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

A. A. T. Johnson¹, B. A. Nault² and R. E. Veilleux¹,³

¹ Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061-0327 USA; ² Eastern Shore Agricultural Research Extension Center, Virginia Polytechnic Institute and State University, Painter, VA, 23420 USA; ³ Corresponding author: E-mail: potato@vt.edu

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Abstract
The study examined the effect of ploidy elevation through unreduced gametes on transgene expression in potato. Tetraploid transgenic progenies were obtained from one tetraploid potato cultivar crossed with 2n pollen producing diploid clones harbouring an exogenous transgene (cry3Aa). Both single- and multiple-insert diploid transgenic lines that were regenerated by Agrobacterium tumefaciens leaf disc inoculation were used in crosses. A DAS–ELISA system and no-choice feeding bioassay enabled characterization of the parental lines as either ‘high’ or ‘low’ expressers of the Cry3Aa protein. High Cry3Aa expression was observed for both single-insert transgenic diploids and their 4x–2x progeny. On the contrary, 68% of 4x–2x progeny derived from a multiple-insert, diploid transgenic had significantly reduced Cry3Aa expression compared with the parent, with 32% demonstrating nearly complete silencing of the transgenic. Multiple copies of a transgene, like homologous native genes, may be susceptible to transgene silencing following polyploidization. Therefore, incorporation of exogenous transgenes into a true potato seed (TPS) production system is feasible if a single-insert diploid parent is used. Gene-centromere mapping of the cry3Aa transgene demonstrated that a non-transgenic refuge might be naturally created in a TPS hybrid system through genetic recombination.

Key words: Solanum spp. — 2n gametes — polyploidy — transgene silencing — true potato seed

Unreduced (2n) gametes commonly occur in solanaceous species (Carpeto et al. 2000) and have been used extensively to transfer useful 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 (Solanum tuberosum) gene pool through 2x–2x and 4x–2x hybridization (Werner and Peloquin 1991, Ortiz et al. 1997, Watanabe et al. 1999). One common strategy for true potato seed (TPS) hybrid production is to employ 4x–2x hybridization to generate a homogeneous pathogen-free crop.

Ploidy increase and its effect on gene expression have emerged as active areas of research, in part, as many important crops, such as maize, have been found to be allopolyploids arising from ancient polyploidization. Ploidy elevation can result in up-regulation or down-regulation of native plant and animal genes. Galitski et al. (1999) generated isogenic lines of yeast (Saccharomyces cerevisiae) ranging from haploid to tetraploid and examined gene expression using oligonucleotide-probe microarrays. Several genes, including CTS1 encoding an endochitinase protein, were up-regulated in haploids compared with tetraploids, as much as 12-fold. Other genes, such as FLO11, were down-regulated 11-fold. The authors speculated that ploidy-dependent regulation of genes in the yeast lines might 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 csa3, encoding a thiol protease, which decreased expression by 28% between the diploid and tetraploid lines.

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. As 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 an 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 with haploid tobacco transformed with the uidA gene. The authors suggested that the increase in chromosome number, combined with homozygosity, could result in higher transgene expression levels.

