avidin in two potato lines: MSE149-5Y, a susceptible potato line, and ND5873-15, a line with *S. chacoense*-derived insect resistance line. Detached leaf bioassays were performed on transgenic and non-transgenic clones of the susceptible and *S. chacoense* lines using first stage Colorado potato beetle larvae. Consumption, survival, and survivor growth were measured after 5 d. Larvae consumed significantly less on the two transgenic lines compared to the non-transgenic lines. Survival was also significantly less for larvae feeding on transgenic avidin lines compared to the non-transgenic lines. The mass of survivors was significantly reduced on two transgenic avidin lines compared to the non-transgenic lines. Further studies examined the development from first stage larvae to adulthood on a greenhouse grown whole plant in a no-choice setting for larvae fed on the four potato lines. Development from first stage to pupation was significantly prolonged for larvae fed on the avidin line compared to larvae fed on the susceptible line. Significantly fewer larvae fed on transgenic avidin plants, avidin or avidin+ *S. chacoense*-derived line survived to adulthood compared to survival

on non-transgenic plants, susceptible or *S. chacoense*-derived line. Avidin-based resistance may be useful in managing Colorado potato beetle populations in commercial planting by reducing the population size.

INTRODUCTION

Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a pest of potatoes in North America, Europe and Asia. As little as 12.5% defoliation can significantly reduce yields (Mailloux et al. 1996). If left uncontrolled, Colorado potato beetle completely defoliates potato crops (Hare 1980). It consistently adapts to insecticides and is currently resistant to over 40 insecticides (Whalon et al. 2006). From 1991 - 1994, Colorado potato beetle was resistant to all available chemical insecticides in regions of Michigan and throughout the northwestern United States. The control costs and crop losses escalated to 9-20% of the crop value during this era, emphasizing the need to develop alternative management strategies for this pest (Grafius 1997).

Genetic engineering is a tool for plant breeders, allowing individual genes or a cassette of genes to be inserted into the genome of a plant. Plant breeders can access resistance genes from any organism in any kingdom. At present, most commercial transgenic crops rely on crystalline (Cry) proteins developed from the bacterium *Bacillus thuringiensis* Berliner for *Bt*-Cry proteins are highly specific, often only effective against a particular insect order and many times act on only some of insect species within the order (Ferre and Van Rie 2002, Whalon and Wingerd 2003). *Bt*-Cry proteins are grouped into classes according to activity and structure of the protein. Generally, *Bt*-Cry3, *Bt*-Cry7, and *Bt*-Cry8 proteins are active against Coleoptera and *Bt*-Cryl proteins are active against Lepidoptera (Herrnstadt et al. 1986, Lambert et al. 1992, and Sato et al. 1994). In order to broaden the range of activity, scientists developed hybrid or chimeric Bt genes with domain regions from different classes of Cry proteins (Naimov et al. 2003; Singh et al. 2004; Chen et al. 2006).

Avidin is a novel protein that confers broad-spectrum resistance to arthropod pests, including Lepidoptera, Coleoptera, Diptera and Acari (Levinson and Bergmann 1959, Levinson et al. 1992, Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002). Avidin is a natural protein derived from the chicken egg white (*Gallus gallus* L.) that sequesters biotin (Izrailev et al. 1997). Biotin is an essential vitamin; it is a cofactor of a number carboxylases involved in important pathway like the citric acid cycle, lipogenesis, gluconeogensis, and fatty acid and amino acid catabolism (Trager 1948, Mistry and Dakshinamurti 1964). Insects require biotin-dependent carboxylases to store and process fat and are particularly sensitive to biotin depletion during molting because of the high-energy requirement of this process (Miura et al. 1967). The addition of avidin to insect diets causes abnormal and retarded larval development, often leading to death (Levinson and Bergmann 1959; Morgan et al. 1993, Marwick et al. 2001, Malone et al. 2002).

The LC_{50} value for avidin for Colorado potato beetle first stage larvae (8 μM at 12 d) is higher than LC_{50} values for other insects such as potato tuberworm, *Phthorimaea operculella* (Zeller) (LC_{50} of 0.1 μM at 9 d), light brown apple moth, *Epiphyas postvittana* Walker (LC_{50} of 2.6 μM at 21 d) and brownheaded leafroller, *Ctenopseustis obliquana* (Walker) (LC_{50} of 2.7 μM at 21 d) (Markwick et al. 2001; Cooper et al. 2006). Therefore, combining avidin with natural host plant resistance factors may be necessary for effective plant protection (Cooper et al. 2004). Furthermore, combining resistance mechanisms into a plant may delay resistance development of insects (Gould 1986; Roush 1998, Zhao et al. 2005).

Potatoes naturally produce compounds called glycoalkaloids that are associated with insect and disease resistance (Maga 1994). *Solanum chacoense* Bitter, a wild relative of potato, produces a number of insect deterrents, including leptine glycoalkaloids, which confer resistance to Colorado potato beetle (Sinden et al. 1986, Lorenzen et al. 2001). ND5873-15 is an elite breeding line from North Dakota State University derived from *S. chacoense* with uncharacterized insect resistance partially attributed to glycoalkaloids. The objectives of this study were to (1) evaluate larval development of Colorado potato beetle larvae fed on transgenic potato plants expressing avidin and (2) determine if combining *S. chacoense*-derived resistance with avidin conferred elevated plant protection by further delaying insect growth under no-choice and greenhouse conditions.

MATERIALS AND METHODS

Plant materials

The potato lines selected were MSE149-5Y, MSE75.7, ND5873-15 and ND75.3 (Table 4.1). 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 reported resistance to Colorado potato beetle derived from *S. chacoense*. The transgenic lines MSE75.7 and ND75.3 were developed in our lab using *Agrobacterium tumefaciens*-mediated transformation to express avidin (Cooper et al. unpublished). If avidin is expressed throughout plant cells, avidin could interfere with biotin-dependent carboxylases and plant cell function. Previous studies demonstrated that avidin may be safely stored in the vacuole of the plant by adding the potato protease inhibitor-I (PPI-I) (Genbank Accession L06606, Beuning et al. 1994, Murray et al. 2002). A fusion gene was constructed PPI-I/avidin driven by constitutive CaMV35S promoter (Fig. 3.1). The selectable marker was neomycin phosphotransferase (*nptII*) under the control of its own nopaline synthase promoter. MSE75.7 and ND75.3 were selected for this study because both the insecticidal activity was high. In addition, the avidin expression in the leaf tissue of MSE75.7 and ND75.3 was high and did not differ significantly differ from each other each other ($F = 203.35$, $df = 49$, $P < 0.0001$), 64.9 ± 0.3 S.E. in MSE75.7 and 63.4 ± 0.3 S.E in ND75.3 line ($LSD_{\alpha=0.5} = 4.8$ μM).

Table 4.1: Potato lines

Potato Line	Designation	Source	Host Plant Resistance Factor
Susceptible	MSE149-5Y	Michigan State University breeding line	No known resistance
Avidin	MSE75.7	MSE149-5Y transformed to express avidin	Avidin
S. chacoense-derived	ND5873-15	North Dakota State University breeding line	*S. chacoense*-derived
Avidin + *S. chacoense*-derived	ND75.3	ND5873-15 transformed to express avidin	Avidin + *S. chacoense*-derived

Molecular characterization

Southern analysis

Total plant genomic DNA was extracted from the fresh leaf tissue (2 g) of greenhouse-grown tissue culture transplants using the CTAB (cetyltrimethylammonium bromide) extraction protocol (Saghai-Maroof et al. 1984), modified by adding 2% beta-mercaptoethanol to the extraction buffer. DNA was quantified using a UV-VIS spectrometer (Genesys 10 series spectrophotometers, ThermoSpectronic, Rochester, NY).

