EFFECT OF PLOIDY ELEVATION, COPY NUMBER AND PARENT-OF-ORIGIN ON TRANSGENE EXPRESSION IN POTATO

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Abstract

Recent advances in plant genetic engineering offer substantial benefits to farmers throughout the world. Genetic research has identified many exogenous genes that could considerably decrease production costs through transgene-mediated resistance to insect, viral, fungal and bacterial pathogens. Potato can be produced from true potato seed (TPS) through a sexual polyploidization step, known as 4*x*-2*x* hybridization. Little is known regarding the stability of transgenes through sexual polyploidization in potato, although studies have associated ploidy elevation with transgene silencing in plants such as *Arabidopsis thaliana*. In the present study, potato was transformed with two different transgenes, *cry3Aa* and *PVY^{o} cp*, and transgene expression was analyzed through 4*x*-2*x* hybridization. Transgene introgression did not affect fertility or agronomic performance (tuber set, average tuber weight, total tuber yield) of the resulting 4*x*-2*x* hybrids; however, reduced seed germination was observed for several transgenic lines in an *in vitro* study. Ploidy elevation did not affect a highly expressed single copy *cry3Aa* transgene, simplex or duplex, transmitted through pollen to 4*x*-2*x* hybrids. By contrast, multiple copies of *cry3Aa* triggered significant transgene silencing in diploids and silencing was further pronounced upon pollen transmission to 4*x*-2*x* hybrids. Crosses between two, single insert plants demonstrated additional evidence that multiple *cry3Aa* transgenes resulted in reduced expression, as well as provided evidence for maternal effects on expression of the *cry3Aa* transgene. Finally, Cry3Aa expression levels of progeny derived from low expressing, multiple copy 4*x*-2*x* hybrids indicated that reduction of transgene number in progeny, through meiotic segregation, could increase Cry3Aa expression. The results suggest that 4*x*-2*x* hybridization using single copy, male parents can result in high expressing, transgenic 4*x*-2*x* hybrids while segregating for a low frequency of non-transgenic hybrids that create a “refuge” to inhibit development of resistance to transgenes in pest populations.

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CHAPTER 1

Genetic engineering of potato (*Solanum tuberosum* L.): potential for improvement of potato production systems in developing countries

Abstract Most potato transgenic research has focused on development of resistance to pathogens and modification of potato physiology. Many transgenes, particularly those conferring pathogen resistance, could substantially lower potato production costs in developing countries. However, transgenes have not been reported in sexually propagated 4*x*-2*x* potato hybrids commonly grown in developing countries. Two transgenes, the *Bacillus thuringiensis cry3Aa* endotoxin protein gene and the PVY^{o} coat protein gene, were engineered into diploid and tetraploid potato using *Agrobacterium tumefaciens*-mediated transformation. Cry3Aa was produced at high levels while the PVY^{o} coat protein was not expressed. Diploid and tetraploid genotypes were crossed to produce transgenic 4*x*-2*x* hybrids. Genetic transformation had no discernable effect on fertility of the parental lines or agronomic performance (tuber set, average tuber weight and total tuber yield) of the 4*x*-2*x* hybrids. However, several 4*x*-2*x* crosses produced seed with reduced germination in an *in vitro* study. Results indicate that Cry proteins could be expressed in 4*x*-2*x* hybrids with little effect on plant phenotype.

Introduction

World production areas of potato, the fourth most important food crop, are in a rapid state of flux. Potato production in developed countries (Europe, former USSR, North America, Australia, Japan, South Africa, Israel) as a whole has not increased significantly over the past three decades. By sharp contrast, potato production in developing countries (Africa, Asia, Latin America) rose from 11% of the global potato output (30 million tons) in the early 1960's to 30% in 2000 (85 million tons). The majority of the world's potato output will be produced in developing countries in less than a generation if this trend continues (Food and Agriculture Organization of the United Nations/International Potato Center, 1995). Increased potato production in developing countries is due partly to improved technology, such as storage facilities, and improved propagation methods such as true potato seed (TPS) technology. Disease control, however, remains a problem for farmers in these parts of the world. It is estimated that developing world farmers spend $700 million U.S. annually on pesticides to combat agents of viral diseases, *Phytophthera infestans*, bacterial wilt and the potato tuber moth (Consultative group on international agricultural research, www.cgiar.org). The modification of potato through genetic engineering holds enormous potential for alleviating these problems, and others, and

could help the developing world to realize its full potential for potato production in the near future.

The importance of potato as a food source has significantly impacted the types of genetic engineering research conducted with this crop. Experimental plant species such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*, with no direct agronomic value, have been genetically modified to explore a wide variety of topics in plant physiology, metabolism and genetics. A review of potato transgenic research, on the other hand, reveals a much more narrow focus revolving around two areas critical to potato production: pathogen resistance and plant physiology. While other transgenic efforts have occurred, the vast majority of research has centered on creating novel resistances to potato pathogens and altering important physiological pathways such as starch synthesis. This trend identifies the most significant problems facing modern potato production, and it is hoped that genetic engineering will complement traditional breeding programs by modifying and enlarging the collection of resistance and physiological traits in the cultivated potato gene pool.

Genetic engineering of potato

Potato Leafroll virus (PLRV), common to many parts of the world, is the most devastating potato virus and yield losses of up to 90% can occur in infected crops. No resistance to PLRV occurred when a full-length cDNA of the virus was engineered into the potato genome (Franco-Lara et al. 1999); however, transformation of potato with the coat protein of PLRV resulted in resistance (Palucha et al. 1998; Derrick and Barker 1997; Kawchuk et al. 1997). Resistance to Potato Virus Y, the second most devastating potato virus causing yield losses of 80%, has been achieved through a variety of transgenic strategies. Transformation with the PVY coat protein gene (Hefferon et al. 1997; Okamoto et al. 1996; Smith et al. 1995), as well as the virally-encoded P1 proteinase sequence of the PVY^{o} strain (Maki-Valkama et al. 2000; Pehu et al. 1995), resulted in high levels of resistance to viral infection. In addition, heterologous immunity to PVY^{o} was achieved through transformation of potato with the Lettuce Mosaic

Potyvirus (LMV) coat protein gene (Hassairi et al. 1998). Using the same coat protein transformation strategy, researchers have developed transgenic plants with resistance to Potato Mop-Top virus (Barker et al. 1999, Barker et al. 1998) and group 3 of Potato Virus X (Spillane et al. 1998). Low transcription levels of transgenes in many of these cases suggest that the viral resistance in transgenic plants was based on gene silencing mechanisms.

A variety of potato insect pests has been the target of genetic engineering schemes, the most high profile case being the NewLeaf® potato cultivars developed by Monsanto (St. Louis, MO) with engineered resistance to the Colorado potato beetle (*Leptinotarsa decemlineata* Say). The Colorado potato beetle is one of the most devastating potato pests in North America (Grafius 1997) and the resistance of NewLeaf potato derives from expression of the insecticidal crystal (Cry) *Bacillus thuringiensis* var. *tenebrionis cry3Aa* endotoxin protein (Reed et al. 2001). The Colorado potato beetle has expanded its range enormously over the past 150 years and now is found in many potato production areas of North America and Europe. The beetle may soon spread its range to include Asia and Mexico. In warmer regions of the world, such as Central America, larvae of the potato tuber moth (*Phthorimaea operculella*) cause major economic losses by feeding on potato tubers. Several different Cry proteins from *B. thuringiensis* are known to control this pest. Mohammed et al. (2000) expressed the *cry5* gene in potato cvs. Lemhi Russet and Atlantic and observed 100% mortality of larvae fed with tuber material from these plants. Li et al. (1999) combined the *cry5* gene with a PVY^{o} coat protein gene to produce transgenic lines of cv. Spunta with high resistance to both tuber moth larval feeding and PVY infection. A modified *cry9Aa2* gene significantly increased mortality of tuber moth larvae when expressed in potato (Gleave et al. 1998). In addition, expression of truncated portions of the *cry1Ab* gene in transgenic tubers gave nearly 100% control of larval feeding during a two month storage bioassay (Jansens et al. 1995). The cotton bollworm (*Helicoverpa armigera*) larva is another serious pest worldwide and in developing countries such as India it is one of the chief pests affecting potato yield. Chakrabarti et al. (2000) transformed potato lines with the *cry1Ab* gene and found that transgenic plants had reduced feeding by the bollworm larvae. Efforts to improve the expression of *B.*

thuringiensis transgenes in plants indicate that removal of polyadenylation sites (Haffani et al. 2000) and single-copy insertions (Chan et al. 1996) can significantly elevate expression.

Insecticidal genes from *B. thuringiensis* are not the only source of transgenic insect resistance in potato. Potato resistant to feeding by tomato moth larvae (*Lacanobia oleracea*) was achieved by transformation with a trypsin inhibitor from cowpea (*Vigna unguiculata*, Bell et al. 2001) and a lectin gene from snowdrop (*Galanthus nivalis*, Bell et al. 1999). The concanavalin A lectin gene from jackbean (*Canavalia ensiformis*) did not affect mortality of tomato moth larvae when expressed in transgenic potato, but decreased consumption by the larvae and decreased overall larval weight (Gatehouse et al. 1999). Snowdrop lectin genes were also transformed into potato to increase resistance to the peach-potato aphid (*Myzus persicae*, Birch et al. 1999; Gatehouse et al. 1996).

Control of late blight (*Phytophthora infestans*), the most serious potato fungal disease, has been explored using transgenic methods. Several groups have engineered the fungal glucose oxidase gene from *Asperigillus niger* into potato, thus elevating hydrogen peroxide levels in plant tissue, and observed significantly increased resistance to late blight (Zhen et al. 2000, Wu et al. 1995). Expression of the tobacco class II catalase gene in transgenic potato led to increased late blight resistance, presumably due to its high affinity for salicyclic acid (Yu et al. 1999). Resistance to *Verticillium dahliae*, another fungal pest of potato, was created through transformation of potato with an antifungal defensin isolated from *Medicago sativa* seeds (Gao et al. 2000). This resistance equaled protection using current fumigants. Resistance to the bacterial pathogens causing soft rot and blackleg diseases during potato storage (*Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi*) was attempted by engineering the antimicrobial genes cecropin B and SB-37 from *Hyalophora cecropia* into potato (Arce et al. 1999). These genes increased resistance to bacterial infection, although cecropin B failed to increase resistance in other studies (Allefs et al. 1995). Increased resistance to soft rot and blackleg diseases was also produced by engineering potato with the lysozyme gene (*chly*) from chicken

(Serrano et al. 2000), while complete resistance was produced by expression of the phage T4 lysozyme in transgenic potato (Ahrenholtz et al. 2000). An antifungal endochitinase gene isolated from *Trichoderma harziantum* gave broad resistance to pathogens such as *Alternaria alternata, A. solani*, *Botrytis cinerea* and *Rhizoctonia solani* when expressed in transgenic potato lines (Lorito et al. 1998).

While much potato transgenic research has involved disease resistance, there is also great economic incentive to modify the potato plant itself. Modification of metabolic and physiological pathways has been achieved through transformation with exogenous genes, however, the majority of studies utilized antisense silencing of endogenous genes. A major goal of contemporary potato breeding is to produce potatoes with more starch (less sucrose) and with modified starch content. Trethewey et al. (1998) engineered the yeast invertase gene into potato to significantly increase sucrose metabolism. Unfortunately, starch production also decreased in their transgenic lines. Even in a case where starch content was increased by 3-fold in leaves (through antisense inhibition of potato hexokinase 1) starch content of tubers was not changed (Veramendi et al. 1999). Researchers have worked to identify native potato genes and pathways important for carbohydrate metabolism and partitioning to ultimately increase starch levels of tubers (van Voorthuysen et al. 2000; Sweetlove et al. 1999; Purcell et al. 1998). Transgenic tubers with reduced amylose content were created by antisense silencing of the granule-bound starch synthase (GBSS) gene (Wolters et al. 1998; Filpse et al. 1996). The branching pattern of potato starch was modified by antisense silencing of the starch branching enzyme A isoform (Jobling et al. 1999), while amylopectin synthesis was altered by antisense silencing of the *SSII* and *SSIII* genes (Edwards et al. 1999).

Research has also been done to identify genes that affect the growth cycle of potato, with the goals of increasing plant productivity and extending potato production to more diverse habitats. Transgenic potato plants expressing the yeast *rolC* gene had increased root growth, tiller number and total biomass (Schmulling and Aksenova, 1998). Biomass was also increased in plants expressing the *Arabidopsis thaliana* phytochrome B gene due to greater photosynthetic rates (Thiele et al. 1999). Antisense silencing of two native

potato genes involved in tuber dormancy and sprouting, (*A2-1* and *G1-1*, respectively) identified *G1-1* as having a significant role in dormancy regulation (Marmiroli et al. 2000). The ability of potato to endure oxidative stresses was increased by expression of a tomato cytosolic Cu,Zn superoxide dismutase (SOD) gene in transgenic lines (Perl et al. 1993). Finally, transgenics helped elucidate the biosynthetic pathways of several compounds important to potato growth such as isoprenoids (Korth et al. 2000), hydroperoxides (Griffiths et al. 2000), polyamines (Kumar et al. 1996), glycine decarboxylase (Heineke et al. 2001), cysteine (Harms et al. 2000), and methionine (Maimann et al. 2000, Tu et al. 1998). Manipulation of pathways producing these compounds, particularly amino acids, may result in a more nutritious food crop.

Potato propagation in developing countries

Clonal propagation of potato cultivars is practiced in developed areas of the world. This process involves the production of tubers, "certified seed," in isolated pathogen-free areas. Pathogen-free conditions are frequently not available in developing countries and have been a limiting factor to certified seed potato production. The production of potato from botanical seed, or true potato seed (TPS), is now allowing farmers to produce potato in many areas where certified seed cannot be grown locally or is prohibitively expensive to import. TPS seed can be produced from open pollination of selected tetraploid potato clones, or directed hybridization between tetraploid and diploid clones (4*x*-2*x* hybridization). Open pollination is the least expensive method of producing TPS, however, selfing that occurs during this process often leads to significant inbreeding depression (Golmirzaei et al. 1998). 4*x*-2*x* hybridization, an alternative to open pollination, utilizes diploid (2*x*) clones that produce unreduced (2*n*) pollen by first division restitution (FDR). First division restitution results from abnormal orientation of spindle fibers prior to anaphase II of meiosis. Rather than orienting in the normal 45° angle, the spindles become fused and result in non-sister chromatids pulled into the same nucleus (Veilleux 1985). The resulting unreduced pollen grains retain 80% or more of the parental heterozygosity. Pollination of *S. tuberosum* tetraploid cultivars (4*x*) with

FDR-derived pollen has frequently resulted in tetraploid progeny that are phenotypically quite uniform and highly vigorous.

TPS, whether derived by open pollination or 4*x*-2*x* hybridization, offers several advantages to farmers of the developing world. Not only are most diseases eliminated by passage through a sexual cycle, but the costs of planting TPS compared to clonal propagation are minimal ($80 per hectare vs. $1,200 U.S. per hectare, respectively). Transport and storage costs are also minimized. The International Potato Center (Lima, Peru) recently elevated TPS production (through 4*x*-2*x* hybridization) to one of ten major research projects and has worked to establish TPS production systems in several countries including India, China and Vietnam. India, where potato output has grown to nearly 30 million tons a year, has established six biocenters to produce TPS. TPS has the potential to increase potato yield in India from 16 to 30 tons per hectare. In addition, the International Potato Center recently reported that 10% of potato acreage in Vietnam is now planted from TPS, resulting in economic benefits estimated at $1.075 million U.S. It is likely that TPS will only increase as a means of potato production, particularly as the developing world produces an ever greater quantity of the world's potato output.

Transgenes and TPS potato

The previous review of potato genetic engineering identified many transgenes that are clearly effective against some of the greatest obstacles to potato production in the developing world. For example, three different Cry protein genes (*cry5*, *cry9Aa2* and *cry1Ab*) have been shown to control larval feeding by the potato tuber moth, one of the greatest pests of potato in India and elsewhere. Resistance to many viruses has resulted from transformation with coat protein genes. Fungal genes such as glucose oxidase can increase late blight resistance. As stated earlier, developing world farmers are spending $700 million U.S. annually on pesticides to control these pathogens. Transgene-mediated resistance to disease, a promising alternative, would be a much less expensive option for these farmers and will likely find widespread use in the near future. Presently, however, all studies of transgenes in potato have used clonally propagated cultivars. Transgenes

may be far more unstable in the sexual systems used for TPS. Not only have plant sexual cycles often decreased expression of transgenes (Meza et al. 2001; Elmayan and Vaucheret 1996), but elevation of ploidy (such as 4*x*-2*x* hybridization) has triggered transgene silencing phenomena in *Arabidopsis thaliana* (Scheid et al. 1996). An examination of transgene expression through TPS could determine the potential of genetic engineering for the improvement of sexually propagated potato.

This chapter describes the development of diploid and tetraploid potato lines transformed with two economically important transgenes, the *Bacillus thuringiensis cry3Aa* endotoxin protein gene and the PVY^o coat protein gene, and their initial performance in a 4*x*-2*x* hybridization scheme. The objectives of this work were to: (1) develop transgenic potato lines via *Agrobacterium tumefaciens*-mediated transformation carrying the two different transgenes; (2) determine the transgene protein levels of primary transformants; (3) evaluate fertility of transgenic lines during 4*x*-2*x* hybridization; (4) determine the effect of transgene incorporation, if any, on germination of 4*x*-2*x* seed, and (5) perform an agronomic evaluation of 4*x*-2*x* hybrid progeny derived from several of these lines.

Materials and methods

Plant material

A highly heterozygous, diploid potato genotype (APM-2) that produces 2*n* pollen by the genetic equivalent of FDR (first division restitution) was obtained by crossing dihaploid *S. andigena* (PI 347773) as female with an F_1 hybrid [S. *phureja* PI 225669 × *S. microdontum* PI 320304] as male. The tetraploid cv. Atlantic, grown commercially throughout North America, was also used for transformation. Prior to transformation, APM-2 and 'Atlantic' were propagated as *in vitro* plantlets for 4 wks on MS (Murashige and Skoog 1962) basal medium w/vitamins, 3% sucrose, 0.01% myo-inositol, 0.7% agar, pH 5.8.

Genetic constructs

A codon modified *Bacillus thuringiensis tenebrionis cry3Aa* gene (GenBank GI# 208152) in the pSPUD8 plasmid (Figure 1), and the PVY^{o} coat protein gene in the pBIPVY plasmid (Figure 2), were kindly supplied by David Douches (Michigan State University, E. Lansing). Each plasmid was carried in *Agrobacterium tumefaciens* strain LBA4404. The pSPUD8 plasmid was developed by David Douches while the pBIPVY plasmid was developed by Tom German (University of Wisconsin, Madison).

Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens cultures were initiated by incubating scrapings of cryopreserved stock in 5 ml Ty Medium (0.5% tryptone, 0.3% yeast extract, 0.05% dihydrate calcium chloride, 1.5% bacto-agar) plus 50 mg l^{-1} kanamycin monosulfate at 30°C with agitation for 2 d. Log phase cultures were initiated by diluting 1 ml of the *Agrobacterium* cultures into 50 ml Ty medium plus kanamycin monosulfate and incubating at 30°C with agitation for 6 h prior to transformation.

Leaves were detached from *in vitro* plantlets of APM-2 and 'Atlantic' and placed adaxial-side down onto callus induction medium (MS basal salt mixture, 0.9 mg l^{-1} thiamine HCl, 3% sucrose, 2.3 μM zeatin riboside, 9 μM 2, 4-D, pH 5.8) for 2 d. The leaves were floated for 10 min in 25 ml of log phase *Agrobacterium* solution and returned to the callus induction medium for 4 d.

Leaves were washed with cefotaxime sodium salt solution (250 mg l^{-1}) for 10 min to kill the *Agrobacterium* and placed onto shoot regeneration medium (MS basal salt mixture, 0.9 mg l^{-1} thiamine HCl, 3% sucrose, 2.3 μM zeatin riboside, 5.8 μM gibberellic acid, 250 mg l^{-1} cefotaxime sodium salt, 50 mg l^{-1} kanamycin monosulfate, pH 5.8) that was replaced every 14 d. Regenerated shoots were removed from leaf explants and rooted on MS basal medium plus 50 mg l^{-1} kanamycin monosulfate.

PCR detection of the transgenes in regenerated plants

DNA from regenerated plants transformed with the pSPUD8 plasmid was amplified with *cry3Aa* specific primers (forward 5'GAG CTG CAA GGC CTT CAA AAC AAT'; reverse 5'TCT AGC ACG GTA AGG GTC ATC TCT') spanning a 440 bp fragment of the gene. DNA from regenerated plants transformed with the pBIPVY plasmid was amplified with PVY coat protein specific primers (forward 5'CTC GGG CAA CTC AAT CAC AGT TT'; reverse 5'TCG GTG GTG TGC CTC TCT GTG TTC') spanning a 500 bp fragment of the gene. PCR amplification cycles consisted of: 1 cycle = 4 min 94°C; 40 cycles = 1 min 94°C, 1 min 58°C, 1.5 min 72°C; 1 cycle = 5 min 72°C. PCR products were electrophoretically separated on 1% agarose gels and visualized with ethidium bromide.

Flow cytometry for ploidy determination of transformants and progeny

Flow cytometry of *in vitro* plant material was performed according to Owen et al. (1988). DNA content in nuclei, relative to standard controls, was determined using a Coulter Epics XL Flow cytometer (Coulter International Corp., Miami, FL).

Establishment of transformants *ex vitro*

A total of ten APM-2 transformants, seven carrying the *cry3Aa* transgene (APM-2 TC1-TC7) and three carrying the *PVY cp* gene (APM-2 TP2-TP4), was acclimated in the greenhouse from *in vitro* shoots. In addition, two Atlantic transformants carrying the *cry3Aa* transgene (Atlantic TC1 and TC2) were acclimated to the greenhouse.

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) to detect transgenic protein expression of primary transformants

DAS-ELISA kits for Bt-Cry3A endotoxin and PVY^{o} coat protein from Agdia (Elkhart, ID) were utilized for this analysis (Figure 3). The youngest, fully expanded leaf from

greenhouse grown plants was ground in extraction buffer and filtered extract was loaded into wells coated with antibody to either the Bt-Cry3A endotoxin or PVY^{o} coat protein. An enzyme conjugate (transgene antibody fused to alkaline phosphatase) was also loaded into the wells. After overnight incubation, the wells were washed clean of extract and p-nitrophenyl was added. Any transgenic protein present in the sample was "sandwiched" between the well antibody and enzyme conjugate, and subsequent p-nitrophenyl addition resulted in a yellow color (p-nitrophenyl catalyzed to p-nitrophenol by alkaline phosphatase). The color intensified with increasing protein concentration, and absorbance was measured using an Emax^{TM} precision microplate reader (Molecular Devices Corporation) at 405 nm (reading capacity ranged from 0-4) and compared to a non-transgenic control or a positive control dilution series of purified transgenic protein to quantify the amount of protein present per well.

