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By

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ABSTRACT

STUDY OF THE INHERITANCE OF THE Bt-CRY I A(c) AND PVY COAT PROTEIN TRANSGENES IN POTATO

By

Rafael Oduardo Mendez Ospino

The production of transgenic plants has recently become a routine practice for many crop species. In potato breeding, genetic transformation plays an important role since traditional breeding is difficult due to the tetraploid nature of its genome. In this study, the inheritance patterns for Bt-*Cry IA(c)*, PVY coat protein (PVY cp) and neomycin phosphotransferase II (NPT II) transgenes in crosses between FL1607-A30 (Bt clone) and ATL-PVY5 (clone with the coat protein gene) and non-transgenic plants (cv. Atlantic and line MS702-80) were determined through PCR , Southern and kanamycin analyses. Southern analysis and the segregation pattern of the Bt gene suggested a single gene locus insert in clone FL1607-A30. In the case of the PVY cp gene and NPT II, Southern analyses indicated the presence of 2 inserted copies of the gene, but the segregation rates suggested tight linkage between the inserted copies.

The kanamycin assay corroborated the segregation pattern of the NPT II gene found in the PCR analysis. It indicated a stable integration and expression of the introduced NPT II gene, that behaved as a single dominant allele.

Forty-seven individuals that have both the Bt-*Cry IA(c)* and PVY cp genes were yielded. However, further research is needed to determine if the resistance observed in the transgenic parents is expressed in this progeny.

To my wife, Rosario E. Mendez

Whose patience, support, understanding and love make me a rich man.

To my son, Rafael Jesus, and my future son,
Ricardo Jose (-1 moth old)

I hope this achievement will give you enough inspiration to reach for high goals in your life

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LIST OF ABBREVIATIONs

Bt..... *Bacillus thuringiensis*

PVY..... Potato virus Y

PTM..... Potato tuber moth

PVY cp gene..... Potato virus Y coat protein gene

NPT II..... Neomycin phosphotransferase

CaMV 35S promoter..... Cauliflower mosaic virus 35S promoter

PCR..... Polymerase chain reaction

RFLP..... Restriction fragment length polymorphism

DNA..... Deoxyribonucleic acid

RNA..... Ribonucleic acid

A, T, C, G..... Adenine, Thymine, Cytosine, Guanine

FM..... Fully modified

PM..... Partially modified

WT..... Wild Type

TMV..... Tomato mosaic virus

AlMV..... Alfalfa mosaic virus

ArMV..... Arabis mosaic virus

CMV..... Cucumber mosaic virus

GCMV..... Grapevine chrome mosaic virus

PLRV..... Potato leafroll virus

PVS..... Potato virus S

PVX..... Potato virus X

PVY^{c}..... Potato virus Y, stipple streak strain

PVY^{n}..... Potato virus Y, tobacco venial necrosis strain

PVY^{0}..... Potato virus Y, common strain

RSV..... Rice stripe virus

SMV..... Soybean mosaic virus

TEV..... Tobacco etch virus

TSV..... Tobacco streak virus

TSWV..... Tomato spotted wilt virus

TYLCV..... Tomato yellow leaf curl virus

INTRODUCTION

Disease resistant crop plants have long been produced by identifying resistant genotypes and then crossing such individuals exhibiting resistance with cultivars that possess the requisite array of agriculturally valuable traits (Grumet et al. 1993). However, in potato because of the tetraploid nature of the genome, classical approaches to breeding and selection for improved properties are especially difficult and laborious when compared with diploid crop species (MacKenzei et al. 1991). In addition, the desired resistance is not always available: it may exist in species that are not fertile with the crop, the gene for resistance may be tightly linked to undesirable traits, or the resistance may be polygenic and thus difficult to transfer (Grumet et al. 1993).

Genetic engineering techniques may be used to overcome some of the factors limiting traditional plant breeding such as fertility barriers among species. This means that we do not have only genes transferred between different plant families, but among individuals of different kingdoms (Grumet et al. 1993).

The application of biotechnology may serve as a key element in the concept of integrated pest management, enhancing the trend toward environmental-compatible pest and disease control strategies (Williams et al. 1992).

The high incidence of diseases and pests on crop plants as compared to wild plant species is most likely due to uninterrupted monoculture production in combination with a

selection for characteristics such as yield rather than for disease and pest resistance in the breeding process. To control these diseases and pests, the industry has developed many chemical products (van den Elzen et al. 1989). However, there is pressure by consumers to reduce pesticide use in all crop production and to minimize the amount of pesticide control. This force drives the development of alternative technologies, including genetic engineering (Martin et al. 1994).

The production of transgenic plants has recently become a routine practice for many species, and an array of genes for resistance is now available. Since crops are often damaged by more than a single pest or disease, lines expressing multiple resistance genes are needed for improved crop protection (Liang et al. 1994).

