Inheritance and expression of *Cry3Aa* and PVY-O coat protein transgenes in diploid and tetraploid potato

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Abstract

The potential benefits of plant genetic engineering for disease and pest resistance have been widely acknowledged in many studies, and although genetically modified crops are still encountering public wariness, these benefits warrant continued exploration. Because of its intrinsic economic benefits, the development of true potato seed (TPS) cropping systems has been instituted in many regions of the world. The incorporation of transgenic resistance could further the economic gain of farmers who are seeking ways to sustain their livelihood in the most efficient way possible. It is, however, largely unresearched how sexual hybridization of a transgenic crop would affect the behavior of a transgene in the resultant progeny. In the initial part of this study, transgenic lines were developed with a *Cry3Aa* transgene. These plants were then used in 4x-4x reciprocal crosses and 4x-2x hybridization schemes to determine the stability of the transgene after sexual hybridization. There was no observed parent of origin effect on transgene expression; however, a highly significant, non-mendelian inheritance of the *Cry3Aa* transgene was seen in the maternally inherited transgene of one set of progeny from a reciprocal cross. Additional transgenic lines of potato were developed with a PVY-O coat protein transgene. These plants were challenged with PVY-O and monitored for symptoms visually and for virus serologically. One transgenic line exhibited complete resistance to PVY-O while two others showed a delay in symptom occurrence. Further

examination of the expression levels of the PVY-O coat protein transgene will be necessary to determine the type and usefulness of the observed resistance.

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Introduction

Recent success in the use of transgenes to promote resistance to plant pathogens and insect pests has created new opportunities for crop improvement. Furthermore, possible benefits (low cost, hybrid vigor, and virus-free propagative material, for example) of using true potato seed (TPS) raise an increased interest in the possible applications of sexually propagated potato cultivars. Plant transformation, a method of crop improvement that was developed in the 1980s involves the transfer of a gene, or segment of DNA, from one organism into another's genome. Among other things, it can be used to create resistance to various plant pathogens and insect pests. The Cry3Aa protein endotoxin from the bacterium *Bacillus thuringiensis*, is harmless to humans, animals, and most insects (Whiteley and Schnepf, 1986). Its toxicity mainly targets members of the insect order Coleoptera, a large taxonomic group including beetles. When expressed in plant tissue, it creates a constitutive insecticide, protecting the plant from herbivory. Pathogen-derived resistance, as it pertains to viruses, is a system used to prevent or inhibit infection and proliferation of virus in a plant by transforming the plant with a small segment of the viral genome. It is thought that the presence of the viral genome in the transformed cell initiates the formation of small interfering RNAs, which disrupt the replication cycle of the virus. The first reported instance of this type of resistance was coat protein mediated, using a tobacco mosaic virus coat protein transgene construct (Powell et al., 1986). Coat protein mediated resistance has since been observed in many

host species including tomato (Sanders et al., 1992), cucumber (Gonsalves et al., 1992), rice (Hayakawa et al., 1992), and potato (Kaniewski et al., 1990 and Kawchuk et al., 1990), to name a few. With 58.7 million hectares of genetically modified crops planted in 2002, transgenic resistance has become a common and realistic means of combating the problems imposed by plant pests. Furthermore, out of six million farmers in 16 countries who use genetically modified crops, five million were small resource poor farmers who rely on the intrinsic benefits of transgenic plants (James, 2003). The incorporation of sexually propagated cropping system for potato could possibly further the economic benefit to this sector of the agricultural community.

True potato seed (TPS)

Although the potato has traditionally been vegetatively propagated, the potential benefits of incorporating true potato seed (TPS) into production methods could be realized. Because of the ease with which true seed can be stored and shipped, the need for elaborate refrigerated storage facilities required for preservation of potato seed tubers can be eliminated. A few hundred grams of TPS is roughly equivalent to twenty 100 kg bags of seed tubers; the amount of propagative material needed to plant one hectare (Chilver et al., 1999). Furthermore, the high potential for plant pathogens to persist from season to season within a vegetatively propagated crop creates a situation in which the use of certified disease-free seed tubers is a requirement to be met by growers. This, however, results in an increased cost which hinders many potato growers who, because of economic or geographic reasons, are not able to obtain certified seed stock. Most plant

viruses are not transmitted through gametes, and thus, virus-free seed can be produced even if the parents are infested with virus. Additionally, seed can be disinfested to eliminate bacterial or fungal pathogens that may reside on the seed coat. The elimination of the certification step through which seed tubers must pass could provide a great economical advantage to smaller growers, such as those in developing countries.

There are several techniques in which TPS can be incorporated into potato production (Pallais, 1991). First, seed is sown in a protected area, such as a greenhouse or glasshouse, and then transplanted into the field after germination. In a second method, TPS seedlings are planted in a field nursery at high density. Small tubers are then harvested and stored and used in the following season as potato seed. The third method, a variation of the second, involves sowing TPS in seed beds with screens to protect the plants from virus vectors and other pathogens. The small tubers are then harvested and planted for the following season's crop. Although the final two methods result in the grower planting seed tubers, the initial cost of purchasing certified potato seed tubers is avoided. A major drawback for TPS is a lack of uniformity in offspring (resulting in poor or inconsistent tuber yield) and inbreeding depression. Inbreeding depression can be overcome through the incorporation of more diverse parental germplasm, but this heightened level of heterozygosity would seem to contribute to the lack of uniformity in the resultant sexual hybrids. A method used to increase hybrid vigor in sexual hybrids is to cross cultivated *S. tuberosum* plants (4x) with extracted dihaploids (2x) or another 2x potato species with a high tendency to produce *2n* pollen. These unreduced pollen and egg cells may result from either first division restitution (FDR) or second division

restitution (SDR). Unreduced gametes resulting from FDR maintain much of the heterozygosity present in the sporophyte and their use in breeding schemes has shown positive results as far as plant yield (Werner and Peloquin, 1991). Additionally, Golmirzaie and Ortiz (2002) have shown that through a simple selection process in which only the more vigorous seedlings are transplanted, any inbreeding depression that may exist can be largely avoided. Although TPS production systems viewed as an economic, pathogen-free means of planting a crop, the incorporation of transgenic resistance to plant pathogens and pests into TPS schemes, which could further reduce the overall cost to farmers, has not been thoroughly researched. It is therefore important to consider the pressures encountered by a transgene which affect its performance.

Variable transgene expression

If transgenic resistance is to be used in conjunction with TPS production it is important to understand the role that sexual hybridization plays in transgene inheritance and expression. In a heterozygous system such as cultivated potato, there are several epigenetic factors that could affect transgene expression. The progressive study of transgenic plants has revealed many instances of dynamic expression levels of a transgene in different lines of plants transformed with identical gene constructs. This is largely due to the randomness with which a transgene construct inserts into the host genome, which creates a "position effect" on transgene expression (Peach and Velten, 1991). A spatial effect on transgene expression seems likely when the variable chromatin events which occur during the cell cycle are considered; however, clonal lines originating

from the same individual through tissue culture have been found to exhibit varying levels of transgenic protein expression as well (Down et al., 2001). With significant expression variability such as this occurring in a clonally propagated line of plants, there would seem to be other factors above and beyond the position effect in operation. These epigenetic factors can alter a (trans)gene's expression without changing its sequence or location, a phenomenon commonly referred to as gene silencing.

Gene silencing

Gene silencing can occur to inhibit transcription (transcriptional gene silencing), as in the methylation of a promoter region (Li et al. 2001), or post-transcriptionally, using a sequence-specific RNA degradation system initiated by the presence of dsRNA in the cell (Waterhouse et al. 2001). Transcriptional gene silencing often occurs as a result of multiple gene copies, or repetitive sequences, and is usually associated with hypermethylation of a promoter region (Matzke and Matzke, 1993). On the other hand, post-transcriptional gene silencing seems to target homologous RNA sequences caused by aberrations in exogenous RNA (Napoli, et al. 1990), transgenes (Elmayan and Vaucheret, 1996), and RNA viruses (English, et al., 1996). Waterhouse et al. (2001) proposed that the formation of dsRNA initiates a defense response in the plant cell, in which the dsRNA is cleaved, and then used as a template for further degradation of homologous ssRNA. This could explain the process through which viral pathogen-derived resistance occurs. Regardless of the method of silencing, the insertion of more than one copy of a transgene during transformation, the inheritance of more than one

transgene copy, or the presence of an invading pathogen whose genome contains a similar gene sequence could reduce, or eliminate expression of the gene altogether. Either of the two forms of silencing could affect transgene expression in successive generations as transcriptional gene silencing is both mitotically and meiotically heritable, while post-transcriptional gene silencing is meiotically reversible (Fagard and Vaucheret, 2000).

Genomic imprinting

A unique manifestation of gene silencing occurs in a phenomenon known as genomic (or gametic) imprinting, where the activity of a gene is reversibly modified depending on the sex of the parent from which it was inherited (Alleman and Doctor, 2000). This example of non-Mendelian genetics can result in a recessive allele being expressed over a silenced dominant one. The first such case was observed in the endosperm of corn, involving the *R-r:std* allele, which results in a pigmented aleurone when the allele is inherited maternally, and a colorless one from a paternally inherited allele. Kermicle (1978) discovered that this was not a result of a dosage effect in the endosperm, but rather was caused by a difference in expression within the maternal and paternal genotypes at the specific locus. In many cases there is a strong maternal influence on phenotype observed in offspring. Luo et al. (2000) have also shown, through the use of reciprocal crosses with two GUS fusion constructs that some paternally inherited genes are not expressed during early seed development in Arabidopsis, but are sometimes turned on as the seed matures. Such a result can be caused by the presence of a maternal gene product in the

egg cell, haplo-insufficiency in the endosperm, or the locus is affected by genomic imprinting (Grossniklaus, et al., 1998).

Segregation Distortion

There are also examples of non-Mendelian segregation in which gene silencing has not been observed. This segregation distortion can be attributed to an unexpected selection process at some stage of plant development. These events would most likely occur early in the plant's life cycle, beginning with meiosis and the formation of gametophytes. An additional opportunity for preferential gene selection occurs during pollination and fertilization. Through a phenomenon known as linkage disequilibrium, certain genes are inherited together more or less frequently than statistically expected. It is then feasible that a transgene could become associated with a group of linked genes due to its point of insertion and be affected by this event. This would be facilitated if the associated genes affect microspore function, such as pollen tube growth or compatibility. In a study involving their own and previously published data, Zamir and Tadmor (1986) observed significant deviations from expected Mendelian segregation ratios in randomly selected genes from several genera, escalating in occurrence as the amount of heterozygosity between parents increase. It is possible that events occurring during seed formation and germination could also have an effect on segregation; however, it would seem to be less of a possibility as the plant develops further from the abovementioned phases of its life cycle.