4*x*-2*x* hybridization

All ten APM-2 transformants were used to pollinate wild-type Atlantic to introduce transgenes from the male parent during 4*x*-2*x* hybridization. The two Atlantic transformants were pollinated with wild-type APM-2 pollen to introduce transgenes from the female parent during 4*x*-2*x* hybridization. Finally, Atlantic TC1 was also pollinated with APM-2 TC1 pollen to introduce transgenes from both parents during 4*x*-2*x* hybridization.

Germination percentage of 4*x*-2*x* hybrid seed

Seed from six different 4*x*-2*x* crosses was used to determine if *cry3Aa* or *PVY^{o} cp* transformation had a significant effect on germination of 4*x*-2*x* hybrid seed. The six crosses were: Atlantic × wild-type APM-2, Atlantic × APM-2 TC1, Atlantic × APM-2 TC2, Atlantic × APM-2 TP2, Atlantic × APM-2 TP3 and Atlantic × APM-2 TP4. A total of 216 seeds from each of the six crosses was surface sterilized (5 sec 80% ethanol, 20 min 30% bleach + 'Tween 20') and imbibed overnight with gibberellic acid (2000 ppm). The seeds were then placed onto plates of MS basal medium with or without 50 mg l^{-1}

kanamycin (36 seeds/plate, six plates total). Plates were arranged into a split plot design in a randomized complete block having three replications with family as the main plot and presence/absence of kanamycin as the subplot. The number of germinated seedlings per plate was determined at 2 and 4 weeks, analyzed by SAS GLM (1985), and grouped by Ryan-Einot-Gabriel-Welsh Multiple Range Test.

Agronomic evaluation of TPS hybrids

Seed from five different 4*x*-2*x* crosses (Atlantic × wild-type APM-2, Atlantic × APM-2 TC1, Atlantic × APM-2 TC2, Atlantic × APM-2 TP2, Atlantic × APM-2 TP3) was collected, dried, imbibed with gibberellic acid (2000 ppm) and planted in the greenhouse on April 10, 2000. Seeds were planted one seed/cell in cell packs of 48 (13.4 cm long × 13.2 cm wide × 5.8 cm deep; Kord Products, Canada, item #K806). After 4 weeks of growth (May 15, 2000), seedlings were transplanted to the field at Kentland Farm (Blacksburg, Va). Forty seedlings from each of the five crosses, as well as cv. Atlantic tubers, were planted in a RCB design with 4 replications (10 plants of each genotype per replication). Seedlings and tubers were planted in rows at 30 cm spacing, with 0.91 m between rows. The field was treated with fungicides on a weekly basis to prevent disease from affecting plant growth. Plants were harvested after roughly three months of growth (August 2000) and total yield (kg) and tuber number per 10 plant plot were recorded. The data were analyzed by SAS GLM (1985) and grouped by Ryan-Einot-Gabriel-Welsh Multiple Range Test.

Fertility of TPS hybrids

TPS hybrids derived from APM-2 TC1 and APM-2 TC2 were used as both male and female parents in sexual crosses to tetraploid cv. Katahdin.

Results

Agrobacterium tumefaciens-mediated transformation, PCR detection of transgenes and ploidy of transformants.

Shoots began to regenerate from leaf discs between 60-90 days after transformation and a total of 12 transgenic lines was obtained (Figure 4). Ten transgenic lines were regenerated from APM-2. Seven were transformed with the pSPUD plasmid while three were transformed with the PBI121-PVY construct. Two transgenic lines transformed with the pSPUD8 construct were regenerated from Atlantic. The transgene was amplified in all regenerated transgenic lines (Figure 5) and flow cytometry determined that ploidy remained stable during transformation. All of the transgenic lines regenerated from APM-2 were diploid whereas both transgenic lines from Atlantic were tetraploid (see Chapter 2, Figure 4).

DAS-ELISA for detection of transgenic protein expression of primary transformants

DAS-ELISA of three APM-2 lines transformed with the PVY coat protein gene (APM-2 TP2-TP4) indicated no transgene expression in all three lines. An extended incubation period of p-nitrophenyl in antibody-coated wells (3 days) also indicated no expression. DAS-ELISA testing of seven APM-2 lines and two Atlantic lines transformed with the *cry3Aa* transgene detected high expression of Cry3Aa protein. Nearly all of the lines (except for APM-2 TC2, APM-2 TC4 and APM-2 TC5) produced strong reactions that surpassed the absorbance reading capability of the microplate reader (4.0) after overnight incubation of p-nitrophenyl in antibody-coated wells. APM-2 TC2, APM-2 TC4 and APM-2 TC5 produced moderate reactions with p-nitrophenyl (absorbance readings of 1.8), indicating lower expression of Cry3Aa in these lines.

4*x*-2*x* hybridization

The ten APM-2 transgenic lines were used to pollinate wild-type cv. Atlantic to generate ten different 4*x*-2*x* hybrid populations (Figure 6A). Fruit set on 'Atlantic' with the various transgenic lines used as male parents varied between 60-90% and an average of 150 seeds/fruit was observed. More than 5,000 seed were regenerated from specific crosses after 50 pollinations. The two Atlantic transgenic lines were damaged by pesticide treatment and produced very few flowers. However, 100% of the pollinations by APM-2 and APM-2 TC1 (Figure 6B) were successful and resulted in several hundred seed from each cross.

Germination percentage of 4*x*-2*x* hybrid seed

ANOVA revealed family and replication to be significant sources of variation for seed germination (Table 1). Only one (Atlantic × APM-2 TC1) of the transgenic 4*x*-2*x* seed lots did not exhibit significantly reduced seed germination compared to w.t. The PVY-transformed 4*x*-2*x* hybrid seed showed only a mean of 10% seed germination compared to 54% for *cry3Aa*-transformed seed and 75% for wild-type seed at two weeks (Table 2). By 4 weeks the PVY-transformed 4*x*-2*x* hybrid showed a mean of 43% seed germination compared to 80% for *cry3Aa*-transformed seed and 96% for wild-type seed. Presence/absence of kanamycin in the medium had no measurable effect on seed germination.

Agronomic evaluation of TPS hybrids

ANOVA revealed family a significant source of variation for tuber number and average tuber weight and both family and replication as significant sources of variation for total yield (Table 3). No significant differences for tuber number, average tuber weight and total yield were detected among the five TPS hybrid populations, indicating that the wild-type TPS population did not differ from the four transgenic TPS populations (Table 4). The TPS populations had significantly smaller tubers than the commercial cv. Atlantic;

however, a corresponding higher tuber number resulted in significantly higher yields for all of the TPS populations relative to cv. Atlantic.

Fertility of TPS hybrids

The ATC1 and ATC2 TPS hybrids (derived from Atlantic × APM-2 TC1 and Atlantic × APM-2, repectively) produced low amounts of pollen that failed to set fruit when used to pollinate cvs. Atlantic or Katahdin. Subsequent acetocarmine staining of pollen from several hybrids showed the pollen to be non-viable. However, the TPS hybrids set fruit when pollinated by cv. Katahdin, indicating female fertility of the 4*x*-2*x* hybrids.

Discussion

The *Bacillus thuringiensis* insecticidal crystal protein Cry3Aa was expressed at high levels in many of the APM-2 and cv. Atlantic transformants, suggesting that these plants may be resistant to the Colorado potato beetle. These results also demonstrated the relative ease with which expression of the *cry3Aa* transgene can be detected in plants. The PVY^{o} coat protein, by contrast, was not expressed in three lines of APM-2 transformed with the gene. Silencing of viral coat protein genes in transgenic potato, both transcriptionally and posttranscriptionally, has often been associated with viral resistance (Maki-Valkama et al. 2000; Hassairi et al. 1998; Smith et al. 1995). It is possible that the APM-2 TP2-TP4 lines are resistant to PVY^{o} infection, however, further examination of the PVY^{o} cp transformants will require virus inoculations and transcriptional analysis.

The successful 4*x*-2*x* hybridizations involving transgenic plants (Figure 6) demonstrated that the transformation procedure did not reduce fertility of the plants. Transgenes inherited through the male or female, or both, parents resulted in high seed set. That more than 5,000 seed could be obtained through 50 pollinations indicates that transgenic potato can produce large amounts of possibly transgenic TPS seed with little effort and may be suitable for commercial production of TPS hybrids. Reduced germination

percentage of transgenic TPS seed, however, could represent a significant obstacle to the incorporation of certain transgenes into TPS hybrids. Seed derived from PVY-transformed parents showed greatly reduced germination in this study (Table 2). One of the PVY seed lines (Atlantic × APM-2 TP4) produced non-viable seed, perhaps caused by insertion of the transgene into a gene required for seed development. In addition, one of the *cry3Aa* transformed seed lines showed significantly reduced germination (APM-2 TC2). The germination difference between seed from *cry3Aa*- and PVY^o coat protein-transformed parents cannot be explained by differences in expression of kanamycin resistance accompanying transformation because there was no effect of kanamycin presence in the medium. Replication, however, was a significant source of variation in the seed germination studies; this effect was likely due to position of the plates in the culture room. Denis et al. (1995) reported that two of 37 *Brassica napus* lines transformed with an albumin seed protein gene had reduced germination, however, this difference was not correlated with transgene presence or expression and was attributed to tissue-culture effects. It is possible that the reduced germination of transgenic lines shown in Table 2 was due to tissue-culture effects (somaclonal variation) although it is notable that the three lowest germination frequencies were observed in seed from PVY^o coat protein-transformed parents. Examination of germination percentage in seed derived from additional transformants, and replication of this result, are needed to verify that transgenes, in particular the PVY^o coat protein gene, significantly impair seed germination of TPS hybrids.

The main advantage of plant genetic engineering is the alteration or addition of a specific plant trait, or set of traits, without otherwise altering phenotype. Thus a commercial line or cultivar can be modified to increase resistance to a pathogen without changing agronomic performance. Several research groups have examined transgenic plants to determine if transgene incorporation has unintended, pleiotropic effects on agronomic performance. Dale and McPartlan (1992) compared 70 independently transformed lines of potato expressing the beta-glucuronidase (GUS) gene with non-transgenic potato lines regenerated from tuber discs. This experiment was designed to separate tissue culture effects from transformation effects on agronomic performance. While somaclonal

variation was evident in both populations, the researchers found that tuber number, average tuber weight and plant height was significantly lower in transgenic plants relative to the non-transgenic, tissue culture-derived plants, providing evidence that transformation had a detrimental effect on plant growth. A similar result reported by Bregitzer et al. (1998) with transgenic barley (*Hordeum vulgare* L.) found that progeny derived from transgenic plants were shorter, lower yielding and produced small seed. Other studies have found little effect of transgene incorporation on agronomic performance. Dongmei et al. (1999) found that less than 20% of transgenic tobacco plants transformed with either the tobacco vein mottling virus coat protein gene or alfalfa mosaic virus coat protein gene had significantly reduced yield compared to non-transgenic plants. Graeber et al. (1999) observed no difference between Bt transgenic corn (*Zea mays* L.) and non-transgenic corn with respect to grain yield, stalk lodging, and test weight. Even when detrimental effects of transgene introgression have been detected, researchers have shown that selective breeding can return performance to that of non-transgenic plants (Horvath et al. 2001).

Any pleiotropic effects of genetic engineering in 4*x*-2*x* hybrids, resulting in decreased agronomic performance, could not be removed by selective breeding because of the nature of 4*x*-2*x* hybridization. These progeny result from hybridization between a tetraploid and diploid plant; no further breeding is necessary. The finding that none of the transgenic 4*x*-2*x* hybrid lines transformed with *cry3Aa* or *PVY^{o} cp* had reduced agronomic performance (as indicated by tuber number, average tuber weight and total yield) relative to the non-transgenic hybrids indicates that transgenic TPS hybrids could be agronomically competitive with wild-type hybrids. The field results also demonstrate that TPS hybrids can be higher yielding than clonally propagated potato in some situations. Male-sterility of TPS hybrids, as observed in this study, could minimize the spread of transgenes to sexually compatible, wild potato relatives in certain areas of the world (Central America) and could therefore slow the development of resistance to transgenes.

These results show that transgenes can be incorporated into diploid and tetraploid potato genotypes used for 4*x*-2*x* hybridization. Expression may depend on the type of transgene used, however, Cry proteins can be expressed at high levels in these genotypes. In addition, transgene incorporation did not affect fertility during 4*x*-2*x* hybridization or agronomic performance of 4*x*-2*x* hybrids. Germination of 4*x*-2*x* seed appeared to be affected by transgene presence, particularly with a viral coat protein gene, but this result must be verified.

The *cry3Aa* transformants were selected over the *PVY*^{o} *cp* transformants for further research and development for several reasons: high expression of the transgene enabled rapid analysis and protein quantification by DAS-ELISA, germination percentage of 4*x*-2*x* seed derived from some Cry expressing plants was similar to wild-type seed, and many different Cry expressing lines were available to work with. Having shown that the *cry3Aa* transgene can be incorporated into a 4*x*-2*x* hybridization system without significant effect on overall plant phenotype, the following chapters explore the effect of 4*x*-2*x* hybridization on Cry3Aa protein expression. The second chapter describes several studies involving the APM-2 *cry3Aa* transformants and their 4*x*-2*x* hybrid progeny (Figure 6A). The third chapter explores the effect of *cry3Aa* inheritance from both parents (Figure 6B), while the fourth chapter analyzes Cry3Aa expression of second generation progeny derived from a low expressing APM-2 transformant.

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Table 1: ANOVAs for number of seeds germinated (of 36 total) at two and four weeks after planting *in vitro*. Seed was derived from crosses between 'Atlantic' and wild-type APM-2 or APM-2 that had been transformed with a *PVY^{o} cp* or *cry3Aa* transgene. Six plates of each cross (36 seeds per plate) were placed in a split plot design in a RCB with 3 replications (2 plates per rep). The number of germinated seeds per plate was recorded at 2 and 4 weeks after planting on MS basal medium with or without kanamycin (1 plate of each per rep).

Time		2 weeks	4 weeks
Source	df	MS	MS
Block	2	185.4**	203.0**
Family	5	669.0**	898.4**
Block*family	10	21.0^{NS}	54.8**
Media	1	0.1^{NS}	3.4^{NS}
Block*media	5	7.1_{NS}	9.4^{NS}
Error	12	23.4	7.3

** indicates significant effect at $p<0.01$; ^{NS} not significant

Table 2: Number of seeds germinated (of 36 total) of 4*x*-2*x* hybrid seed derived from crosses between 'Atlantic' and wild-type APM-2 or APM-2 that had been transformed with a *PVY*^{o} *cp* (TP) or *cry3Aa* (TC) transgene. Six plates of each cross (36 seeds per plate) were placed in a split plot design in a RCB with 3 replications (2 plates per rep). The number of germinated seeds per plate was recorded at 2 and 4 weeks after planting on MS basal medium with or without kanamycin (1 plate of each per rep).

Cross	Average number seeds germinated			
	2 weeks		4 weeks	
Atlantic × w.t. APM-2	26.7	a[1]	34.7	a
Atlantic × APM-2 TC1	22.0	ab	31.3	a
Atlantic × APM-2 TC2	16.7	b	26.2	b
Atlantic × APM-2 TP2	5.5	c	25.3	b
Atlantic × APM-2 TP3	5.8	c	20.5	c
Atlantic × APM-2 TP4	0.0	c	0.2	d

[1]Means followed by the same letter within columns do not differ significantly by Ryan-Einot-Gabriel-Welsh Multiple Range Test.

Table 3: ANOVAs for mean tuber number, average tuber weight and total yield for five 4*x*-2*x* hybrid potato families and the parental cv. Atlantic transplanted in four replications of 10 plant plots at Kentland Farm (Blacksburg, Va). Four of the hybrid families were derived from a transgenic (*cry3Aa* or *PVY*^{o} *cp*) 2*x* parent.

Trait		tuber number	average tuber weight	total yield
Source	df	MS	MS	MS
Family	5	37103.56**	6932.08**	19.77*
Rep	3	8665.11^{NS}	7.38^{NS}	18.55*
Error	15	3860.54	50.24	4.78

*, ** indicates significant effect at $p<0.05$ and $p<0.01$, respectively; ^{NS} not significant

Table 4: Field evaluation of 4*x*-2*x* hybrids derived from crosses between Atlantic and wild type APM-2 or APM-2 that had been transformed with either PVY cp (TP) or Cry3Aa (TC) genes. Seedlings of the 5 crosses were transplanted to the field at Kentland Farm (Blacksburg, Va) in May, 2000 and arranged in a RCB design with three replications (10 plants per rep). 'Atlantic' was planted from tubers. Plants were harvested after three months and total yield (kg) and tuber number per 10 plant plot was recorded.

Genotype	Tuber number		Average tuber weight (g)		Total yield (kg)	
Atlantic × wild-type APM-2	228	a[1]	47	b	11	ab
Atlantic × APM-2 TC1	268	a	48	b	12	ab
Atlantic × APM-2 TC2	281	a	46	b	13	a
Atlantic × APM-2 TP2	324	a	42	b	13	a
Atlantic × APM-2 TP3	288	a	49	b	14	a
Atlantic	54	b	148	a	8	b

[1]Means followed by the same letter within columns do not differ significantly by Ryan-Einot-Gabriel-Welsh Multiple Range Test

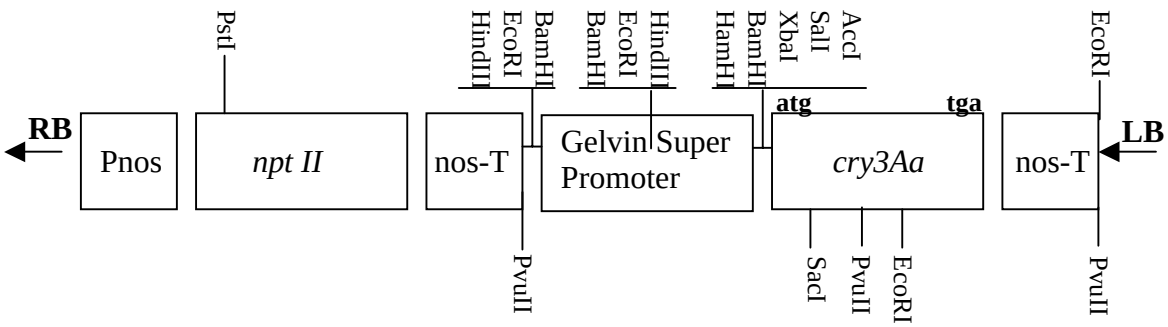

Figure 1: Structure of the pSPUD8 genetic construct containing the *cry3Aa* gene (1.79 kb) and the kanamycin resistance selectable marker. The *cry3Aa* was codon modified for high expression in plants (Sutton et al. 1992).

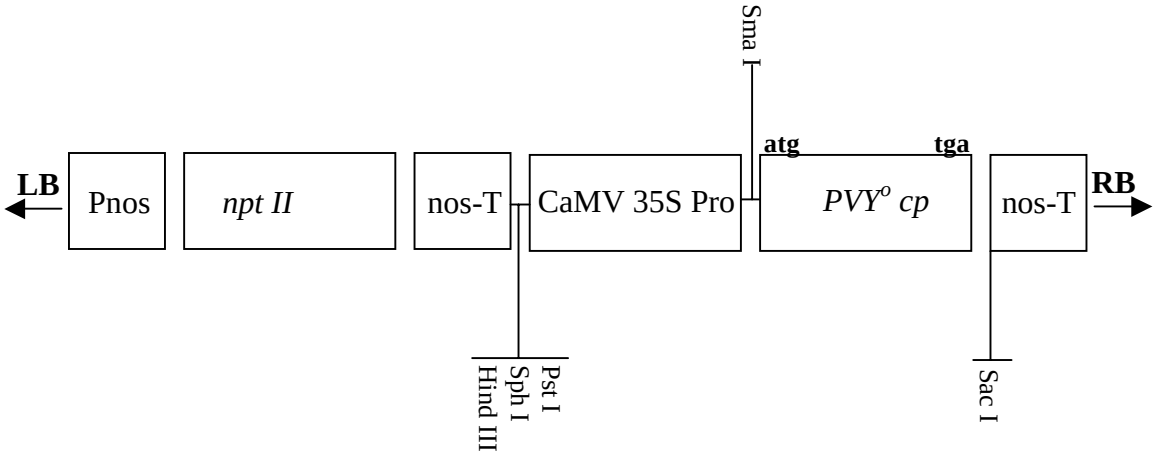

Figure 2: Structure of the PBI121-PVY construct containing the PVY^{o} coat protein gene and the kanamycin selectable resistance marker. A glycine in the *PVY^{o} cp* transgene was changed to a glutamate to inactivate the aphid transmission site.

