Combining Engineered Resistance, Avidin, and Natural Resistance Derived From *Solanum chacoense* Bitter to Control Colorado Potato Beetle (Coleoptera: Chrysomelidae)

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ABSTRACT The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), 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. A preliminary study was conducted to determine the bioactivity of the transgene in each background. A single transgenic line was selected in each background for further studies. Detached leaf bioassays were performed on transgenic and nontransgenic clones of the susceptible and *S. chacoense* lines by using first-stage Colorado potato beetle larvae. Consumption, survival, and survivor growth were measured after 5 d. Larvae consumed significantly less on the two avidin-expressing lines compared with the nontransgenic lines. Survival was also significantly less for larvae feeding on transgenic avidin lines compared with the nontransgenic lines. The mass of survivors was significantly reduced on two transgenic avidin lines compared with the nontransgenic lines. Further studies examined the development from first-stage larvae to adulthood on greenhouse-grown whole plants 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 with 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 with survival on nontransgenic 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.

KEY WORDS *Solanum tuberosum*, *Leptinotarsa decimlineata*, host plant resistance, transformation

Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a pest of potatoes, *Solanum tuberosum* (L.), 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 1990). It consistently adapts to insecticides and is currently resistant to >40 insecticides (Whalon et al. 2008).

At present, most commercial transgenic crops rely on crystalline (Cry) proteins developed from the bacterium *Bacillus thuringiensis* (Bt) Berliner for Bt-Cry proteins are highly specific, often only effective against a particular insect order and many times act on only some insect species within the order (Ferré 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-Cry1 proteins are active against Lepidoptera (Herrnstadt et al. 1986, Lambert et al. 1992, Sato et al. 1994). 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, Kramer et al. 2000, Burgess et al. 2002). Avidin is a natural protein derived from the chicken (*Gallus gallus* L.) egg white. It has a strong affinity for biotin, with the strongest noncovalent bond found in nature ($K_d = 10^{-15}$ M) and is able to sequester biotin (Izrailev et al. 1997). Biotin, also called vitamin H or B_8, is critical for all organisms, including insects (Trager 1948). Many 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 some plants, bacteria, and fungi synthesize biotin. Hence, most organisms, in-

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cluding insects, must acquire biotin from their diet or environment (Trager 1948). Insects require biotin-dependent carboxylases to store and use fat (Miura et al. 1967). Insects with little biotin available die because of 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). Because of the universal dependence of biotin, avidin is effective against a broad range of plant pests such as Diptera, Lepidoptera, and Coleoptera (Morgan et al. 1993, Markwick et al. 2001, Malone et al. 2002).

Colorado potato beetle larvae are less sensitive to avidin (lower LC_{50} values) than other insects (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 multiple resistance mechanisms into a plant may delay resistance development of insects (Gould 1998, 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 Colorado potato beetle and disease resistance (Maga 1994). The wild species *Solanum chacoense* Bitter 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 a breeding line from North Dakota State University derived from *S. chacoense* with insect resistance attributed to glycoalkaloids (Lorenzen et al. 2001). Engineering ND5873-15 to express avidin may enhance resistance to Colorado potato beetle.

The objectives of this study were to 1) transform potato plants to express avidin, 2) evaluate the performance of Colorado potato beetle larvae feeding on transgenic potato plants expressing avidin, and 3) determine whether combining *S. chacoense*-derived resistance with avidin conferred elevated plant protection by further delaying insect growth under no-choice and whole plant greenhouse studies.

Materials and Methods

Preliminary Trials with Multiple Lines. *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 with partial resistance to Colorado potato beetle derived from *S. chacoense.* The potato lines were maintained in tissue culture by nodal propagation in 25- by 150-mm culture tubes or GA-7 Magenta vessels (Magenta Corp., Chicago, IL) in modified Murashige and Skoog (1962) (MS) medium (MS salts at 4.3 g liter^{-1}, 3% sucrose, 1.4 mM sodium phosphate, 1.1 μM thiamine, 0.55 mM *myo*-inositol, pH 6.0, and Bactoagar at 8 g liter^{-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 photoperiod of 16:8 (L:D) h.