To determine the number of PPI-I/avidin gene insertion events, the DNA was digested with *XbaI*. Agarose-gel electrophoresis, Southern blotting, membrane hybridization, and detection were performed as per Li et al. (1999), with the exception of the PPI-I/avidin RNA probe, which was made by in vitro SP6 RNA polymerase transcription of the PPI-I/avidin gene cut from pSP73 with *BamHI* as per manufacturer's instructions (Roche, Indianapolis, IN).

Enzyme-linked immunosorbent assay for quantification of avidin

Indirect sandwich Enzyme linked immunosorbent assay (ELISA) was conducted on the leaves of PCR-positive 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 leaf by grinding 1g of tissue in 1 ml of 50mM 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 determined with para-nitrophenyl phosphate at 1mg/ml at 37°C. Absorbance was measured at 405 nm after 60 min incubation 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. Mean protein expression levels were compared using Fisher's least significant difference test ($P = 0.05$) (SAS Inst. 2005). A single transgenic line with comparable avidin expression was selected from each parent line (MSE149-5Y and ND5873-15) to be further characterized by Southern analysis and detached leaf bioassays with Colorado potato beetle larvae.

Colorado potato beetle

Colorado potato beetle egg masses were obtained from the New Jersey Department of Agriculture's Phillip Alampi Beneficial Insect Rearing Laboratory, West Trenton, NJ. This strain was originally collected in 1983 from potato and eggplant fields in New Jersey and has been continuously reared without exposure to insecticides.

Detached-leaf bioassays

No-choice detached-leaf bioassays were performed using MSE149-5Y (susceptible) and MSE75.7 (avidin), ND5873-15 (*S. chacoense*-derived), and ND75.3 (avidin + *S. chacoense*-derived) lines (Table 4.1). The potato lines were maintained in tissue culture as previously described (Coombs et al. 2002). When tissue culture plants reached about 60 mm ht, they were transferred to soil in seedling trays (50 cells per tray, 3 cm diam.) in the greenhouse. After a month, seedlings were transferred into a single

plastic pot (2.5 L). Young, fully expanded leaves of similar age and size were removed from greenhouse transplants. The petiole was immersed in a water filled vial (3.5 ml), sealed with Parafilm and placed into a Petri dish (125mm diam) lined with filter paper. Ten first instars were gently transferred from egg masses to each leaf. The first instars had no feeding on the foliage before the detached leaf bioassay. Detached-leaf bioassays were maintained at 25 ± 2 °C with constant light of 25 μmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps. The detached-leaf bioassays were conducted as a completely randomized design with two trials with five replications per trial (100 individuals per potato line).

Consumption, percent survival, and mass of survivors were recorded after 5 d. Consumption was visually estimated with mm^2 grid paper and recorded for each group of larvae (Coombs et al. 2002). Larvae were considered dead if missing or no movement was observed after being gently touched with a fine-tipped paintbrush. Percentage survival was transformed with the arcsine of the square root to homogenize variance. The data sets (consumption, survivor mass, arcsine square root survival) were analyzed using Fisher's protected least significant difference test ($P = 0.05$) in the general linear models procedure of SAS (SAS Inst. 2005). Reported mean arcsine square root survival values were retransformed into percentages for presentation.

Whole Plant Bioassays

The potato lines, MSE149-5Y, MSE75.7, ND5873-15 and ND75.3 were maintained in tissue culture as previously described (Coombs et al. 2002). When tissue culture plants reached about 60 mm ht, they were transferred to soil in seedling trays (50

cells per tray, 3 cm diam.) in the greenhouse. After a month, the 5 seedlings were transferred into a single plastic pot and grown to ~0.5m in height (3.78 L). Leaves were removed from each plant and fed to 20 first stage larvae for 5 d in filter lined Petri dishes to ensure larvae would be large enough and could not escape through the holes in the screen. Each plant was placed into a sleeve cage. Twenty larvae were transferred from the leaves to a plant of the same line. After all the fourth instars burrowed into the soil for pupation, the plants were trimmed to approximately 2cm above the soil and the pot was covered with a screen.

This procedure was repeated for each potato line (MSE149-5Y, MSE75.7, ND5873-15 and ND75.3). Two trials were conducted with four replications per trial. Larval development was recorded every 3 d through pupation. Adult emergence was recorded every 2 d. The duration of each study was 56 d. The percent of larvae surviving to adulthood, number of days to entering pupation and adult emergence were recorded. Percent survival was transformed with the arcsine of the square root to homogenize variance. The data sets (arcsine square root survival, days to pupation, days to emergence) were analyzed using Fisher's protected least significant difference (LSD, $P = 0.05$) in the general linear models procedure of SAS (SAS Inst. 2005). Reported mean arcsine square root survival values were retransformed into percentages for presentation.

RESULTS AND DISCUSSION

Molecular characterization

Southern Analysis

Southern analysis showed that MSE75.7 had one copy of the avidin gene and ND75.3 had three copies of the avidin gene (Fig. 4.1)

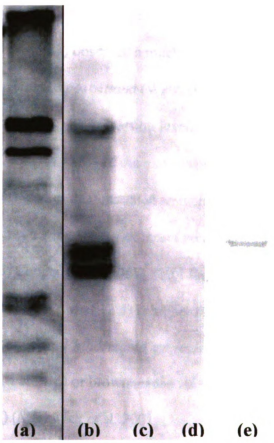

Figure 4.1: Southern analysis of total plant DNA from avidin transgenic lines digested with XbaI and hybridized with avidin RNA probe. (a) Roche DIG-molecular weight marker III (b) ND75.3 (c) ND5873-15 (d) MSE149-5Y (e) MSE75.7.

ELISA for quantification of avidin

The level of avidin expression for MSE75.7 (45.0 μM ± 5.36 S.E.) was not significantly different from ND75.3 (45.1 μM ± 5.36 S.E.) at 1d ($F = 1.26$, $df = 7$, P

=0.3088) ($LSD_{\alpha=0.05}$ = 15.7 μM). The level of avidin expression for MSE75.7 (46.96 μM ± 5.36 S.E.) was not significantly different from ND75.3 (39.6 μM ± 5.36 S.E.) at 35d (F= 1.26, df = 7, P =0.3088) ($LSD_{\alpha=0.05}$ = 15.7 μM).

Detached-leaf bioassays

First stage larvae fed the susceptible line had significantly higher survival (92% ± S.E.) than first stage larvae fed the avidin line (42% ± 6 S.E.) (F=10.39, df=3, P=0.0005) (Fig. 4.2). Markwick et al. (2003) observed a much higher survival rate (>75%) for lightbrown apple moth, *Epiphyas postvittana* (Walker), feeding on transgenic apples tissues expressing avidin at 7 d. The expression levels of avidin in apple tissues were between 1.9 – 11.2 μM, which is <18% of the expression level of our avidin line (63.8 μM ± 0.25 S.E.). Previous studies demonstrated an increased mortality with increased avidin concentration against a wide variety of insect pests (Kramer et al. 2000; Burgess et al. 2002; Markwick 2003; Cooper et al. 2006). The LC_{50} value of avidin for Colorado potato beetle first stage larvae (8 μM at 12 d) is higher than the LC_{50} value for other insects such as potato tuberworm, *Phthorimaea operculella* (Zeller) (0.1 μM at 9 d), light brown apple moth (2.6 μM at 21 d) or brownheaded leafroller, *Ctenopseustis obliquana* (Walker) (2.7 μM at 21 d) (Markwick et al. 2001; Cooper et al. 2006). Therefore, the low survival of Colorado potato beetle larvae fed the avidin line is likely due to high avidin expression in the plant.