Our research addresses ploidy increase and its effect on transgene expression using 4x–2x hybridization of potato, a method of ploidy elevation that has not been examined in transgenic systems. Nearly all plant studies addressing this topic have used tissue culture-induced chromosome doubling or inter-ploidy crosses to elevate ploidy. Most 4x–2x 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. Finally, our research explores the feasibility of incorporating transgenes into 4x–2x hybrids – a technology that holds potential for lowering costs of potato hybrid...
production. Our objectives were: (1) to express an economically important transgene in a diploid potato genotype producing unreduced gametes by first division restitution (FDR); (2) to transmit the transgene to tetraploid progeny using 4x–2x hybridization; and (3) to analyze the effect of ploidy elevation 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 a mechanism genetically equivalent to FDR was obtained by crossing diploid S. andigena (PI 347773) as female with an F1 hybrid (S. phureja PI 225669 × S. microdontum PI 320304) as male. Heat tolerance, field performance and glycoalkaloid content of tetraploid progeny derived from 4x–2x 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 weeks on MS (Murashige and Skoog 1962) basal medium w/vitamins, 3% sucrose, cry3Aa was amplified with David Douches (Michigan State University, E. Lansing). The gene (GenBank GI# 208152) in the pSPUD8 vector (Fig. 1) carried in the gene was engineered for high expression in plants (Sutton et al. 1992). Information came from in vitro described (Veilleux et al. 1997). Leaves of APM-2 were initiated by incubating scrapings of cryopreserved stock in 5 ml Ty medium (0.5% tryptone, 0.3% yeast extract, 0.05% dihydro calcium chloride and 1.5% bacto-agar) plus 50 mg/l kanamycin monosulphate at 30°C with agitation for 2 days. Log phase cultures were initiated by diluting 1 ml of the Agrobacterium cultures into 50 ml Ty medium plus kanamycin monosulphate 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 days. The leaves floated for 10 min in 25 ml Agrobacterium solution and returned to the callus induction medium for 4 days.

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

PCR detection of the cry3Aa transgene: DNA from regenerated plants was amplified with cry3Aa specific primers (forward 5GAC TGC TGA TAA CAA CAC GCy; reverse 5ATG TAG ACC TTA TCT CCG GCy) resulting in a 1761 bp fragment of the 1794 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. Southern analysis to detect transgene copy number: Total genomic DNA extracted from regenerated plants was restricted with Promega restriction enzymes BanHI or XhoI, electrophoretically separated and transferred to a charged nylon membrane using alkaline capillary transfer. The nylon membrane was hybridized overnight with a P32 labelled 440 bp fragment of the cry3Aa gene (prepared by nick translation), washed and the hybridization signal was visualized using a Storm® gel (Amersham Biosciences, Piscataway, NJ, USA) and blot imaging system (Molecular Dynamics, Inc.).

Establishment of transformants ex vitro and 4x–2x hybridization: In vitro plantlets of seven transgenic lines (TC1–TC7) were acclimated to the greenhouse. 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. (1998). DNA content in nuclei, relative to standard controls, was determined using a Coulter Epics XL Flow cytometer (Coulter International Corp., Miami, FL, USA).

Double antibody sandwich–enzyme-linked immunosorbent assay (DAS–ELISA) to detect Cry3Aa of primary transformants: A DAS–ELISA test for Bt-Cry3A endotoxin from Agdia (Elkhart, ID, USA) was used to detect Cry3Aa expression levels of the seven transformants established in the greenhouse. Cry3Aa expression was measured by the absorbance of each sample after the addition of p-nitrophenyl phosphate using an Emax™ precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at 405 nm. Absorbance ratios were calculated by dividing the absorbance of each transgenic sample by the absorbance 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 of primary transformants: 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 a control were incubated in Petri dishes (9 cm in diameter) lined with moistened filter paper. There were four dishes (each dish is 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 mm2) was calculated prior to, and after, beetle feeding for 24 h. The average leaf area consumed per beetle was analysed by SAS general linear models (GLM) procedure (SAS 1985) and genotypes were grouped by a Ryan–Einot–Gabriel–Welsh Multiple Range Test.

Cry3Aa expression of tetraploid progeny: Replicated analysis of Cry3Aa expression was conducted with 25 4x–2x progeny derived from crosses between ‘Atlantic’ and a single-insert transgenic plant (TC1) and 25 progeny derived from crosses between ‘Atlantic’ and a multiple-insert plant (TC2). Seeds were germinated and propagated...
in vivo on MS basal plus kanamycin monosulphate (50 mg/l) and three copies 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 extraction buffer) whereas the TC2 progeny were ground at a 1 : 5 dilution. Absorbance ratios of progeny and parental genotypes were analysed by SAS GLM and grouped by a Ryan–Einot–Gabriel–Welsh Multiple Range Test.