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 within the cell and inhibit cell function (Murray et al. 2002). A 111-bp oligonucleotide 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 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 nontranscribed strand with 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. 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. 1). The plasmid pSPUD75 was introduced into *Agrobacterium tumefaciens* Smith and Townsend strain LBA4404 (Clontech, Palo Alto, CA) by triparental mating (Bevan 1984).

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 mg liter^{-1}) in 25- by 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 main-

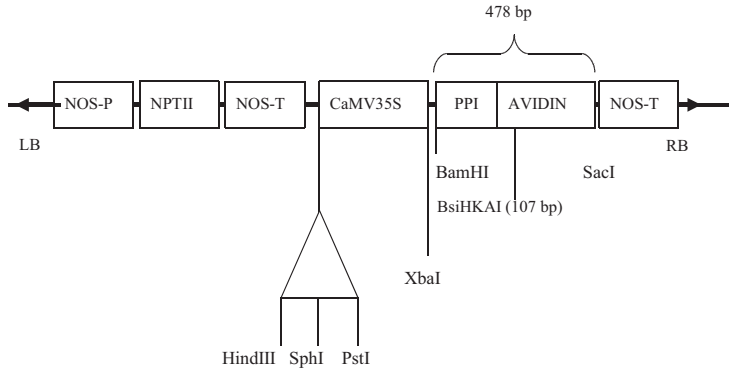

Fig. 1. Schematic of the T-DNA region of the gene construct pSpud75.

tained by micropropagation and were transplanted to trays in the greenhouse for further analyses. Rooted putative transgenic plants in the MSE149-5Y background number were denoted as MSE*AV*- followed by the shoot number, while rooted putative transgenic plants in the ND5873-15 background number were denoted as ND*AV*- followed by the shoot number.

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-wk-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) (1× PCR buffer, 0.2 mM dNTP mixture, 1.0 μM of each primer, 100 ng of template DNA, and 1 U of *Taq*DNA polymerase).

The length of the synthetic PPI-I/avidin gene is 383 bp. A 25-base primer and a 26-base primer were chosen to amplify the 383-bp 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 nontranscribed strand with 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% (wt:vol) agarose gel containing ethidium bromide at 0.5 μg ml^{-1} in 1× Tris-acetate/EDTA, pH 8.0, buffer at 100 mV for 1 h (Sambrook et al. 1989) and viewed under UV light (254 nm).

Enzyme-Linked Immunosorbent Assay (ELISA) for Quantification of Avidin. Indirect sandwich 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) overnight at 4°C. Protein was extracted from the leaf by grinding 1 g of tissue in 1 ml of 50 mM phosphate-buffered saline (PBS), pH 7.0, containing 0.05% Tween (Sigma) before being adjusted to a final dilution of 1:10 (wt:vol). The avidin protein from the leaf extracts was captured overnight at 4°C. The avidin protein reacted with rabbit anti-avidin antibody (Sigma) (1.25 h at 37°C). Finally, the plates were incubated with an anti-rabbit conjugated to alkaline phosphatase (Sigma) (1.25 h at 37°C). The alkaline phosphatase was determined with *para*-nitrophenyl phosphate at 1 mg/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, PerkinElmer Life and Analytical Sciences, Boston, MA). The ELISA analysis was replicated three times for each line. Data were analyzed by analysis of variance (ANOVA), and mean protein expression levels were compared using Fisher least significant difference (LSD) ($P = 0.05$) (SAS Institute 2005).

Southern Analysis. Total plant genomic DNA was extracted from the fresh leaf tissue (2 g) of greenhouse-grown tissue culture transplants using the cetyltrimethylammonium bromide extraction protocol (Saghai-Maroof et al. 1984), modified by adding 2% β-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, 20 mg of each DNA line 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 Diagnostics, Indianapolis, IN).

Avidin Detached Leaf Assay. 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.

Table 1. Bioactivity of Colorado potato beetle first stage larvae after 3 d

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

[a] Fisher $LSD_{\alpha = 0.05} = 4.8$ μM.
[b] Fisher $LSD_{\alpha = 0.05} = 23\%$.
[c] Fisher $LSD_{\alpha = 0.05} = 11.0$ mg/larva.