Some major constraints to commercial potato production are insect pests and viruses. Among the insect pests, *Phthorimaea operculella* (Zeller) or potato tuber moth (PTM) is an important pest both in the field and in storage in tropical and sub-tropical regions (Trivedi et al. 1992). In addition, virus problems in potato are well recognized, and virus control by chemical products has been ineffective. Viruses make the seed certification scheme expensive in potato as in vegetatively propagated crops (van den Elzen et al. 1989). Fortunately, some genes are available to control these problems such as the *Cry IA(c)* gene that codes for a lepidoteran specific Bt protein toxin, and PVY coat protein gene that confers resistance to homologous and heterologous viruses (van den Elzen et al. 1989, Barton and Miller 1993, Liang et al. 1994).

The practical application of plant transformation technology to potato improvement can have two directions. First, transgenes can be introduced directly into

suitable breeding lines and these lines can be used to generate new varieties by standard crossing and selection procedures. Second, transgenes can be introduced directly into existing cultivars to confer specific genetic changes. The second approach has the advantage of producing transgenic lines within established backgrounds (Belknap et al. 1994).

POTATO TUBER MOTH, *Phthorimaea operculella* (Zeller)

The potato tuber moth (PTM), *Phthorimaea operculella* (Zeller) (Lepidoptera:Gelechiidae) is an oligophagous insect widely distributed in almost all tropical and subtropical regions. The species is a serious pest of potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabacum* L.), and sporadically strikes tomato (*Lycopersicum esculentum* Mill) (Das et al. 1994). Potato tuber moth is a serious pest of potato under both field and storage conditions. The infestations start in the field on the leaves and tubers, and infested tubers are carried to storage and act as the initial source of infestation (Das et al. 1994).

Feeding damage caused by this pest drastically reduces the quality and market value of tubers and creates sites for infection by pathogens that cause complete destruction of the tubers (Das et al. 1994). Losses up to 50% and more have been recorded in commercial crops in Peru, storage losses may be up to 100% in India and Philippines, 90% in Kenya, 86 % in Tunisia, Algeria and Turkey (Das et al. 1995).

The larvae of PTM mine in the mesophyll layer of the leaves and shoots, and additionally potato tubers. Tunneling damage in tubers caused by PTM is a severe problem, especially in traditional, non-refrigerated storage systems (Jansens et al. 1995).

Under field conditions, the pest deposits a high proportion of eggs in the soil adjacent to host plants rather than directly on the plants themselves and some eggs are laid under the skin of partially exposed tubers. Post-harvest infestation also occurs if the tubers are left unprotected in the field. The larvae hatch under the tuber skin and feed inside until the 5th instar when they exit through holes to pupate in the soil. The bored tubers are then contaminated with fungi and bacteria, leading to rotting and destruction (Siddig 1988, Fenemore 1988).

Until recently, the use of insecticides was a widely accepted practice to control PTM; however, the use of insecticides (especially for stored table potatoes) is a potential health risk, resistance to commonly used insecticides is likely to develop, and chemical control in storage has been inefficient (Siddig 1988, Das et al. 1995 and Kroschel et al. 1996).

Bacillus thuringiensis

Commercial formulations of *Bacillus thuringiensis* (Bt) have demonstrated insecticidal effect against PTM in potato storage (Kroschel et al. 1996). However, Bt sprays have limited field efficacy, since they may be washed off the leaves by rain and are most effective on young larvae. Therefore, the topically applied Bt protein must be synchronized with good weather conditions and susceptible larval stages. These drawbacks of Bt sprays make the introduction of Bt genes into plants a better use of this biological pest control (Stewart et al. 1996, Cheng et al. 1992).

Bt is a gram-positive, free-living soil bacterium that accumulates high levels of insecticidal proteins during sporulation. The bacteria have been utilized for several

decades in insecticidal formulations, which have shown high specificity toward certain insect pests. Such high specificity of Bt for target pests presents a potential ecological advantage over many chemical insecticides: Bt toxins are not toxic to beneficial insects, plants and vertebrates, and chemical insecticide may be persistent well after application (Barton and Miller 1993, Salamitou et al. 1996, Stewart et al. 1996, Adang et al. 1993).

Reports on bacteria as causative agents of insect disease date back to the 1900s. Their use as control agent began in the 1930s. For example, *Bacillus popilliae* has been used to control Japanese beetle grubs. Bt was originally isolated from infected silkworm colonies in 1901 in Japan and was initially named *B. sotto* (*B. thuringiensis* subsp. *sotto*, in the current nomenclature). The identification of intracellular proteinaceous inclusions as a major source of the insecticidal agents produced by these bacteria dates back to the 1940's (Aronson 1994, Whitely and Schnepf 1986). The introduction of Bt as a biological pesticide started in the early 1960's (Baum et al. 1994).