Effect of ploidy change on transgene expression

Expression of (trans)genes can be influenced by many factors; including but not limited to cis-elements, intrinsic qualities of the gene itself which only change through rare irreversible mutations, trans-acting regulator factors which act in response to some external stimuli to reversibly alter gene expression, and epigenetic factors such as gene silencing which stably change gene expression, but are more common than mutations (Scheid et al., 1996). As previously mentioned, gene silencing is thought to be initiated by multiple copies of a gene, or an increased gene load. An increase in transgene copy number due to polyploidization could be a possible trigger for a silencing event. In a study involving monoploid, diploid, and tetraploid maize, the translational levels of some proteins was inversely proportional to the chromosome copy number (Birchler and Newton, 1981). In many plants polyploidization can occur through 4x-2x crosses, involving a pollen donor with a high tendency to produce 2n pollen. In potato, this type of pollination most often results in tetraploid progeny. Johnson et al. (2003) found that 4x-2x progeny resulting from a transgenic diploid pollinator incurred far less transgene silencing in lines derived from a single-insert diploid pollinator rather than progeny produced from a multiple-insert pollinator. As 4x-2x hybridization is a commonly used technique to produce TPS hybrids, it is important to consider its possible implications on transgene expression.

Summary

This paper examines the potential of transgenic potato lines in different breeding strategies. The *Cry3Aa* and *PVYcp* transgenes have been shown to provide resistance to insect herbivory and virus pathogenicity, respectively, and thus potentially have widespread economic value. The objectives of this study were to: (1) compare *Cry3Aa* expression levels in sexual hybrids of potato; (2) examine aberrations in transgene segregation ratios, and (3) determine the effectiveness of coat protein-mediated resistance in several lines of transgenic potato.

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CHAPTER 2:
Expression and inheritance of transgenic *Cry3Aa* in sexual hybrids of potato

Abstract

Transgenic resistance to plant pathogens and insect pests, although temporarily on hold for potato because of political reasons, offers promise for the future of both asexually and sexually propagated potato. For a system that exploits both true potato seed (TPS) and transgenic resistance, factors that affect transgene expression during and after gametogenesis must be understood. Gene silencing is an epigenetic phenomenon affecting transgene expression that may be influenced by the parent-of-origin of a transgene. Using PCR to determine transgene segregation, and ELISA to determine transgene expression in reciprocal crosses of tetraploid potato lines, one of which carried the *Cry3Aa* gene as a single insert, we found that expression levels of progeny bearing the transgene did not differ. However, in crosses between transgenic Atlantic and wild type Katahdin plants, the ratio of expressers to nonexpressers varied significantly between reciprocals and differed significantly from the expected 1:1 segregation ratio ($n=70$, $\chi^2=8.2$). A lack of variation in transgene expression indicated that there was no parent of origin effect in the reciprocal progeny tested. An excess of transgenic progeny when the *Cry3Aa* gene was maternally inherited suggests differential gametic selection or an epigenetic effect on transgene expression during megagametogenesis and/or pollination and fertilization.

Introduction

As the world's population rises, the pressures on current agriculture production systems to meet the needs of their consumers using available cultivated land will become increasingly challenging. The most obvious means to counteract this potential problem is to concentrate efforts in yield increase. This, of course, has always been a goal of the agricultural community whether through selective breeding and pesticide development or more recently, the direct genetic manipulation of a plant. The traditional process of introducing a resistance gene or genes involves first identifying the source of resistance, and then attempting to introduce that source into the desired plant line. This requires a lengthy breeding scheme that involves many generations, and is limited to sexually compatible germplasm. A process which inserts a segment of DNA directly into the plant genome, such as transformation, would bypass many of the time consuming steps involved with classical plant breeding. This, along with a growing public wariness towards chemical pesticides, has opened the door for transgenic resistance to combat the economic losses caused by pressure from insect pests.

Insect control proteins

Soon after successful plant transformation was reported by Horsch et al. (1984), efforts began to use this technique to develop plant lines with genetic resistance mechanisms to insect herbivory. Many of the proteins with putative insect control qualities that have been targeted for use in transformation originate in the plant kingdom. Throughout

evolution plants have evolved natural defense mechanisms to deal with the problems that phytophagous insects present, and the genes that are responsible for these defenses have become targets for transformation into susceptible plants. One class of insect control proteins are protease inhibitors, which restrict the insect's ability to feed through the inhibition of protein digestion. A study involving tobacco transformed with a cowpea trypsin inhibitor showed increased mortality and reduced plant damage from corn earworm (*Helicoverpa zea*) in transgenic plants compared to untransformed control lines (Hoffmann et al., 1992). Similarly, a group of proteins that impede the insect's carbohydrate metabolism known as alpha-amylase inhibitors have been involved in several studies (Carbonero et al., 1993; Morton et al., 2000). Many of these studies also report an increased protection against insect feeding in plants expressing these proteins. The abovementioned types of proteins are but a few of many types of natural plant genes that have been transformed into susceptible plant populations; however, genes from other organisms have also been looked at as possible sources of insect resistance in transgenic plant lines. Many of these studies involve the *Cry* gene from the bacterium *Bacillus thuringiensis*.

Bt and the Cry *gene*

The organism *Bacillus thuringiensis* (Bt), is a Gram-positive, endospore-forming bacteria that produces a crystalline protein with insecticidal properties. It has been isolated from soil, insects, stored-product dust, and phylloplane surfaces (Schnepf et al., 1998). Because of its ubiquitous nature, its ecological niche has been under some debate;

however, its possible role as an entomopathogen is what has led it into the field of biotechnology. The bacterium's ability to produce proteins that are harmful to insects has led to the cloning of the responsible genes and subsequent transformation of susceptible plants. This insect toxicity can come from various gene products, most notably the *Cry* gene. The proteins produced from these genes, also known as a δ-endotoxins, have shown significant diversity among different strains of *B. thuringiensis*. There are currently over 350 reported *Cry* genes with new accessions frequently added to the list (Crickmore et al., 2002). This level of diversity is largely attributed to the nature and location of the *Cry* gene in the bacterium's genome. The *Cry* gene is commonly found on bacterial plasmids which could facilitate gene transfer between different strains that come into contact with each other. Additionally, Lereclus et al. (1992) report that many *Cry* genes are flanked by repetitive sequences that are actually insertion sequences for transposable elements. Evolutionarily speaking, the combination of these two factors would seem to make *Cry* an extremely adaptable gene.

Toxicology of the Cry toxin

The mode of action of the *B. thuringiensis* Cry toxin begins with the ingestion by a susceptible host insect. The crystalline protein is then solubilized in the insect midgut, where it then undergoes a recognition interaction with receptors in the epithelial wall of the midgut. In a susceptible insect the toxin is then inserted directly into the apical membrane where it creates ion channels or pores (Schnepf et al., 1998). This eventually leads to cell lysis in the wall of the insect midgut. The severity of response to this

pathway is largely dependent then on the specific strain, or species, of insect which could affect feeding behavior, solubility of the protein in the midgut, as well as availability of suitable receptors.

Applications of the Cry *gene in transgenic plants*

To date, many plants have been transformed with *Cry* genes and shown resistance to insect feeding. Chinese japonica rice (*Oryzo sativa* L.) expressing a synthetic *Cry1Ab* transgene showed significant resistance to feeding by the stripe stem borer (*Chilo suppressalis*) in a laboratory study by Wang et al. (2002). Leaf tissue consumption was significantly reduced in transgenic lines, with larval mortality as high as 100% in some instances. Similar results have been seen with resistance to insect feeding in *Cry* expressing transgenic eggplant (Arpaia et al., 1997), broccoli (Cao et al., 2002), and potato (Nault, 2001). Successes such as these with regard to insect feeding resistance have led to the development and release of a number of commercial transgenic plant lines. The most prevalent types of commercial Bt crops are currently corn and cotton with many commercial lines available. Bt crops are becoming increasingly popular for their intrinsic insecticidal qualities. In a study by Reed et al. (2001), insect resistance of a commercially available transgenic potato cultivar (Newleaf®, expressing the Cry3Aa protein) was compared to traditional control regimes involving chemical pesticide applications. They found that transgenic resistance in Newleaf® potatoes was more effective in controlling Colorado potato beetle populations and reducing the impact of insect feeding than conventional insecticide applications. Although the 'Newleaf®'

cultivars were removed from distribution in 2001, largely because of consumer wariness of genetically modified food products their ability to reduce yield loss to insect feeding was proven. Despite the successes in developing insect resistance mentioned above, little effort has been made to determine any usefulness of transgenic lines in a true potato seed production system. If the potential benefits of these two cropping schemes are to be attained concurrently, additional research into the effects of sexual propagation on transgene expression needs to occur.

Summary

This chapter will examine the activity of the *B. thuringiensis Cr3Aay* gene in sexual hybrids of potato. In order for a commercial production scheme incorporating transgenics and true potato seed to be implemented it is necessary to look at the effects sexual hybridization has on transgene performance. In many previous studies involving the Cry toxin, a significant reduction of damage caused by insect feeding has been observed in clonally propagated transgenic lines. It is only after the *Cry* gene's behavior has been significantly evaluated in sexual hybrids that efforts can be made to match the accomplishments in insect resistance seen in vegetativley propagated lines.

Materials and Methods

Plant material

Transformed potato (*Solanum tuberosum* L.) lines containing a codon modified *Bacillus thuringiensis tenebrionis Cry3Aa* gene (GenBank GI# 208152) from the pSPUD8 plasmid (Figure 1) have been obtained through the previous work of Alexander Johnson, a former PhD student in the Virginia Tech Department of Horticulture. The pSPUD8 plasmid was developed and contributed by Dr. David Douches (Michigan State University, E. Lansing). *Agrobacterium*-mediated transformation was used to deliver the constructs, following the procedure outlined by Horsch et al. (1988). In brief, *in vitro* plant material was inoculated with a pSPUD8 containing *Agrobacterium* solution and regenerated plantlets were transferred to a kanamycin monosulphate selective media. Reciprocal crosses were done between the primary transformant lines and untransformed cultivars to produce the following hybrids (where TC1 following the cultivar name designates transformation with *Cry3Aa*); Atlantic × Atlantic TC1/Atlantic TC1 × Atlantic; Katahdin × Atlantic TC1/Atlantic TC1 × Katahdin. Additional hybrids of Atlantic TC1 × APM-2 TC1, Atlantic TC1 × wt APM-2, and self-pollinated Atlantic TC1 were also investigated. APM-2 is a diploid line derived from pollination of dihaploid *Solanum andigena* by an F_1 hybrid of *S. phureja* × *S. microdontum*. It produces *2n* pollen through first division restitution (FDR) and has been used in crosses with tetraploid cultivars to produce highly heterozygous 4x-2x hybrids to avoid the inbreeding

depression commonly seen in 4x-4x crosses. 'Atlantic' and 'Katahdin' are commercially grown tetraploid cultivars used for processing and table stock, respectively. Each of the reciprocal crosses as well as the self-pollinated transgenic line is a 4x-4x hybridization and involves no ploidy change. Atlantic TC1 × APM-2 TC1 and Atlantic TC1 × wt APM-2 are 4x-2x crosses involving unreduced microspores from APM-2. The parent plants used for hybridization were grown from stored tubers collected from greenhouse acclimatized T_0 plants. When flowering began, pollen was collected from each male parent and used to hand pollinate the emasculated female parent prior to anthesis.