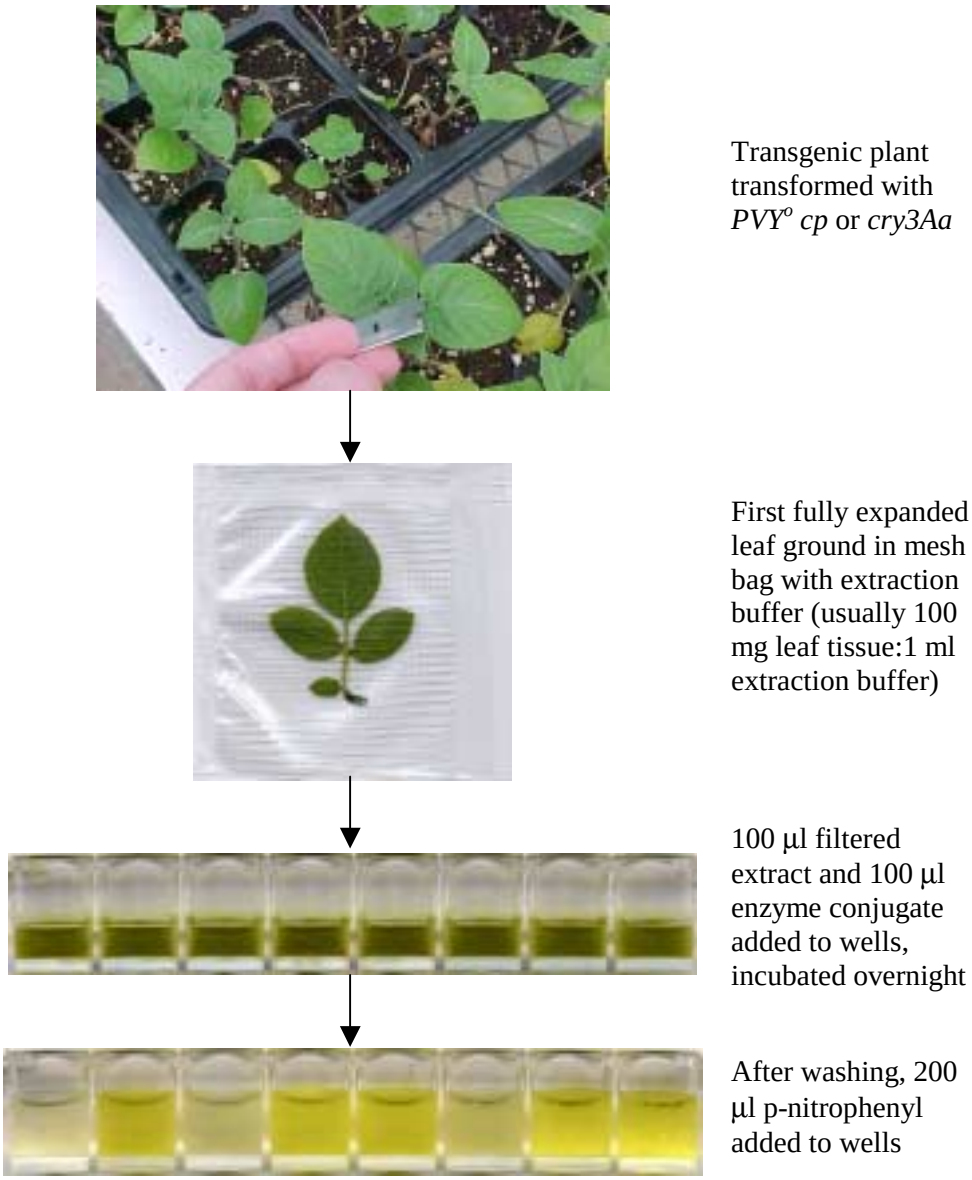

Figure 3: DAS-ELISA flowchart for transgenic plants transformed with the *PVY^o cp* or *cry3Aa* transgenes. The youngest, fully expanded leaf of greenhouse plants was ground in mesh bags with extraction buffer (phosphate buffer solution, 0.4% nonfat dried milk, 0.5% Tween 20) usually at a 1:10 w/v dilution. Filtered extract was loaded into microtiter wells coated with polyclonal antibody to the transgenic protein along with an enzyme conjugate (antibody to the transgenic protein fused to alkaline phosphatase). After overnight incubation, the wells were washed and p-nitrophenyl was added. A positive reaction was indicated by yellow color (p-nitrophenyl catalyzed to p-nitrophenol by alkaline phosphatase), with greater protein level indicated by darker color (more enzyme conjugate bound to transgenic protein). The absorbance was quantified using a microplate reader at 405 nm (reading capacity ranged from 0-4) and compared to a non-transgenic control or a positive control dilution series of purified transgenic protein to quantify the amount of protein present per well. The test could detect transgenic protein expression levels ranging from 1 ng ml^{-1} extract to 128 ng ml^{-1} extract.

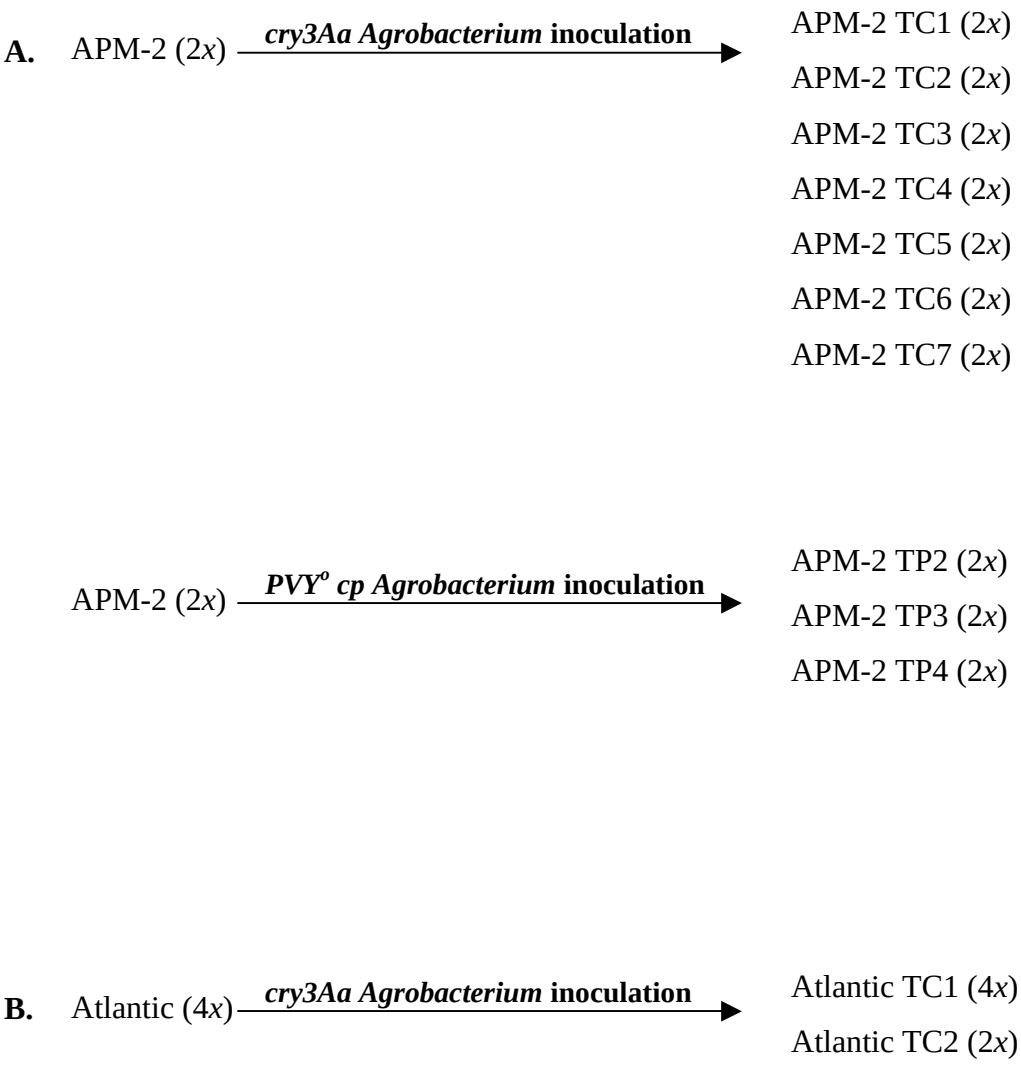

Figure 4: Results of *Agrobacterium tumefaciens*-inoculation of APM-2 (A) and Atlantic (B) *in vitro* leaf discs. Twenty leaf discs were used for each of the three inoculations. Ploidy of the genotypes as determined by flow cytometry is indicated parenthetically.

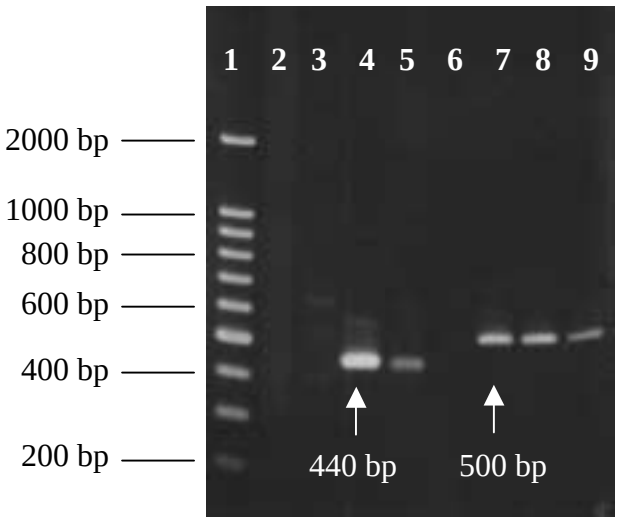

Figure 5: PCR analysis of DNA extracted from wild-type APM-2 (lanes 3 and 6), the two *Agrobacterium* strains used to create transgenic plants (*cry3Aa* Agro and *PVY^{o} cp* Agro, lanes 4 and 7 respectively), one transgenic plant transformed with the *cry3Aa* gene (lane 5) and two transgenic plants transformed with the PVY^{o} cp transgene (lanes 8 and 9). PCR amplification with the *cry3Aa* specific primers resulted in the expected 440 bp fragment whereas amplification with the *PVY^{o} cp* specific primers resulted in the expected 500 bp fragment. Lane 1 contains a 100 bp ladder ranging from 100 bp to 2000 bp and lane 2 is a negative control containing PCR primers with no DNA.

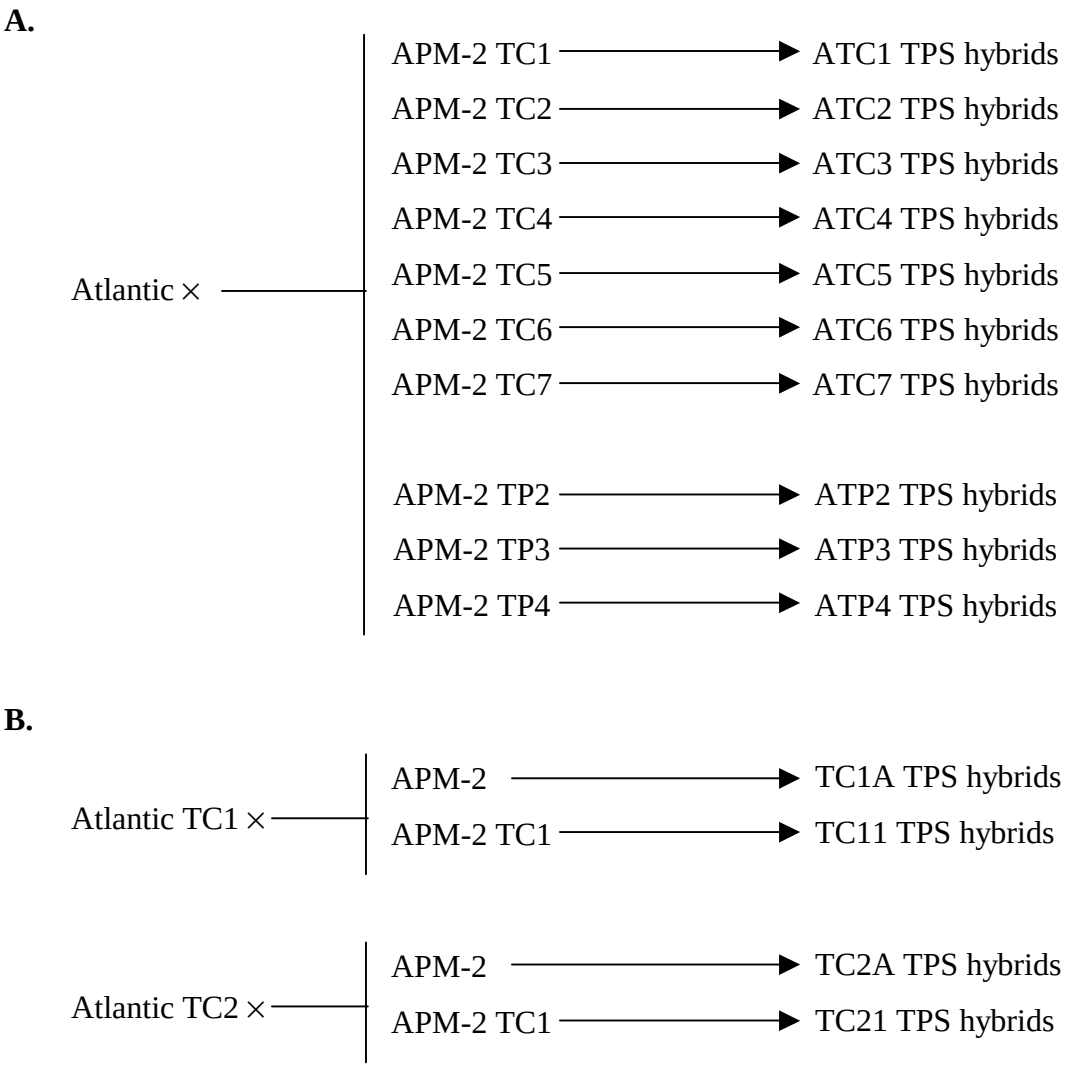

Figure 6: TPS hybrid populations produced using different transgenic parents. (A) The seven lines of APM-2 transformed with the *cry3Aa* transgene (TC1-TC7) and the three lines transformed with the PVY^{o} cp gene (TP2-TP4) were used to pollinate wild-type Atlantic to produce TPS hybrids inheriting transgenes from the male parent. (B) The two lines of Atlantic transformed with the *cry3Aa* transgene (TC1 and TC2) were crossed to wild-type APM-2 and APM-2 TC1 to produce TPS hybrids inheriting transgenes from the female parent or both parents, respectively.

CHAPTER 2

Transmission of a *Bacillus thuringiensis cry3Aa* transgene from diploid to tetraploid potato using 4*x*-2*x* hybridization: effect of ploidy increase on transgene expression and implications for TPS hybrid production

Abstract Ploidy elevation has altered the expression of native genes and transgenes in plant and animal studies. Unreduced gametes were used to produce tetraploid, transgenic progeny from diploid, transgenic parents to explore the effect of ploidy increase on the expression of an exogenous transgene (*cry3Aa*) in potato. Both single- and multiple-insert transgenic lines were regenerated using *Agrobacterium tumefaciens* leaf disc inoculation. A DAS-ELISA system and no-choice feeding bioassay enabled characterization of the lines as either "high" or "low" expressers of the Cry3Aa protein. Ploidy elevation did not have an effect on the high expression of a single-insert transgene, both in the hemizygous and duplex state. However, ploidy elevation caused significant silencing of a multiple-insert transgene configuration. Sixty-eight percent of tetraploid progeny derived from a multiple-insert, diploid parent had significantly reduced Cry3Aa expression relative to the parent. In addition, 32% of progeny derived from the multiple-insert parent demonstrated near complete silencing of the transgenes. Multiple copies of a transgene, like homologous native genes, may be particularly susceptible to transgene silencing following polyploidization. Results of this study indicate that incorporation of exogenous transgenes into a TPS hybrid potato production system is feasible assuming that a single-insert diploid parent is used. Gene-centromere mapping of the *cry3Aa* transgene in one of the single-insert plants demonstrates that a transgenic refuge can be created in a TPS hybrid system through genetic recombination.

Introduction

Unreduced gametes commonly occur in solanaceous species (Carputo et al. 2000) and have been used extensively to transfer agronomic traits such as bacterial wilt (*Pseudomonas solanacearum*), late blight (*Phytophthora infestans*), and potato cyst nematode (*Globodera pallida*) resistance from diploid *Solanum* species to the cultivated, tetraploid potato (*S. tuberosum*) gene pool through 2*x*-2*x*, 2*x*-4*x* and 4*x*-2*x* hybridization (Werner and Peloquin 1991; Ortiz et al. 1997; Watanabe et al. 1999). Unreduced diploid gametes produced through first division restitution (FDR) retain 80% or more of parental heterozygosity and, when used in pollination, often result in vigorous, tetraploid hybrids. True potato seed (TPS) hybrid production is a sexual method of producing potato cultivars that may employ 4*x*-2*x* hybridization to generate a pathogen-free crop. TPS hybridization is considered superior to open pollination of selected tetraploid clones as a means of producing potato botanical seed because selfing, and the resulting inbreeding

depression, is avoided. The International Potato Center (www.cipotato.org) recently elevated TPS hybrid production to one of ten major areas of current research and has successfully implemented TPS production systems in developing countries such as India where it is estimated that 250,000 hectares of potato will be TPS hybrid-derived by 2015.

Ploidy increase and its effect on gene expression have emerged as active areas of research in part because many of the world's most important crops, such as maize, have been found to be allopolyploids arisen from ancient polyploidization during which two different plant genomes were combined. Several studies have found that ploidy elevation can result in up-regulation or down-regulation of certain plant and animal native genes. Galitski et al. (1999) generated isogenic lines of yeast (*Saccharomyces cerevisiae*) ranging from haploid to tetraploid and examined expression of all genes in the yeast lines using oligonucleotide-probe microarrays. While the majority of genes remained consistent in expression across the ploidy lines, several genes, including *CTS1* encoding an endochitinase protein, were up-regulated in haploids compared to tetraploids, as much as 12-fold. Other genes, such as *FLO11* involved in filamentation, were downregulated 11-fold. The authors speculated that ploidy-dependent regulation of genes in the yeast lines may have occurred due to an increase in total cellular DNA content. Guo et al. (1996) examined expression of 18 genes in a maize ploidy series ranging from monoploid to tetraploid. While expression of most of the 18 genes increased correspondingly with ploidy, there were exceptional cases such as *csu 5*, encoding a thiol protease, which decreased expression by 28% between the diploid and tetraploid lines. Certain plant and animal native genes are apparently subject to expression alteration as a result of ploidy increase.

Foreign genes introduced into plant genomes through genetic engineering are also subject to expression changes as a result of ploidy increase. Scheid et al. (1996) crossed tetraploid *Arabidopsis thaliana* to transgenic diploid *A. thaliana* lines (homozygous for hygromycin resistance) and found that 18% of the resulting triploid hybrids lost expression of the transgene. Because all triploid hybrids had received only a single copy of the transgene, loss of expression could not have been due to silencing of multiple

transgene copies; rather, it was attributed to a silencing mechanism triggered by increase in chromosome number. The authors speculated that elevation to the tetraploid, rather than triploid, level could result in even more pronounced silencing of the transgene. Beaujean et al. (1998) found that GUS levels were 2.9-fold higher in homozygous diploid plants compared to haploid tobacco transformed with the *uidA* gene. The authors suggested that increase in chromosome number, combined with homozygosity, could result in higher transgene expression levels.

This research addresses ploidy increase and its effect on transgene expression using sexual polyploidization (4*x*-2*x* hybridization of potato), a method of ploidy elevation that as yet has not been examined in transgenic systems. Nearly all studies addressing this topic have used tissue culture-induced chromosome doubling or inter-ploidy crosses to elevate ploidy. Most 4*x*-2*x* hybrids derived from a transgenic diploid parent would have double the number of somatic chromosomes (relative to the diploid parent), yet retain the same number of transgenes, and thus, changes in transgene expression can be attributed primarily to ploidy increase. The 4*x*-2*x* hybridization system also serves as an appropriate model for allopolyploid formation and thus the results may be relevant to the gene silencing often seen in recently formed allopolyploids. Finally, this research explores the feasibility of incorporating transgenes into 4*x*-2*x* hybrids—a technology that holds great potential for lowering costs of potato hybrid production in developing countries but one that has not been described until now. The objectives were: 1) to express an economically important transgene in a diploid potato genotype producing unreduced gametes by FDR; 2) transmit the transgene to tetraploid progeny using 4*x*-2*x* hybridization; and 3) analyze the effect of ploidy elevation, if any, on transgene expression in the tetraploid progeny.

Materials and methods

Plant material

A highly heterozygous, diploid potato genotype (APM-2) that produces 2n pollen by the genetic equivalent of FDR (first division restitution) was obtained by crossing dihaploid *S. andigena* (PI 347773) as female with an F_1 hybrid [S. *phureja* PI 225669 $\times$ *S. microdontum* PI 320304] as male. Heat tolerance, field performance and glycoalkaloid content of tetraploid progeny derived from 4*x*-2*x* hybridization of cv. Atlantic and APM-2 have been previously described (Veilleux et al. 1997). Leaves of APM-2 used for transformation came from *in vitro* plantlets grown for 4 wks on MS (Murashige and Skoog 1962) basal medium w/vitamins, 3% sucrose, 0.01% myo-inositol, 0.7% agar, pH 5.8.

Vector

A codon modified *Bacillus thuringiensis tenebrionis cry3Aa* gene (GenBank GI# 208152) in the pSPUD8 vector (Fig. 1) carried in *Agrobacterium tumefaciens* strain LBA4404 was kindly supplied by David Douches (Michigan State University, E. Lansing). The *cry3Aa* gene was engineered for high expression in plants (Sutton et al. 1992).

Transformation protocol

Agrobacterium tumefaciens cultures were initiated by incubating scrapings of cryopreserved stock in 5 ml Ty Medium (0.5% tryptone, 0.3% yeast extract, 0.05% dihydrate calcium chloride, 1.5% bacto-agar) plus 50 mg l^{-1} kanamycin monosulfate at 30°C with agitation for 2 d. Log phase cultures were initiated by diluting 1 ml of the *Agrobacterium* cultures into 50 ml Ty medium plus kanamycin monosulfate and incubating at 30°C with agitation for 6 h prior to transformation.

Leaves were detached from *in vitro* plantlets and placed adaxial-side down onto callus induction medium (MS basal salt mixture, 0.9 mg/l thiamine HCl, 3% sucrose, 2.3 μM zeatin riboside, 9 μM 2, 4-D, pH 5.8) for 2 d. The leaves were floated for 10 min in 25 ml of log phase *Agrobacterium* solution and returned to the callus induction medium for 4 d.

Leaves were washed with a cefotaxime sodium salt solution (250 mg l^{-1}) for 10 min to kill the *Agrobacterium* and placed onto shoot regeneration medium (MS basal salt mixture, 0.9 mg l^{-1} thiamine HCl, 3% sucrose, 2.3 μM zeatin riboside, 5.8 μM gibberellic acid, 250 mg l^{-1} cefotaxime sodium salt, 50 mg l^{-1} kanamycin monosulfate, pH 5.8) that was replaced every 14 d. Regenerated shoots were removed from leaf explants and rooted on MS basal medium plus 50 mg l^{-1} kanamycin monosulfate.

PCR detection of the *cry3Aa* transgene

DNA from regenerated plants was amplified with *cry3Aa* specific primers (forward 5'GAC TGC TGA TAA CAA CAC GG3'; reverse 5'ATG TAG ACC TTA TCT CCG GC3') resulting in a 1,761 bp fragment of the 1,794 bp coding sequence. PCR amplification cycles consisted of: 1 cycle = 4 min 94°C; 40 cycles = 1 min 94°C, 1 min 58°C, 1.5 min 72°C; 1 cycle = 5 min 72°C. PCR products were electrophoretically separated on 1% agarose gels and visualized with ethidium bromide.

Southern analysis to detect transgene copy number

Total genomic DNA from regenerated plants was extracted using a CTAB procedure and suspended in TBE. A 50 μl digestion reaction was carried out on 10 μg of DNA using Promega restriction enzymes *BamH* I or *Xba* I, appropriate buffers and acetylated BSA. The pSPUD8 construct has *BamH* I and *Xba* I restriction sites directly before the *cry3Aa* coding sequence but not within the coding sequence (Figure 1). Restricted DNA was electrophoretically separated on a 2.5% agarose gel in 1× TAE running buffer and transferred overnight to a charged nylon membrane using alkaline capillary transfer. The

nylon membrane was hybridized overnight with a P^{32} labeled (prepared by random priming) 440 bp fragment of the *cry3Aa* gene (base numbers 252-692, GenBank #208152), washed and the hybridization signal was visualized using a Storm® gel and blot imaging system (Molecular Dynamics, Inc.).

Establishment of transformants *ex vitro* and 4*x*-2*x* hybridization

*In vit*ro plantlets of seven transgenic lines were acclimated to the greenhouse and, upon flowering, pollen from TC1 and TC2 was used to pollinate the tetraploid cv. Atlantic.