The Bt group produces protoxins designated delta (δ) endotoxins and in some cases secrete α or β exotoxins (Aronson 1994). These designations refer to the type of toxic activity involved. While α or β exotoxins are toxic either to a variety of insect orders or to many cell types, the crystal protein, or (δ) endotoxins, have a more limited host range (Whitely and Schenepf 1986). The crystalline proteins are accumulated in the cytoplasm of the cells during the stationary phase or sporulation to form crystal inclusions which can account for 25% of the dry weight of the cell, and *B. thuringiensis* can synthesize one or more crystal inclusions (Salamitou et al. 1996, Bora et al. 1994). At the end of the sporulation process, crystals and spores are liberated by lysis of the cell (Salamitou et al. 1996). These crystals are composed of proteins called δ-endotoxins or

Cry proteins. A single crystal may contain one million protein subunits held together by interchain disulfide bonds, and thus the cleavage of those bonds is a critical step in crystal solubilization. Many *Bacillus thuringiensis* strains have multiple *Cry* genes; however, there are some exceptions such as *B. thuringiensis* HD-73, which produces crystals from only a single *Cry1A(c)* gene (Du et al. 1996, and Herrera et al. 1994). In addition, some strains show non-toxic effects toward known susceptible insects, such as the isolated LBIT-113 which has a flat, square parasporal crystal composed of two proteins with sizes of 88 and 54 kDa (Lopez-Meza et al. 1996).

Cry proteins are synthesized as protoxins, which must be solubilized and activated through proteolytical cleavage by trypsin-like proteases in the alkaline environment of the insect gut. The activated toxin binds to receptors, which are located in the apical microvilli of susceptible larval midgut epithelial cells. After binding, the toxin inserts itself into plasma membrane and forms a pore or lesion by a process that is not yet fully understood. Pore formation allows net uptake or leakage of ions and water leading to osmotic lysis of the epithelial target cells, cessation of feeding and finally insect death (Ceron et al. 1995, Adang et al. 1993, Orduz et al. 1994, Meza et al. 1996, de Maagd et al. 1996, Dean et al. 1996). The specificity of a particular toxin depends on the efficiency of each of the steps mentioned above. Hence, the ability to solubilize and activate protoxins has been shown to influence toxicity. The activity of the protease-activated toxin is dependent on the type of gut protease involved and the present of a specific receptor in the brush border membrane (de Maagd et al. 1996).

***Bacillus thuringiensis* (*CRY*) genes**

Many thousands of *Bacillus thuringiensis* strains have been isolated, and they exhibit a great diversity in the spectrum of their toxicities including toxic activity against lepidopteran, dipteran, coleopteran insects and against nematode and aphids (Lopez-Meza et al. 1996). To date, nearly 100 distinct crystal protein gene sequences have been published either in the general scientific literature or in patent applications (Du et al. 1996, Lambert et al. 1996, Baum et al. 1996). To search for and to characterize novel *Cry* genes is a worldwide project (Ceron et al. 1995).

These *Cry* genes have been classified into six different groups based on their amino acid sequence similarities and ranges of specificities (Ceron et al. 1995, Barton and Miller 1993, Du et al. 1996). They are: *Cry* I, lepidopteran-specific; *Cry* II, lepidopteran- and dipteran-specific; *Cry* III, coleopteran-specific; *Cry* IV, dipteran-specific; *Cry* V, coleopteran and lepidopteran-specific; and *Cry* VI, specific against nematodes, but the origin of its protein is different from those of the other *Cry* proteins (Ceron et al. 1995, Kenneth et al. 1993). Each class of *Cry* genes is grouped into subclasses (A, B, C...; and a, b, c...) according to amino acid sequence (Agaisse and Lereclus 1995, Kuo et al. 1996).

The Cry genes code for proteins with molecular masses from approximately 50 to 140 kDa, which release insecticidal crystal proteins (delta-endotoxins) of 27 to 140 kDa in the larval midgut (Grochulski et al. 1995, Blizzard et al. 1991). *Cry* I, *Cry* III, *Cry* IV proteins are closely related and their activated toxins share five segments of significant homology, and the *Cry* II and *Cry* V proteins are more distantly related to the other *Cry* proteins and homology in these five regions is weaker, while *Cry* VI proteins display no

detectable homology sequences at all. In spite of the differences in insecticidal spectra and divergence in amino acid sequences among the *Cry* proteins, their mode of action appears to be very similar (Grochulski et al. 1995).

To identify the different *Cry* gene classes, several methods have been employed. One of them is the multiplex PCR *Cry* gene typing method (Ceron et al. 1995). However, this method is not sharpen to identify novel *Cry* type genes. So far, a new method termed the PCR-RFLP typing system has demonstrated to be efficient in detecting both known and novel *Cry* genes. In this method, two pairs of universal oligonucleotide primers, which have the most highly conserved nucleotide sequences, based upon published gene sequences, were designed for PCR amplification. Following PCR amplification, restriction fragment length polymorphism (RFLP) analysis was employed to identify the origin of the *Cry*-type genes (Lopez-Meza et al 1996).