Experimental design

Progeny from the above mentioned pollinations were planted in four trials. The first two trials were planted during Fall/Winter of 2002/2003 in a completely random design in the greenhouse with 16 plants per hybrid family. The third repetition was a field trial, transplanted to the VPI&SU research farm at Whitethorne in the Summer of 2003, involving 16 plants per family in a randomized complete block design (four plants per plot) with four blocks. The hybrid families involved in each trial are shown in Table 1. A fourth greenhouse trial was examined in the Fall of 2003 to include the remaining seed from hybrid families Atlantic TC1 × Katahdin and Katahdin × Atlantic TC1.

PCR analysis

Screening of hybrid progeny was carried out with a Sigma-Aldrich Extract-N-Amp-Red™ plant PCR kit. A DNA template was obtained through incubation of plant tissue in the included extraction solution, and was then cycled with the Sigma-Aldrich REDTaq™ solution and primers (forward 5'GAG CTG CAA GGC CTT CAA AAC AAT'; reverse 5'TCT AGC ACG GTA AGG GTC ATC TCT') designed to amplify a 440 bp fragment of the codon modified *Cry3Aa* gene. The program consisted of the following cycles; 1 cycle = 4 min 94°C; 40 cycles = 1 min 94°C, 1 min 58°C, 1.5 min 72°C; 1 cycle = 4 min 72°C. The products were then separated on 1% agarose gels at 90V and visualized under UV light after treatment with ethidium bromide.

Chi-square analysis of segregation ratios

Chi-square analysis was used to determine the significance of observed segregation ratios in hybrid lines. Expected segregation ratios are derived from the expectation of Mendelian segregation of a hemizygous transgene and are shown in Table 2.

Screening progeny for transgene expression via DAS-ELISA

Expression levels of transgenic *Cry3Aa* were examined using an Agdia PathoScreen® kit (Agdia, Inc. Elkhart, IN). Plants were sampled at the late seedling stage approximately 3

weeks after germination (Figure 2). Leaf tissue from each of the hybrids was placed in an Agdia mesh sample bag (Catalog # ACC 00930) and ground in extraction buffer for 1 min, after which, the tissue was fully macerated. Additional extraction buffer was then added to bring the extract concentration to 1 g leaf tissue : 100 ml extraction buffer. Diluted extract from each sample was then loaded into individual wells of a 96-well test plate coated with Cry3A toxin antibodies and incubated overnight at 4º C. Secondary Cry3A toxin antibodies linked to alkaline phosphatase were then added trapping any toxin present in the sample between the primary and secondary antibodies. After overnight incubation, a wash step was performed to remove any un-bound, enzyme-linked secondary antibodies, and p-nitrophenyl phosphate was added. Any remaining alkaline phosphatase in the well (i.e., that bound to the primary antibody/Cry toxin/secondary antibody complex) would convert p-nitrophenyl to p-nitrophenol which results in the development of a yellow color in the well. This assay is quantitative, and was then measured in an Emax™ precision microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 405 nm after 30 minutes of incubation. Results are expressed as optical density (OD), the values of which were used for statistical analysis within plant lines and trials. Data from trial four were compared to standards of known Cry protein concentration which were included in the assay in an attempt to quantify expression levels in the plant tissue.

Field analysis of hybrid lines

Seed from five hybrid transgenic lines (Katahdin × Atlantic TC1, Atlantic TC1 × Katahdin, Atlantic TC1 × APM-2 TC1, Atlantic TC1 × wt APM-2, and a selfed Atlantic TC1 line) were collected from available fruit in the fall of 2002. In the following spring they were sown in the greenhouse and transplanted into cell packs after germination. The seedlings were not transplanted to the field at Kentland Farm in Blacksburg, Virginia until June, 2003 due to an unusually rainy spring. In addition to the seedlings, tubers of Atlantic, Katahdin, and Atlantic TC1 were planted as controls. Sixteen plants from each line were planted in a randomized complete block design with four replications of four plants per plot. The plants were then harvested in September, 2003 and tuber number per plant and tuber weight per plant were then determined.

Results

PCR with Cry-specific primers

PCR performed with primers designed to amplify a 440-bp segment of the codon-modified *Cry3Aa* gene successfully amplified the *Cry3*Aa transgene in seedlings (Figure 3). The plants from three of the four hybrid families in Trial 1 showed no deviation from the expected 1:1 Mendelian segregation ratio. However, plants from the hybridization Atlantic TC1 X Katahdin exhibited a much higher tendency to inherit the *Cry3Aa*

transgene than other families (Table 3). A ratio exceeding 4:1 transgenic:nontransgenic was seen in that instance. Although there were no significant departures from the expected ratios in trial two, the ratio of transgenic:nontransgenic progeny was still skewed towards more transgenic offspring in the Atlantic TC1 X Katahdin family (Table 4). In trial three, Atlantic TC1 X Katahdin progeny again displayed a significantly greater number of transgenic offspring than expected whereas the other families did not (Table 5). None of the families in trial four showed a significant difference from the expected ratios (Table 6). However, the pooled data from all four trials show that the segregation ratio observed in the Atlantic TC1 X Katahdin hybrid family exhibited a highly significant deviation from the expected 1:1 ratio of transgenics:nontransgenics (Table 7).

DAS-ELISA on sexually propagated progeny

To confirm PCR results, as well as determine whether the *Cry3Aa* transgene was expressed in the sexual hybrids, we performed DAS-ELISA to examine Cry3Aa protein accumulation in leaves from *Cry3Aa* positive progeny. There was no significant difference in Cry3Aa transgene expression levels observed in TPS offspring produced from reciprocal crosses or among hybrid families within each trial. However, since a strong trial effect was observed from optical density values (Table 8), each trial was examined independently. Trial one, which took place in a greenhouse, showed no significant difference in transgene expression in *Cry3Aa* positive individuals, with mean OD values ranging from 3.30 to 3.48 (Table 9). These values are similar to the OD of

3.40 seen in the transgenic parental control, Atlantic TC1, which was included in the assay. The comparison between reciprocal crosses Atlantic × Atlantic TC1/Atlantic TC1 × Atlantic showed no significant differences in Cry expression. Because of obvious inbreeding depression (stunted plants, with poor growth), we excluded these lines from further trials. Greenhouse trial two showed a similar trend, with statistically similar expression levels in the observed hybrid families (Table 10). The OD values observed in trial two were significantly lower than those of trial one, ranging from 2.58 to 3.17. The mean OD values for trial three were also significantly lower than those of trial one; in addition, *Cry* expression varied significantly among families (Table 11). Progeny of the Atlantic TC1 × wt APM-2 cross exhibited decreased Cry expression compared to the reciprocal hybrids of Atlantic TC1 and Katahdin. Trial four, which involved the remaining available seed from each reciprocal cross also failed to display any significant variation among families included in the trial (Table 12). The inclusion of positive controls of known Cry protein concentration allowed a standard curve to be calculated to which sample values were compared. Cry protein concentration levels in leaf tissue were not significant by different families and were in a range of 2.24 to 2.55 µg Cry per g leaf tissue (Table 13) in trial 4.

Yield analysis of hybrid lines

Statistical analysis of field data revealed significant variation for yield per plant, tubers per plant, and average tuber weight. ANOVA showed a significant family and replication effect for all parameters (Table 14). Agronomic performance with regard to

average tuber weight revealed that cvs. Atlantic and Katahdin produced significantly larger tubers than hybrid families as well as the transformed line Atlantic TC1 (Table 15). In addition, the yield per plant was highest in the two cultivars. Although there was no significant difference between the reciprocal families, a dramatic increase in tuber number per plant was observed in the two hybrid lines containing APM-2 germplasm (Atlantic TC1 × wt APM-2 and Atlantic TC1 × APM-2 TC1) compared to the *S. tuberosum* hybrids and cultivars, which did not seem to be affected by the occurrence of a pollen-borne *Cry* transgene in the Atlantic TC1 × APM-2 TC1 hybrid. Despite the increase in tuber production, the overall yield per plant was not significantly higher than that of the parental line Atlantic TC1.

Discussion

In order to determine whether a parent-of-origin effect was influencing inheritance and expression of the *Cry3Aa* transgene, we examined the progeny from two reciprocal crosses (Atlantic TC1/Atlantic, Atlantic TC1/Katahdin, resulting in four families) that inherited allelic copies of the transgene from Atlantic TC1. Additionally, this same transgenic line was used in three 4x-2x crosses (a common method of producing TPS) for comparison to the reciprocals, as well as in an attempt to validate previous data. One of these three 4x-2x families (Atlantic TC1 × APM-2 TC1) included plants expected to inherit non-allelic copies of the *Cry3Aa* transgene from both parents. Segregation of the transgene in hybrid families was compared to the expected segregation ratios from putative single-insert transgenic lines (Atlantic TC1 and APM-2 TC1), while expression

levels observed through DAS-ELISA were examined for significant differences among families.

Hybrid families generated from crosses involving at least one transgenic parent were examined over the course of four trials in an effort to determine how transgene expression is affected during and after sexual hybridization. Seed was divided into trials in order to accommodate as many plants as could be managed in a single day's analysis. Through PCR analysis, the expected segregation ratios were upheld in four of five families through four trials. However, a highly significant, non-Mendelian segregation ratio of *Cry3Aa* expressers to non-expressers was observed in trials one and three for the Atlantic TC1 × Katahdin family, and confirmed in the pooled data of all four trials. All of the seed had been extracted from the same berry. The Atlantic TC1/ Katahdin reciprocals showed significantly more expressers when the transgene was inherited maternally. In previous crosses as well as in the majority of the hybridizations performed in this study, the progeny of Atlantic TC1 and an untransformed line have resulted in a 1:1 segregation ratio. This had led us to believe that Atlantic TC1 is a hemizygous transgenic line harboring a single copy of the *Cry3Aa* transgene. Segregation distortion in the progeny of a cross involving transgenic parents is not uncommon; however, in most reported cases the observed ratio of expressers to non-expressers has been skewed towards non-expressers (Scott et al., 1998; Wu et al., 2002). This makes the observed 2:1 ratio of transgenic progeny in the Atlantic TC1 × Katahdin line unusual, and raises the possibility that Atlantic TC1 does have multiple insertions of the transgene. However, when the uniquely segregating line was compared to a similar cross in this study where

the same Atlantic TC1 transgenic was pollinated by an untransformed plant (wt APM-2), the phenomenon was not repeated (Figure 4). Furthermore, although the sample population in which Atlantic TC1 was crossed to untransformed Atlantic was relatively small (n=10), the transgene segregated with no significant difference from a 1:1 ratio.