Flow cytometry to determine ploidy of transformants and progeny

Flow cytometry of *in vitro* plant material was performed according to Owen et al. (1988). DNA content in nuclei, relative to standard controls, was determined using a Coulter Epics XL Flow cytometer (Coulter International Corp., Miami, FL).

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) to detect Cry3Aa expression of primary transformants

A DAS-ELISA test for Bt-Cry3A endotoxin from Agdia (Elkhart, ID) was used to detect Cry3Aa expression levels of the seven transformants established in the greenhouse. Cry3Aa expression was measured by the absorbance reading of each sample after the addition of p-nitrophenyl phosphate using an Emax™ precision microplate reader (Molecular Devices Corporation) at 405 nm. Absorbance ratios were calculated by dividing the absorbance reading of each transgenic sample by the absorbance reading of the non-transgenic control. A dilution series of purified Cry3Aa protein enabled quantification of Cry3Aa production (µg per g of leaf tissue) for two of the seven lines (TC1 and TC2) both in the greenhouse and field.

Colorado potato beetle no-choice feeding assay

Ten genotypes (treatments) were used in a replicated no-choice feeding assay: Non-transgenic APM-2, seven transgenic lines derived from APM-2 (TC1-TC7), 'NewLeaf® Atlantic' (commercial variety engineered with a *cry3Aa* transgene) and non-transgenic 'Atlantic.' NewLeaf Atlantic and Atlantic plants were initiated from tubers. In vitro plantlets of the APM-2 transgenic lines were acclimated to the greenhouse and cuttings were taken of each genotype. After 6 weeks (19 May 2000), the APM-2 cuttings and 'Atlantic' and 'Newleaf Atlantic' plants were transplanted to the field at the Eastern Shore Agricultural Research and Extension Center, near Painter, VA. Plots consisted of 2 plants per genotype and treatments were arranged in a randomized complete block design replicated 6 times. On 13 June, several leaflets from each plant were excised and placed into petri dishes (9 cm in diameter) lined with moistened filter paper. There were 4 dishes (each dish = an experimental unit) or subsamples per plot. A single, unfed, first-generation Colorado potato beetle adult, *Leptinotarsa decemlineata* (Say), was placed into each dish and the dish was sealed with parafilm. Leaf area (in mm^2) was calculated prior to, and after, beetle feeding for 24 h. The average amount of leaf area consumed per beetle was analyzed by SAS general linear models (GLM) procedure (SAS 1985) and the genotypes were grouped by Ryan-Einot-Gabriel-Welsh Multiple Range Test.

Cry3Aa expression of tetraploid progeny

Replicated analysis of Cry3Aa expression was conducted with 25 progeny derived from crosses between 'Atlantic' and a single-insert APM-2 transgenic line (TC1) and 25 progeny derived from crosses between 'Atlantic' and a multiple-insert APM-2 transgenic line (TC2). Seeds were germinated and propagated *in vitro* on MS basal plus kanamycin monosulfate (50 mg l^{-1}) and three plantlets of each progeny genotype were acclimated to the greenhouse. The progeny were arranged in a randomized complete block design with one plant of each genotype per replication (three replications total). Six weeks after acclimation the youngest, fully expanded leaf of each plant was collected for DAS-ELISA testing. The TC1 progeny were ground at a dilution of 1:100 (g leaf tissue ml^{-1}

extraction buffer) whereas the TC2 progeny were ground at a 1:5 dilution. Absorbance ratios of progeny and parental genotypes were analyzed by SAS GLM and grouped by Ryan-Einot-Gabriel-Welsh Multiple Range Test.

Gene-centromere mapping of a single-insert transformant

Additional seedlings of Atlantic × APM-2 TC1 were planted in the greenhouse without prior selection for kanamycin resistance. Six weeks after germination, 283 were screened for Cry3Aa expression. All non-expressers were screened by PCR for presence of the transgene. Map distance (in centimorgans) was calculated by doubling the frequency of non-expressers (nulliplex individuals) and multiplying by 100 (Veilleux 1985).

Results

Incorporation and molecular configuration of the *cry3Aa* transgene in transgenic plants

Seven lines regenerated from *Agrobacterium*-inoculated APM-2 leaf discs (TC1-TC7) between 68 and 112 days after inoculation. TC1, 3, 6 and 7 regenerated from separate leaf discs while TC2, 4 and 5 regenerated from the same disc. All lines showed the expected 1,761 bp fragment when examined by PCR, verifying that they were transgenic (Fig. 2). Southern analysis of five lines (TC1, 2, 4, 5, and 7) after *Xba* I or *BamH* I restriction revealed a single insertion in TC1 and TC7 but at least four insertions in TC2, TC4 and TC5 (Fig. 3). TC1 and TC7 arose from independent transformation events whereas TC2, TC4 and TC5 apparently arose from the same transformation event on the same leaf disc.

Establishment of TC1-TC7 in the greenhouse and 4*x*-2*x* hybridization

All seven primary transgenics acclimated to the greenhouse and flowered normally; TC1 (single-insert) and TC2 (multiple-insert) were used as pollen donors for 4*x*-2*x* hybridization with cv. Atlantic. Pollen from TC1 resulted in 89% fruit set (average of

145 seeds per fruit) while pollen from TC2 resulted in 67% fruit set (average of 141 seeds per fruit).

Ploidy analysis of TC1-TC7 and 4*x*-2*x* hybrids

Flow cytometry determined that all seven primary transformants were diploid whereas six randomly selected hybrids derived from 4*x*-2*x* hybridization with cv. Atlantic were tetraploid (Fig. 4).

DAS-ELISA to determine Cry3Aa expression levels of TC1-TC7

The seven transgenic lines separated into two groups based on Cry3Aa expression estimated by DAS-ELISA (Fig. 5). Four lines (TC1, TC3, TC6 and TC7) were "high-expressers" with absorbance ratios ranging from 24-30 whereas three lines (TC2, TC4 and TC5) were "low-expressers" with absorbance ratios ranging from 1-5. A dilution series of purified Cry3Aa protein showed that one of the high expressers, TC1, produced 230 μg Cry3Aa g^{-1} leaf tissue in the greenhouse whereas one of the low expressers, TC2, produced 4 μg Cry3Aa g^{-1} leaf tissue in the greenhouse.

Colorado potato beetle feeding assay

To determine if DAS-ELISA results correlated with resistance to the Colorado potato beetle, a no-choice feeding assay was performed. The ten genotypes (APM-2 TC1-TC7, NewLeaf Atlantic, wild-type Atlantic and wild-type APM-2) separated into three statistically distinct groups based on ANOVA of leaf area consumed by Colorado potato beetle first-generation adults (Fig. 6). The two non-transgenic genotypes, 'Atlantic' and APM-2, supported the most leaf feeding with an average of 473 mm^2 consumed after 24 h. The three "low-expressers" (TC2, TC4 and TC5) had partially reduced feeding with an average of 262 mm^2 consumed after 24 h. The four "high expressers" (TC1, TC3, TC6 and TC7) had the least amount of feeding after 24 h, an average of 87 mm^2, and this value was not significantly different from 79 mm^2 consumed by the beetles of the

commercially resistant cv. NewLeaf Atlantic. A correlation analysis of absorbance ratios obtained by DAS-ELISA for TC1-TC7 and the results from the Colorado potato beetle feeding assay showed the two assays to be negatively correlated ($r^2 = -0.90$; $p<0.01$).

Cry3Aa expression of 4*x*-2*x* progeny derived from TC1 (single-insert parent) and TC2 (multiple-insert parent)

None of the 25 tetraploid progeny derived from Atlantic × TC1 had significantly lower absorbance ratios than TC1 (Fig. 7), indicating that a raise in ploidy (diploid to tetraploid) did not affect expression of a single-insert *cry3Aa* transgene. However, 17 of 25 similarly treated tetraploid progeny (68%) derived from Atlantic × TC2 had significantly lower absorbance ratios than TC2 itself (Fig. 8). In addition, eight progeny (32%) had near complete silencing of the multiple *cry3Aa* transgenes (< 0.1 μg Cry3Aa g^{-1} leaf tissue). In the case of multiple transgene copies in the potato genome, ploidy elevation had a negative effect on overall *cry3Aa* expression.

Gene-centromere mapping of TC1

Of 283 tetraploid progeny examined, 36 showed no Cry3Aa expression by DAS-ELISA. PCR failed to detect the *cry3Aa* transgene in all of the non-expressing progeny, indicating that these individuals arose from nontransgenic gametes produced by TC1 as a result of genetic recombination during meiosis. Assuming a single crossover on sister chromatids (one carrying the transgene and one without) between the transgene and the centromere, the single-insert locus of TC1 can be mapped at 26 cM from the centromere: 2×0.13 (frequency of nulliplex individuals) $\times 100 = 26$ cM

Discussion

Gene silencing of both native and foreign genes can occur transcriptionally or posttranscriptionally (TGS and PTGS, respectively). In many plant transformation studies introduction of several copies of a transgene, or a transgene showing homology to

a native gene sequence, has resulted in silencing via methylation of all or some gene copies (Demeke et al. 1999, Neuhuber et al. 1994). This type of "homology-dependent" gene silencing does not require that transgenes be physically connected (cis) but can occur when they are on separate chromosomes (trans). The fact that the multiple-insert plants (TC2, TC4 and TC5) have severely reduced levels of Cry3Aa expression relative to the single-insert plants (4 vs. 230 μg Cry3Aa protein g^{-1} leaf tissue) is likely due to methylation of the transgene sequences. The single insert plants, such as TC1 and TC7, could not trigger homology-dependent silencing by the host genome. The tetraploid progeny derived from 4*x*-2*x* hybridization of the single-insert parent (TC1) to cv. Atlantic showed stable, high expression of the *cry3Aa* transgene (Fig. 7) whereas the majority of similarly derived 4*x*-2*x* hybrids from the multiple-insert parent (TC2) showed even greater transgene silencing than that observed in TC2 (Fig. 8). Polyploidization may have been responsible for this increase in silencing of *cry3Aa* between diploid TC2 and its tetraploid 4*x*-2*x* hybrids.

Polyploidization causes rapid alterations in genome structure and gene expression (Leitch and Bennet 1997; Comai 2000, Comai et al. 2000). One of the most notable effects of polyploidization is “gene silencing” of duplicate or highly similar genes that result from the combination of two related genomes, thus halting the production of unnecessary message and/or freeing up duplicate genes to evolve novel functions. To carry out such gene silencing the plant genome must detect duplicate or highly similar genes. Although the exact mechanisms of detection are currently unknown, one can theorize that these mechanisms are perhaps most active directly following polyploidization when the greatest frequency of duplications occur. With this rationale, one can speculate that a single-insert transgene is more likely to avoid silencing by the host genome after polyploidization than multiple inserts of the same transgene.

The single-insert transgenic plant, TC1, primarily donates highly heterozygous 2n gametes containing a single *Bacillus thuringiensis* transgene (hemizygous transgenic gametes) during sexual polyploidization in crosses to cv. Atlantic (4*x*-2*x* hybridization). Therefore, while many of the TC1 native genes are duplicated or at least partially

homologous with respect to those in cv. Atlantic, the introduced *B. thuringiensis* transgene has no homologous match in the Atlantic genome and may therefore escape silencing by the host genome. The multiple-insert plant (TC2), however, donates highly heterozygous 2n gametes containing multiple, partially silenced copies of the *B. thuringiensis* transgene during the sexual polyploidization step. Identical copies of the transgene may serve as obvious targets for the genome's homologous gene silencing systems and could therefore be subjected to extensive silencing. Assuming that homology-dependent gene silencing is especially active in first generation sexual polyploids, one could expect many partially and completely silenced progeny as were observed in 4*x*-2*x* populations derived from TC2.

High expression of exogenous transgenes, such as bacterial genes encoding insecticidal or herbicide-resistance proteins, in a TPS hybrid production system for potato is possible provided that a single-insert diploid (diplandroid) parent is used. All of the high-expressing lines (as determined by ELISA, Fig. 5) showed similar resistance to feeding by the Colorado potato beetle as cv. NewLeaf Atlantic (Fig. 6), and one of those lines, TC1, transmitted equally high Cry3Aa expression to TPS potato hybrids (Fig. 7). The high negative correlation between ELISA testing of greenhouse grown plants and leaf-feeding bioassays of field-grown plants demonstrates that a simple laboratory analysis of greenhouse grown leaf material can be used to identify Colorado potato beetle-resistant diploid parents for use in 4*x*-2*x* hybridization.

A major concern with the widespread use of *B. thuringiensis*-derived (Bt) insecticidal proteins, particularly with the advent of recombinant plants producing these proteins, is the development of resistance to Bt within target insect populations. In the United States, a 20% refuge of non-Bt potato is required alongside all transgenic potato fields. This requirement follows research showing that structured blocks of non-transgenic potato grown adjacent to blocks of transgenic potato allow for mating between susceptible and resistant insects, thus mitigating the buildup of homozygous resistant individuals (Hoy 1999). The suggestion of incorporating Bt transgenes into TPS hybrids, that are expected to be commercially grown primarily in developing countries where planting of special

refuge areas may not be practical, could therefore pose a threat to the continued effectiveness of Bt-mediated resistance.

A single-insert transgenic diploid used in TPS hybrid production could alleviate such concerns because such plants produce non-transgenic progeny at varying frequencies due to genetic recombination during meiosis (nontransgenic gametes). Upon examination of 283 progeny derived from one of the single-insert plants, 13% were found to be non-transgenic and thus the transgene was mapped at 26 cM from the centromere. An examination of larger numbers of single-insert transgenics would likely identify a line producing non-transgenic progeny at or near a frequency of 20%. Incorporation of seed marker genes such as the green fluorescence protein (GFP) along with the target transgene would allow clear visual identification of the non-transgenic seed. Molinier et al. (2000) was able to separate transgenic from non-transgenic tobacco seed based on green fluorescence of the embryo visible through the seed coat. Thus, a potato diploid with a single transgene at 40 cM from the centromere would produce TPS hybrid seed with the currently recommended percentage of refuge needed for transgenic potato production. Visual separation of the seed could be done quickly and cheaply and would enable the construction of structured refuge areas consisting of genetically equivalent hybrids without the transgene.

Lastly, for every non-transgenic gamete that is produced by a diploid with 2n pollen production by FDR, a corresponding homozygous transgenic (duplex) gamete is also produced. Thus, roughly 13% of the progeny derived from single-insert TC1 are duplex for the *cry3Aa* transgene. This increase in dosage of the transgene appears to have had no effect on transgene expression in the sample of tetraploid progeny as no significant differences in expression among 25 progeny were found (Fig. 7). In that respect, the results do not agree with the results by Beaujean et al. (1998) who found a positive correlation between transgene dose and expression in transgenic tobacco. In conclusion, the results offer a new perspective regarding interaction between ploidy and transgene expression in plants. Unlike Scheid et al. (1996) no transgene silencing upon ploidy elevation was observed with a single-insert transgene. However, substantial transgene

silencing was detected when a genome possessing multiple transgenes underwent ploidy elevation. The multiple transgene silencing that was observed may result from gene silencing mechanisms intended to aid in the evolution of polyploid plant species.

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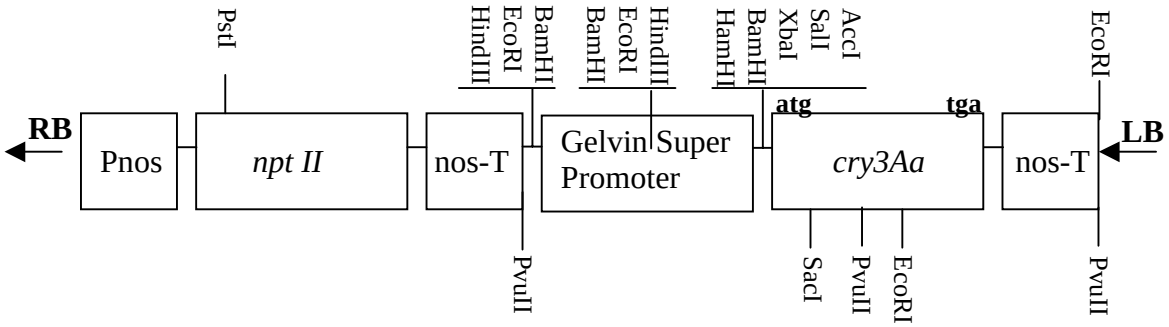

Figure 1: Structure of the pSPUD8 genetic construct (designed by David Douches, Michigan State University) harboring the *cry3Aa* gene (1.79kb). The *cry3Aa* gene was modified for high expression in plant systems.

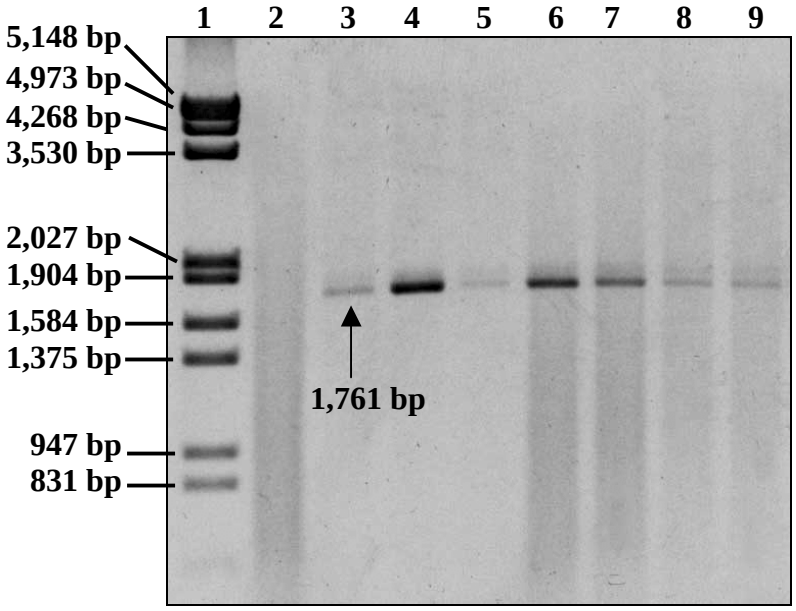

Figure 2: PCR amplification of a 1,761 bp fragment of the 1,794 bp *cry3Aa* transgene in seven lines (TC1-TC7) regenerated from *Agrobacterium*-inoculated leaf discs of diploid potato clone APM-2 (lanes 3-9, respectively). Lane 1 contains a DNA ladder of lambda DNA digested by *Eco*R I and *Hin*d III. Wild-type APM-2 (lane 2) is the non-transgenic control.

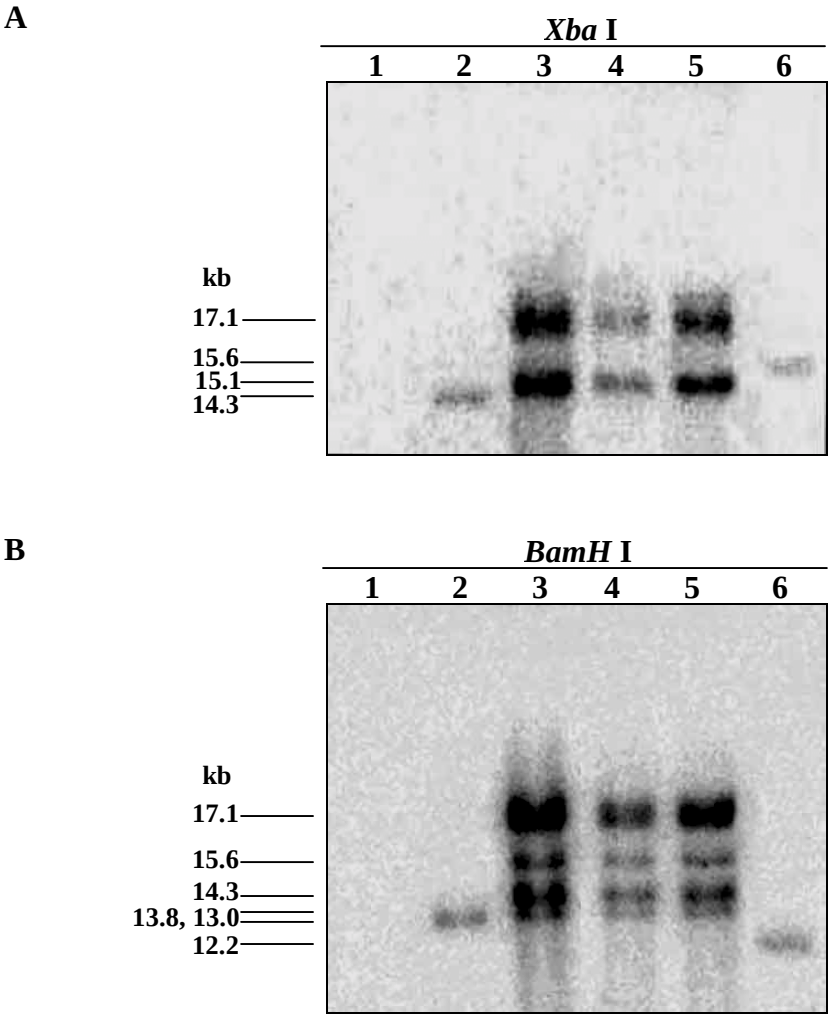

Figure 3: Southern blot analysis of wild-type APM-2 (lanes 1) and transgenic lines TC1, TC2, TC4, TC5 and TC7 (lanes 2-6, respectively) with restriction enzymes *Xba* I (blot A) and *BamH* I (blot B) reveals single- and multiple-copy transgenic lines; molecular weights of fragments are listed on left.

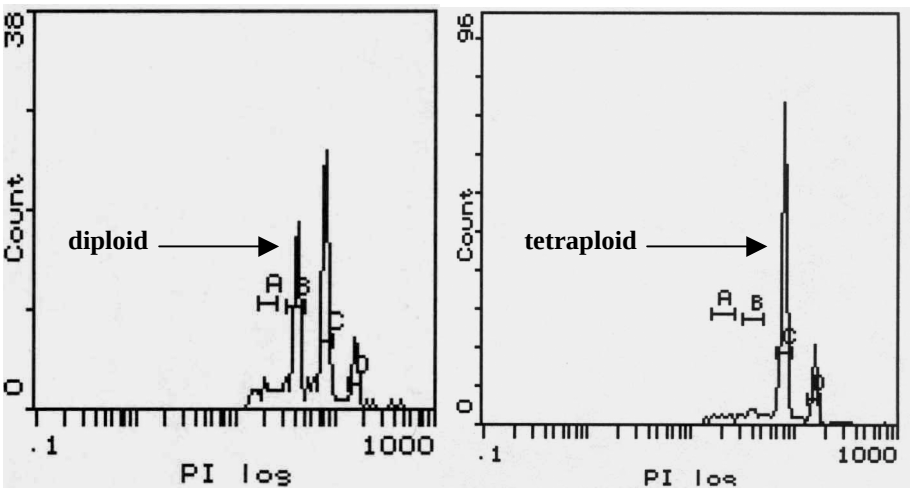

Figure 4: Flow cytometric histograms of a primary transformant (TC1, left) and a progeny genotype (right) resulting from 4*x*-2*x* hybridization of TC1 to cv. Atlantic show that the parent is diploid (2*n*=2*x*=24) whereas the progeny is tetraploid (2*n*=4*x*=48). The A, B, C, and D gates represent the potato monoploid, diploid, tetraploid and octoploid DNA contents, respectively, that were set by running a monoploid (2*n*=1*x*=12) control. The count on the y-axis is the number of propidium iodide stained cell nuclei that fall into particular channels (PI log) corresponding to DNA content. The first peak (furthest to the left) in each histogram indicates the true ploidy of the plant. Subsequent peaks result from endomitosis.