No-choice detached leaf bioassays were performed using MSE149-5Y, 14 transgenic avidin lines in parental background MSE149-5Y, ND5873-15, and seven transgenic avidin lines in parental background ND5873-15. The potato lines were maintained in tissue culture as described previously (Coombs et al. 2002). Plants from tissue culture were grown in 2.5-liter pots in a greenhouse. 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 (125 mm in diameter) lined with filter paper. Leaf tissue was harvested for ELISA and detached leaf bioassay simultaneously. ELISA analysis was replicated three times for each line. Data were analyzed by ANOVA, and means of protein expression were compared using Fisher LSD in the GLM procedure of SAS ($P = 0.05$) (SAS Institute 2005).

Ten newly hatched first instars were 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).

Mass of survivors and percent survival were recorded after 3 d. Consumption was visually estimated with square millimeter 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 percentage of survival and survivor mass) were analyzed by ANOVA, and means were compared using Fisher LSD ($P = 0.05$) in the GLM procedure of SAS (SAS Institute 2005). Reported mean arcsine survival values were retransformed into percentages for presentation.

Detached Leaf Assays with Selected Lines. Four potato lines were selected for no-choice detached leaf bioassays and whole plant assays based on level avidin expressed as determined by ELISA. The plants selected were MSE149-5Y (susceptible) and MSE*AV*-7 (avidin), ND5873-15 (*S. chacoense*-derived), and ND*AV*-3 (avidin + *S. chacoense*-derived) lines (Table 1).

The detached leaf bioassay was performed as stated earlier except the assay was extended to 5 d. The detached leaf bioassays were conducted as a completely randomized design with two trials of five replications per trial (100 individuals per potato line).

ELISA analysis was replicated three times for each replicated line to confirm protein expression level. Data were analyzed using ANOVA, and mean protein expression levels were compared using Fisher LSD ($P = 0.05$) in the GLM procedure of SAS (SAS Institute 2005).

Whole Plant Assay with Selected Lines. The potato lines, MSE149-5Y, MSE*AV*-7, ND5873-15 and ND*AV*-3 were maintained in tissue culture. When tissue culture plants reached ≈60 mm in height, they were transferred to soil in seedling trays (50 cells per tray, 3 cm diameter) in the greenhouse. After a month, five plants per line were transferred into individual 3.78-liter pots and grown to ≈0.5 m in height. ELISA analysis was replicated three times for each replicated line to confirm protein expression level. Data were analyzed using ANOVA, and mean protein expression

levels were compared using Fisher LSD ($P = 0.05$) in the GLM procedure of SAS (SAS Institute 2005), 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 ≈2 cm above the soil, and the pot was covered with a cage.

The larval instar was recorded every 3 d through pupation. Adult emergence was recorded every 2 d. The duration of each study was 56 d. The percentage of larvae surviving to adulthood, number of days to entering soil for pupation and adult emergence were recorded. Two trials were conducted with four replications per trial. Percentage of 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 ANOVA, and means were compared using Fisher protected LSD (LSD, $P = 0.05$) in the GLM procedure of SAS (SAS Institute 2005). Reported mean arcsine square root survival values were retransformed into percentages for presentation.

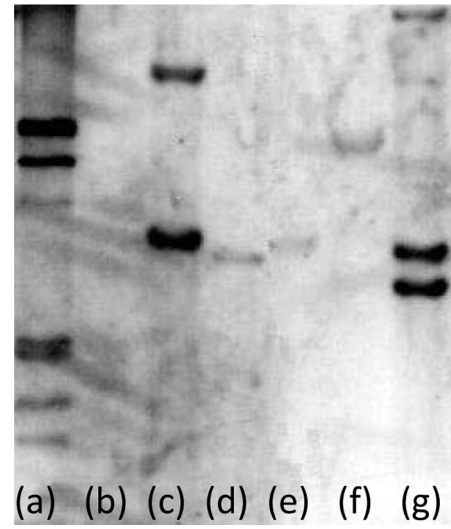

Fig. 2. Southern analysis of total plant DNA from avidin transgenic lines digested with XbaI and hybridized with avidin RNA probe. The avidin plasmid *pSPUDAV* also was digested. (a) Roche DIG-molecular weight marker III. (b) MSE149-5Y. (c) MSE*AV*-21. (d) MSE*AV*-7. (e) MSE*AV*-25. (f) MSE*AV*-27. (g) ND*AV*-3.