Assuming a single gene insert based on an observed 1:1 segregation ratio in crosses between Atlantic TC1 and other pollinators (Table 7), there would appear to be a reciprocal effect caused by the interaction between Atlantic TC1 and Katahdin gametophytes that affects transmission of the transgene into hybrid progeny. A strong maternal effect seems to have occurred as the transgene was preferentially transmitted through the female gametes. It is well-known that competition among pollen grains occurs, and is a result of speed of pollen tube growth, environmental factors (Johannsson and Stephenson, 1998), as well as the genotypes of pollen donors and recipients (Mangelsdorf and Jones, 1926). However, competition among egg cells is not a widely reported phenomenon. A parent-of-origin effect has been reported to occur in certain plants, which manifests as a dominance of one parent's genetic influence over the other. This usually begins during double fertilization when the two sperm cells fuse with the egg cell and the bi-nucleate central cell. In this study, the pathway which concludes in an increased transmission of the *Cry3Aa* transgene seems to lie in the development of the female gametes.

With only a single exception, PCR positive plants (n=136) exhibited Cry3Aa protein expression as measured by DAS-ELISA, thus confirming the legitimacy of the

segregation ratios. Although there was significant variation in OD values among trials, expression levels for families within the trials, with only one exception, remained relatively constant. This was not unexpected, as transgenic progeny inherited identical copies of the transgene from Atlantic TC1. The one exception to the constant expression levels was seen in trial three (field trial), where Atlantic TC1 × wt APM-2 progeny expressed less Cry3Aa protein than the Atlantic TC1/Katahdin reciprocal hybrid offspring (Table 11). Although a similar trend of lower expression in the Atlantic TC1 × wt APM-2 family was seen in trial four, the difference was not statistically significant. In previous data comparing hybrid families Atlantic TC1 × wt APM-2 and wt Atlantic × APM-2 TC1 Alexander Johnson (2001) found a significant difference in transgene expression between the two families where the paternally inherited transgene from APM-2 TC1 was expressed at much higher levels. Although these two crosses are not true reciprocals it is interesting to see that the transgene was expressed at different levels depending on whether it was inherited through pollen or the egg cell. The theory that this was caused by a parent of origin effect is not supported by data from the reciprocal hybrids presented here. Surprisingly, the steady level of expression seen in most of the families also existed in the hybrid family which had the opportunity to inherit either of two independent copies of the transgene (Atlantic TC1 × APM-2 TC1, Table 10). It was expected that 43.5% of these offspring (37% T_1000/T_20, 6.5% T_1000/T_2T_2) would carry two independent copies of the transgene, subjecting them to possible transgene silencing. However, DAS-ELISA data gave no indication that any silencing had occurred.

There are several proposed factors which may affect the expression of a foreign gene. Dunsmuir et al. (1988) describe a source of variation know as a position effect, in which the expression of an introduced gene is affected by its inserted location in the existing plant genome. Originally, this effect was attributed to the DNA regions surrounding the inserted gene; however, this variability in transgene expression was not reduced when the transgene construct included as much as an 8 kb flanking region around the gene of interest (Dunsmuir et al., 1987). Thus, the factors that cause this type of variation must be able to operate over longer genomic distances. Somaclonal variation has also been attributed to variation in transgene expression levels. The pressures exerted by callus growth and regeneration in tissue culture often alter plant development which could affect the performance of a transgene directly or indirectly as in the methylation of a promoter region. However, since the hybrid lines examined in this study are all F_1 plants derived from a parental primary transformant, neither of these factors would be expected to affect their expression. Most of the hybrid lines involved in this study have inherited the same transgene from the same parent (Atlantic TC1), and thus the most notable source of variation from a plant breeding standpoint would come from meiotic recombination. In a relatively large sample population, the effect of recombination on transgene expression would presumably be insignificant.

When examining the mean expression levels from the individual trials in this study, it seems that the *Cry3Aa* transgene is stably expressed in the hybrid progeny that harbor the transgene. This is not unusual, as stable transgene expression in sexual hybrids has been reported in many instances. In a study examining inheritance and expression of *neo* and

gusA genes in transgenic rice, transgene expression did not significantly differ within hybrid progeny groups of generations T_2 and T_3 (Peng et al., 1995). Another study involving transgenic rice showed that *Cry1Ab* transgene expression was quantitatively stable through six sexual generations (Wu et al., 2002). Fearing et al. (1997) reported that expression of Cry1Ab protein was shown to be stable over four backcross generations in a study examining protein concentrations in both leaves and pollen of transgenic maize. Because of these examples, the uniform protein expression seen in this study is not unique to the crosses involved.

It is, however, unexpected that the four trials would exhibit such a high level of variation when compared to each other. Each of the four trials showed mean OD values that significantly differed from each other. As identical DAS-ELISA kits were used to assay each trial and each sample was ground in the same weight to volume dilution, the variation could be attributed to slight differences in the execution of the kit protocol. Variation caused by slight differences in incubation time/temperature and time to load each well could contribute to OD differences among trials. However, these differences are supposedly minimized by the inclusion of transgenic (Atlantic TC1) and untransformed (Atlantic) controls in each assay. It is interesting to note that trials one, two, and four, from which the highest OD values were recorded, were grown and sampled in the cooler months of November, February, and December while trial three was grown during the summer. Trial three, in which the seedlings were started in the greenhouse and transplanted into the field, exhibited a significantly higher level of variation. This was likely caused by the increased stress of being transferred from

greenhouse conditions into the field, as well as the fluctuating environmental conditions the seedlings encountered as they developed. Hart et al. (1992) observed that seedlings that were transferred to a glasshouse after being raised in controlled conditions exhibited far greater variation in transgene expression than seedlings that had been raised in a glasshouse. Similar results of consistent transgene expression under controlled environmental conditions are reported by Down et al. (2001). Transgene activity in both seedlings and adult plants has been linked to the temperature at which the plants are grown. Neumann et al. (1997) showed that seedlings transformed with kanamycin resistance and luciferase genes expressed their transgenes at a much lower level when grown at 37°C rather than 25°C. Expression at high temperatures was even completely eliminated in some instances. It is likely that the variation observed between trials is a result of unavoidable differences in the assaying technique employed for each trial. However, it is possible that the lower OD seen in trial three was a result of the warmer temperatures to which the plants were exposed. For this to be determined a more extensive time course study monitoring seasonal temperature, solar radiation, and moisture regime to which the plants are exposed would be necessary to investigate an environmental effect on transgenic *Cry3Aa* expression.

During DAS-ELISA analysis of the hybrid progeny included in this study, one plant out of 228 examined was observed to harbor a copy of the *Cry3Aa* transgene that was not expressed. This plant was unexpectedly an Atlantic TC1 × Katahdin hybrid. Transgene silencing is usually seen in multiple-insert transgenic lines; however, it has been reported in single-insert transgenics. In a study involving the selfed progeny of a single insert

transgenic tobacco plant expressing the GUS protein, silencing was observed in offspring that were homozygous and hemizygous for the transgene (Elmayan and Vaucheret, 1996). Because silencing can occur to inhibit transcription, as well as post-transcriptionally, it would require transcript analysis of the plant harboring the silenced transgene to determine at what stage the event is taking place.

Because of its desirable economic benefits and convenience, TPS systems incorporating transgenic resistance to insects could be instituted. Examination of tuber yield in trial three showed, in this case, that the TPS hybrids did not perform as well as the cultivars planted from seed tubers (Table 15). As expected, there was significant inbreeding depression in the Atlantic TC1 self pollinated line. A similar phenomenon occurred in the Atlantic TC1/Katahdin reciprocals, presumably from a lower level of heterozygosity between the two cultivars in the 4x-4x cross. The highest yielding test lines were those coming from 4x-2x crosses, supporting the use of this breeding scheme in TPS production. In order for a transgenic TPS line to be useful, it would have to produce plants with consistent yield and mostly strongly expressing, transgenic individuals. One way to achieve this would be to use a multi-insert parent, or two transgenic parents. In either case past studies have shown that this opens the door for transgene silencing to occur. In this study we examined a hybrid line (Atlantic TC1 × APM-2 TC1) that demonstrated a high segregation of the *Cry3Aa* transgene (93% transgenic) with similar yields to commercial cultivars planted from seed tubers.

Although there was no verified parent-of-origin effect on transgene expression in this study, an event did occur that resulted in a far greater number of transgenic offspring produced from the Atlantic TC1 × Katahdin cross. Whether this observation involves a complex interaction between gametes from the two parents, or is merely an aberration caused by outside influences, bears further investigation. If the cause of this increased transgene transmission can be identified it could provide an additional tool in the effort to incorporate transgenic resistance into TPS production.

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Table 1: Hybrid families involved in each of three trials. Plants were arranged in a completely randomized design for the first two trials, and in a randomized complete block for the third. Each trial involved 16 plants per family.

Trial	Location	Families involved
1	Greenhouse	Atlantic TC1 X Katahdin Katahdin X Atlantic TC1 Atlantic TC1 X Atlantic Atlantic X Atlantic TC1
2	Greenhouse	Atlantic TC1 X Katahdin Katahdin X Atlantic TC1 Atlantic TC1 selfed Atlantic TC1 X wt APM-2 Atlantic TC1 X APM-2 TC1
3	Field	Atlantic Katahdin Atlantic TC1 Atlantic TC1 X Katahdin Katahdin X Atl TC1 Atlantic TC1 X wt APM-2 Atlantic X APM-2 TC1 Atlantic TC1 X APM-2 TC1 Atlantic TC1 selfed

Table 2: Expected segregation (excluding possible occurrence of double reduction gametes) of transgenic: non-transgenic progeny for single insert transgenics used in hybridizations. T_1 indicates the same *Cry3Aa* insertion as in Atlantic TC1; while T_2 indicates a single *Cry3Aa* insertion into APM-2 TC1; 0 indicates a null allele on homologues.

Hybrid	Ploidy	Parental genotypes	Expected progeny genotypes	Transgenic:non-transgenic
Atlantic TC1 X Katahdin	4x-4x	T_1 000 X 0000	1 T_1 000 : 1 0000	1:1
Katahdin X Atlantic TC1	4x-4x	0000 X T_1 000	1 T_1 000 : 1 0000	1:1
Atlantic TC1 X Atlantic	4x-4x	T_1 000 X 0000	1 T_1 000 : 1 0000	1:1
Atlantic X Atlantic TC1	4x-4x	0000 X T_1 000	1 T_1 000 : 1 0000	1:1
Atlantic TC1 X wt APM-2	4x-2x	T_1 000 X 00	1 T_1 000 : 1 0000	1:1
Atlantic TC1 self-pollinated	4x-4x	T_1 000 X T_1 000	1 T_1 T_1 00 : 2 T_1 000 : 1 0000	3:1
Atlantic TC1 X APM-2 TC1	4x-2x	T_1 000 X T_2 0*	6.5% T_1 000 X T_2 T_2 37% T_1 000 X T_2 0 6.5% T_1 000 X 00 6.5% 0000 X T_2 T_2 37% 0000 X T_2 0 6.5% 0000 00	14.3:1

*Segregation of T2 is based on predicted gametic frequencies considering that 13% of the unreduced gametes produced by APM-2 TC1 are homozygous for the transgene based on gene-centromere mapping (Johnson, 2001).

Table 3: Chi square values of observed segregation ratios in greenhouse trial one. Ratios are compared to the expected 1:1 ratio of expressers to non-expressers based on PCR detection of the transgene. Chi square values followed by an asterisk (*) indicated results significantly different from the null hypothesis at $p<0.05$.