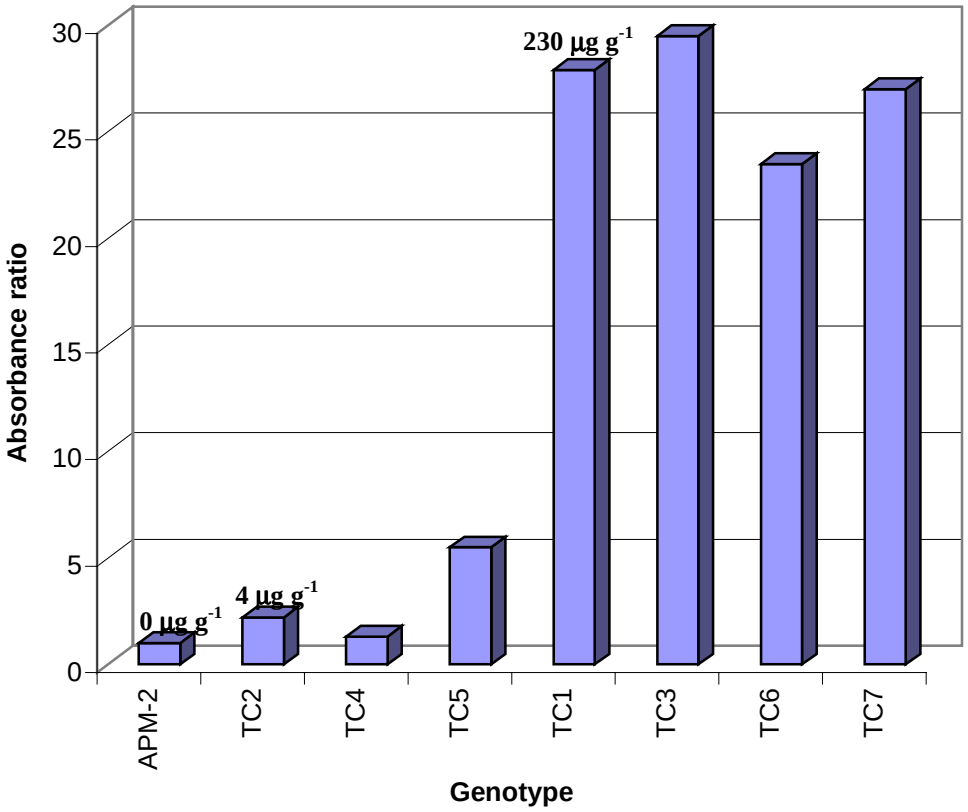

Figure 5: DAS-ELISA determined Cry3Aa expression of seven transgenic lines (TC1-TC7) indicated by the absorbance reading of each sample after the addition of p-nitrophenyl phosphate using an Emax^{TM} precision microplate reader at 405 nm. Absorbance ratios were calculated by dividing the absorbance of the transgenic samples by the absorbance of the non-transgenic control (APM-2). A dilution series of purified Cry3Aa protein was used to quantify the amount of transgenic protein (µg Cry3Aa g^{-1} leaf tissue) produced by TC1 and TC2 in the greenhouse.

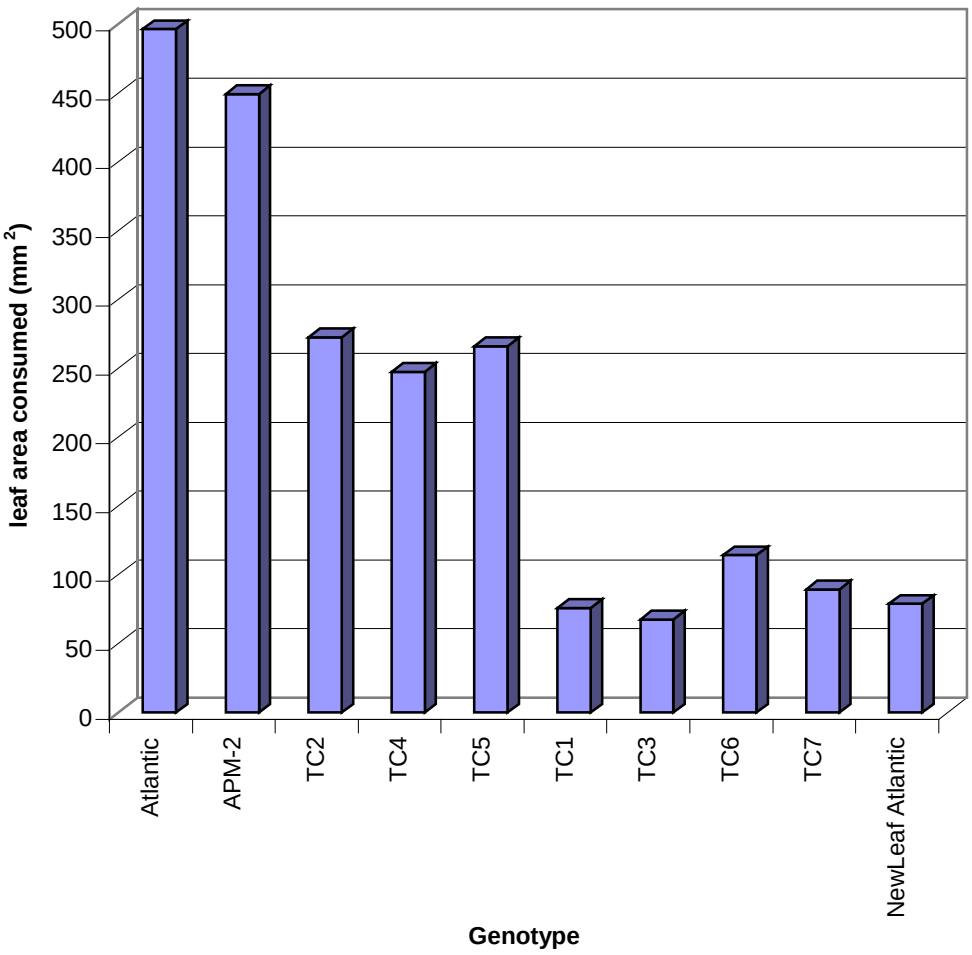

Figure 6: No-choice Colorado potato beetle feeding assay. First-generation adults were allowed to feed for 24 h on foliage from each genotype. The results (ten genotypes × six replications × four experimental units = 240 observations) were analyzed by Ryan-Einot-Gabriel-Welsh Multiple Range Test and significant groupings are indicated by letter above the genotype bars.

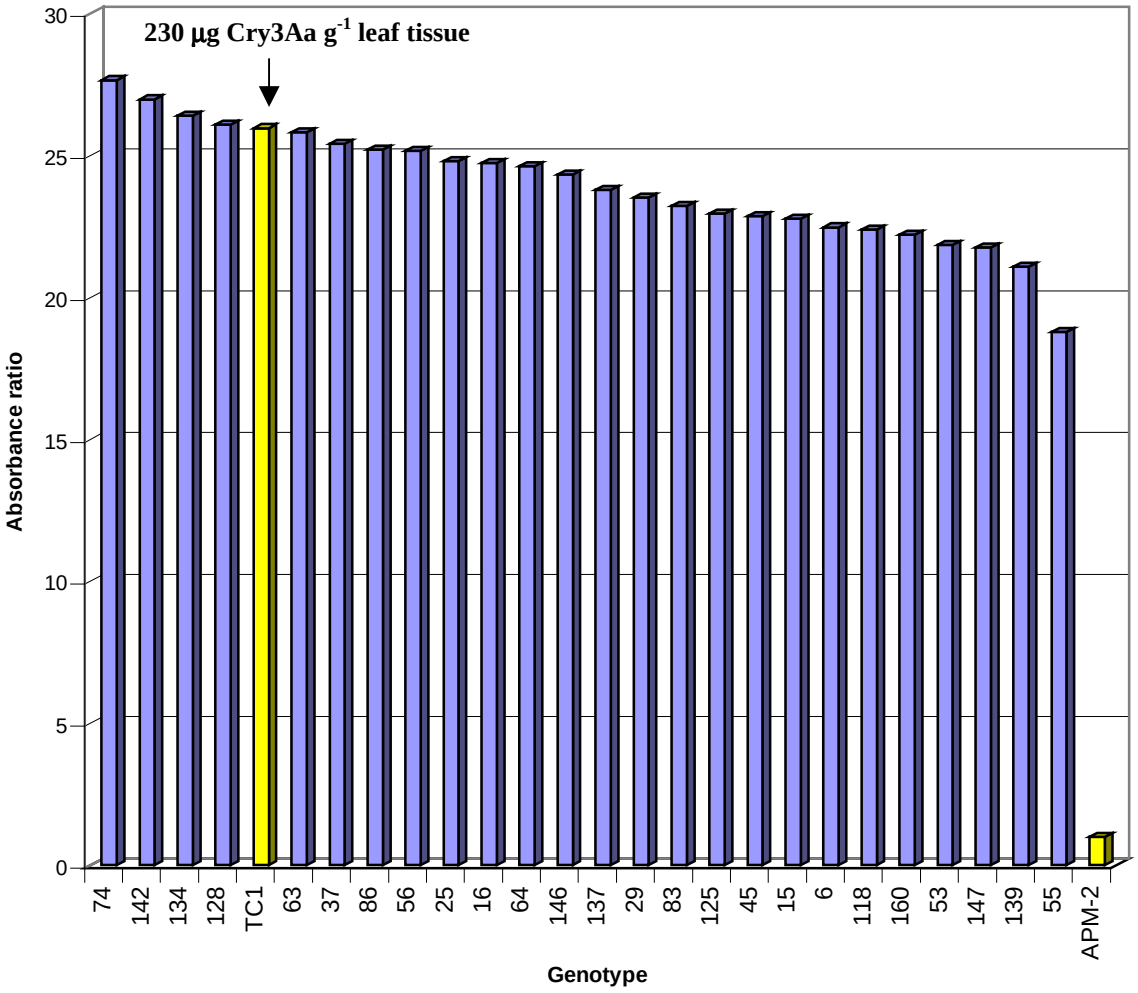

Figure 7: Cry3Aa expression of the single insert transgenic TC1 (5th bar from left) and 25 of its tetraploid (4*x*-2*x*) progeny detected by DAS-ELISA absorbance readings (three replications) using an Emax™ precision microplate reader at 405 nm. Absorbance ratios were calculated by dividing the absorbance of the transgenic samples by the absorbance of the non-transgenic control APM-2 (last bar on right).

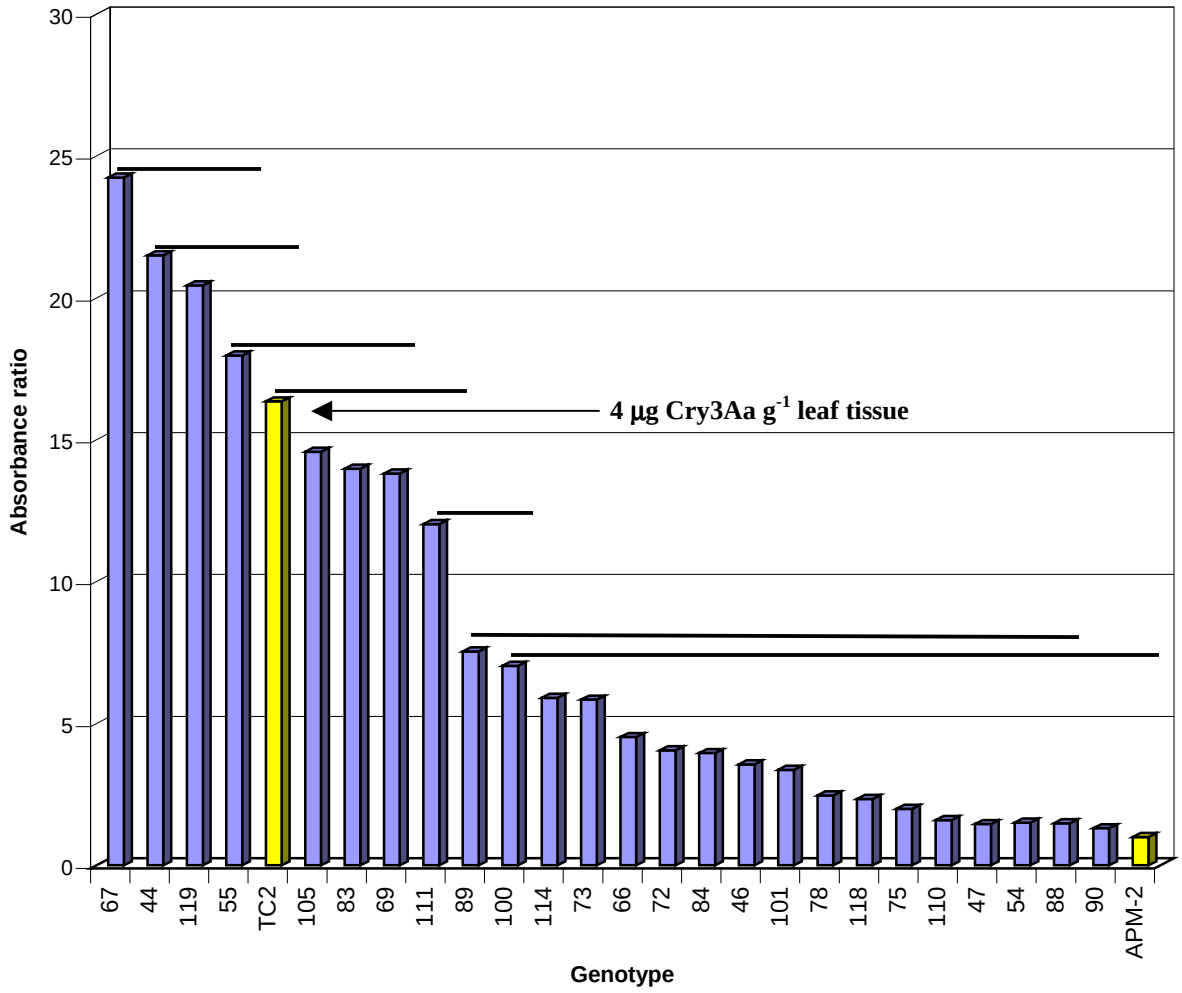

Figure 8: Cry3Aa expression of the multiple-insert transgenic TC2 (5th bar from left) and 25 of its tetraploid (4*x*-2*x*) progeny detected by DAS-ELISA absorbance readings (three replications) using an Emax™ precision microplate reader at 405 nm. Absorbance ratios were calculated by dividing the absorbance of the transgenic samples by the absorbance of the non-transgenic control APM-2 (last bar on right). Genotypes occurring under the same horizontal bar are not significantly different by Ryan-Einot-Gabriel-Welsh Multiple Range Test.

CHAPTER 3

Parent-of-origin effect on expression of a *Bacillus thuringiensis cry3Aa* transgene in 4*x*-2*x* hybrids of potato (*Solanum tuberosum*)

Abstract Parental imprinting, an epigenetic phenomenon typically associated with endosperm development, may also have effects on the expression of transgenes in genetically modified organisms. Two copies of a *cry3Aa* transgene were combined in potato tetraploid hybrids using transgenic male and female parents in a 4*x*-2*x* hybridization breeding scheme. The results suggest that non-allelic copies of the transgene interacted in hybrids to cause reduced expression. The tetraploid, single-insert transgenic plant consistently generated low Cry3Aa expressing 4*x*-2*x* hybrids and transgene silencing appeared to transmit maternally through the cytoplasm when this plant was used as female during 4*x*-2*x* hybridization. Wild-type eggs (no *cry3Aa*) derived from the tetraploid plant caused silencing in progeny of a highly expressed, single-insert *cry3Aa* transgene inherited from the paternal parent. Transmission of maternal methyltransferases, such as *MET1*, through the cytoplasm of eggs to progeny could be responsible for the parent-of-origin effects on transgene expression observed in this study.

Introduction

Parent-of-origin effects, or parental imprinting, on male and female gametes typically occurs by parent-specific DNA methylation and is essential to normal development in both mammalian and plant reproductive cycles (Ruvinsky 1999, Matzke and Matzke 1993). Parental imprinting in plants has been researched most extensively with respect to endosperm development in angiosperms. Several maize genes, such as *R-r:std*, encoding an aleurone pigment, have been identified that show differential methylation and expression depending on parental origin (Alleman and Doctor 2000). In addition, some genes have been discovered that show parental imprinting not only in the endosperm but also the embryo (Luo et al. 2000). It is believed that genetic engineering may create genetic configurations, such as inverted repeats, that mimic characteristics of imprinted genes and thus foster the possibility of parental imprinting, via methylation, on transgene expression (Alleman and Doctor, 2000; Ruvinsky 1999). Differential methylation of transgenes through mice reproductive cycles has been reported. In all cases, hypermethylation of the transgene by passage through female gametes resulted in transgene silencing (Swain et al. 1987, Reik et al. 1987), sometimes resulting in an irreversibly silenced imprint on the transgene that was subsequently transmitted through

male and female gametes (Hadchouel et al. 1987). Evidence for parental effects on transgene expression in plants, however, remains scarce in transgenic research.

Sexual polyploidization of potato (*Solanum tuberosum* L.) resulting in 4*x*-2*x* hybrids involves pollination of a tetraploid (4*x*) potato cultivar with unreduced pollen from a diploid (2*x*) potato line (Veilleux 1985, Carputo et al. 2000). The resulting tetraploid progeny can be highly vigorous, uniform and free of disease, making this an economical method of potato production via true seed (TPS) in developing countries. Recently it was demonstrated that a single-insert, *cry3Aa* transgene was expressed at high levels in a diploid potato line (APM-2 TC1) that produces unreduced pollen by the genetic equivalent of first division restitution (FDR). Similar high expression of *cry3Aa* was also observed in the tetraploid hybrids derived from APM-2 TC1 through 4*x*-2*x* hybridization with tetraploid cv. Atlantic (Johnson et al. 2001). APM-2 TC1 produced approx. 230 μg Cry3Aa protein g^{-1} leaf tissue and 4*x*-2*x* hybrids, either simplex or duplex for the transgene, did not differ significantly in transgene expression from the diploid parent, as determined by DAS-ELISA (double antibody sandwich-enzyme linked immunosorbent assay). In addition, tetraploid progeny derived by crossing high-expressing 4*x*-2*x* hybrids as female parents with non-transgenic tetraploid cv. Katahdin (second generation progeny) produced Cry3Aa at levels as high as 238 μg g^{-1} leaf tissue, indicating stable, high expression of the single *cry3Aa* insert (present in APM-2 TC1) initially through male (2*n* pollen from a 2*x* plant) and subsequently through female (2*n* eggs from a 4*x* plant) sexual cycles. Multiple copies of the *cry3Aa* transgene, in contrast to a single copy, exhibited severe reduction in transgene expression (approx. 4 μg g^{-1} leaf tissue) and 4*x*-2*x* hybrids derived from a multiple-copy, diploid line showed even more drastic silencing of the transgenes, with 32% of the hybrids having virtually no expression of the transgene.

To further explore the effect of multiple transgene copies and ploidy manipulation in potato, the previously described high expressing, single-insert diploid line (APM-2 TC1) was crossed to a high expressing, single-insert tetraploid line (Atlantic TC1) to yield 4*x*-2*x* hybrids. These hybrids are genetically equivalent to the Atlantic × APM-2 TC1

hybrids described by Johnson et al. (2001) except that some will receive two *cry3Aa* copies, one each from the maternal and paternal parent. Because Atlantic TC1 is a tetraploid plant undergoing normal reductive meiosis, half of the egg cells do not receive a copy of *cry3Aa* (wild-type eggs). The single-insert in APM-2 TC1 was mapped at 26 cM from the centromere and thus APM-2 TC1 produces unreduced, wild-type pollen at a frequency of 13% (Figure 1). If the combination of two *cry3Aa* transgenes, one originating from each parent, in hybrids causes a reduction of transgene expression, one would expect roughly 44% of the 4*x*-2*x* hybrids from Atlantic TC1 × APM-2 TC1 to have reduced Cry3Aa expression. An additional 44% of progeny should receive only a single transgene from the high-expressing paternal parent (APM-2 TC1) and would be expected to show high expression, as observed in Atlantic × APM-2 TC1 hybrids. Table 1 summarizes the expected segregation of transgene inserts in a 4*x*-2*x* cross between Atlantic TC1 and APM-2 TC1 and the expected expression profiles of the tetraploid hybrids.

The original objective of this study was to explore the effect of ploidy elevation through 4*x*-2*x* hybridization on expression of duplicate transgene copies, originating from both male and female parents, in potato hybrids; however, unanticipated results of the crosses provide strong evidence for parent-of-origin effects on expression of a *cry3Aa* transgene. The results also suggest a mechanism for how maternal-specific imprinting of a transgene, or an endogenous gene, can be transmitted to progeny in plants.

Materials and methods

Plant material

APM-2 is a diploid (2*n*=2*x*=24) potato clone that produces 2*n* pollen by the genetic equivalent of FDR and was obtained by crossing dihaploid *S. andigena* L. (PI 347773) as female with an F_1 hybrid [S. *phureja* Juz & Buk. PI 225669 × *S. microdontum* Bitt. PI 320304] as male. The tetraploid (2*n*=4*x*=48) cv. Atlantic is a commercial potato grown widely throughout North America. APM-2 TC1 and Atlantic TC1 were similarly

transformed with a *Bacillus thuringiensis cry3Aa* transgene (codon modified for high expression in plants) via *Agrobacterium tumefaciens*-inoculation of *in vitro* leaf discs as described by Johnson et al. (2001).

Atlantic TC1 was determined to be a single-insert line based on segregation ratios of Cry3Aa expression in 85 randomly selected 4*x*-2*x* hybrid progeny (Figure 2). DAS-ELISA analysis of greenhouse grown Atlantic TC1 detected expression of Cry3Aa at 178 $\mu g\ g^{-1}$ leaf tissue. APM-2 TC1 is also known to have a single copy of the *cry3Aa* transgene by southern blot analysis using both the *BamH* I and *Xba* I restriction enzymes. DAS-ELISA analysis of greenhouse grown APM-2 TC1 detected expression of Cry3Aa at 230 $\mu g\ g^{-1}$ leaf tissue.