Results and Discussion

Preliminary Trials with Multiple Lines. *Transformation and Characterization. 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 ≈75 MSE149-5Y explants. Of the 34 shoots, 92% rooted in MS medium with kanamycin at 50 mg/liter. Twenty-six shoots were removed from ≈75 ND5873-15 explants of which 100% rooted in MS medium with kanamycin at 50 mg/liter.

Eighty percent of the rooted MSE149-5Y avidin lines and 88% of the rooted ND5873-15 avidin lines were PCR positive after DNA amplification of the 383-bp *avidin* fragment. PCR was not possible on nine of the putative transgenic ND5873-15 because of fungal contamination of the tissue culture tubes. The copy number ranged from one to three copies for transgenic plants expressing avidin (Fig. 2). All of the transgenic avidin plants seemed phenotypically normal in test tubes. There was no relationship between copy number and avidin expression ($r = -0.07$, $P = 0.5879$).

Avidin expression of the PCR positive lines ranged from 0.0 to 63.8 ± 0.3 μM ($F = 203.35$, df = 49, $P < 0.0001$) comparable or higher than previous studies in other plants. However, the level of avidin expression was undetectable (0.0 ± 0.3 μM) in three other transgenic lines MSE*AV*-18, MSE*AV*-28, and MSE*AV*-30. Avidin expression ranged from 3.1 to 4.6 μM in transformed tobacco and from 1.9 to11.2 μM in apple; ≈160 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).

Detached Leaf Bioassays. First-stage larvae fed on any of the transgenic avidin lines were significantly smaller than first-stage larvae fed on the nontransgenic susceptible line MSE149-5Y (Table 1). Larvae fed on the transgenic lines MSE*AV*-18, MSE*AV*-28, and MSE*AV*-30 were significantly smaller than MSE149-5Y even though avidin expression was undetectable in the transgenic lines. Although ELISA was unable to detect avidin, low levels may be present that inhibited the larval growth. There was small significant negative correlation between avidin expression and the mass of the surviving larvae fed on the transgenic avidin lines ($r = -0.4485$, $P < 0.0001$).

First-stage larvae fed on the transgenic avidin + *S. chacoense*-derived resistance lines ND*AV*-2, ND*AV*-3, and ND*AV*-7 were significantly smaller than first-stage larvae fed on the nontransgenic *S. chacoense*-derived resistance line ND5873-15 (Table 1). There was no correlation between avidin expression and the mass of the surviving larvae fed on avidin + *S. chacoense*-derived resistance lines ($r = -0.1802$, $P = 0.2657$). 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 1).

There was not a significant correlation between avidin expression and mortality for larvae fed on the transgenic avidin lines ($r = -0.1199$, $P = 0.3056$). First-stage larvae fed on the susceptible line MSE149-5Y had significantly higher survival (96.0 ± 8.0%, mean ± SE) than first-stage larvae fed all the avidin lines, except MSE*AV*-30 (90.0 ± 8.0%) that did not have detectable avidin expression (Table 1) ($F =$

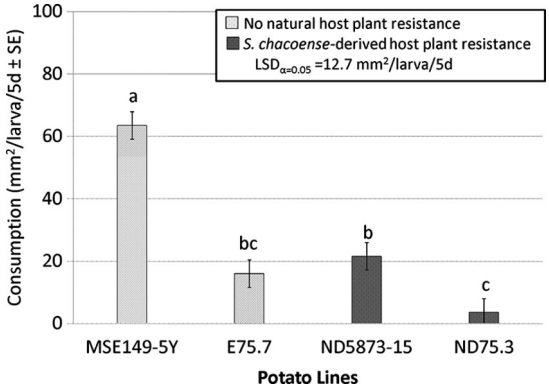

Fig. 3. Mean consumption by Colorado potato beetle first stage larvae fed on four potato lines: MSE149-5Y (susceptible), MSE*AV*-7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND*AV*-3 (avidin + *S. chacoense*-derived) at 5 d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different ($P < 0.05$) and were separated using Fisher LSD.

1.70, $n = 22$, $P = 0.0291$). First-stage larvae fed on MSE*AV*-18 (72.0 ± 8.0%) or MSE*AV*-2 (72.0 ± 8.0%), with undetectable avidin levels, had significantly lower survival than larvae fed on the susceptible line and is likely because of the variability naturally associated with assay. 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. 2004).