Family	df	Ratio (expressers:non)	Chi square (1:1)
Atlantic TC1 X Katahdin	**1**	**13:3**	**6.25***
Katahdin X Atlantic TC1	**1**	**9:7**	**0.25**
Atlantic TC1 X Atlantic	**1**	**5:7**	**0.33**
Atlantic X Atlantic TC1	**1**	**5:11**	**2.25**

Table 4: Chi square values of observed segregation ratios in greenhouse trial two. Expected ratios to which observed values were compared are included in the table. There was no significant derivation from the expected ratios in this trial, however a high number of Cry expressers were observed in the first family.

Line	df	Expected ratio (expressers:non)	Observed ratio (expressers:non)	Chi Square
Atlantic TC1 X Katahdin	**1**	**1:1**	**10:6**	**1.00**
Katahdin X Atlantic TC1	**1**	**1:1**	**7:9**	**0.25**
Atlantic TC1 self	**1**	**3:1**	**11:5**	**0.33**
Atlantic TC1 X APM-2 TC1	**1**	**14.3:1***	**15:1**	**0.25**
Atlantic TC1 X wt APM-2	**1**	**1:1**	**6:10**	**1.00**

*unique segregation ratio due to high frequency of 2n pollen in APM-2 TC1

Table 5: Chi square values of observed segregation ratios in trial three (field trial). Ratios are compared to the expected 1:1 ratio of expressers to non-expressers. Chi square values followed by an asterisk (*) indicated results significantly different from expected values at $p<0.05$.

Line	df	Ratio (expressers:non)	Chi Square
Atlantic TC1 X Katahdin	**1**	**11:3**	**4.60***
Katahdin X Atlantic TC1	**1**	**6:4**	**0.40**
Atlantic TC1 X wt APM-2	**1**	**7:7**	**0.00**

Table 6: Chi square values of observed segregation ratios in trial four. Ratios are compared to the expected segregation of expressers to non-expressers included in the table. Observed chi square values were not significantly different from expected values in trial four.

Line	df	Expected ratio (expressers:non)	Observed ratio (expressers:non)	Chi Square
Atlantic TC1 X Katahdin	**1**	**1:1**	**13:11**	**0.17**
Katahdin X Atlantic TC1	**1**	**1:1**	**4:3**	**0.14**
Atlantic TC1 self	**1**	**3:1**	**9:1**	**1.40**
Atlantic TC1 X wt APM-2	**1**	**1:1**	**6:3**	**1.00**

Table 7: Pooled segregation data from all four trials. A highly significant ($p<0.01$) deviation from the expected 1:1 ratio of expressers to non-expressers is seen in the family Atlantic TC1 × Katahdin (denoted by the asterisk).

Line	df	Expected ratio (expressers:non)	Observed ratio (expressers:non)	Chi Square
Atlantic TC1 X Katahdin	**1**	**1:1**	**47:23**	**8.20***
Katahdin X Atlantic TC1	**1**	**1:1**	**26:23**	**0.18**
Atlantic TC1 X wt APM-2	**1**	**1:1**	**19:20**	**0.03**
Atlantic TC1 self	**1**	**3:1**	**20:6**	**0.05**
Atlantic TC1 X APM-2 TC1	**1**	**14.3:1**	**15:1**	**0.01**

Table 8: Mean optical density (OD) values of *Cry3Aa* expressing plants from each of four trials. OD was determined after 30 min incubation. N indicates the number of positive individuals in each trial. Mean values followed by the different letters are significantly different (Tukey-Kramer HSD, $p<0.05$).

Trial	N	Mean OD	SE
1	32	3.43 a	0.08
2	49	2.93 b	0.07
3	23	0.93 c	0.10
4	32	2.50 d	0.08

Table 9: Mean optical density (OD) values of hybrid families in first trial of 16 plants per family. Mean values include only *Cry3Aa* positive plants. Results are not significantly different at $p<0.05$.

Hybrid family	N	Mean OD	SE
Atlantic TC1 X Atlantic	**5**	**3.30**	**0.09**
Atlantic X Atlantic TC1	**5**	**3.37**	**0.09**
Atlantic TC1 X Katahdin	**13**	**3.48**	**0.06**
Katahdin X Atlantic TC1	**9**	**3.46**	**0.07**

Table 10: Mean optical density (OD) values of hybrid families in second trial of 16 plants per family. Mean values include only *Cry3Aa* positive plants. Results are not significantly different at $p<0.05$.

Hybrid family	N	Mean OD	SE
Atlantic TC1 X Katahdin	**10**	**2.66**	**0.18**
Katahdin X Atlantic TC1	**7**	**2.58**	**0.22**
Atlantic TC1 self	**11**	**3.00**	**0.18**
Atlantic TC1 X APM-2 TC1	**15**	**3.17**	**0.15**
Atlantic TC1 X wt APM-2	**6**	**3.07**	**0.24**

Table 11: Mean optical density (OD) values of hybrid families in trial three, the field trial, of 16 plants per family. An increase in variation is observed among the hybrid families in this trial. Mean values include only *Cry3Aa* positive plants. Means followed by the same letter are not significantly different from each other ($p<0.10$).

Hybrid family	N	Mean OD	SE
Atlantic TC1 X Katahdin	**10**	**1.11 a**	**0.13**
Katahdin X Atlantic TC1	**6**	**0.96 ab**	**0.17**
Atlantic TC1 X wt APM-2	**7**	**0.65 b**	**0.15**

Table 12: Mean optical density (OD) values of hybrid families in trial four which included the remaining seed from the reciprocal hybrids. N values represent the Cry expressing plants for each family. Results are not significantly different at $p<0.05$.

Hybrid family	N	Mean OD	SE
Atlantic TC1 X Katahdin	**13**	**2.48**	**0.14**
Katahdin X Atlantic TC1	**4**	**2.59**	**0.24**
Atlantic TC1 self	**9**	**2.61**	**0.16**
Atlantic TC1 X wt APM-2	**6**	**2.31**	**0.20**

Table 13: Actual Cry protein levels in plant tissue from sampled plants in trial four. Means were not significantly different from each other ($p<0.05$).

Hybrid family	N	Mean Cry concentration (μg/g leaf tissue)	SE
Atlantic TC1 X Katahdin	**13**	**2.41**	**0.16**
Katahdin X Atlantic TC1	**4**	**2.53**	**0.18**
Atlantic TC1 self	**9**	**2.55**	**0.18**
Atlantic TC1 X wt APM-2	**6**	**2.24**	**0.17**

Table 14: ANOVA of tubers per plant, average tuber weight, and yield per plant for five hybrid families, cultivars Atlantic and Katahdin, and Atlantic TC1 included in field trial. Seedlings were planted in four replications of four plants within the field plot. An asterisk(*) following a value denotes a significance at $p<0.05$.

Effect		**Tubers/plant**	**Average tuber weight**	**Yield/plant**
Source	df	MS	MS	MS
Family	7	843.3*	5274.9*	282119.5*
Replication	3	443.7*	1343.9*	249347.5*
Error	20	68	635.2	40148.3

Table 15: Analysis of field performance of five hybrid lines, transformed parental line Atlantic TC1, and cultivars Atlantic and Katahdin. Values followed by the same letter are not significantly different from each other ($p<0.05$).

Hybrid family	N	Mean yield per plant (g)	Tubers per plant	Average tuber weight (g)
Atlantic[1]	15	1019 a	13.6 b	99.7 a
Katahdin[1]	15	946 a	11.1 b	105.4 a
Atlantic TC1	10	661 ab	15.7 b	40.6 b
Atl TC1 X Katahdin	13	407 b	17.4 b	23.8 b
Katahdin X Atl TC1	10	341 b	14.9 b	27.6 b
Atl TC1 X wt APM-2	16	715 ab	45.5 a	17.3 b
Atl TC1 X APM-2 TC1	16	723 ab	45.5 a	16.2 b
Atlantic TC1 self	8	323 b	13.0 b	29.9 b

[1]**Planted from tubers**

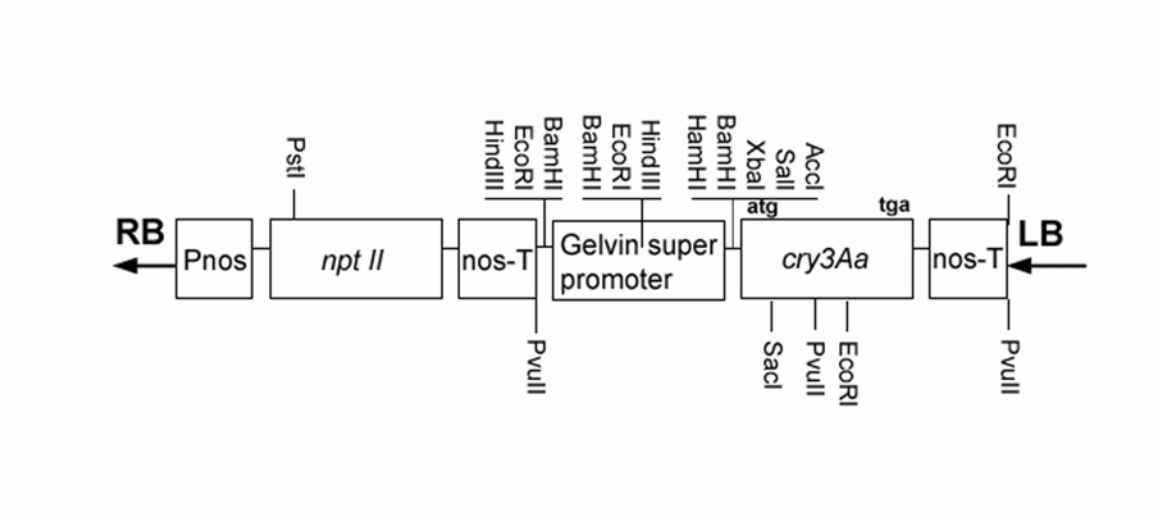

Figure 1: Diagrammatic representation of the 1.79 kb *Cry3Aa* gene and the kanamycin resistance selectable marker within the pSPUD8 plasmid.

Figure 2: Hybrid seedlings at time of sampling for DAS-ELISA to measure *Cry3Aa* expression. Seed was sown in pots with germination media, and transplanted to cell-packs after germination.