Gene-centromere mapping of a single-insert transformant: Additional seedlings of Atlantic × 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 leaf discs (TC1–TC7) between 68 and 112 days after inoculation. TC1, TC3, TC6 and TC7 regenerated from separate leaf discs while TC2, TC4 and TC5 regenerated from the same disc. All lines showed the expected 1761 bp fragment when examined by PCR, verifying that they were transgenic (Fig. 2). Southern analysis of five lines (TC1, TC2, TC4, TC5, and TC7) after XbaI or BamHI 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 4x–2x 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 4x–2x hybridization with cv. Atlantic: TC1: 89% fruit set, average 145 seeds/fruit; TC2: 67% fruit set, average 141 seeds/fruit.

Ploidy analysis of TC1–TC7 and 4x–2x hybrids

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

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

The seven transgenic lines were separated into two groups based on Cry3Aa expression estimated by DAS–ELISA (Fig. 4). Four lines (TC1, TC3, TC6 and TC7) were ‘high expressers’ with absorbance ratios ranging from 24 to 30 whereas three lines (TC2, TC5 and TC6) were ‘low expressers’ with absorbance ratios ranging from 1 to 5. A dilution series of purified Cry3Aa protein showed that one of the high expressers, TC1, produced 230 μg Cry3Aa/g leaf tissue in the
Welsh Multiple Range Test and significant groupings are indicated with an average of 262 mm² consumed after 24 h. The four genotypes were separated into three statistically distinct groups based on ANOVA of leaf area consumed by Colorado potato beetle first-generation adults (Fig. 5). The two non-transgenic genotypes, ‘Atlantic’ and APM-2, supported the highest leaf feeding with an average of 473 mm² consumed after 24 h. The three ‘low expressers’ had partially reduced feeding with an average of 262 mm² consumed after 24 h. The four ‘high expressers’ had the least amount of feeding after 24 h, an average of 87 mm², and this value was not significantly different from 79 mm² 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 4x–2x progeny derived from TC1 (single-insert parent) and TC2 (multiple-insert parent)

After in vitro selection on kanamycin and propagation in the greenhouse, none of the 25 tetraploid progeny derived from TC1 had significantly lower absorbance ratios than the parent plant, indicating that a raise in ploidy (diploid to tetraploid) did not affect expression in this small population of single-insert cry3Aa transgenics (Table 1: the single plant in the 11–20 AR range was not significantly lower than the parent). However, 17 of 25 similarly treated tetraploid progeny (68%) derived from TC2 had significantly lower absorbance ratios than TC2 itself (Table 1). Of these 17, eight (32%) had absorbance ratios lower than 2.5, indicating near complete silencing of the multiple cry3Aa transgenes (<0.1 µg Cry3Aa/g 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 the 283 tetraploid progeny examined qualitatively for the presence/absence of Cry3Aa expression, 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 non-transgenic 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, we can map the single-insert locus of TC1 at 26 cM from the centromere: $2 \times 0.13 \times 100 = 26$ cM.

Table 1: Number of genotypes within ranges of DAS–ELISA absorbance ratios for Cry3Aa expression of 25 4x–2x progeny from S. tuberosum cv. Atlantic crossed to both a single-insert (TC1, 230 µg/g leaf tissue) and a multiple-insert (TC2, 4 µg/g leaf tissue) 2n pollen-producing transgenic hybrid. The average absorbance reading of each of the 50 genotypes was based on three replications and each progeny classified into broad ranges depending on the mean. The samples were read after the addition of p-nitrophenyl phosphate 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 (0.1 dilution) by the absorbance of the non-transgenic control APM-2 (0.1 dilution) by the absorbance of the non-transgenic control APM-2 (0.1 dilution). The results (10 genotypes × six replications × four experimental units = 240 observations) were analyzed by a Ryan–Einot–Gabriel–Welsch Multiple Range Test and significant groupings are indicated by letters within the genotype bars.
**Discussion**