Development of 4*x*-2*x* hybrid progeny populations

Atlantic TC1, APM-2 TC1 and wild-type APM-2 were established in the greenhouse from *in vitro* plantlets. Upon flowering, pollen from APM-2 TC1 and wild-type APM-2 was used to pollinate Atlantic TC1. Two different 4*x*-2*x* crosses were performed: (1) Atlantic TC1 × wild-type APM-2 and (2) Atlantic TC1 × APM-2 TC1. Seeds were collected, dried, imbibed with gibberellic acid (2000 ppm) and planted in the greenhouse on May 22, 2001. Seeds were planted one seed/cell in cell packs of 48 (13.4 cm long × 13.2 cm wide × 5.8 cm deep; Kord Products, Canada, item #K806).

Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) to detect Cry3Aa expression of the two 4*x*-2*x* hybrid progeny populations

Approximately 6 weeks after planting, a total of 85 progeny from the Atlantic TC1 × wild-type APM-2 cross and 188 progeny from the Atlantic TC1 × APM-2 TC1 cross were screened using a DAS-ELISA test for Bt-Cry3Aa endotoxin from Agdia (Elkhart, ID) to determine Cry3Aa expression levels. The youngest fully expanded leaf of each progeny was collected for the analysis. Cry3Aa expression was measured by the absorbance reading of each sample following the addition of p-nitrophenyl phosphate

using an Emax™ precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 405 nm. Absorbance ratios were calculated by dividing the absorbance of each transgenic sample by the absorbance of a non-transgenic control.

Leaf material of eight progeny (four from each cross) was initially ground at a 1:100 (g leaf tissue ml^{-1} extraction buffer) dilution. Subsequently, a 1:5 dilution was used to analyze all the remaining progeny. A dilution series of purified Cry3Aa protein enabled quantification of Cry3Aa expression (µg per g of leaf tissue) for all 85 Atlantic TC1 × wild-type APM-2 progeny and for 95 of the 188 Atlantic TC1 × APM-2 TC1 progeny.

Results

Selection of a suitable dilution (g leaf tissue ml^{-1} extraction buffer) for ELISA testing of the two 4*x*-2*x* hybrid populations

A 1:100 dilution had been used to quantify Cry3Aa expression for both the Atlantic TC1 and APM-2 TC1 parents, but initial results with eight hybrid progeny revealed this to be too dilute to obtain detectable microplate readings. Subsequently, a 1:5 dilution enabled detection of Cry3Aa levels ranging from 0 to 4.5 µg g^{-1} leaf tissue among the eight progeny. The remaining progeny were screened for Cry3Aa expression using a 1:5 dilution.

Distribution and quantification of Cry3Aa expression in Atlantic TC1 × wild-type APM-2 hybrids

The 85 Atlantic TC1 × wild-type APM-2 hybrid progeny segregated at a ratio of 51 non-expressers of Cry3Aa to 34 expressers of Cry3Aa (Figure 2). The chi-square value for independent segregation (1:1) of the *cry3Aa* transgene in these 85 progeny (derived from crossing a single-insert, tetraploid parent to a non-transgenic diplandroid) was not significant ($\chi^2 = 3.8$, $p > 0.05$), thus Atlantic TC1 was confirmed as a single-insert plant. Absorbance ratios of the 34 Cry3Aa expressers ranged from 10.5 to 21.5.

On two different dates (separated by 5 days) the Cry3Aa expressers averaged 4.2 µg Cry3Aa g^{-1} leaf tissue and 3.7 µg Cry3Aa g^{-1} leaf tissue, giving an average of 4 µg Cry3Aa g^{-1} leaf tissue for the transgenic 4*x*-2*x* hybrids derived from crossing Atlantic TC1 to wild-type APM-2. This level of expression was unexpectedly low compared to 178 µg g^{-1} leaf tissue for the Atlantic TC1 parent.

Distribution and quantification of Cry3Aa expression in Atlantic TC1 × APM-2 TC1 hybrids

In the progeny of Atlantic TC1 × APM-2 TC1, where transgene transmission was possible from both parents, 17 of 188 hybrid progeny (9%) were identified as non-expressers of Cry3Aa (Figure 3). Absorbance ratios of the 171 Cry3Aa expressers ranged from 4.2 to 19.7, indicating unexpectedly low expression of Cry3Aa similar to that observed in the Atlantic TC1 × wild-type APM-2 progeny. Of the Cry3Aa expressing progeny, 86 (50%) had lower absorbance ratios than the lowest recorded absorbance ratio of the Atlantic TC1 × wild-type APM-2 progeny.

On two different dates (separated by 5 days) the Cry3Aa expressers averaged 2.1 µg Cry3Aa g^{-1} leaf tissue and 2.0 µg Cry3Aa g^{-1} leaf tissue, giving an average of 2 µg Cry3Aa g^{-1} leaf tissue for the transgenic 4*x*-2*x* hybrids derived from crossing Atlantic TC1 to APM-2 TC1. This level of expression was unexpectedly low compared to 178 µg g^{-1} leaf tissue for the Atlantic TC1 parent.

The low Cry3Aa expression levels of Atlantic TC1 × APM-2 TC1 progeny precluded testing the predicted expression ratios in Table 1 (no high expressing progeny). However, using the assumption that progeny with only one parental transgene (T_1 or T_2, 50%) represent the higher expressing progeny (similar in expression to Atlantic TC1 × wild-type APM-2 progeny) and those with both parental transgenes (43.5%) represent the lower expressing progeny, a 50 high: 43.5 low; 6.5 zero Cry3Aa expression model (χ^2 = 2.95, $p > 0.20$) was not rejected. Reasons for making this assumption follow.

Discussion

One major difference in Cry3Aa expression between the two 4*x*-2*x* hybrid populations is that, when transgenes were transmitted by both parents (Atlantic TC1 × APM-2 TC1), half of the Cry3Aa expressing hybrids had lower absorbance ratios (Figure 3) than the lowest recorded absorbance ratio of Cry3Aa expressers in the population where the transgene was only inherited maternally (Atlantic TC1 × wild-type APM-2; Figure 2). While the highest absorbance ratio for each cross differed by less than 2, the lowest ratio differed by more than 6. In other words, the Atlantic TC1 × APM-2 TC1 cross differed from the Atlantic TC1 × wild-type APM-2 cross by the presence of a low expressing "class" of progeny. That these progeny make up half of the total Cry3Aa expressers explains why the average Cry3Aa expression level of the Atlantic TC1 × APM-2 TC1 progeny was half that of the Atlantic TC1 × wild-type APM-2 progeny (2 vs. 4 µg Cry3Aa g^{-1} leaf tissue, respectively). Because the low expressing class of progeny was seen only in the Atlantic TC1 × APM-2 TC1 cross, and because the critical difference between this cross and the Atlantic TC1 × wild-type APM-2 cross was the possibility of combining two, non-allelic *cry3Aa* transgenes, it is highly probable that the low expressing progeny represent the 43.5% of individuals ($\chi^2 = 2.95$, $p > 0.20$) predicted to inherit both a maternal and paternal copy of the *cry3Aa* transgene (Table 1). As such, the results agree with previous findings that multiple copies of the *cry3Aa* transgene trigger homology dependent transgene silencing during 4*x*-2*x* hybridization of potato.

In several plant studies where non-allelic transgene copies have been combined through sexual crosses, transgene silencing has been the result (Vaucheret et al. 1995; Matzke et al. 1994). For example, De Wilde et al. (2001) observed significant silencing of two different antibody fragment expression cassettes in *Arabidopsis thaliana* after two lines, each homozygous for one of the two expression cassettes, were crossed to create hemizygous progeny. Although the expression cassettes encoded different antibody chains, they shared several homologous functional regions that were sufficient to induce

silencing. Silencing was correlated with cytosine methylation in coding regions of the transgenes.

Silencing of transgenes through sexual cycles has been a frequently reported occurrence, sometimes even the norm, in genetic engineering studies. Methylation of the promoter and/or the transcribed sequence has been associated with silencing in nearly all cases. Recently Meza et al. (2001) developed 111 second generation *A. thaliana* lines engineered with one of two different T-DNA constructs (the primary transformants, created by root-transformation, were selfed to the T2 generation). Up to 56% of the lines displayed some degree of silencing and a detailed analysis of 20 lines revealed three of them to be single-insert lines. Multiple-copy lines, unlike single-copy, were shown to have partial methylation of the promoter sequence. Kilby et al. (1992) examined stability of kanamycin resistance in seven single-insert *A. thaliana* lines over four cycles of selfing. Two lines developed methylation of the promoter sequence and gradually lost expression of the transgene; however, treatment with 5-azacytidine (demethylation agent) could restore transgene expression. Elmayan and Vaucheret (1996) examined progeny derived from 11 single-insert tobacco lines (transformed with the bacterial *UidA* gene) and found silencing of the transgene in all progeny lines, although onset of silencing (in terms of plant development) varied among lines.

With these previous reports of transgene silencing through sexual cycles in mind, one should not be so surprised to find only low expressing progeny (4 μg g^{-1} leaf tissue) from the cross of high expressing parent Atlantic TC1 (178 μg g^{-1} leaf tissue) to a wild-type genotype (Figure 2). Although Atlantic TC1 segregated as a single-insert plant, it could have multiple repeats of the *cry3Aa* transgene at a single locus, thus triggering homology-dependent gene silencing (Buck et al. 2001), or a single copy may have inserted into a gene-poor genomic region that triggers silencing (Iglesias et al. 1997). Wolters et al. (1998) examined T-DNA insertion sites in four antisense potato lines developed by *Agrobacterium tumefaciens* inoculation and found that all had multiple T-DNA inserts positioned as inverted or direct repeats. While lacking structural knowledge of the T-DNA insertion site in Atlantic TC1, it is known that this genotype consistently produced

progeny with strongly reduced expression of Cry3Aa. Based on current models of transgene silencing, this reduction was likely caused by methylation of either the *cry3Aa* coding sequence and/or promoter.

The fact that all Atlantic TC1 × APM-2 TC1 progeny had reduced expression levels of Cry3Aa protein, however, was unexpected (Figure 3). The expected segregation of transgenes for this cross (Table 1) was that 44% of the progeny should inherit only the paternal *cry3Aa* transgene (from APM-2 TC1). Because transgenic hybrids from the cross of wild-type Atlantic to APM-2 TC1 have shown high expression of the transgene not significantly different from APM-2 TC1, it was anticipated that 44% of progeny from this cross (Atlantic TC1 × APM-2 TC1) would produce high levels of Cry3Aa in the same range as APM-2 TC1. In contrast, all transgenic progeny of Atlantic TC1 × APM-2 TC1 showed similar or lower Cry3Aa expression compared to hybrids from the Atlantic TC1 × wild-type APM-2 cross. These results cannot be explained by segregation of a partially silenced *cry3Aa* transgene from the maternal parent (Atlantic TC1); rather, it appears that *cry3Aa* silencing was transmitted through the egg itself, causing silencing of all (maternal and paternal) transgenes in the progeny.

Adams et al. (2000) produced transgenic *Arabidopsis thaliana* plants carrying an antisense copy of the *METHYLTRANSFERASE I* (*METI*) gene. *MET1*, which facilitates preferential methylation of cytosines in CpG sequences (Finnegan and Kovac, 2000), has been found to be the primary methyltransferase in *A. thaliana*. Plants homozygous for the *MET1* antisense construct showed 13% reduced DNA methylation compared to wild-type plants. Crosses were performed using homozygous, antisense *MET1* plants as both male and female parents to determine the effect of hypomethylation on parental imprinting in plants. Seed traits such as weight and size were examined and the results mirrored those seen in interploidy crosses of *A. thaliana*. In 2*x*-4*x* crosses of *A. thaliana*, heavier, larger, triploid seeds result from a double dose of paternal genomes while in 4*x*-2*x* crosses, smaller, lighter, triploid seeds result from a double dose of maternal genomes. Thus, maternal specific alleles contribute to small, light seeds while paternal specific alleles contribute to large, heavy seeds. In crosses of diploid, wild-type females to

diploid, *MET1* antisense males, seed phenotypes resembled a double dose of maternal genomes (small, light) indicating that normally silenced maternal alleles in the pollen had been derepressed by hypomethylation. Likewise, crosses of *MET1* antisense females to wild-type males produced seeds resembling a paternal genome double dose (large, heavy), indicating derepression of normally silenced paternal alleles in the eggs.

Most relevant to the results of this study, the authors found similar size and weight trends of seeds with parents hemizygous, rather than homozygous, for the *MET1* antisense construct. For example, crosses of hemizygous antisense *MET1* females to wild-type males yielded a single class of large, heavy seeds despite the fact that only half of the eggs in this cross inherited a *MET1* antisense gene. To explain this unexpected result, the authors considered two possibilities. One was that the parent established methylation patterns, or imprints, on gametes before meiotic nuclear divisions began. The other possibility was that gametes did not produce MET1 autonomously; rather, parental MET1 protein was transmitted to gametes through diploid spore mother cells.

Jones et al. (2001) investigated the role of *MET1* in transgene silencing by generating transgenic tobacco (*Nicotiana benthamiana*) with single copies of 35S-*GFP* (green fluorescent protein). Transgenic plants were then inoculated with tobacco rattle virus (TRV) carrying portions of either the GFP gene or the 35S promoter in order to induce posttranscriptional gene silencing (PTGS) or transcriptional gene silencing (TGS), respectively. TGS was identified by promoter methylation and lack of transcription while methylation of GFP coding sequence identified PTGS. Regardless of the silencing mechanism, no GFP protein was produced in the plants upon virus inoculation. After the silenced plants had been selfed, TRV-free progeny were generated that no longer had the silence-inducing agent (the virus). Methylation of the promoter sequence (TGS), and the associated GFP transgene silencing, was retained in such progeny while GFP coding methylation (PTGS) was not. The transgene silencing caused by promoter methylation was stable through several generations of progeny but could be alleviated by suppression of the tobacco *MET1* gene. Results of these experiments directly implicated *MET1* with transgene silencing (in this case TGS) and also showed that *MET1* was capable of

maintaining methylation patterns of silenced transgenes through sexual cycles even after the cause of silencing (in this case the TRV virus) was gone.

Uniformly low Cry3Aa expression levels in the Atlantic TC1 × APM-2 TC1 progeny, where 50% of the progeny did not inherit a transgene from Atlantic TC1, could be explained by the transmission of maternal MET1 protein to the eggs of Atlantic TC1. Transgene silencing, and gene silencing in general, is associated with methylation of DNA sequences by methyltransferases. Furthermore, recent studies have shown *MET1* to be directly involved with methylation of parent-specific alleles (Adams et al. 2000) and transgene promoters (Jones et al. 2001). In both of these cases, altered methylation states of the parents were transmitted to, and maintained in, progeny even in the absence of factors that initially caused altered methylation. If MET1 protein, primed to recognize *cry3Aa* as a methylation target, were transmitted to the cytoplasm of eggs derived from Atlantic TC1, uniform silencing of *cry3Aa* would be expected in all progeny regardless of transgene origin. The silencing effect observed in this cross could not have been programmed before the start of meiosis, or anytime during megagametogenesis, because wild-type eggs lacking a *cry3Aa* transgene (50% of the Atlantic TC1 eggs) did not possess a gene on which the silencing imprint could be passed. The use of an exogenous, rather than an endogenous, transgene strongly suggests that the silencing agent was present in the cytoplasm of eggs where it caused silencing of all *cry3Aa* transgenes (maternal or paternal). Evidence for cytoplasmic transmission of maternal methyltransferases to progeny comes from animal studies. Cardoso and Leonhardt (1999) found high levels of maternal DNA methyltransferase (*Dnmt1*) in the cytoplasm of early mouse embryos, where it may play a role in development and/or protect against foreign DNA. *MET1* encodes a similar protein to *Dnmt1*, and in fact, *MET1* was cloned from *Arabidopsis* using degenerate primers based on conserved regions of *Dnmt1*.

As previously shown by Adams et al. (2000) this work describes an instance where parent-of-origin, already a recognized phenomenon in animals such as mice, has been observed to have an effect on transgene expression in plants. Cytoplasmic transmission of methyltransferases causing transgene silencing, such as *MET1*, to gametes could

influence the design of sexual hybridizations with transgenic plants. Regarding 4*x*-2*x* hybridization, use of high expressing transgenic parents as pollen donors, thereby minimizing cytoplasmic transfer, would ensure high expression in hybrids. Indeed, high expressing 4*x*-2*x* hybrids were only obtained when a high expressing, transgenic male was used to pollinate a wild-type female (Chapter 2). Cytoplasmic transfer of gene silencing could also be exploited to produce non-transgenic plants with altered methylation patterns. For example, a single copy transgenic plant with antisense silencing of an endogenous gene could be crossed as female to a wild-type plant. Half of the progeny would not inherit a transgene (wild-type progeny), however, transmission of the silencing via methyltransferases in the cytoplasm could cause a permanent imprint on the endogenous gene that subsequently would be maintained by methyltransferases such as *MET1*. Additional examples of parent-of-origin effects on transgene expression are needed to clarify the types of gene silencing (PTGS or TGS) that can be transmitted in this manner, and by what mechanisms, in plants.

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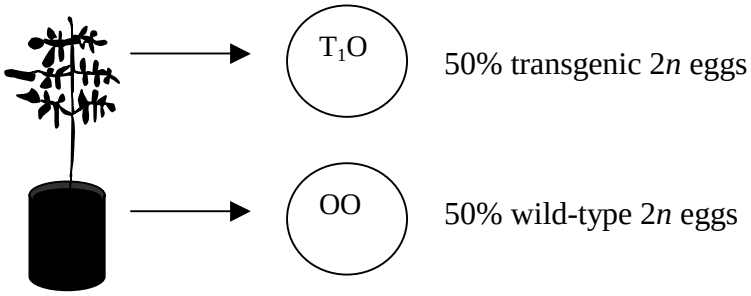
Table 1: Punnette square of 4*x*-2*x* hybridization between Atlantic TC1 and APM-2 TC1 with the assumptions that single copies of *cry3Aa* lead to high expression in tetraploid hybrids while the combination of two, non-allelic copies of *cry3Aa* leads to reduced expression. The single insert in Atlantic TC1 is represented by T_1 while the single insert in APM-2 TC1 is represented by T_2. O represents chromosomes with no copy of *cry3Aa*. Expected phenotypic expression of *cry3Aa* is in parentheses. Frequencies of homozygous and hemizygous 2n pollen produced by APM-2 TC1 are based on gene-centromere mapping (Johnson et al. 2001).

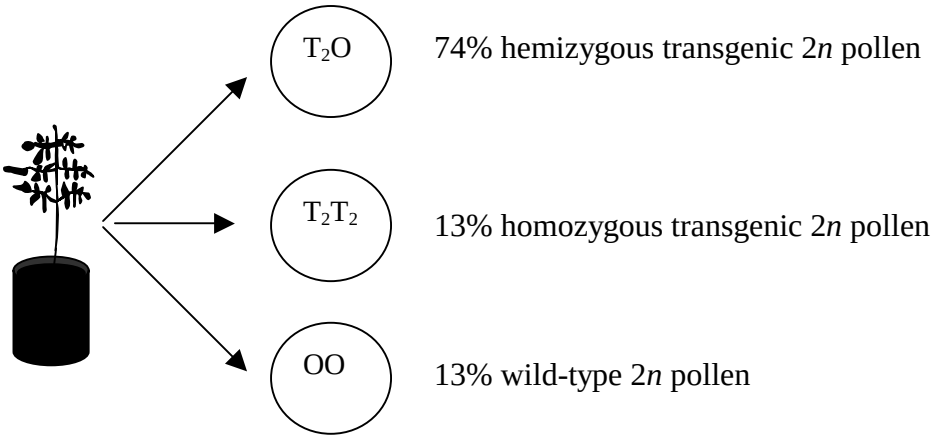
	APM-2 TC1		
	13% OO pollen	74% T_2O pollen	13% T_2T_2 pollen
Atlantic TC1			
50% T_1O eggs	6.5% T_1OOO (high)	37% T_1T_2OO (reduced)	6.5% $T_1T_2T_2O$ (reduced)
50% OO eggs	6.5% OOOO (none)	37% T_2OOO (high)	6.5% T_2T_2OO (high)

A.



Atlantic TC1 (T_1OOO)
(*cry3Aa* single insert, tetraploid maternal parent)

B.



APM-2 TC1 (T_2O)
(*cry3Aa* single insert, diploid paternal parent with unreduced pollen by FDR)

Figure 1: Distribution of transgenic and non-transgenic (wild-type) eggs and pollen produced by Atlantic TC1 (A) and APM-2 TC1 (B), respectively. The single insert in Atlantic TC1 is represented by T_1 while the single insert in APM-2 TC1 is represented by T_2. O represents chromosomes with no copy of *cry3Aa*. APM-2 produces unreduced pollen by the genetic equivalent of FDR (first division restitution). Gene-centromere mapping of APM-2 TC1 indicated that the *cry3Aa* transgene is located 26 cM from the centromere (Johnson et al. 2001). Thus, genetic recombination between the centromere and the *cry3Aa* transgene produces 13% wild-type, and 13% homozygous transgenic, pollen. Tetraploid hybrids (4*x*-2*x* hybrids) result from pollination of cv. Atlantic with unreduced pollen from APM-2.

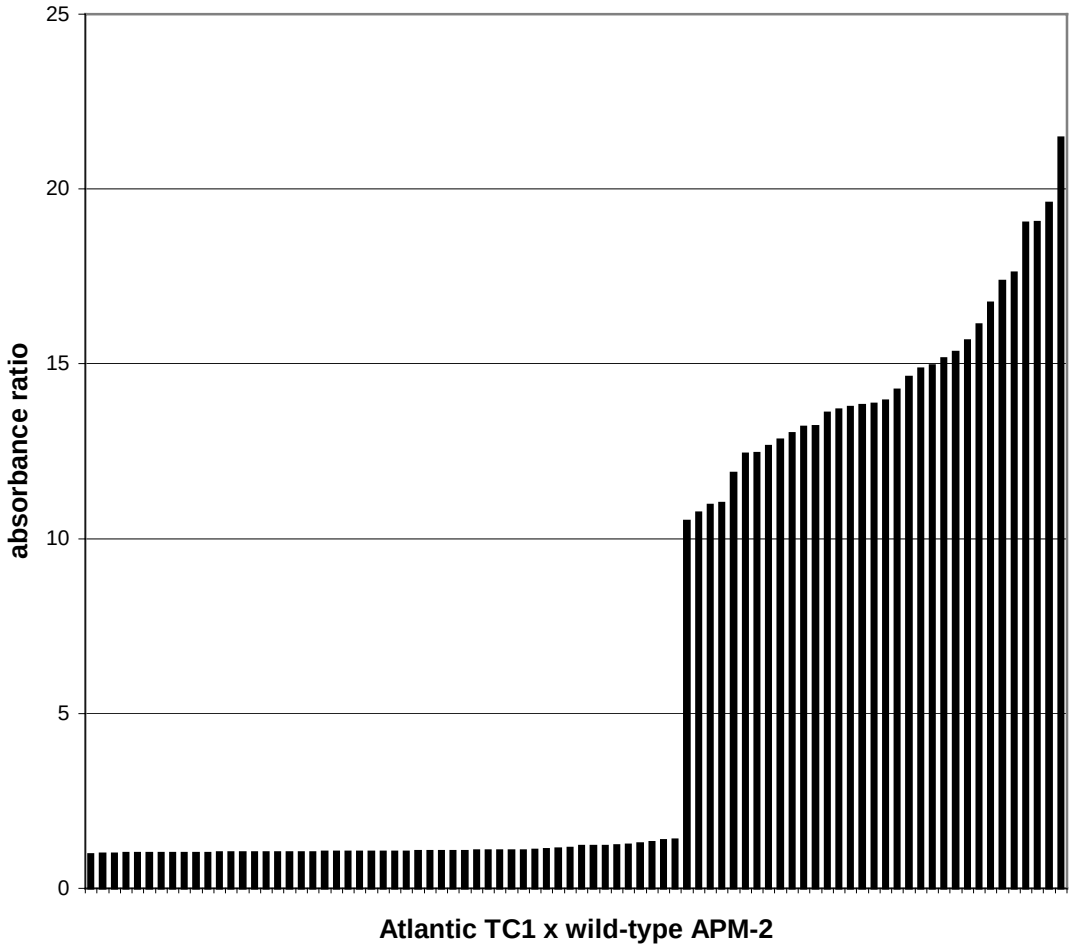


Figure 2: DAS-ELISA absorbance ratio distribution, sorted in ascending order, of the 85 hybrids derived from the Atlantic TC1 × wild-type APM-2 cross (progeny genotypes on x-axis and absorbance ratios on y-axis). Leaf material was ground at a 1:5 (g leaf tissue/ml extraction buffer) dilution. Absorbance ratios were calculated by dividing the absorbance of each transgenic sample by the absorbance of a non-transgenic control following the addition of p-nitrophenyl phosphate using an Emax^{TM} precision microplate reader at 405 nm. A total of 51 progeny did not express cry3Aa whereas 34 progeny expressed the transgene.