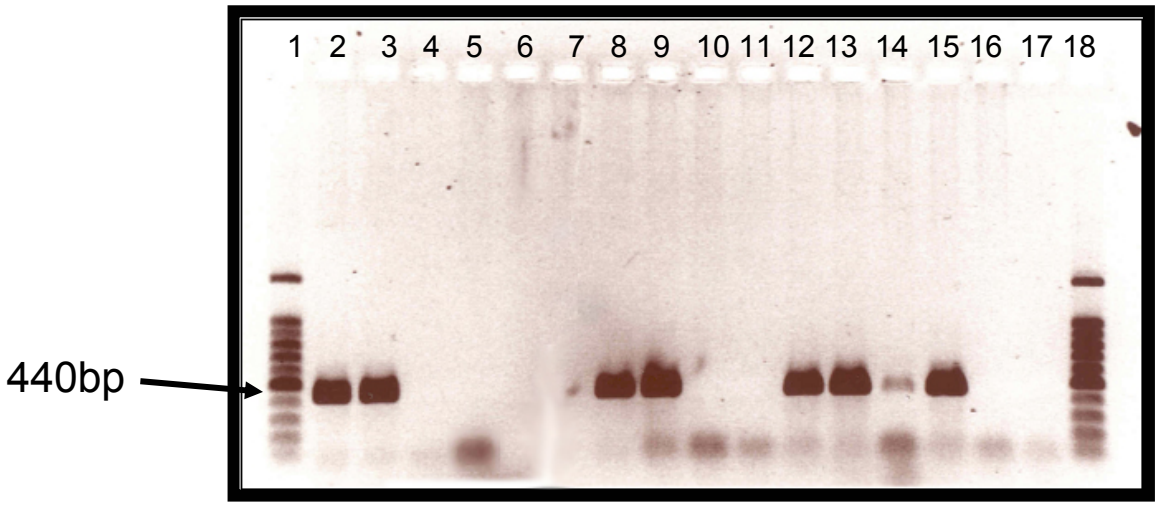

Figure 3: PCR results using Cry-specific primers resolved on 1% agarose gel. The expected 440 bp fragment is seen in plants expressing the Cry protein Lanes 1, 18, 100 bp ladder; lanes 2,3,8,9,12,13,15, plants positive for *Cry3Aa*; lanes 4,5,6,7,10,11,16,17, plants negative *Cry3Aa*; The plant represented by lane 14 was shown to be negative in subsequent gels.

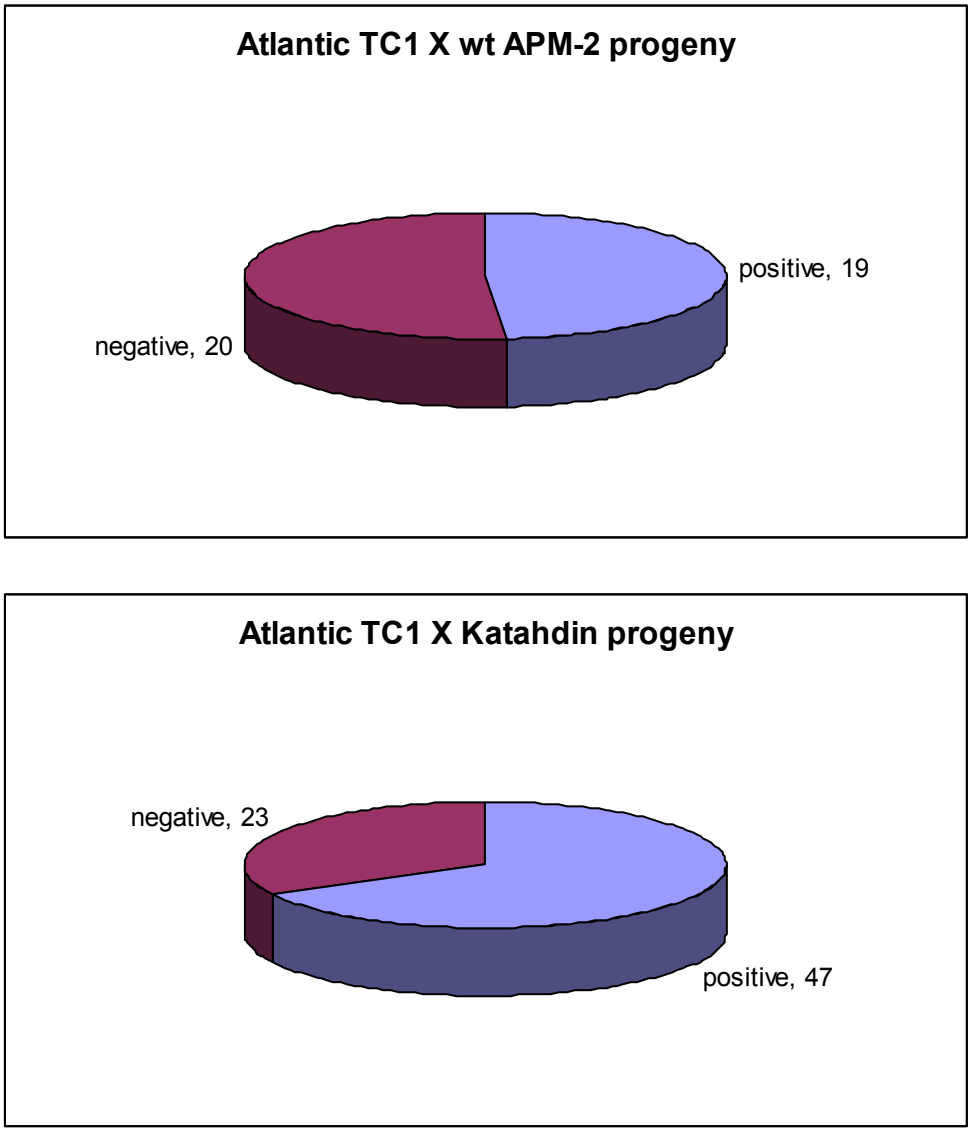

Figure 4: Graphical representation of expression patterns of transgenic progeny in hybrid families Atlantic TC1 × wt APM-2 and Atlantic TC1 × Katahdin. Data have been pooled from all four trials. The number of positive or negative individuals is indicated. The second hybrid family shows an unexpected segregation ratio of almost 2:1.

CHAPTER 3:
Analysis of transgenic resistance to PVY-O

Abstract

Potato Virus Y (PVY), the type member of the potyvirus family, is responsible for significant yield losses to potato and other crops worldwide. Coat protein-mediated resistance has been described in many reports as an effective means to protect plants against infection by many viruses, including PVY. In this study, seven transgenic lines were produced via *Agrobacterium*-mediated transformation with a PVY-O coat protein transgene and tested for response to infection after mechanical inoculation with PVY-O. One of the transgenic lines expressed complete resistance to infection as no symptoms were observed visually nor was virus detected through the use of serology. Two other transgenic lines showed partial resistance, manifested as a delay in the occurrence of symptoms compared to the wild type control.

Introduction

Potato Virus Y (PVY) is the type member of the genus *Potyvirus* and is responsible for substantial economic losses in potato crops (De Bokx and Huttinga, 1981). It is a single stranded RNA virus transmitted by aphids in a non-persistent manner. Reported symptoms in *Solanum tuberosum* include stunting, leaf mottling or crinkling, and the

development of necrotic streaks or lesions. The response of each plant relies heavily on the cultivar and specific strain of the virus involved. There are several strains of the virus that are divided into three groups; the necrotic strains (PVY-N), the stipple-streak strains (PVY-C), and the common strains (PVY-O). Although all three groups can cause significant crop loss, PVY-O is the only group with worldwide distribution. The agricultural industry has developed ways to combat most types of plant pests through chemical pesticides when a natural resistance is inadequate; however, the availability of a chemical means to control plant viruses is severely limited. Instead, control measures for viruses have focused on obtaining certified virus-free planting material, reducing the existing vectors of a virus (predominantly insects), and developing resistant plant lines. Because of a desire to reduce or even eliminate the cost and environmental effect of applying chemical pesticides for vector control, this development of resistant plant lines through the incorporation of natural resistance genes or transgenic pathogen-derived resistance has become increasingly appealing.

Pathogen-derived resistance

Pathogen-derived resistance is an event in which a plant transformed with a segment of viral nucleic acid, functional gene or otherwise, is able to inhibit or even prevent infection by a virus. The concept of pathogen-derived resistance was pioneered by Sanford and Johnston (1985), and has since been the focus of many efforts to produce virus resistant plants. There are generally two accepted methods in which transgenic resistance is exhibited in a plant; that in which the transgenic protein product physically

interferes with the replication cycle of the virus, and also an event leading to a disruption at the nucleic acid level involving sequence homology between the virus and transgenic nucleic acid. Although efforts have been made to confer virus resistance to plants by transforming them with various viral genes such as virus movement protein and replicase genes, as well as non-coding sequences, most studies have involved the coat protein gene.

Shortly after the concept of pathogen-derived resistance was proposed, Powell et al. (1986) showed that transgenic tobacco plants expressing the coat protein from tobacco mosaic virus (TMV) exhibited as much as a 60% delay in symptom development. Similar instances of transgenic plants showing coat protein-mediated resistance have been seen in sweet potato (*Ipomoea batatas* L.) to sweet potato feathery mottle virus (SPFMV; Okada et al., 2001), muskmelon (*Cucumis melo* L.) to cucumber mosaic virus (CMV; Gonsalves et al., 1994), soybean (*Glycine max* L.) to soybean mosaic virus (SMV; Wang et al., 2001), and potato to potato leafroll virus (PLRV) and potato mop-top virus (PMTV; Presting et al., 1995; Barker et al., 1998, respectively). Register and Beachy (1988) proposed that in the case of TMV, the transgenic coat protein apparently interferes with the disassembly of the TMV particle and thus prevents infection. This could lead to the conclusion that a strongly expressed, constitutive transgene would provide the greatest protection against infection; however, there are many examples of highly resistant lines which accumulate relatively low levels of transgene product (Smith et al., 1995). In their potato lines transformed with a PVY coat protein transgene, Smith et al. (1995) found that the more resistant lines accumulated far less transgene RNA, with a complete absence of translation product in many instances. This inverse relationship

between resistance and transgene product accumulation would seem to indicate an RNA-mediated event rather than one resulting in the interference of the virus replication cycle by transgenic protein.

Summary

PVY is an economically detrimental virus, and any resistance, whether a delay in appearance of symptoms, or complete inhibition of infection, could benefit potato growers. In this study the effectiveness of coat protein-mediated resistance against PVY is examined in a selection of transgenic potato lines derived from three genetically diverse sources of germplasm. After mechanical inoculation, plants will be monitored both phenotypically and serologically.

Materials and Methods

Plant material

Agrobacterium-mediated transformation was utilized to produce transgenic lines containing the PVY-O coat protein gene (Horsch et al., 1988). The transgene is contained in the pBIPVY plasmid (Figure 1), which was developed by Tom German from the University of Wisconsin in Madison, and supplied by David Douches from Michigan State University, East Lansing. Transgenic lines were produced from cv. Atlantic, and

the diplandroids APM-2 and 05-10. In addition to the transgenic lines, PVY resistant potato cvs. Bison and Corine, obtained from USDA/ARS Potato Introduction Station (NRSP-6), Sturgeon Bay, WI, were included to compare natural resistance to any observed transgenic resistance. These cultivars exhibit high resistance to the effects of PVY according to The European Cultivated Potato Database (http://www.europotato.org/index.htm). The plant lines included in this study are shown in Table 1. Each plant line was maintained and propagated *in vitro* and transferred to soil for acclimatization to greenhouse conditions. After 2 weeks, four plants from each line, 48 total, were transplanted into 4-inch pots and moved to a separate greenhouse.