The survival (88.0 ± 8.0%) 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 1). First-stage larvae fed on the *S. chacosense*-derived resistance line had significantly higher survival than first-stage larvae fed all avidin + *S. chacoense*-derived resistance lines except ND*AV*-5 (72.0 ± 8.0%), the low expression line (0.2 ± 0.3 μM , mean ± SE). There was not a significant correlation between avidin expression and mortality for larvae fed on avidin + *S. chacoense*-derived resistance lines ($r = -0.2035$, $P = 0.2079$).

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 1). The duration of the present assay (3 d) was too brief to determine efficacy of combining *S. chacoense*-derived resistance with avidin. Colorado potato beetle larvae fed on transgenic avidin plants had higher insect mortality and lower larval mass. Combining multiple host plant resistance factors can increase the efficacy and effective life of individual host plant resistance factors (Gould 1998, 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 were needed to elucidate the potential impacts of avidin alone and in combination with other resistance factors.

ELISA for Quantification of Avidin. The level of avidin expression for MSE*AV*-7 (45.0 ± 5.36 μM) was not significantly different from ND*AV*-3 (45.1 ± 5.36 μM) at 1d ($F = 1.26$, df = 7, $P = 0.3088$) ($LSD_{\alpha = 0.05} = 15.7$ μM) of the whole plant assay. The level of avidin expression for MSE*AV*-7 (46.96 ± 5.36 μM) was not significantly different from ND*AV*-3 (39.6 ± 5.36 μM) at 35 d ($F = 1.26$, df = 7, $P = 0.3088$) ($LSD_{\alpha = 0.05} = 15.7$ μM). The avidin level was lower than measured previously in the initial molecular characterization. The difference may be attributed to the different time of year that the plants were grown in the greenhouse.

Select Line Trials. *Detached Leaf Bioassays.* 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, $P < 0.0001$) (Figs. 3 and 4). 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 with large areas consumed on the other lines (Fig. 4).

Surviving larvae fed on the susceptible line gained the greatest mass over 5 d, with an average mass of 7.4 mg per larva ± 0.7 (SE) after 5 d (Fig. 5). The mass of surviving larvae fed on the avidin line was signifi-

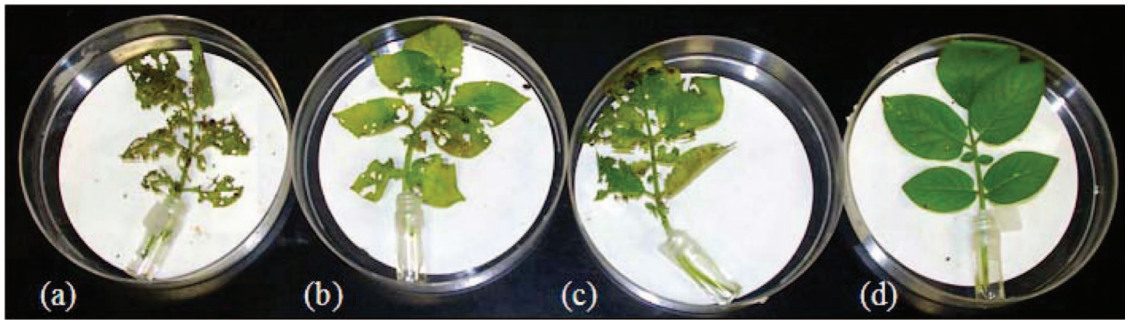

Fig. 4. Examples of damage caused by Colorado potato beetle first-stage larvae fed potato leaves after 5 d. (a) MSE149-5Y (susceptible). (b) MSE*AV*-7 (avidin). (c) ND5873-15 (*S. chacoense*-derived). (d) ND*AV*-3 (avidin + *S. chacoense*-derived). (Online figure in color.)