The survival of first stage larvae fed *S. chacoense*-derived resistance line (80% ± 6 S.E.) did not differ significantly compared to the survival of first stage larvae fed the susceptible line (Fig. 4.3). The survival of first stage larvae fed the *S. chacoense*-derived

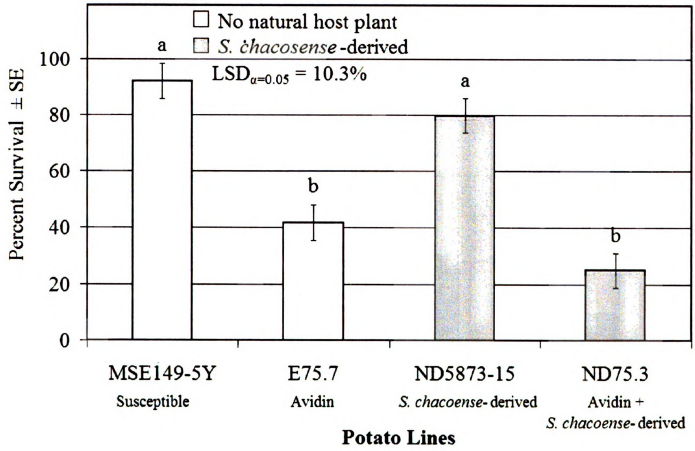

Figure 4.2: Mean percentage surviving Colorado potato beetle first stage larvae fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense* -derived), orND75.3 (avidin + *S. chacoense* -derived) at 5 d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different (P<0.05) based on analysis of arcsine square-root transformed data. Means were separated using Fisher's least squared differences test. Untransformed data is presented.

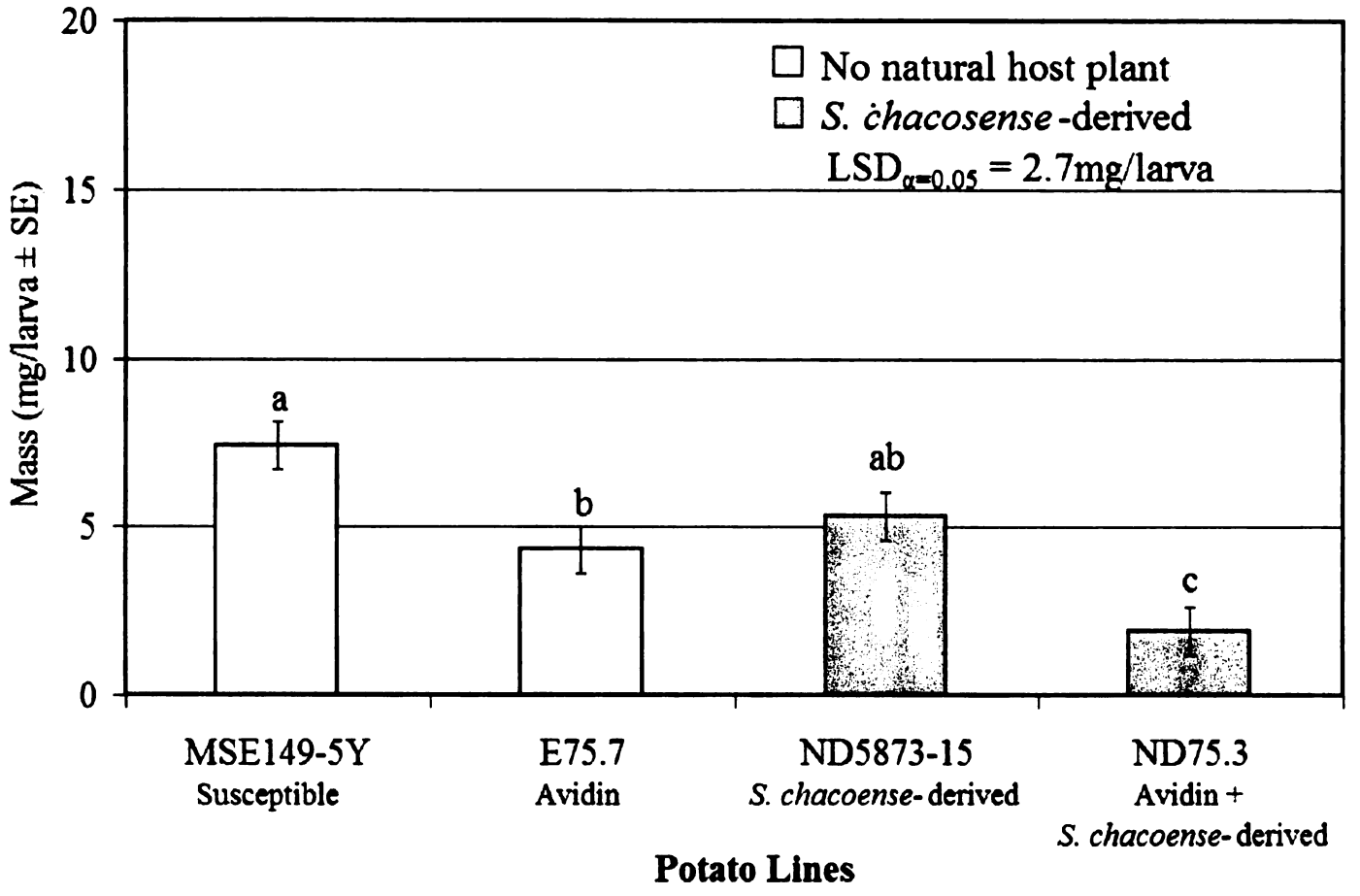

Figure 4.3 : Mean mass of surviving Colorado potato beetle first stage larvae fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense* -derived), or ND75.3 (avidin + *S. chacoense* -derived) after 5d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different (P<0.05) and were separated using Fisher's least squared differences test.

resistance line was significantly higher than the survival of first stage larvae fed avidin + *S. chacoense*-derived resistance line, suggesting avidin is detrimental to the survival of first stage larvae. The survival of first stage larvae fed the combined resistance avidin + *S. chacoense*-derived resistance line did not significantly differ from the survival of first stage larvae fed on the avidin line, even though they ate a little less and were significantly smaller after 5 d. Avidin is anti-nutritional; it retards the development of larvae, eventually leading to death of insects (Levinson et al. 1992). Therefore, if the assay were extended, the survival of first stage larvae fed on the avidin +*S. chacoense*-derived resistance line would likely be lower than survival of first stage larvae feeding on the avidin line.

Surviving larvae fed on the susceptible line gained the greatest mass over 5 d, with an average mass of 7.4 mg/larva ± 0.7 S.E. after 5 d (Fig. 4.3). The mass of surviving larvae fed on the avidin line was significantly reduced compared to that of surviving larvae fed on the susceptible line ($F=10.40$, $df=3$, $P=0.0005$). Avidin is effective against a number of insects (European corn borer [*Ostrinia nubilalis* Hübner], red flour beetle [*Tribolium castaneum* (Herbst)], lightbrown apple moth [*Epiphyas postvittana* (Walker)], cotton bollworm [*Helicoverpa armigera* (Hubner)], and the cluster cutworm [*Spodoptera litura* (Fabricius)]) (Morgan et al. 1993, Burgess et al. 2002, Markwick et al. 2003). In a previous study, the growth of Colorado potato beetle first stage larvae fed on potato foliage treated with avidin (8 μM) was not inhibited after 6 d (Cooper et al. 2006). Although duration of the current assay was shorter than the foliar application study, the avidin line reduced larval mass after 5d because the avidin expression level was almost 8 times higher (63.8 ± 0.25 μM) than the foliar application (8

μM) (Cooper et al. 2006). Moreover, the avidin protein was expressed throughout the leaf compared to surface of the leaf in the foliar application used by Cooper et al. (2006); therefore, the effective dose was much higher.