Experimental design

In order to reduce the amount of bias during screening, the 48 plants were arranged in a completely randomized design. Plant tags from each pot were hidden to conceal the identity of each individual until the observed data were collected and analyzed. One plant from each group of four was designated for mock inoculation, leaving the remaining three to be challenged with the virus.

Inoculation with PVY-O

PVY-O inoculum was supplied by Sue Tolin (Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute & State University, Blacksburg) in the

form of an inoculated tobacco (*Nicotiana tabacum* cv. NC 95) plant exhibiting severe vein-clearing and mosaic symptoms (Figure 2). Leaf tissue from the infected tobacco plant was ground in neutral 0.01 M sodium phosphate inoculation buffer at a 1:10 weight to volume ratio using a mortar and pestle. Homogenized tissue extract was then applied to carborundum dusted leaves of seedlings by gently rubbing the leaf surface with the pestle. This treatment was applied to the first two fully expanded leaves on three of the four plants from each plant line. Fresh inoculum was prepared every 12 plants. The remaining plant from each line was mock inoculated in a similar manner, applying fresh inoculation buffer with a clean pestle to carborundum dusted leaves.

Phenotypic examination of virus symptoms

An arbitrary 1-5 scale was developed to monitor the visual symptoms in inoculated plants. The parameters for each value are as follows; 1-no visual symptoms, 2-beginnings of vein-clearing or mottle, 3-distinct mottle, 4-severe mosaic and/or necrosis, 5-extreme plant decline and/or death. Plants were examined after 2 days to record any damage to the leaf surface caused by inoculation with carborundum. Screening for virus symptoms began 3 days post-inoculation and was continued every 2 days for 25 days.

Leaf tissue immunoblot

An immunoblot serological procedure was employed to monitor the presence of PVY in the inoculated leaf, as well as movement into uninoculated tissue. The procedure involves the direct transfer of antigen from plant tissue to a nitrocellulose membrane by "blotting" cut or torn tissue onto the surface of the membrane. The green tissue blots can be spaced through the use of a homemade template with uniform holes punched in it. The green color was removed by treatment with the detergent 5% Triton X-100 (Srinivasan and Tolin, 1992), followed by a rinse in 1X potassium phosphate buffered saline (KPS) with 0.05% Tween-20 (Lin et al., 1990). The membrane was then soaked in a blocking solution (5% non-fat dry milk, 0.5% BSA, in 1X KPS) to discourage non-specific binding of the primary antibody. The membrane was then transferred to primary antibody in the form of rabbit anti-PVY antiserum at a concentration of 1:10,000 for 45 min with agitation. The antiserum was from the collection of S.A. Tolin and was designated PVY-Hebert (R342-343), received from G.V. Gooding, North Carolina State University, Raleigh. After three rinses, the membrane was placed in a 1:15,000 solution of secondary antibody (enzyme labeled goat anti-rabbit, Zymed® Laboratories, Inc.) for 30 min. A substrate/chromophore solution (one part each of BCIP and NBT to eight parts water, Zymed® Laboratories, Inc.) was then responsible for the development of a blue color in positive blots. This assay is qualitative and was largely used to monitor movement of PVY out of the inoculated plant leaves. In this study, three leaf blots were taken from each plant; one from the inoculated leaf 8 days post inoculation, and blots from the next youngest leaf from the inoculated leaf at 15 and 22 days post inoculation.

By taking samples over a period of time from different leaves, movement of the virus out of the inoculated leaf was monitored.

PTA-ELISA

PTA-ELISA (Plate-trapped antigen) was used to determine relative quantities of PVY in inoculated plant leaves as well as in the next three highest leaves. The protocol used was developed by Koenig (1981), and optimized for use with soybean mosaic virus (SMV), also a potyvirus (Tolin, unpublished). Tissue was sampled 33 days post inoculation and ground in extraction buffer (1.59 g sodium carbonate, 2.93 g sodium bicarbonate per liter, pH 9.6) at a w:v ratio of 1:50 in an Agdia® mesh sample bag (Catalog # ACC 00930). Extract was then loaded into duplicate wells of a 96-well ELISA plate and incubated overnight at 4ºC. The extract was then removed and the plate rinsed with 1X PBS-Tween (PBST, Agdia®, Inc.) three times. Primary antibody (1:10,000 rabbit anti-PVY antiserum) was added to each well and incubated at 37ºC for 2 h. The plate was then emptied and rinsed again before addition of enzyme-linked secondary antibody (1:15,000 goat anti-rabbit antiserum from Sigma Chemical Co., St. Louis (Product # A3687), in 1X PBS-Tween with 0.2% bovine serum albumin and 2% polyvinylpyrrolidone (PVP, MW 40,000)), and incubated at 37ºC for 2 h. The contents of the plate were then shaken out and it was again rinsed three times. Substrate was then added to each well (1 mg/ml paranitrophenyl phosphate (Sigma Chemical Co., Sigmafast™ PNP tablets) in 0.2 M Tris) and the plate read at 15 min intervals using a Spectramax™ (Molecular Devices Corp. Sunnyvale, Ca) plate reader at 405 nm. Relative quantities of PVY-O in each

sample were determined by comparing the absorbance (A) readings from each well after 45 min incubation with substrate. Positive values were defined as any sample with an absorbance reading of more than twice that of an uninoculated plant used as a negative control. Two samples were taken from each plant; one including whole inoculated leaves, and the second from the next three highest leaves.

Results

Phenotypic examination of virus symptoms

Of the seven transgenic lines examined for resistance, only 05-10 TP3 showed high levels of resistance to PVY based on phenotypic observation. None of the three 05-10 TP3 plants inoculated with PVY exhibited any symptoms of infection after 25 days, and their growth and development was similar to that of the control plant (Figure 3). The presence of the PVY-O coat protein transgene in two transgenic lines of APM-2 seemed to give some protection to PVY infection as symptom appearance was delayed by as much as a week. However, symptoms did still occur and were visually similar to APM-2 wt after 25 days post inoculation (Figure 4). The results from the visual inspection are presented in Table 2. A distinct difference in plant response was noticed between the different families shortly after inoculation with PVY. Both transgenic and untransformed 'Atlantic' plants developed necrotic lesions (local lesions) on the inoculated leaves at 7 days post inoculation. In contrast, transgenic and wild-type APM-2 and 05-10 plants

exhibited stunting and systemic leaf mottling (Figure 5). Although the development of local lesions was unique to plants in the 'Atlantic' group, there was no significant difference in time of appearance or quantity of lesions between transgenic and non-transgenic lines of 'Atlantic' (data not shown).

Presence of virus

The first serological technique employed was a leaf tissue immunoblot procedure that, in method, was a rapid and convenient means to monitor PVY-O as it moved from the inoculated leaf into the rest of the plant (Table 3). The transgenic line 05-10 TP3, which showed no phenotypic symptoms of PVY-O infection, gave no positive results in any of the three immunoblots taken from each plant. The remaining 05-10 plants (TP2 and wild-type) showed systemic infection with PVY-O by 15 days. The plant material which tested positive for PVY-O was from tissue other than the inoculated leaf, indicating the virus had begun to spread throughout the entire plant. Additionally, the plants that exhibited a local lesion response (transgenic and wild-type 'Atlantic' plants) yielded no positive results from blots taken from the inoculated leaves or non-inoculated younger leaves. The three APM-2 clones (wild-type, TP2, and TP3) each included at least one plant which tested positive for PVY-O through the immunoblot assay. The phenotypic observation of an apparent delay in the development of virus symptoms mentioned above was supported in the APM-2 TP3 family which did not test positive until day 22 of the study. The mock-inoculated control plants from each plant line, as well as the two

resistant lines, ‘Bison’ and ‘Corine’ showed negative results in all of the blots from these plants.

PTA-ELISA

Qualitative results from PTA-ELISA support the data obtained by leaf tissue immunoblot assays in that positive results were conserved across the two immunoassays. As in the immunoblot assay, 05-10 TP3, transgenic and wild-type ‘Atlantic,’ and resistant lines ‘Bison’ and ‘Corine’ showed no detectable PVY-O accumulation by PTA-ELISA (Table 3). This lack of detectable virus accumulation was also observed in the mock-inoculated control plants from each plant line. There does not appear to be an obvious effect of plant family on virus accumulation in PVY-O positive plants. In the APM-2 plant lines there seems to be a greater amount of virus in the younger, non-inoculated leaves than those inoculated with PVY-O, indicating a higher virus accumulation in systemically infected leaves (Table 4). A similar relationship was difficult to observe in other families as the inoculated leaves of some plants had begun to senesce by the conclusion of the study; however, one 05-10 wt plant whose inoculated leaves were tested showed a higher accumulation of virus in the inoculated leaves rather than those above them.

Discussion

Seven transgenic lines of unknown transgene copy number were examined for their coat protein-mediated resistance to virus with the expectation that the presence of the PVY-O coat protein transgene would lend some level of protection against the virus. The work presented in this study shows that coat protein-mediated resistance, although it was not established in each transgenic line, can be effective in lessening the impact of PVY-O infection. One of the seven transgenic lines (05-10 TP3) expressed no phenotypic symptoms or serological indication of virus accumulation (Table 2). The wild type 05-10 plants showed not only the most severe visible symptoms (leaf distortion, leaf mottle, stunting) but the highest concentration of virus in the inoculated leaves after 33 days (Table 4). Similar examples of complete resistance were found by Hefferon et al. (1997) in potato lines transformed with a PVY coat protein. High levels of resistance were not seen in the transgenic lines APM-2 TP2 and TP3; however, visible symptoms appeared 6 and 7 days, respectively, after APM-2 wt began to display symptoms. This occurred despite the detection of virus through immunoblot in the inoculated leaves of both transgenic lines. A similar resisatnce response was observed by Fuchs et al. (1998) in transgenic lines of squash (*Cucurbita pepo* L.) expressing a single CP gene from either cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV), or watermelon mosaic virus 2 (WMV 2) to its respective virus. They found that these single insert lines showed a delay in symptom development of 2 to 4 weeks compared to the control plants. Although the delay seen in this study is interesting, for it to benefit production there would presumably need to be a much greater lag in symptom initiation, allowing the

plant to grow and develop at a normal rate before symptoms such as stunting begin. Confirmation of this would require further study, however.

The 'Atlantic' lines showed a considerably different response to PVY-O. Rather than developing mottle and leaf distortion the inoculated leaves developed local lesions, a defensive hypersensitive response developed, in which the invaded cells were destroyed by the plant to restrict movement of material to additional cells. The three transgenic lines of 'Atlantic' showed virtually no difference from the untransformed plants in response to inoculation with PVY-O. Neither the appearance, nor number of local lesions varied among the four families. The difference in response of 'Atlantic' plants to inoculation with PVY, which has been reported to include mild to severe mosaic, crinkle, and mild to severe necrosis (Mihovilovich et al., 1998). Mosaic was not seen in the transgenic or wild type 'Atlantic' lines, and the unexpected development of local lesions on the inoculated leaves is likely a plant response resulting from the specific strain of PVY used in this study.