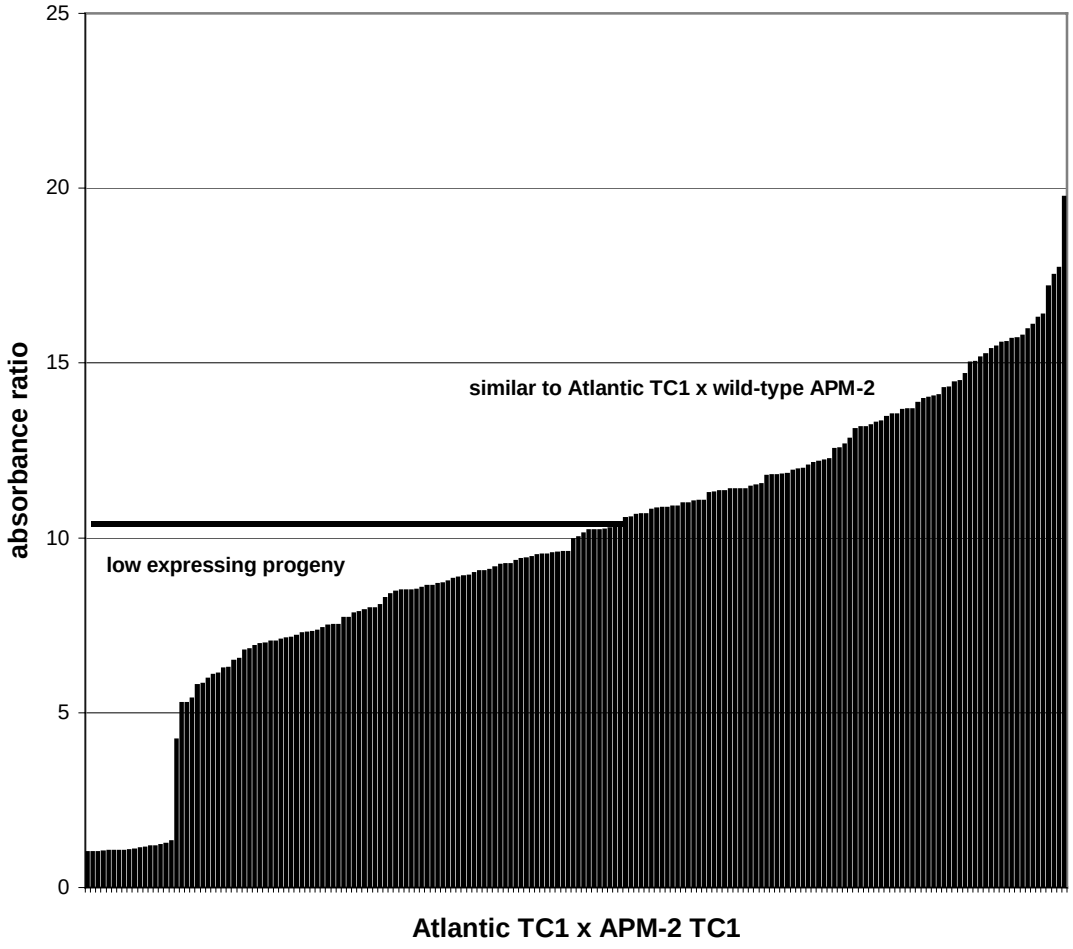


Figure 3: DAS-ELISA absorbance ratio distribution, sorted in ascending order, of 188 hybrids derived from Atlantic TC1 × APM-2 TC1 (progeny genotypes on x-axis and absorbance ratios on y-axis). The bold horizontal line indicates the lowest absorbance ratio observed in the Atlantic TC1 × wild-type APM-2 progeny (Figure 2). The progeny can be divided into 17 with no Cry3Aa expression, 86 with lower expression than Atlantic TC1 × wild-type APM-2 progeny, and 85 with Cry3Aa expression in the same range as Atlantic TC1 × wild-type APM-2 progeny. Sample preparation and data presentation as in Figure 2.

CHAPTER 4

Effect of transgene segregation on Cry3Aa expression of second generation tetraploid progeny derived from a multiple insert diploid potato line

Abstract Previous studies of APM-2 TC2, a line transformed with four *cry3Aa* loci, have indicated that low expression of Cry3Aa results from homology dependent gene silencing. High and low expressing 4*x*-2*x* hybrids derived from 'Atlantic' × APM-2 TC2 were pollinated by 'Katahdin' to produce second generation progeny. High Cry3Aa expression of some individuals in the progeny populations indicated that meiotic segregation of the multiple inserts of APM-2 TC2 led to higher expression. The results also suggest that certain APM-2 TC2 *cry3Aa* loci have greater silencing effects on expression than others.

Introduction

The previous chapter examined transgene expression in a cross between two single insert transgenic plants that could produce 4*x*-2*x* progeny with none, one, or two, non-allelic copies of the *cry3Aa* transgene. Reduced expression was observed in a large proportion of those progeny (49%) and was believed to result from silencing of the two, homologous *cry3Aa* loci (homology-dependent gene silencing). The impetus for that study was to associate copy number with reduced expression, as observed in the low Cry3Aa expression of a four copy, diploid line (APM-2 TC2) relative to single copy lines (APM-2 TC1, APM-2 TC7). An additional route to establishing multiple *cry3Aa* copies as the causal agent of low expression in APM-2 TC2 is to generate progeny lacking some of the parental transgene copies by segregation through meiosis. The 4*x*-2*x* hybrids derived from APM-2 TC2 are expected to inherit most of the multiple parental *cry3Aa* copies because of their derivation from unreduced FDR gametes retaining 80% of heterozygosity in APM-2. The hybrids may lose some of the transgene copies through genetic recombination between the transgene insertion site and a centromere. Sexual crosses of the tetraploid 4*x*-2*x* hybrids to non-transgenic tetraploid genotypes, however, is expected to allow normal segregation of transgenes among progeny. If loss of some of the transgenes, or specific transgenes, leads to higher expression then one would expect some high expressing progeny to result from these crosses. Such a result would provide further evidence that homology dependent gene silencing accounts for the reduced Cry3Aa expression of APM-2 TC2.

Fujiwara et al. (1993) created single copy transgenic lines of tobacco (*Nicotiana tobacum* L.) that expressed kanamycin resistance (*npt II*). Two expressing lines were selected for second transformation with a highly homologous plasmid that also encoded kanamycin resistance. Half of the resulting double transformants subsequently lost expression of kanamycin resistance, indicating homology dependent gene silencing. Tobacco transformed with only the second plasmid expressed kanamycin resistance. The researchers then produced progeny from the doubly transformed, kanamycin sensitive lines and observed reactivation of kanamycin resistance in several progeny lines where the second plasmid was eliminated by meiotic segregation. This study provided evidence that transgenes can silence other homologous sequences in *trans*, and this effect can be alleviated following independent segregation of transgenes through meiosis. Vaucheret (1993, 1994) created a "silencing" line of tobacco transformed with a plasmid carrying antisense tobacco nitrite reductase under control of the 35S promoter and neomycin phosphotransferase (*npt II*) under control of the 19S promoter. Sexual crosses of the "silencing" line to other transgenic lines resulted in promoter methylation and silencing of any transgene, regardless of coding sequence, under control of the 35S or 19S promoter. Several high expressing transgenic lines were silenced by crosses to the "silencing" line. Selfing of these silenced lines, and elimination of the "silencing" locus, resulted in a gradual reactivation of silenced transgenes that at 4 months was between 4-72% of the original level. The authors concluded that an unstable, methylation "imprint" on the silenced promoters was gradually lost after elimination of the "silencing" locus.

Digestion of APM-2 TC2 genomic DNA with *BamH* I restriction enzyme, followed by hybridization with a 440 bp fragment of *cry3Aa*, revealed four copies of the transgene in APM-2 TC2 (Figure 1, C_1-C_4). Two of the bands (C_1 and C_3) hybridized with probe more intensely than the other two, suggesting the presence of multiple *cry3Aa* inserts (or fragments of inserts) in these bands. Tandem repeats of transgenes often occur as a result of *Agrobacterium* transformation (Tinland 1996). Tandem repeats are particularly prone to homology-dependent gene silencing and can transmit this silencing to other copies in

trans (Buck et al. 2001). Thus, of the four *cry3Aa* loci in APM-2, C_1 and C_3 may have a more powerful silencing effect than C_2 and C_4 on overall Cry3Aa expression.

APM-2 TC2 was initially crossed as male to cv. Atlantic (through 4*x*-2*x* hybridization) to generate tetraploid hybrids. Cry3Aa expression among 25 hybrids varied, with 2/3 of the hybrids producing significantly less Cry3Aa than the transgenic parent, APM-2 TC2 (Figure 2). Seven of those tetraploid hybrids, three high and four low producers of Cry3Aa, were crossed as female parents to cv. Katahdin to generate second generation progeny (Figure 2, darkened bars). These seven, second generation progeny populations are the focus of this study.

Materials and methods

Southern analysis to detect transgene copy number of 4*x*-2*x* hybrids

Total genomic DNA from three high and four low Cry3Aa-producing hybrids (parents of the second generation progeny populations) was extracted using a CTAB procedure and suspended in TBE. A 50 µl digestion reaction of 10 µg DNA was performed using restriction enzymes *BamH* I or *Xba* I (Promega Corporation, Madison, WI), appropriate buffers and acetylated BSA. Restricted DNA was electrophoretically separated on a 2.5% agarose gel in 1× TAE running buffer and transferred overnight to a charged nylon membrane using alkaline capillary transfer. The nylon membrane was hybridized overnight with a P^{32} labeled 440 bp fragment of the *cry3Aa* gene (prepared by random priming), washed and the hybridization signal was visualized using a Storm® gel and blot imaging system (Molecular Dynamics, Inc.).

Sexual hybridization of APM-2 TC2 derived 4*x*-2*x* hybrids and development of seven, second generation progeny populations

Three 4*x*-2*x* hybrids with Cry3Aa expression not significantly different from APM-2 TC2 (ATC2 67, ATC2 83, ATC2 69) and four 4*x*-2*x* hybrids with virtually no production of

Cry3Aa (ATC2 110, ATC2 47, ATC2 88, ATC2 90) were established from *in vitro* plantlets and acclimated to the greenhouse. Upon flowering, the hybrids were crossed as female to cv. Katahdin. Seed was collected, dried, imbibed with gibberellic acid (2000 ppm) and planted (32 seed of each cross) in the greenhouse. Seeds were planted one seed/cell in cell packs of 48 (13.4 cm long × 13.2 cm wide × 5.8 cm deep; Kord Products, Canada, item #K806).

Cry3Aa analysis of second generation progeny

Six weeks after planting, 204 second generation progeny (an average of 29 progeny from each of the seven crosses) were screened using a DAS-ELISA test for Bt-Cry3Aa endotoxin from Agdia (Elkhart, ID). The youngest, fully expanded leaf of each progeny was used for analysis and leaf tissue was ground at a 1:5 (g leaf tissue ml^{-1} extraction buffer) dilution. Cry3Aa expression was measured by the absorbance reading of each sample following the addition of p-nitrophenyl phosphate using an Emax™ precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 405 nm. A dilution series of purified Cry3Aa protein was used to quantify Cry3Aa production (μg g^{-1} leaf tissue) of the seven progeny populations.

Results

Genomic DNA of seven selected 4x-2x hybrids between Atlantic and APM-2 TC2 was restriction digested with *BamH* I and subjected to southern analysis (Figure 2). One of the high expressing hybrids (ATC2 69) has lost the two upper bands (C_1 and C_2) present in APM-2 TC2 and all of the other hybrids. In addition, one of the low expressing hybrids (ATC2 88) has lost the C_2 band. It appears that the other five 4*x*-2*x* hybrids inherited, most of bands present in the APM-2 TC2 parent.

Figures 3-5 show Cry3Aa levels (μg g^{-1} of leaf tissue) of progeny derived from high-expressing hybrids, while Figures 6-9 show Cry3Aa levels of progeny derived from low-expressing hybrids. Some progeny derived from the three high expressers, ATC2 67,

ATC2 83 and ATC2 69, produced two fold or more Cry3Aa than the respective parents, at levels of 7 μg g^{-1} leaf tissue or higher. These high expressing progeny occurred at similar frequencies in the three populations: ATC2 67 had 20% high expressers, ATC2 83 had 18% high expressers and ATC2 69 had 21% high expressers. Some progeny derived from the four low expressers, ATC2 110, ATC2 47, ATC2 88 and ATC2 90, were also high expressing (7 μg g^{-1} of leaf tissue or higher) but they occurred less frequently: ATC2 110 had 7% high expressers, ATC2 47 had 4% high expressers, ATC2 88 had no high expressers and ATC2 90 had 3% high expressers.

Six of the seven populations had low expressing progeny that produced 3 μg Cry3Aa g^{-1} of leaf tissue or less (Figures 3, 4, 6-9). The low expressing hybrids, however, produced a greater frequency of these low expressing progeny than high expressing hybrids. The high expressing hybrid that had lost bands on the Southern blot, ATC2 69, was the only hybrid that produced no low expressing progeny (Figure 5). The low expressing hybrid that lost a band on the Southern blot, ATC2 88, produced no high expressing progeny (Figure 8). The frequency of non-expressing progeny was similar for most of the families ranging from 47% to 59%, regardless of derivation from high expressing or low expressing parent. Only the ATC2 83 progeny population had an unusually small percentage (24%) of non-expressing progeny.

Discussion

Segregation of Cry3Aa expression clearly occurred in the seven progeny populations derived from APM-2 TC2. Progeny that expressed more Cry3Aa than the 4*x*-2*x* hybrids and APM-2 TC2 were observed in all populations. In addition, a similar range of Cry3Aa expression was observed for most of the crosses, varying from less than 1 μg g^{-1} of leaf tissue to more than 7 μg g^{-1} of leaf tissue. The only exceptions were ATC2 88, which lacked high expressing progeny, and ATC2 69, which lacked low expressing progeny. Although the range of expression was similar for most crosses, the frequency of high and low expressing progeny varied among the populations. This result indicates that the potential for producing high and low expressing progeny existed in most of the parent

4*x*-2*x* hybrids, however, the rate at which those progeny were produced was a factor that differed among parents.

The Southern blot results (Figure 2) suggest that, with the exceptions of ATC2 69 and ATC2 88, most of the hybrids inherited the same number of *cry3Aa* loci. So what could explain the higher overall expression of progeny derived from high-expressing 4*x*-2*x* hybrids relative to low expressing hybrids? Even if most of the 4*x*-2*x* hybrids inherited four *cry3Aa* loci from APM-2 TC2, differences could exist among those loci particularly if some of them, such as C_1 and C_3, have tandem repeats of transgenes. A loss of repeats through genetic recombination at such a locus could weaken the ability of that locus to silence other transgenes (Buck et al. 2001). If this occurred at one of the loci in the high expressing 4*x*-2*x* hybrids, not only would expression in the hybrid itself be higher but overall expression in the progeny population would also be higher. The fact that ATC2 69, which does not appear to possess the C_1 or C_2 loci from APM-2 TC2, produced no low expressing progeny suggests that the C_1 locus may be responsible for much of the low expression of progeny observed in other crosses. ATC2 88, which lost the C_2 locus, produced only low expressing progeny giving further evidence that the C_1 locus causes low expression in progeny.

Figure 1 depicts segregation of four *cry3Aa* loci in progeny of 4*x*-2*x* hybrids derived from APM-2 TC2 assuming independent assortment, i.e., that the four transgenes or transgene complexes had inserted on different chromosome arms. The potential for transgene assortment in progeny is large (16 possible) ranging from all four to none. If all of the inserts (C_1-C_4) had similar expression levels, then one would expect any progeny receiving at least one insert (95%) to have low expression similar to APM-2 TC2. The variable Cry3Aa expression levels observed in progeny populations of this experiment make this hypothesis unlikely. It appears that different transgene assortments result in differential Cry3Aa expression. A more plausible possibility is that expression is dose-dependent, with fewer trangene copies resulting in higher expression. Perhaps the expressing individuals represent the one and two transgene configurations (62%) while the non-expressers have 3, 4 or no transgenes (38%). This idea seems to fit the observed

data more accurately, although the frequency of non-expressers in most populations exceeds what is expected.

The most likely explanation is that transgene expression is partly dosage dependent, but also dependent on which particular inserts are inherited. As stated previously the C_1 and C_3 loci of APM-2 TC2 (Figure 1) were more intensely staining than C_2 and C_4, indicating that they may contain tandem repeats of *cry3Aa* and therefore may induce homology-dependent gene silencing. Assuming that C_1 and C_3, by themselves or together, result in no expression of Cry3Aa, then a higher percentage of non-expressers (44%) would be expected in the dosage dependent hypothesis discussed in the previous paragraph.

In conclusion, segregation of Cry3Aa expression in these seven, second generation progeny populations indicates that independent assortment of the four transgenes present in APM-2 TC2 through meiosis increases expression in progeny that inherit fewer copies. The results support the conclusions of chapters 2 and 3, with respect to homology-dependent gene silencing, but also suggest that some of the transgene loci in APM-2 TC2 have a greater silencing effect than others.

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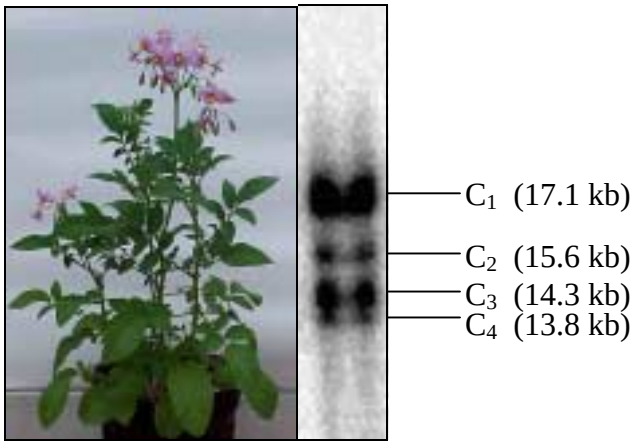

OOOO

C_1OOO

C_2OOO

C_3OOO

C_4OOO

C_1 C_2OO

C_1 C_3OO

C_1 C_4OO

C_2 C_3OO

C_2 C_4OO

C_3 C_4OO

C_1 C_2 C_3O

C_2 C_3 C_4O

C_1 C_3 C_4O

C_1 C_2 C_4O

C_1 C_2 C_3 C_4

Figure 1: APM-2 TC2 has four *cry3A* inserts visible by southern blot analysis of copy number using restriction enzyme *BamH* I (upper right). Independent assortment of the four transgene inserts (labeled C_1-C_4) in progeny of 4*x*-2*x* hybrids derived from APM-2 TC2 would produce 16 different possibilities (listed below the arrow) all with a 6.25% chance of occurrence. Molecular weight is listed parenthetically by each of the four *cry3Aa* inserts. O represents chromosomes with no copy of *cry3Aa*.

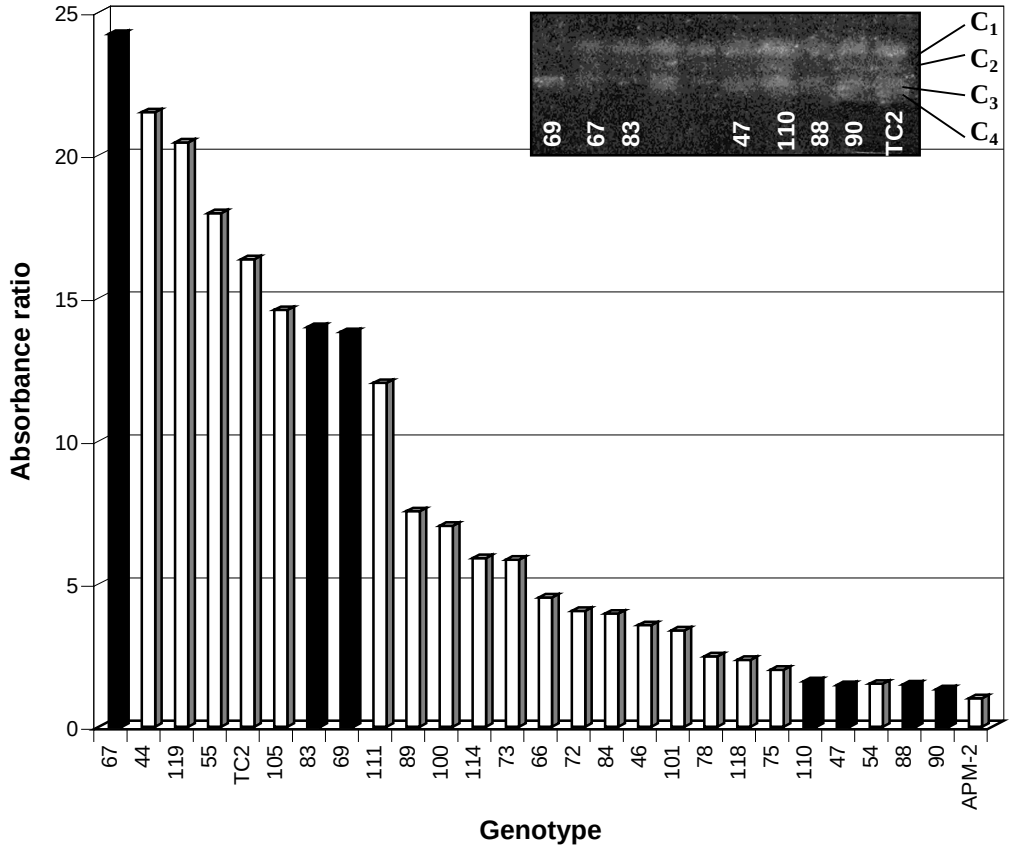

Figure 2: Absorbance ratio distribution for Cry3Aa protein production of 25 4x-2x progeny derived from Atlantic × APM-2 TC2 (4-insert parent). Darkened bars represent selected progeny that were crossed to cv. Katahdin to produce second generation tetraploid progeny. The three selections on the left were not significantly different from APM-2 TC2 for Cry3Aa production (approx. 4 µg Cry3Aa g^{-1} leaf tissue) whereas the four selections on the right had significantly lowered Cry3Aa production (less than 0.l µg Cry3Aa g^{-1} leaf tissue). Southern blot analysis using *BamH* I for the seven selections compared to the APM-2 TC2 parent is presented in the inset. Numbers listed on the southern blot correspond to progeny genotypes on the chart x-axis. The four bands of APM-2 TC2 are labeled C_1-C_4. A loss of bands is evident for progeny 69 and progeny 88.