Surviving larvae fed on the *S. chacoense*-derived resistance line were not significantly smaller than surviving larvae fed on the susceptible line (Fig. 4.3). Surviving larvae fed on avidin+*S. chacoense*-derived resistance line were significantly smaller than surviving larvae fed on the susceptible, avidin or *S. chacoense*-derived resistance lines. Although first stage larvae fed on plants with combined resistance factors (avidin + *S. chacoense*-derived) did not have significantly higher mortality than first stage larvae fed on plants with the single resistance factor (avidin), first stage larvae fed on plants with combined resistance factors (avidin + *S. chacoense*-derived) were significantly smaller than larvae fed on plants with either single resistance factor, avidin or *S. chacoense*-derived resistance.

First stage larvae consumed significantly less leaf area of the avidin line or the *S. chacoense*–derived resistance line than of the susceptible line ($F=19.95$, $df=3$, $P<0.0001$) (Fig. 4.4-4.5). First stage larvae consumed significantly less leaf area of the avidin + *S. chacoense*–derived resistance line than the susceptible line or the *S. chacoense*–derived resistance line. The feeding observed on avidin+*S. chacoense*-derived resistance line was only pinhole size compared to large areas consumed on the other lines (Fig. 4.5).

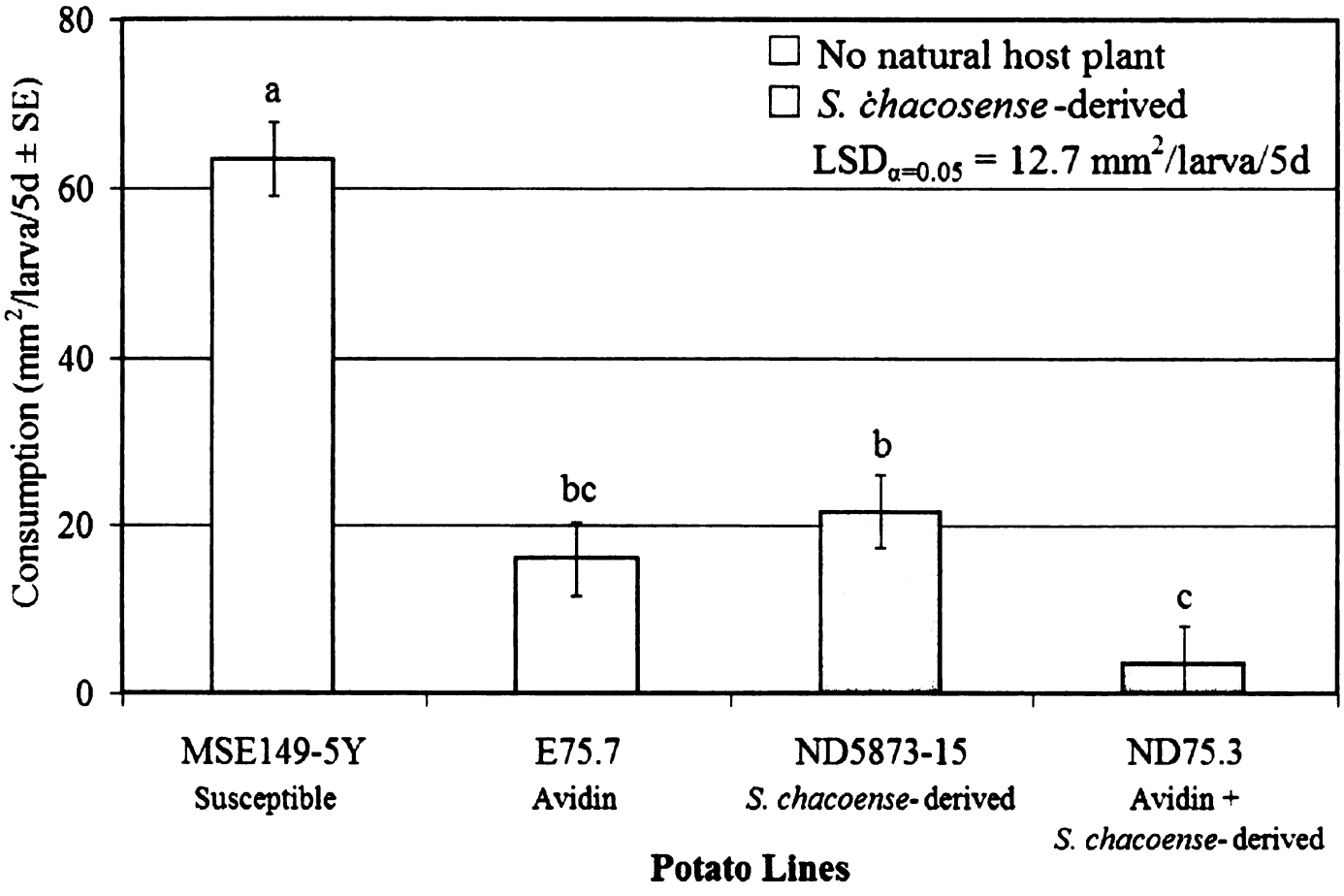

Figure 4.4: Mean consumption by Colorado potato beetle first stage larvae fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND75.3 (avidin + *S. chacoense* -derived) at 5 d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different (P<0.05) and were separated using Fisher's least squared differences test.

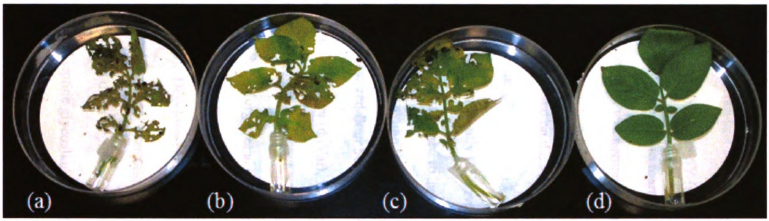

Figure 4.5: Examples of damage caused by Colorado potato beetle first stage larvae fed potato leaves after 5 d. (a) MSE149-5Y (susceptible), (b) MSE75.7 (avidin) (c) ND5873-15 (*S. chacoense*-derived), and (d) ND75.3 (avidin + *S. chacoense*-derived). Image presented in color.

Whole Plant Bioassay

Larval development

The duration from first stage to the pre-pupal stage was the shortest (16.4 d ± 1.0 S.E.) for larvae fed on MSE149-5Y (susceptible line) (Fig. 4.6). Generally, Colorado potato beetle mature from first to fourth instar within 15-20 d at 20° C, therefore, the duration of development was normal under the greenhouse conditions (Walgenbach and Wyman 1984). The duration from first stage to pre-pupal stage was prolonged significantly for larvae fed on the avidin line (21.9 d ± 1.4 S.E.) compared to larvae fed on the susceptible line ($F = 4.04$, $n = 3$, $P = 0.0096$). The duration from first stage to pre-pupal did not differ significantly for larvae fed on the *S. chacoense*-derived resistance line (18.2 d ± 1.2 S.E.) compared to larvae fed on the susceptible line (Fig. 4.6).