It is difficult to determine the exact nature of the resistance seen in some of the transgenic plant lines in this study because levels of transcription and translation have not been measured. In many cases of resistance it is not essential that detectible levels of transgenic protein accumulate in plant tissue (Lindbo and Dougherty, 1992; van der Vlugt et al., 1992). This has led many to the conclusion that transgenic virus resistance is an RNA-mediated event, often involving the degradation of RNA sequences homologous to transgenic RNA (Mueller et al., 1995). Determination of levels of transgenic

transcriptional and translational products would help determine if the resistance seen in this study is RNA- or protein-mediated.

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Table 1: Plant lines included in this study. Transgenic lines are delineated by 1R* or TP* following the name of their parental germplasm. 'Bison' and 'Corine' are untransformed cultivars with reported resistance to PVY.

Plant line	Ploidy	Known resistance
Atlantic	4x	no
Atlantic 1R1	4x	no
Atlantic 1R2	4x	no
Atlantic 1R3	4x	no
wt APM-2	2x	no
APM-2 TP2	2x	no
APM-2 TP3	2x	no
wt 05-10	2x	no
05-10 TP2	2x	no
05-10 TP3	2x	no
Bison	4x	yes
Corine	4x	yes

Table 2: Results of phenotypic observation of inoculated plants in study. Symptom occurrence is based on the mean days post inoculation on which symptoms were observed in each group of plants. Because of the difference in symptom development the 'Atlantic' lines were not monitored using this scale. Transgenic line 05-10 TP3 and resistant lines 'Bison' and 'Corine' showed no symptoms at 25 days post inoculation (the final observation). The phenotypic score is based on an arbitrary 1-5 scale, with a score of 1 indicating no observed symptoms.

Family	Symptom appearance (Days post inoculation)	Score at final observation	Resistance
Atlantic	7	na	none
Atlantic 1R1	7	na	none
Atlantic 1R2	7	na	none
Atlantic 1R3	7	na	none
05-10	15	3.3	none
05-10 TP2	10	3.0	none
05-10 TP3	ns	1.0	high
APM-2	14	2.3	none
APM-2 TP2	20	2.3	low*
APM-2 TP3	21	2.3	low*
Bison	ns	1.0	high
Corine	ns	1.0	high

*****Based on a delay in appearance of symptoms rather than a reduction of symptom severity.

[ns]No symptoms observed

[na]Arbitrary scale not applicable

Table 3: Serological detection of PVY in potato leaf tissue by immunoblot or indirect ELISA. Immunoblot data include one set of data from the inoculated leaf, and two from the first leaf above the inoculated leaves (systemic accumulation). Days after inoculation at which each blot was taken are given for each sampling. The two tissue samples from each plant used for PTA-ELISA include the remaining tissue from the inoculated leaves and the three oldest leaves above the inoculated leaves (systemic accumulation). Positive (+) indicates positive test results in at least one of the three inoculated plants. A lack of positive results in the 05-10 plants sampled through the immunoblot assay at 8 days is likely due to inadvertent blotting of localized healthy tissue from the inoculated leaves. Virus was not detected in any of the mock-inoculated control plants from each family.

	Immunoblot			**PTA-ELISA**	
	Inoculated leaf	**Systemic accumulation**			
Family	**8 days**	**15 days**	**22 days**	**Inoculated leaves**	**Systemic accumulation**
Atlantic	--	--	--	--	--
Atlantic 1R1	--	--	--	--	--
Atlantic 1R2	--	--	--	--	--
Atlantic 1R3	--	--	--	--	--
05-10	--	**+**	**+**	**+**	**+**
05-10 TP2	--	**+**	**+**	--	**+**
05-10 TP3	--	--	--	--	--
APM-2	+	**+**	**+**	**+**	**+**
APM-2 TP2	**+**	**+**	**+**	**+**	**+**
APM-2 TP3	**+**	--	**+**	**+**	**+**
Bison	--	--	--	--	--
Corine	--	--	--	--	--

Table 4: Quantitative data from PTA-ELISA on transgenic lines with individuals positive for PVY-O. Values are absorbance (A) readings 45 min after addition of substrate. Samples with A readings greater than twice those of the negative controls were considered positive. Data from negative samples were omitted from this table. Inoculated leaf tissue was not available from 05-10 TP2 plants as inoculated leaves had senesced.

Family	Accumulation in inoculated leaves	Systemic accumulation
APM-2 wt	0.256	0.861
APM-2 TP2	0.371	0.590
APM-2 TP3	0.570	0.664
05-10 wt	0.913	0.679
05-10 TP2	n/a	0.364
control (-)	0.109	0.139

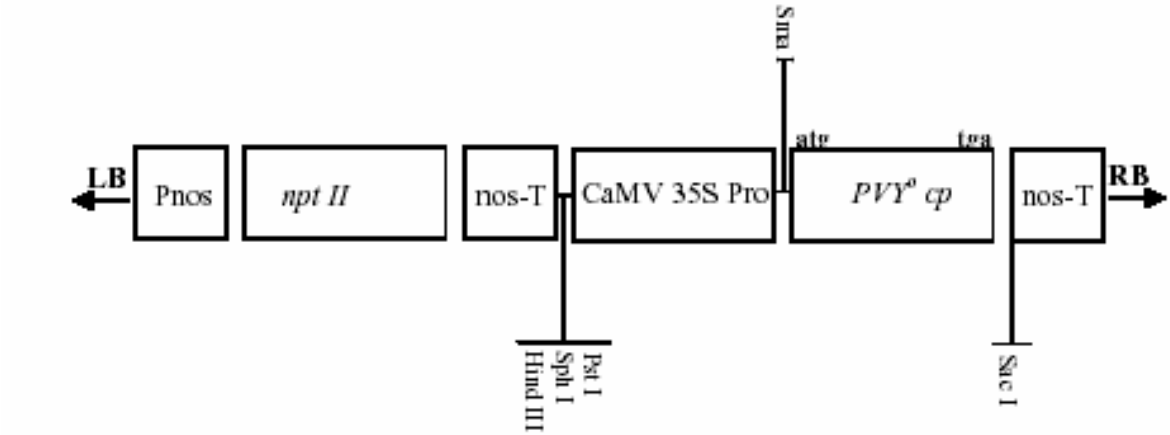

Figure 1: Diagram of the gene construct containing the PVY-O coat protein gene and kanamycin selectable marker.

Figure 2: PVY-O inoculated tobacco (*Nicotiana tabacum* L.) plant used to produce inoculum for study. The plant exhibits the classic symptoms of vein-clearing and leaf mottle, typical for PVY infection in this species.

Figure 3: 05-10 TP3 plants exhibiting no symptoms of PVY-O infection. The mock inoculated plant is on the left while the three on the right were inoculated with PVY-O.

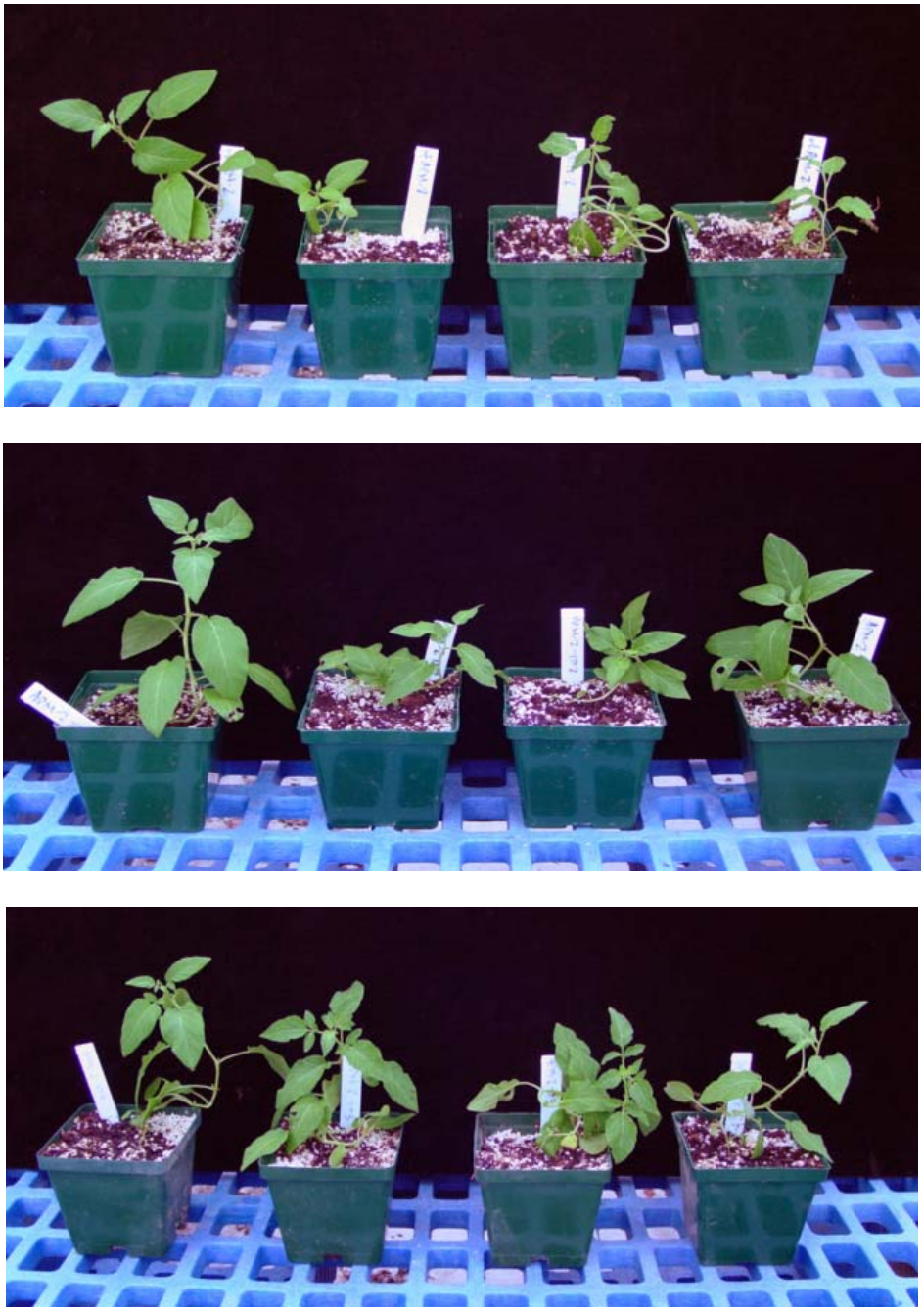

Figure 4: From top to bottom; APM-2 wt, APM-2 TP2, APM-2 TP3. Plants on the left are mock-inoculated controls, followed by three inoculated plants on the right.

Figure 5: Above; local necrotic lesions occurring on inoculated leaf of 'Atlantic' plant seven days post inoculation. Lesions are typical of those seen on inoculated leaves of both transgenic and wilt-type 'Atlantic' plants in this study. Below; mottling and leaf crinkling seen in symptomatic plants of APM-2 and 05-10 families.

Vita

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Personal History

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Professional Experience

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