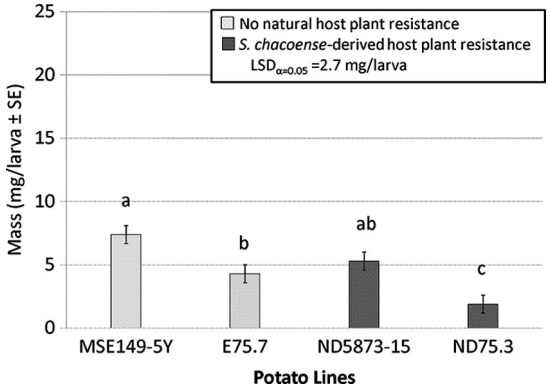

Fig. 5. Mean mass of surviving Colorado potato beetle first-stage larvae fed on four potato lines: MSE149-5Y (susceptible), MSE*AV*-7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND*AV*-3 (avidin + *S. chacoense*-derived) after 5 d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different ($P < 0.05$) and were separated using Fisher LSD.

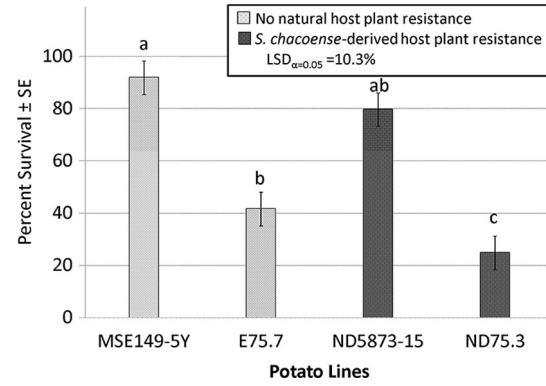

Fig. 6. Mean percentage of surviving Colorado potato beetle first-stage larvae fed on four potato lines: MSE149-5Y (susceptible), MSE*AV*-7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND*AV*-3 (avidin + *S. chacoense*-derived) at 5 d in a no-choice detached leaf bioassay. Means followed by different letters are significantly different ($P < 0.05$) based on analysis of arcsine square-root transformed data. Means were separated using Fisher LSD. Untransformed data are presented.

cantly reduced compared with 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); *Helicoverpa armigera* (Hübner), and *Spodoptera litura* (F.) (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 prior foliar application study, the avidin line reduced larval mass after 5 d because the avidin expression level was almost 5 times higher (45.0 ± 5.36 μM) than the foliar application (8 μM) (Cooper et al. 2006). Moreover, the avidin protein was expressed throughout the leaf compared with 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. 5). Surviving larvae fed on the 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 that were fed on plants with combined resistance factors (avidin + *S. chacoense*-derived) did not have significantly higher mortality than the 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 fed the susceptible line had significantly higher survival (92%, ± SE) than first-stage larvae fed the avidin line (42%, ± 6 SE) ($F = 10.39$, df = 3, $P = 0.0005$) (Fig. 6). Markwick et al. (2003) observed a much higher survival rate (>75%) for lightbrown apple moth feeding on transgenic apple tissues expressing avidin at 7 d. The expression levels of avidin in apple tissues were between 1.9 and 11.2 μM, which is <20% of the expression level of our avidin line (45.0 ± 5.36 μM). Previous studies demonstrated an increased mortality with increased avidin concentration against a variety of insect pests (Kramer et al. 2000, Burgess et al. 2002, Markwick et al. 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), lightbrown apple moth (2.6 μM at 21 d), *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 because of high avidin expression in the plant.

The survival of first-stage larvae fed the *S. chacoense*-derived resistance line (80 ± 6%) did not differ significantly compared with the survival of first-stage larvae fed the susceptible line (Fig. 6). The survival of first-stage larvae fed the *S. chacoense*-derived 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 antinutritional; it retards the development of larvae, eventually leading to death of insects (Levinson et al. 1992). Therefore,

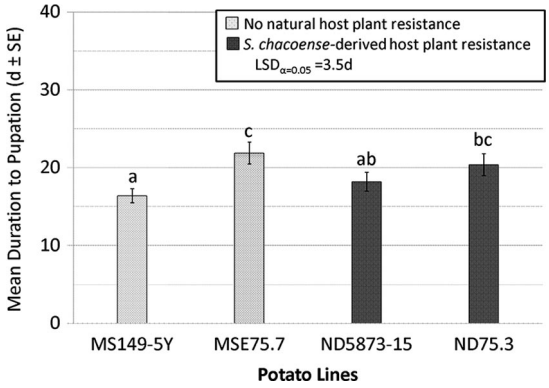

Fig. 7. Mean duration of surviving Colorado potato beetle first-stage larvae to pupation fed on four potato lines: MSE149-5Y (susceptible), MSE*AV*-7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND*AV*-3 (avidin + *S. chacoense*-derived) after 56 d in a no-choice bioassay. Means followed by different letters are significantly different ($P < 0.05$) and were separated using Fisher LSD.