In similar studies avidin retards development in a variety of arthropods, including the following beetles: red flour beetle (*Tribolium castaneum* (Herbst), confused flour beetle (*Tribolium confusum* (duVal), sawtooth grain beetle (*Oryzaephilus surinamensis* L.), rice weevil (*Sitophilus oryzae* L.) and lesser grain borer (*Rhyzopertha dominica* F.) (Levinson et al. 1992, Morgan et al. 1993, Burgess et al. 2002, Markwick et al. 2003).

S. chacoense based resistance is due to deterred feeding by Colorado potato beetle (Sinden et al. 1980). Development is retarded for larvae fed on leaves with high levels of glycoalkaloids and leptine glycoalkaloids (331-496 mg/100g fresh wt tissue), but development does not differ significantly for larvae fed on leaves with low levels of glycoalkaloids and leptine glycoalkaloids (206 mg / 100g fresh wt tissue) compared to

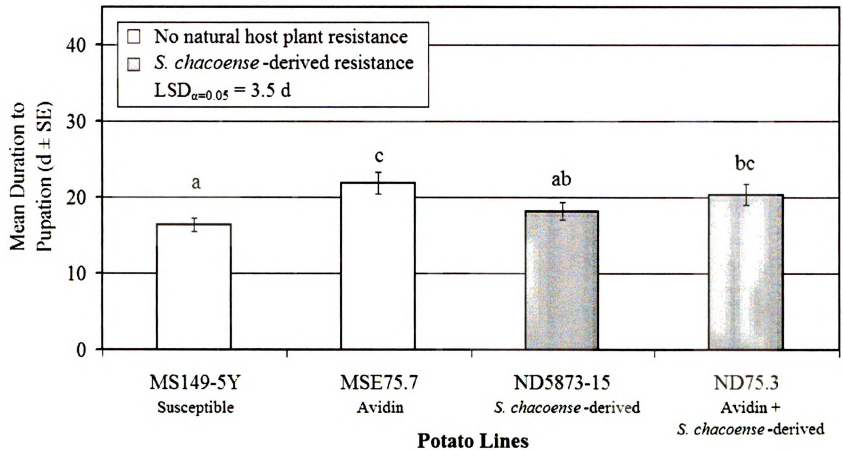

Figure 4.6: Mean duration of surviving Colorado potato beetle first stage larvae to pupation fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND75.3 (avidin + *S. chacoense*-derived) after 56 d in a no-choice bioassay. Means followed by different letters are significantly different (P<0.05) and were separated using Fisher's least squared differences test.

larvae fed on leaves of *S. tuberosum* with no host plant resistance (Sinden et al. 1986). ND5873-15 glycoalkaloid levels are lower than 331mg/100g fresh wt tissue, therefore, it is not expected to slow larval growth (Douches unpublished).

The duration from first stage to the pre-pupal stage did not differ significantly for larvae fed on the avidin + *S. chacoense*-derived resistance line (20.4 d ± 1.4 S.E.) compared to larvae fed on the susceptible line, avidin line or *S. chacoense*-derived resistance line (Fig. 4.6). Development was monitored every 3 d. If development was monitored more frequently, development rates for larva feeding on *S. chacoense*-derived resistance and avidin + *S. chacoense*-derived resistance lines may have differed. Additionally, consumption was measured for each plant. The plants were large and the damage caused by the 20 insects was never greater than 5%. Therefore, it was difficult in this study to determine, but *S. chacoense*-derived resistance may have deterred insect feeding, reducing the dose of avidin received by the larvae. The effects of avidin are dosage dependent for Colorado potato beetle (Cooper et al. 2006). If larvae fed less on the avidin + *S.* chacoense-derived resistance line than the avidin, the larvae may have not received a sufficient dose of avidin to retard development.

Transgenic avidin plants may have implications to plant protection in addition to antibiosis. Insects with prolonged development due to temperature have greater exposure to natural enemies, which subsequently leads to an increase in mortality in the field (Pincebourde and Casas 2006). Likewise, avidin-fed Colorado potato beetle larvae may have a greater risk of being attacked by predators, such as stink bugs (*Euschistus servus* (Say) or parastic wasps, thereby increasing mortality of larvae in the field. Also, an increase in generation time affects population growth rate and may reduce the number of

generations of Colorado potato beetle can complete in a season. Our experiment was performed under controlled environmental conditions. To assess the impact of natural enemies and generation time, additional studies should be conducted under field conditions.

Adult Emergence

Avidin did not significantly delay adult emergence. A majority of the adults emerged between 37 d – 47 d from the onset of the study. The total duration from first stage larvae to newly emerged adults was shortest (41.5 d ± 0.6 S.E.) for larvae fed on the susceptible line, but did not significantly different the duration for to larvae fed on the avidin (42.1 d ± 1.0 S.E.), *S. chacoense*-derived resistance (42.2 d ± 0.8 S.E.), or avidin + *S. chacoense*-derived resistance (43.0 d ± 0.8 S.E) lines (Fig. 4.7) ($F = 0.74$, $n = 3$, $P = 0.5306$).

Survival

Significantly fewer larvae survived to adulthood fed on the avidin line (26% ± 6 S.E.) compared to survived the susceptible line (59% ± 6 S.E.) (Fig 4.8) ($F = 4.88$, $n = 3$, $P = 0.0109$). Larvae fed on *S. chacoense*-derived resistance line had the highest Survival to adult (75% ± 17 S.E.), but the percentage of survival of larvae fed on the *S. chacoense*-derived resistance line did not differ significantly from the percentage of survival of larvae fed the susceptible line. The percentage of emerging adults from larvae fed on the avidin + *S. chacoense*-derived resistance line (43% ± 17 S.E.) was significantly lower

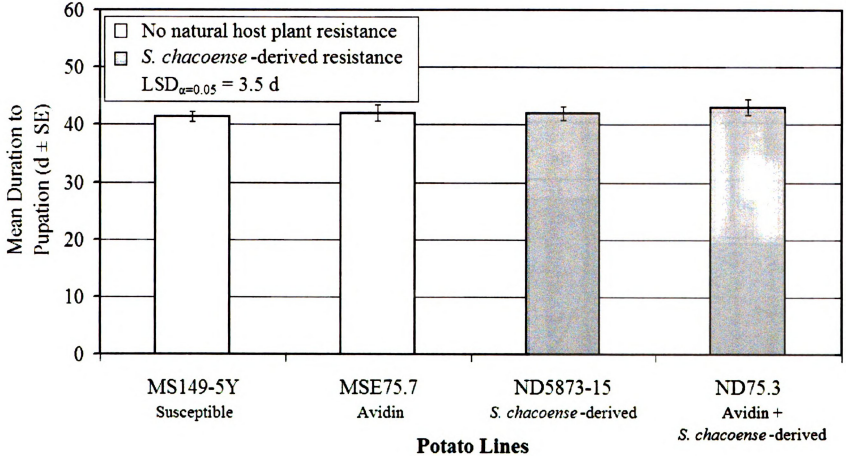

Figure 4.7: Mean duration of surviving Colorado potato beetle first stage larvae to newly emerged adults fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense* -derived), or ND75.3 (avidin + *S. chacoense* -derived) after 56 d in a no-choice bioassay. Means followed by different letters are significantly different (P<0.05) and were separated using Fisher's least squared differences test.