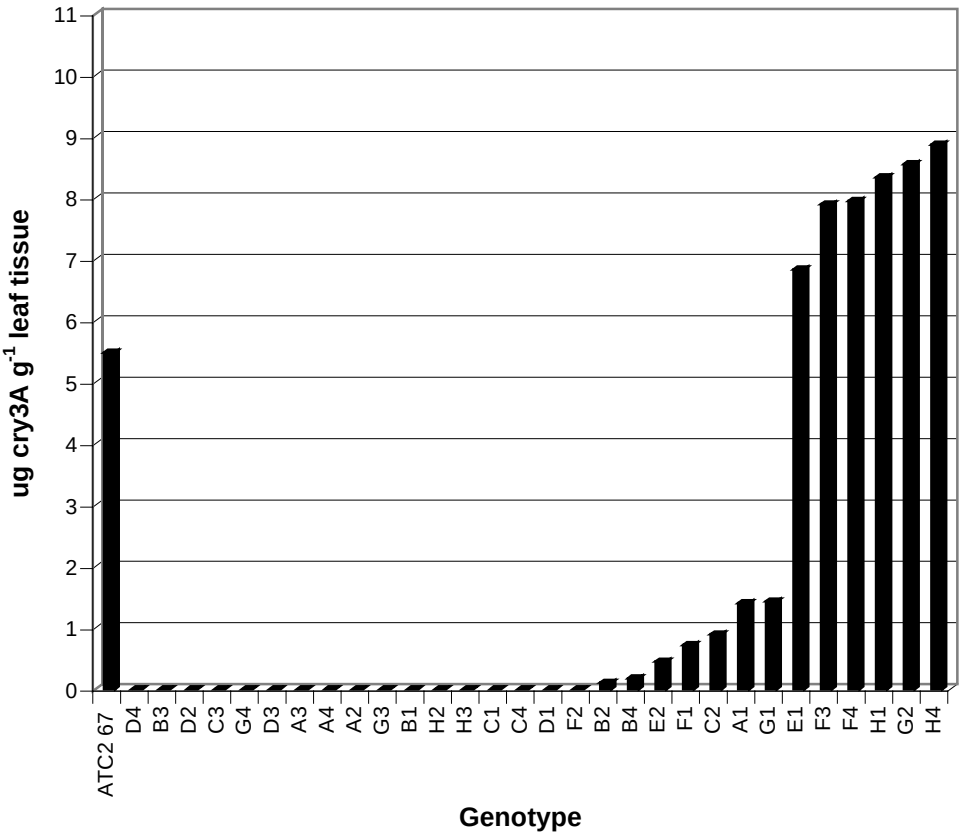

Figure 3: Distribution of Cry3Aa expression among 30 tetraploid progeny derived from ATC2 67 × Katahdin. The parent is represented by the first bar on the left.

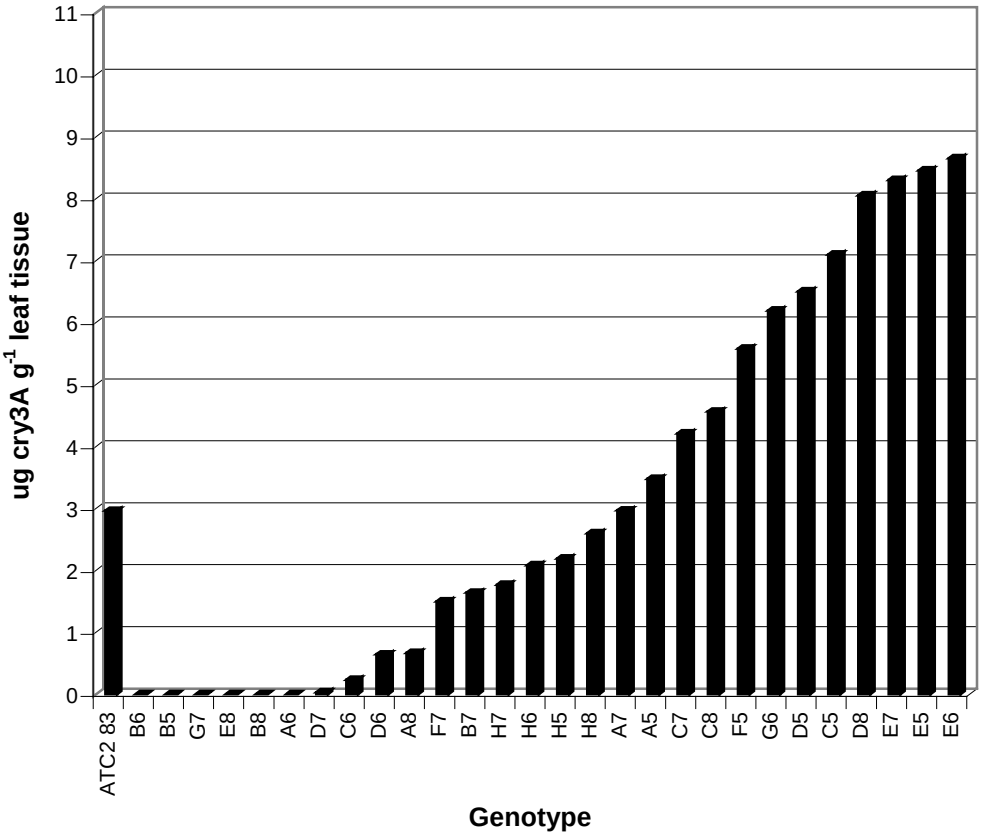

Figure 4: Distribution of Cry3Aa expression among 28 tetraploid progeny derived from ATC2 83 × Katahdin. The parent is represented by the first bar on the left.

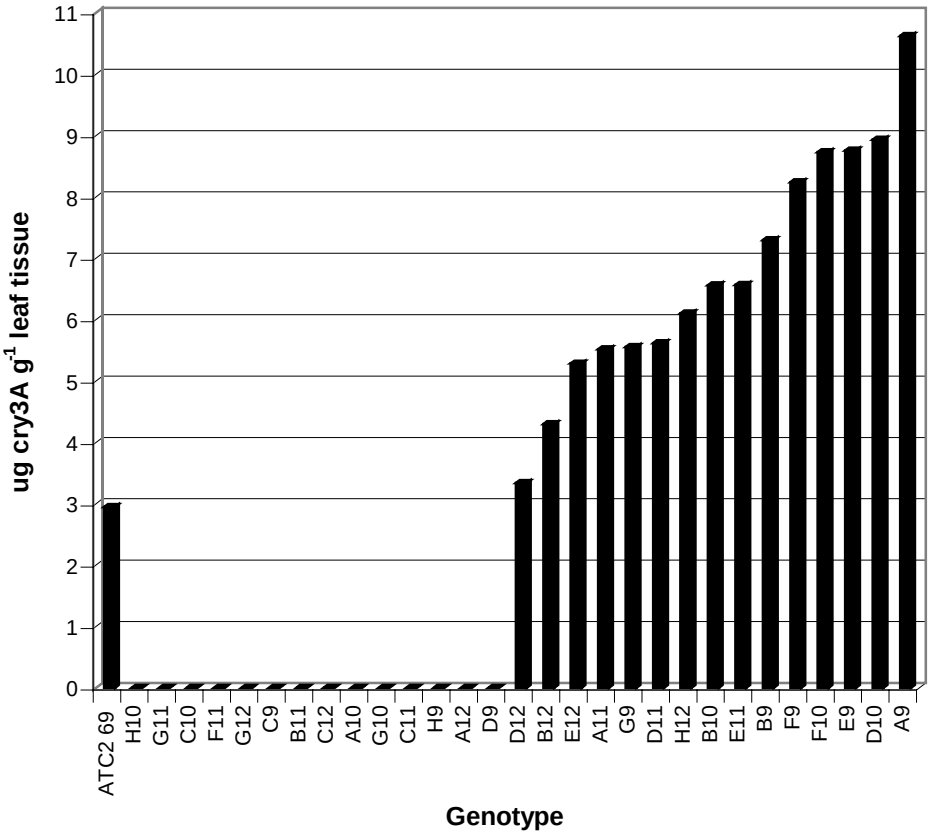

Figure 5: Distribution of Cry3Aa expression among 29 tetraploid progeny derived from ATC2 69 × Katahdin. The parent is represented by the first bar on the left.

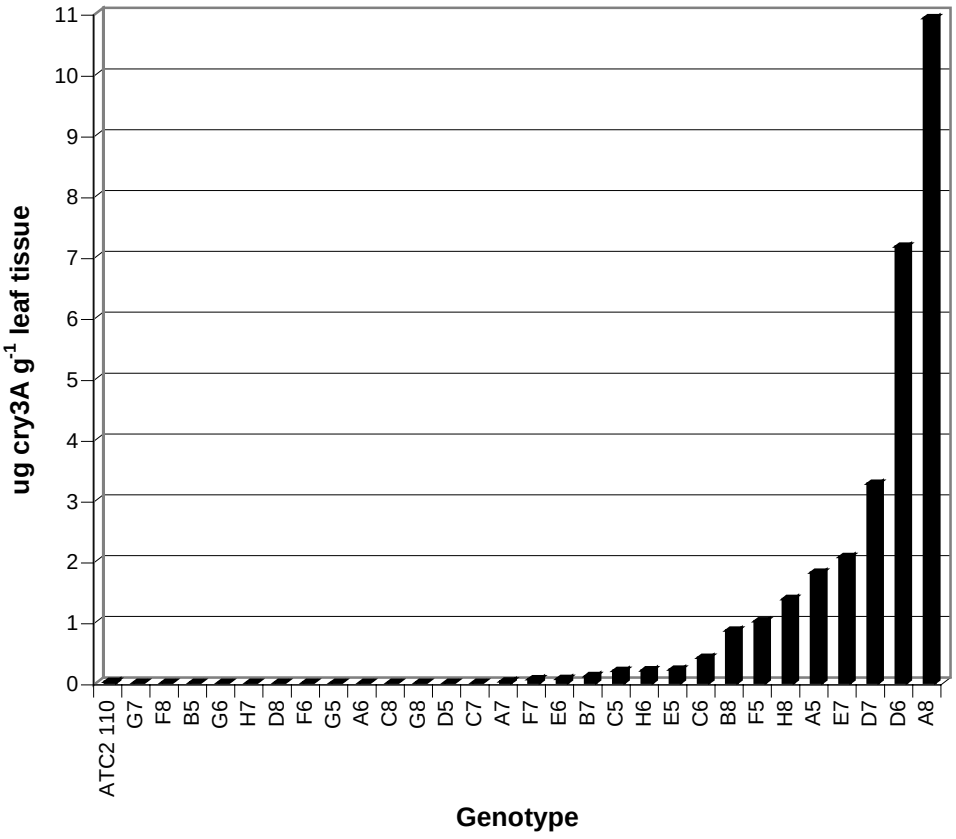

Figure 6: Distribution of Cry3Aa expression among 29 tetraploid progeny derived from ATC2 110 × Katahdin. The parent is represented by the first bar on the left.

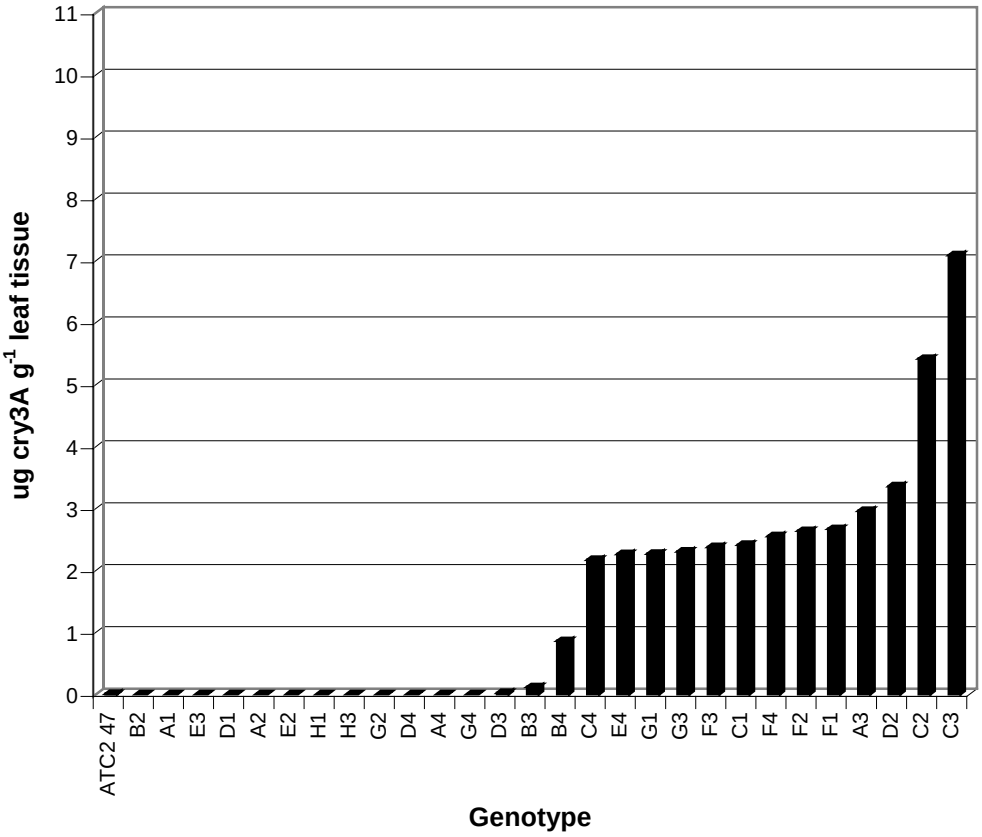

Figure 7: Distribution of Cry3Aa expression among 28 tetraploid progeny derived from ATC2 47 × Katahdin. The parent is represented by the first bar on the left.

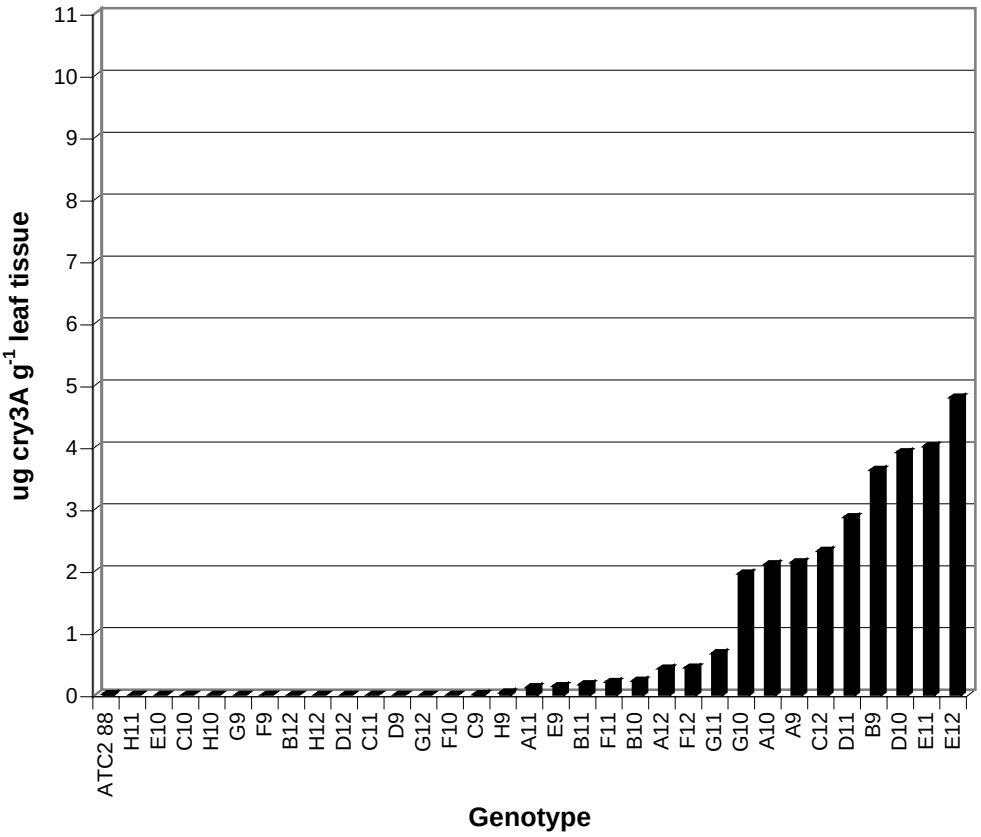

Figure 8: Distribution of Cry3Aa expression among 32 tetraploid progeny derived from ATC2 88 × Katahdin. The parent is represented by the first bar on the left.

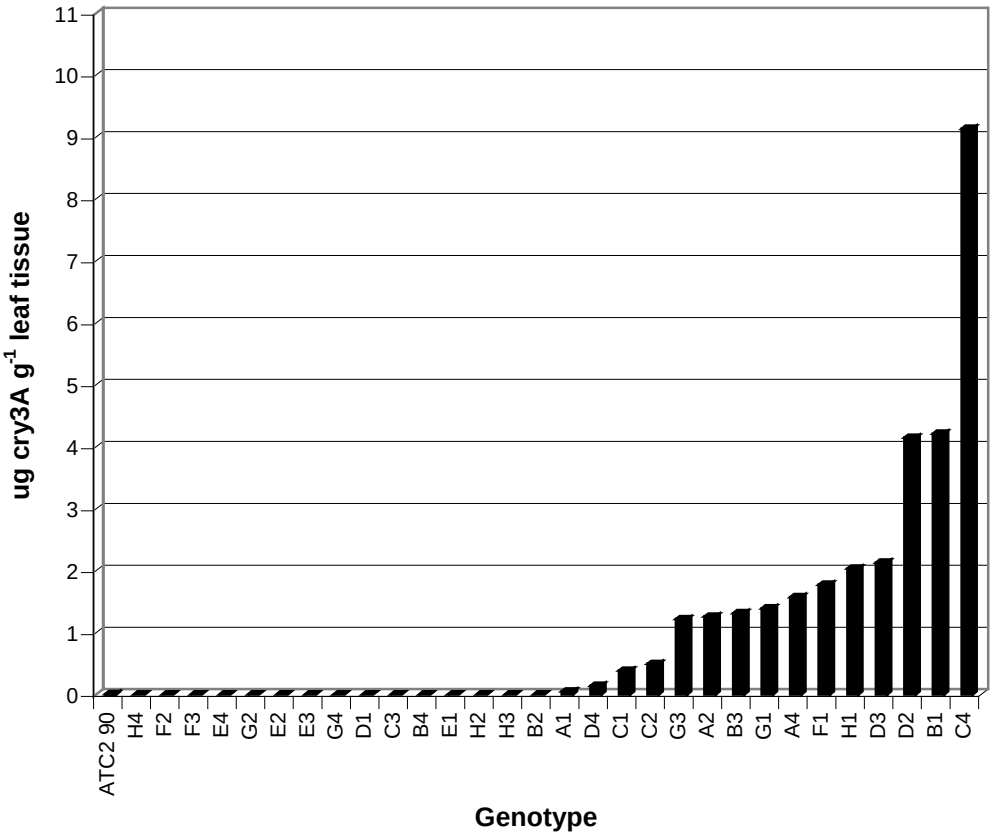

Figure 9: Distribution of Cry3Aa expression among 30 tetraploid progeny derived from ATC2 90 × Katahdin. The parent is represented by the first bar on the left.

CONCLUSIONS

These results do not indicate that ploidy elevation, through 4*x*-2*x* hybridization, has a general silencing effect on transgene expression. However, multiple copies of transgenes appear to be targets of silencing upon ploidy elevation. This was demonstrated by three different studies examining expression of the *cry3Aa* transgene. The first (Chapter 2) showed that multiple copies of the *cry3Aa* transgene resulted in low Cry3Aa expression of diploid genotypes and even lower expression of 4*x*-2*x* hybrids derived from a multiple copy line. The second study (Chapter 3) provided evidence that the combination of two, non-allelic *cry3Aa* transgenes in 4*x*-2*x* hybrids resulted in reduced Cry3Aa expression. This experiment also suggested the transmission of a maternal transgene silencing factor, perhaps the methyltransferase *MET1*, through the cytoplasm of eggs to progeny. In the third study (Chapter 4) higher Cry3Aa expression was detected among some second generation progeny derived from a low expressing, multiple copy line indicating that segregation of transgenes among progeny, and reduction of transgene copy number, resulted in elevation of Cry3Aa expression.

The results indicate that transgenes can be expressed at high levels in TPS potato derived by 4*x*-2*x* hybridization. Most important is that single copy transgenic lines be used. Multiple copies of transgenes are typically silenced by homology-dependent gene silencing, and this silencing effect may be increased in 4*x*-2*x* hybrids because of ploidy elevation. Newly formed polyploids, such as 4*x*-2*x* hybrids, have been shown to undergo rapid silencing of duplicate or highly homologous native gene sequences, and homologous transgene sequences are likely targets as well. Transgenes should be transmitted through the male parent not only because this will result in a large proportion of transgenic progeny (unreduced FDR gametes retain 80% or more of parental heterozygosity) but also because this limits the transfer of cytoplasm from the transgenic parent. The results demonstrate that transgene silencing can be transmitted through the cytoplasm of eggs from a transgenic parent (Chapter 3) and thus using the transgenic parent as pollen donor would minimize this effect. Finally, the introduction and

expression of multiple transgenes in 4*x*-2*x* hybrids could be achieved if homology between the transgenic sequences (promoter and coding regions) is avoided.

VITA

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Personal History

Born in Alexandria, Virginia on June 16, 1974

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Professional Experience

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3. Member of Phi Kappa Phi and The Scientific Research Society of Sigma Xi