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.

Whole Plant Bioassay. *Larval Development.* The duration from first stage to the prepupal stage was the shortest (16.4 ± 1.0 d, mean ± SE) for larvae fed on MSE149-5Y (susceptible line) (Fig. 7). Colorado potato beetle mature from first to fourth instar within 15–20 d at 20°C and was normal under the greenhouse conditions (Walgenbach and Wyman 1984). The duration from first stage to prepupal stage was prolonged significantly for larvae fed on the avidin line (21.9 ± 1.4 d) compared with larvae fed on the susceptible line ($F = 4.04$, $n = 3$, $P = 0.0096$). The duration from first stage to prepupal did not differ significantly for larvae fed on the *S. chacoense*-derived resistance line (18.2 ± 1.2 d) compared with larvae fed on the susceptible line (Fig. 7). In similar studies, avidin retards development in a variety of arthropods, including the beetles that infest grains (Levinson et al. 1992, Morgan et al. 1993, Burgess et al. 2002, Markwick et al. 2003).

S. chacoense-based resistance is attributed to deterred feeding by Colorado potato beetle (Sinden et al. 1980). In addition, the development of Colorado potato beetle is slowed for larvae fed on leaves with high levels of glycoalkaloids and leptine glycoalkaloids (331–496 mg/100 g fresh weight tissue) but is not inhibited larvae fed on leaves with low levels of glycoalkaloids and leptine glycoalkaloids (206 mg/100 g fresh weight tissue) (Sinden et al. 1986). Resistance to Colorado potato beetle in ND5873-15 did not seem to affect developmental time, perhaps because glycoalkaloid levels in ND5879-15 are low (331 mg/100 g fresh weight tissue), relatively compared with other *S. chacoense*-derived lines.

The duration from first stage to the prepupal stage did not differ significantly for larvae fed on the avidin + *S. chacoense*-derived resistance line (20.4 ± 1.4 d) compared with larvae fed on the susceptible line, avidin line or *S. chacoense*-derived resistance line (Fig. 7). Development was monitored every 3 d. The lack of differences may be because of the frequency of monitoring development. If development was monitored daily, differences in development rates for larva fed on *S. chacoense*-derived resistance and avidin + *S. chacoense*-derived resistance lines may be elucidated.

Transgenic avidin plants may have implications to plant protection in addition to antibiosis. Insects with prolonged development because of 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, or parasitic wasps, thereby increasing mortality of larvae in the field. Also, an increase in generation time affects population growth rates and may reduce the number of generations the Colorado potato beetle can complete in a season. Our experiment was performed under greenhouse 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 and 47 d from the onset of the study. The total duration from first-stage larvae to newly emerged adults was shortest (41.5 ± 0.6 d) for larvae fed on the susceptible line but did not significantly differ from the duration for larvae fed on the avidin (42.1± 1.0 d), *S. chacoense*-derived resistance (42.2 ± 0.8 d), or avidin + *S. chacoense*-derived resistance (43.0 ± 0.8 d) lines ($F = 0.74$, $n = 3$, $P = 0.5306$).

Survival. Significantly fewer larvae survived to adulthood fed on the avidin line (26 ± 6%) compared with larvae that survived the susceptible line (59 ± 6%) (Fig. 8) ($F = 4.88$, $n = 3$, $P = 0.0109$). 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).

Larvae fed on *S. chacoense*-derived resistance line had 75 ± 17% survival to adulthood, 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%) was significantly lower compared with the percentage of emerging adults from larvae fed on *S. chacoense*-derived resistance (Fig. 8). The natural resistance from *S. chacoense*-derived line is associated with deterred feeding (Sanford et al. 1997, Rangarajan et al. 2000). In addition, large vigorous haulms of ND5873-15 (*S. chacoense*-derived) may limit the percentage of 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.