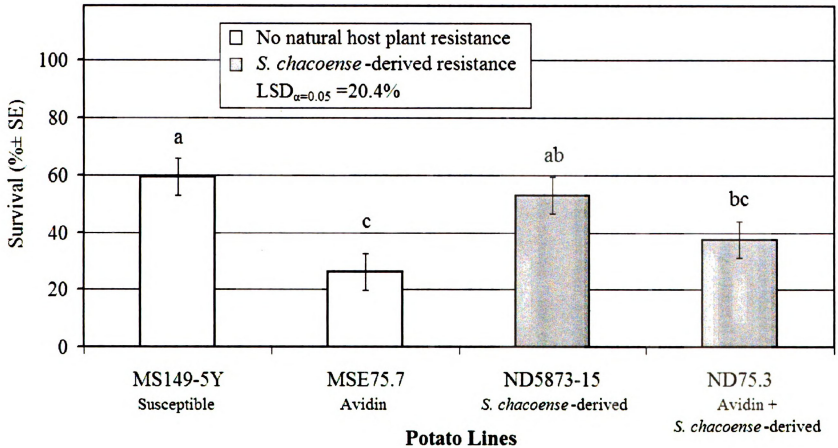

Figure 4.8: Mean percentage surviving Colorado potato beetle first stage larvae to adults fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense* -derived), orND75.3 (avidin + *S. chacoense -derived*) at 56 d in a no-choice bioassay. Means followed by different letters are significantly different (P<0.05) based on analysis of arcsine square-root transformed data. Means were separated using Fisher's least squared differences test. Untransformed data is presented.

compared to the percentage of emerging adults from larvae fed on *S. chacoense*-derived resistance (Fig. 4.8).

The natural resistance from *S. chacoense*-derived resistance line is associated with deterring feeding (Sanford et al. 1997, Rangarajan et al. 2000). In addition, large vigorous haulms of ND5873-15 (*S. chacoense*-derived) may limit the percent defoliation and the economic damage. The plant vigor does not reflect the ability of Colorado potato beetle larvae to feed and thrive on the plant. Pupation is an energy intensive process. Avidin sequesters biotin; without adequate biotin, a pupa may be unable to sufficiently access stored fat and may not survive pupation (Miura et al. 1967). The percentage of emerging adults from larvae fed on the avidin line did not differ significantly compared to percentage of emerging adults from larvae fed on avidin + *S. chacoense*-derived resistance line (Fig. 4.8). Although elevated resistance was not observed in the no-choice situation of the present study, ND5873-15 (*S. chacoense*-derived) confers resistance in field conditions that was not evident in the present study (Coombs et al. 2005).

Additionally, the level of glycoalkaloids is positively correlated with the intensity of light; glycoalkaloid levels for a particular cultivar are often higher under field conditions than greenhouse conditions (Dimenstein et al. 1997). Therefore, avidin + *S. chacoense*-derived resistance line may have elevated resistance compared to avidin line under field conditions due to predators and higher levels of glycoalkaloids (Dale et al. 1993).

Avidin is also effective against a variety pests in other crops, including Lepidoptera, Coleoptera, Diptera, and Arcari; therefore, it may confer plant protection to other potato pests like wireworms, Elateridae ssp. and variegated cutworm, *Peridroma*

saucia (Hübner) (Levinson et al. 1992, Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Markwick et al. 2003, Yoza et al. 2005). Additionally, biotin is required for other organisms like fungi and bacteria. The growth of a number of *Fusarium* species is stunted without sufficient biotin (Robbins and Ma 1942). Many fungi require biotin to stimulate sporulation (Yoshida & Shirata 2000). Hence, transgenic avidin potatoes may inhibit proliferation of pathogens by sequestering available biotin.

Humans consume avidin in the form of egg whites on a daily basis. The allergens in egg are well documented. The primary egg allergens are ovomucoid, ovalbumin, ovotransfferin, and lysozyme; avidin is not highly allergenic (Subramanian and Adiga 1997). Avidin loses its ability to bind to biotin after cooking (Durance 1991). In transgenic rice, only 3% of the avidin was able to bind to biotin after cooking (Yoza et al. 2005). Finally, humans eat a diverse diet. Each day the average person consumes 35-70 μg of biotin from varied food sources, including vegetables and nuts and potato is a primary source of biotin (Hardinge 1961).

Avidin is a promising insecticidal protein. It is broad-spectrum and can target crop pests by expressing it in the plant. Additionally, avidin does not appear to affect non-target pests. Only 10 – 28% of the avidin extracted from tobacco cutworms, *Spodoptera litura* Fabricius, fed on transgenic tobacco plants expressing avidin was active and able to bind to biotin (Christeller et al. 2005). Hence, predators of tobacco cutworm are unlikely to be negatively impacted by small amounts of avidin.

Avidin-based resistance may be a useful in managing Colorado potato beetle populations in commercial planting by reducing the population size. Although the present greenhouse data does not support combined resistance reducing survival, further

test are needed to test efficacy in the field due with higher glycoalkaloid levels and predator effects and reduced population growth rate and generation time. Additionally, avidin combined with other resistance factors including engineered factors, such as *Bt*-Cry proteins, and natural, such as leptines, may confer durable and broad spectrum resistance.

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CHAPTER V:

ENHANCED RESISTANCE BY COMBINING ENGINEERED RESISTANCE, AVIDIN, AND NATURAL RESISTANCE DERIVED FROM *Solanum chacoense* TO CONTROL POTATO TUBERWORM, *Phthorimaea operculella* (Zeller)

ABSTRACT

Potato tuberworm, *Phthorimaea operculella* (Zeller), is a destructive insect pest of potato, *Solanum tuberosum* (L.), 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 demonstrated 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, thereby 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 ± 2.7 S.E. in MSE75.7 (avidin) and 12.5 μM ± 2.7 S.E. in ND75.3 (avidin + *S. chacoense*-derived) in the tuber. Potato tuberworm bioassays were performed 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% ± 9 S.E.) was observed with larvae fed on ND75.3 (avidin + *S. chacoense*-derived) tubers than the mortality of larvae fed on MSE149-5Y (susceptible). Expressing avidin in combination with natural host plant resistance may be of value in managing potato tuberworm.

INTRODUCTION

Potato tuberworm is distributed throughout the world, including the Americas, Europe, Africa, Asia, and Australia (Trivedi and Rajagopal 1992). Potato tuberworm, *Phthorimaea operculella* (Zeller), is chiefly considered a tropical pest. In 2002, a field near Hermiston, Oregon suffered severe damage due to potato tuberworm (Alvarez et al. 2005). At present, it is a major problem for potato growers in the Pacific Northwest. Potato tuberworm established itself in California as early as 1855 and in Texas as early as 1917 (Berthon 1855, Graf 1917). Due to mild winters and dry summer conditions, potato tuberworm has expanded its geographic range to the Columbia Basin (Alvarez et al. 2005).

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. The greatest damage occurs in storage. 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). Potato tuberworm does not have discrete generations in tropical climates; multiple generations occur throughout the year and all stages of larvae and adults may be present at any one time (Trivedi and Rajagopal 1992).

Potato tuberworm causes 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). Insecticides remain

the chief means of control for potato tuberworm (Alvarez et al. 2005). Cultural practices, such as irrigation regimens and planting depth, reduce infestation in the field (Shelton and Wyman 1979, Coll et al. 2000). Utilization of host plant resistance factors, both natural and engineered, may augment current pest management practices.

Natural host plant resistance factors from *Solanum* species can be introgessed 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 an elite 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 in introgress resistance genes from any organism into potato. Crystal (Cry) proteins derived from the soil-borne bacterium *Bacillus thuringiensis* are among the most well studied class of insecticidal proteins and are commonly engineered into plants to confer insect resistance (Whalon and Wingerd 2003). A myriad of *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, with the strongest non-covalent bond found in nature (K_d=10^{-15} M) (Green 1990). Biotin, also called vitamin H or B_8, is an essential vitamin for all organisms (Trager 1948). It 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, Burgess et al. 2002, Markwick et al. 2003).