Gene silencing of both native and foreign genes can occur transcriptionally or post-transcriptionally (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 (Neuhuber et al., 1994, Demeke et al. 1999). 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 our multiple-insert plants (TC2, TC4 and TC5) have reduced Cry3Aa expression relative to the single-insert plants (4 vs. 230 μg Cry3Aa protein per g 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 4x–2x hybridization of the single-insert parent (TC1) to cv. Atlantic showed stable, high expression of the cry3Aa transgene (Table 1) whereas the majority of similarly derived 4x–2x hybrids from the multiple-insert parent (TC2) showed even greater transgene silencing than that observed in TC2 (Table 1). Polyploidization may have been responsible for this increase in silencing of cry3Aa between diploid TC2 and its tetraploid 4x–2x 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, we 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 B. thuringiensis transgene (hemizygous transgenic gametes) during sexual polyploidization in crosses to cv. Atlantic (4x–2x 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 sexual polyploidization. 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 we observed in 4x–2x populations derived from TC2.

High expression of exogenous transgenes, such as bacterial genes encoding insecticidal or herbicide-resistant proteins, in a TPS hybrid production system for potato is possible provided that a single-insert diploid (diploidand) parent is used. All of our high-expressing lines (as determined by ELISA, Fig. 4) showed similar resistance to feeding by the Colorado potato beetle as cv. NewLeaf Atlantic (Fig. 5), and one of those lines, TC1, transmitted equally high Cry3Aa expression to potato hybrids (Table 1). The high negative correlation between results of ELISA testing of 6-week-old greenhouse seedlings and the leaf-feeding bioassay of 11-week-old field grown plants demonstrate that a simple laboratory analysis of greenhouse grown leaf material can be used to identify high-expressing diploid parents for use in 4x–2x hybridization. This correlation also indicates that relative expression levels of Cry3Aa do not differ between immature and mature potato plants, and that the Cry3Aa protein is stable in potato plants throughout development. A similar result has been observed for other transgenic proteins in potato, such as bean chitinase, while others such as snowdrop lectin show little expression correlation between immature and mature plants (Down et al. 2001).

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. A 20% refuge of non-Bt potato has been recommended for planting alongside transgenic potato fields. These structured blocks of non-transgenic potato allow for mating between susceptible and resistant insects, thus mitigating the build-up of homozygous resistant individuals (Hoy 1999). The suggestion of incorporating Bt transgenes into TPS hybrids, that are expected to be commercially grown primarily where planting of special refuge areas may not be practical, could 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 (non-transgenic gametes). Upon examination of 283 progeny derived from one of our single-insert plants, we found that 13% were non-transgenic and thus the transgene was mapped at 26 cm from the centromere. An examination of larger numbers of single-insert transgensics would probably identify a line producing non-transgenic progeny at any desired frequency.

For every non-transgenic gamete that is produced by a diploid with 2n pollen production by FDR, a corresponding homozygous transgenic (duplex) gamete should also be produced. Thus, we expect that 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 our sample of tetraploid progeny as no significant differences in expression among 25 progeny that had been selected on kanamycin were found (Table 1). We did not expect nulliplex among these 25 progeny because selection on kanamycin would have eliminated them. With regard to gene dosage, our results do not support those of Beaujean et al. (1998) who found a positive correlation between transgene dose and expression in transgenic tobacco. In conclusion, our results offer a new perspective regarding interaction between ploidy and transgene expression in plants. Unlike Scheid et al. (1996) we did not observe transgene silencing upon ploidy elevation with a single-insert transgene. We did, however, observe substantial transgene silencing when a genome possessing multiple transgenes underwent ploidy elevation. The multiple transgene silencing that we observed...
may have resulted from gene silencing mechanisms that facilitate the evolution of polyploid plant species.

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