Although elevated resistance was not observed in the no-choice situation of the current study, ND5873-15 (*S. chacoense*-derived) confers resistance

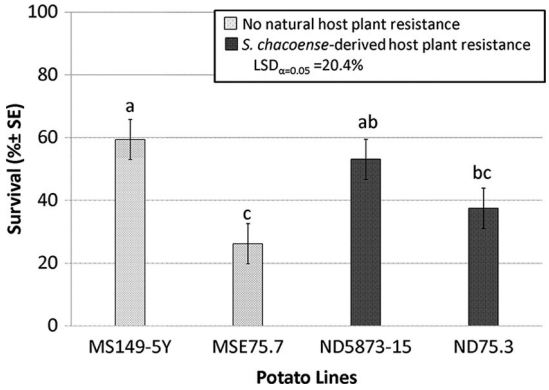

Fig. 8. Mean percentage of surviving Colorado potato beetle first-stage larvae to adults fed on four potato lines: MSE149-5Y (susceptible), MSE*AV*-7 (avidin), ND5873-15 (*S. chacoense*-derived), or ND*AV*-3 (avidin + *S. chacoense*-derived) at 56 d in a no-choice bioassay. Means followed by different letters are significantly different ($P < 0.05$) based on analysis of arcsine square-root transformed data. Means were separated using Fisher LSD. Untransformed data are presented.

in field conditions that was not evident in the current 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 with avidin line under field conditions because of predators and higher levels of glycoalkaloids (Dale et al. 1993).

Avidin is also effective against a variety of pests in other crops, including Lepidoptera, Coleoptera, Diptera, and Arcari; therefore, it may confer plant protection to other potato pests like wireworms, Elateridae spp., and variegated cutworm, *Peridroma saucia* (Hübner) (Levinson et al. 1992; Kramer et al. 2000; Markwick et al. 2001, 2003; Burgess et al. 2002; Yoza et al. 2005). Moreover, biotin is a required nutrient for other organisms such as fungi and bacteria. For example, the growth of a number of *Fusarium* species is stunted without sufficient quantities of biotin (Robbins and Ma 1941). Additionally, many fungi require biotin to stimulate sporulation (Yoshida and Shirata 2000). Therefore, transgenic avidin potatoes may inhibit proliferation of pathogens by sequestering available biotin.

Currently, commercial transgenic crops largely rely on crystal toxins (Cry) from the bacterium *B. 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). Because of 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). In particular, avidin may help protect potatoes against many lepidopteran and coleopteran pests such as wireworms, variegated cutworms and potato tuberworm (Kramer et al. 2000; Markwick et al. 2001, 2003; Burgess et al. 2002; Malone et al. 2005; Yoza et al. 2005). More broadly, avidin could be deployed in combination with a Bt-based resistance factor in a number of different crops, such as maize and cotton to provide strong, broad spectrum resistance. Furthermore, combining host plant resistance factors with different modes of action can increase insecticidal activity and effective life of individual toxins (Gould 1998, 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 such as leptines may increase both the effectiveness and longevity of the resistance factors.

Avidin-expressing potato tubers are safe for human consumption. Humans consume avidin in the form of egg whites on a daily basis. In addition, avidin is not an allergen. 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). Furthermore, 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). Similarly, potatoes are cooked before being consumed; the cooking process should denature the small quantities of biotin present in the potato. Finally, humans consume a diverse diet and do not depend on potatoes as a source of biotin. The average person eats 35–70 μg of biotin daily from varied food sources, including many vegetables and nuts (Hardinge 1961).

Avidin is a promising insecticidal protein with broad spectrum activity. Transgenic crops can target plant pests by expressing avidin in its tissue and can target crop pests by expressing it in the plant. Additionally, avidin is not persistent in the insects and will not cause negative tritrophic effects. For example, Christeller et al. (2005) was only able to extract 10–28% of active avidin consumed from *S. litura* fed on transgenic tobacco plants. Therefore, predators of tobacco cutworm are unlikely to be negatively impacted by small amounts of avidin.

Avidin-based resistance may be 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 experiments are needed to test efficacy under field conditions. The combination of higher glycoalkaloid levels within the plant reduced population growth rate and generation time and natural predators may provide plant protection. Additionally, avidin combined with other resistance factors including engineered factors such as Bt-Cry proteins, and natural factors such as leptines, may confer durable and broad-spectrum resistance.

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