Insect mortality, as a result of biotin depletion, is slow (Levinson et al. 1992, Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Markwick et al. 2003). 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 1986; Roush 1998, Zhao et al. 2005).

The objectives of this study were: (1) to evaluate the performance of potato tuberworm first stage larvae on ND5873-15 (*S. chacoense*-derived) and transgenic tubers

expressing avidin and (2) examine the combined effects of avidin with the natural host plant resistance derived from *S. chacoense*.

MATERIALS AND METHODS

Plant materials

The potato lines selected were MSE149-5Y, MSE75.7, ND5873-15 and ND75.3 (Table 3.1). 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 reported resistance to Colorado potato beetle (Coombs et al. 2002). The transgenic lines MSE75.7 and ND75.3 were developed in our lab using *Agrobacterium tumefaciens*-mediated transformation to express avidin. If avidin is expressed throughout the plant cell, it may interfere with biotin-dependent carboxylases and cell function (Murray et al. 2002). Previous studies demonstrated that avidin could be safely stored in the vacuole of the plant by adding the signal sequence potato protease inhibitor-I (PPI-I) (Genbank Accession L06606, Beuning et al. 1994, Murray et al. 2002). A fusion gene was constructed PPI-I/avidin driven by constitutive CaMV35S promoter (Fig. 3.1). The selectable marker was neomycin phosphotransferase (*nptII*) under the control of its own nopaline synthase promoter. MSE75.7 and ND75.3 were selected for this study because high insecticidal activity to Colorado potato beetle larvae and the avidin expression in the leaf tissue was high and did not significantly differ from each other ($F = 203.35$, $df = 49$, $P < 0.0001$), 63.8 μM ± 0.25 S.E. in MSE75.7 and 61.6 μM ± 0.25 S.E in ND75.3 line ($LSD_{\alpha=0.5} = 4.8$ μM).

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 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 1g of tissue in 1 ml of 50mM 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 4C. 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-nitrophenyl phosphate (pNPP) at 1mg/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. The colony is maintained on potato tubers as previously described in Mohammed et al. (2000). Eggs laid on the No. 1 Whatman filter paper (Whatman, Hillsboro, OR) were placed on top of sliced potato tubers in the Petri dish until larvae emerge.

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 were 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 tuber. Missing individuals were considered dead. Percentage mortality was transformed with the arcsine of the square root to homogenize variance and analyzed using Fisher's protected least significant difference test (LSD, P = 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 5 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 ± 2.7 S.E.) did not differ significantly compared to the level of avidin expression in tubers of ND75.3 (12.5 μM ± 2.7 S.E.) ($F = 6.75$, $df = 3$, $P < 0.0064$) ($LSD_{\alpha=0.5} = 28.19$ μM). The level of avidin expression was much lower in the tuber than previously observed in the leaf tissue of the same potato lines (63.8 μM ± 0.25 S.E. for MSE75.7 line and 61.6 μM ± 0.25 S.E for ND75.3 line) (Cooper et al. unpublished).

Potato tuberworm bioassays

The susceptible, avidin and *S. chacoense*-derived line tubers were rotting with obvious mining damage at 28 d (Fig. 5.1). Larvae fed on the susceptible line had the lowest mortality (16.0% ± 9.0 S.E) (Fig. 5.2).

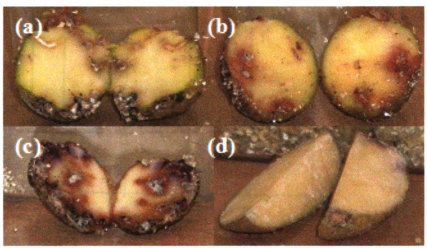

Figure 5.1: Examples of damage caused by potato tuberworm larvae fed potatoes after 28 d. (a) MSE149-5Y (susceptible), (b) MSE75.7 (avidin) (c) ND5873-15 (*S. chacoense* -derived), and (d) ND75.3 (avidin + *S. chacoense* -derived). Image presented in color.

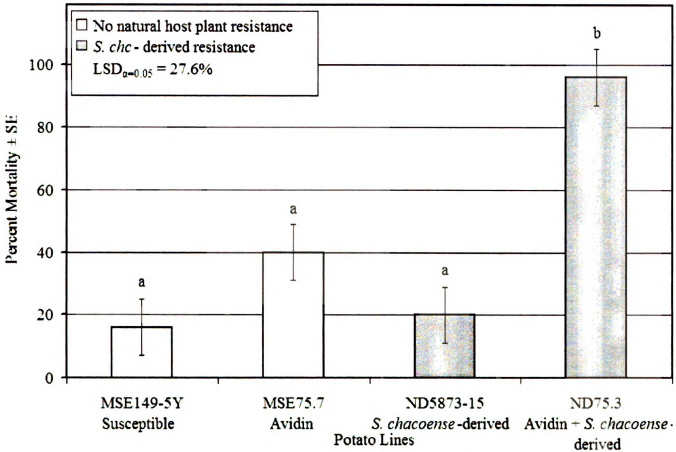

Figure 5.2: Mean percentage mortality of potato tuberworm larvae fed on four potato lines: MSE149-5Y (susceptible), MSE75.7 (avidin), ND5873-15 (*S. chacoense* -derived), or ND75.3 (avidin + *S. chacoense* -derived) at 28 d in a no-choice tuber bioassay. Means followed by different letters are significantly 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 is presented.

The mortality for larvae fed on the avidin line was higher (40.0 % ± 9.0 S.E.), but did not differ significantly compared to the mortality for larvae fed on the susceptible line ($F=13.01$, $df = 3$, $P < 0.0001$). Larval damage was evident inside the tubers with avidin. Markwick et al. (2003) observed much higher mortality (~97%) in a shorter time (9 d) with transgenic avidin tobacco. Avidin expression was much lower in transgenic tobacco (3.1 – 4.6 μM) compared to transgenic potato (10.6 μM ± 2.7 S.E.) (Markwick et al. 2003). Additionally, potatoes produce low levels of biotin (0.14 μg/100g) compared to leafy vegetable crops such as lettuce (0.62 μg/100g) or cabbage (0.83 μg/100g), 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 did not confer appreciable resistance against potato tuberworm. The mortality for larvae fed on the *S. chacoense*-derived resistance line was low (20.0% ± 9.0 S.E.) and did not differ significantly from the mortality for larvae fed on the susceptible line (Fig. 5.2) ($F=13.01$, $df = 3$, $P < 0.0001$). Mining damage was apparent on the *S. chacoense*-derived resistance line tubers (Fig. 5.1).

Although avidin or *S. chacoense*-derived resistance alone did not appear to provide plant protection, the combination avidin and *S. chacoense*-derived resistance provided a high level of protection (Fig. 5.1-2). The mortality (98.0 ± 9.0 S.E.) for larvae fed on the avidin+*S. chacoense*-derived resistance line was significantly higher than the mortality for larvae fed on the susceptible line, the MSE75.7 avidin line, or the *S. chacoense*-derived resistance line. 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. 5.1).

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 elite breeding line ND5873-15 does not have leptine glycoalkaloids (Douches unpublished). The current literature is limited regarding potato tuberworm resistance in *Solanum*. 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, like 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 ssp., and variegated cutworms, *Peridroma saucia* (Hübner) (Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Markwick et al. 2003, Yoza et al. 2005).

Avidin does not appear to negatively impact such non-target organisms as young honeybees, *Apis mellifera* L.; Malone et al. (2002) found the growth and development was not retarded for newly emerged honeybees fed pollen containing avidin (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).

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; transgenic avidin potatoes may delay the development of Fusarium dry rot, *Fusarium sambucinum* (Robbins and Ma 1941). 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.

Impacts of avidin from transgenic potatoes on consumers will be negligible. Humans regularly consumer 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).

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, such as 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 *Solanum berthaultii* Hawkes, *S. commersonii* Dunal, *S. sparsipilum* Bitter, *S. sucrense* Hawkes or *S. tarijense* Hawkes may provide superior protection.

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CHAPTER VI:

CONCLUSIONS

Avidin is a natural protein produced in the egg white of chicken eggs (*Gallus gallus* L.). Avidin protects the chicken embryo by sequestering biotin from disease-causing microorganisms (Green 1990, Stevens 1991). Biotin is an essential vitamin for all organisms; it is involved in such critical biosynthetic pathways as the citric acid cycle, lipogenesis and gluconeogenesis (Alban et al. 2000). Without sufficient biotin, these pathways and cell viability is severely compromised (Alban et al. 2000). In insects, biotin depletion from avidin causes stunted growth, abnormal development and death (Morgan et al. 1993, Marwick et al. 2001, Malone et al. 2002).

Avidin, especially in combination with other host plant resistance factors, has potential as an insecticidal transgene. Currently, commercial transgenic crops largely rely on crystal toxins (Cry) from the bacterium *Bacillus thuringiensis* for insect control (Ferry et al. 2006). *Bt*-Cry toxins are highly specific and generally only effective against select groups of insects (Ferré and Van Rie 2002). Avidin has been expressed in a number of crops, including maize (*Zea mays* L.), tobacco (*Nicotiana tabacum* L.), and rice (*Oryza sativa* L. var. Nipponbare) and is resistant to a wide spectrum of insect pests (Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Malone et al. 2005, Yoza et al. 2005). The current studies demonstrate Colorado potato beetle larvae are sensitive to biotin depletion via avidin (Cooper et al. 2006).

Even so, avidin is less toxic than *Bt*-Cry3A to Colorado potato beetle larvae (Naimov et al. 2003; Cooper et al. 2006). Colorado potato beetle first stage larvae fed on MSE75.7 (avidin line) consumed significantly less, were significantly smaller, and had

higher mortality than larvae fed on MSE149-5Y (susceptible line) at 5 d. Additionally, the development time from first stage to pre-pupal stage for Colorado potato beetle larvae fed on MSE75.7 (avidin line) was significantly longer than Colorado potato beetle larvae fed on MSE149-5Y (susceptible line). In general, larvae with longer development times have greater incidences of predation and attacks by pathogens in the field (Pincebourde and Casas 2006). Additionally, increases in generation time may affect the population growth rate and reduce the number of generations of Colorado potato beetle in season. Lastly, significantly fewer Colorado potato beetle larvae fed on MSE75.7 (avidin line) survived to adults compared to Colorado potato beetle larvae fed on MSE149-5Y (susceptible line). Potatoes expressing avidin may suppress Colorado potato beetle numbers in the field.

Both modeling and biological data suggest that combining host plant resistance factors with different modes of action can increase insecticidal activity and effective life of individual host plant resistance factors (Gould 1986, Roush 1998, Zhao et al. 2005). Avidin is an excellent resistance factor for stacking because of its unique activity. Additionally, avidin is not likely to confer complete insect protection alone due to the slow mode of action inherent with an anti-nutritional protein. Combining avidin with stronger toxins like Bt-Cry or natural host plant resistance like leptines may increase both the effectiveness and longevity of the resistance factors. For example, Colorado potato beetle third stage larvae fed *Bt*-Cry3A leaves dipped in 8μM avidin had higher mortality than larvae fed on untreated *Bt*-Cry3A leaves at 3 d (Cooper et al. 2006). Additionally, avidin confers broad spectrum resistance. Potatoes with both *Bt*-Cry3A and avidin could

effectively combat the primary pest, Colorado potato beetle, and secondary pests perhaps reducing the need for insecticide treatments.

The present studies are the first reports of combining avidin with any natural host plant resistance factor. ND5873-15 derives its resistance from *S. chacoense*; the insect resistance is attributed to glycoalkaloids and an uncharacterized resistance factor, but is not attributed to leptine glycoalkaloids previously described from *S. chacoense* (Sinden et al 1986). Colorado potato beetle first stage larvae fed on ND75.3 (avidin + *S. chacoense*-derived resistance line) consumed significantly less and were significantly smaller than larvae fed on MSE149-5Y (susceptible line), MSE75.7 (avidin line) and ND5873-15 (*S. chacoense*-derived resistance line) at 5 d. However, the development time from first stage to pre-pupal stage for Colorado potato beetle larvae fed on ND5873-15 (avidin+ *S. chacoense*-derived resistance line) did not significantly differ from the development time for Colorado potato beetle larvae fed on MSE149-5Y (susceptible line), MSE75.7 (avidin line), or ND5873-15 (*chacoense*-derived resistance line). Moreover, the number of Colorado potato beetle larvae fed on MSE75.7 (avidin) that survived to adults did not differ significantly from the number of Colorado potato beetle larvae fed on MSE75.7 (avidin line).

The present studies were conducted under greenhouse conditions; the lines should be further evaluated under field conditions. Glycoalkaloid levels are highly affected by light intensity; a potato clone grown in the field will often have higher glycoalkaloid levels than the same clone grown in the greenhouse (Dimenstien et al. 1997). Similarly, transgene expression level can be affected by light; for example, *Bt*-Cry3A potatoes grown in the field will also often have higher expression than the same clone grown in

the field (Cooper unpublished). Therefore, the avidin and avidin+*S. chacoense*-derived resistance lines should be evaluated in the field due to the likelihood of higher avidin and glycoalkaloid levels.

Avidin is effective against a range of insect pests, including Lepidoptera, Coleoptera and Diptera (Kramer et al. 2000, Markwick et al. 2001, Burgess et al. 2002, Malone et al. 2005, Yoza et al. 2005). The present studies investigated the effect of avidin against two potato insect pests; further studies are needed to examine the activity of avidin against other potato pests. MSE75.7 (avidin line) tubers were susceptible to potato tuberworm larvae, *Phthorimaea operculella* (Zeller). These findings contradict previous studies in tobacco (Markwick et al. 2003). ND75.3 (avidin+*S. chacoense*-derived resistance line) was highly resistant against potato tuberworm infestations. Further studies are needed to ascertain the use of avidin, singly and in combination with other natural resistance factors, against potato tuberworm.

Flour mite, *Acarus siro* L, consuming avidin have suppressed fertility (Levinson et al. 1992). Although a number of Colorado potato beetle and potato tuberworm were able to survive and develop from first stage to adults fed on potatoes expressing avidin, the reproductive ability of the survivors may be compromised. Therefore, further studies are needed to evaluate the fecundity of insects fed on plant expressing avidin.

Biotin is involved in pathways, such as the citric acid cycle, that are present in all organisms. Hence, transgenic avidin potatoes may inhibit proliferation of pathogens by sequestering available biotin. The growth of a number of *Fusarium* species is stunted without sufficient biotin (Robbins and Ma 1942). Many fungi require biotin to stimulate sporulation (Yoshida and Shirata 2000). Therefore, studies should be conducted to

evaluate incidences of diseases caused by bacterial and fungal pathogens on transgenic potatoes expressing avidin.

Avidin may not have high toxicity against Colorado potato beetle larvae compared to *Bt*-Cry3A, but is an exciting transgene due to its potential broad spectrum resistance against arthropods pests and pathogens.

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