CRY I GENE

Cry I genes have lepidopteran insecticidal activity and are the most thoroughly studied of all δ-endotoxin genes. The *Cry* I genes encode proteinaceous protoxins of 130-140 kDa, which are assembled as bipyramidal crystal inclusions within the bacterial spore. The proteins of these crystals would disperse when exposed to alkaline medium (pH 9-12), as found in the gut of lepidopteran insects. Proteolytic activity from various sources, including both bacterial residue and insecticidal gut juices, is capable of degrading the high molecular weight protoxins by undergoing specific trypsin cleavage to smaller, insecticidally active peptides or the N-terminal toxin of 65-70 kDa (Barton and Miller 1993, Regev et al. 1996).

The resulting toxin comprises three structural domains. The N-terminal domain disrupts the midgut epithelial cell membrane by forming ion pores. The immediate terminus is contained within amino acids 1-29, and the carboxil terminus is contained approximately within amino acids 603-1179 on *Cry* I gene products. The two latter domains are involved in specific folding and interaction with membrane receptors, which is a prerequisite for pore formation and dictates toxin specificity (Barton and Miller 1993, Regev et al 1996).

The individual *Cry* I genes generally display broad toxicity toward a number of different lepidopteran species. In spite of the substantial relatedness of the *Cry* I genes, variations in the *Cry* I protein sequences contribute to differing levels of toxic activity against susceptible lepidopteran insects (Kenneth and Miller 1993, Von Tersch et al. 1991). Six different genes coding for lepidopteran-specific protein have been identified on the basis of DNA sequence. They are *Cry* IA(a), *Cry* IA(b), *Cry* IA(c), *Cry* IB, *Cry* IC and *Cry* ID. The first three genes are closely related, the remaining three genes are not as widely distributed (Blizzard et al. 1991).

***Bacillus thuringiensis* (*Cry*) *Toxin* in Plants**

Formulations of *B. thuringiensis* crystals and spores have been used as biopesticides for over 20 years, but their instability in the environment and relatively high cost incurred by multiple applications have limited grower acceptance (Barton and Miller 1993, Herrera et al. 1994, Li et al.1995). On the other hand, major insect pests are developing resistance to most classes of chemical insecticides and these insecticidal chemicals cause serious environment problems (Li et al. 1995). These facts make the

production of protein with insecticidal activity by the plant itself an attractive alternative for protection (Barton and Miller 1993, Herrera et al. 1994, Li et al. 1995, Wunn et al. 1996).

Recently, some researchers have introduced the crystal protein genes (*Cry* genes) into different plants such as tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicum esculentum* Mill.), cotton (*Gossypium hirsutum* L.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), American cranberry (*Vaccinium macrocarpon* Ait), plants of rutabaga (*Brassica napobrassica* L.), apple (*Malus pumila* cv. *Greensleeves*), strawberry (*Fragaria spp.*), canola (*Brassica napus* L.), amelanchier (*Amelanchier laevis*), populus (*Populus spp.*), walnut (*Juglans regia* L.), cabbage (*Brassica oleracea* var. *capitata*), white clover (*Trifolium spp*), common bean (*Phaseolus vulgaris* L.), chrysanthemum (*Chrysanthemum morifolium*), soybean (*Glycine max* [L.]Merr.), alfalfa (*Medicago sativa* L.) eggplant (*Solanum melongena* L.), rapeseed in order to produce genetically engineered insect resistance plants (Li et al. 1995, Serres et al. 1993, James et al. 1993, Sims et al. 1996, Iannocone et al. 1995, Dolgov et al. 1995, Kleiner et al. 1995, Strizhov et al. 1996, Parrot et al. 1994, Barton and Miller 1993, Herrera et al. 1994, Stewart et al. 1995, Von Tersch et al.1991, Van Aarssen et al. 1995). A common problem encountered by most research groups has been the difficulty in achieving a high level of expression of these prokaryotic *Cry* genes in higher plants (Barton and Miller 1993). Without exception, typically, chimeric *Cry* plant genes have about 1000-fold lower levels of protein than chimeric bialophos resistance (BAR), neomycin phosphotransferase II (NPT II), chloramphenicol acetyltransferase (CAT), or glucoronidase (GUS) genes even being flanked by the same plant regulatory expression

signal (Van Aarssen et al. 1995).

Although, the underlying mechanism restricting *Cry* expression is still not understood, it is believed that plants do not have many of the tRNAs required by bacterial gene codons, and also the low level of *Cry* gene expression in plants is due to a higher A+T (adenine-thymine) content in native *Cry* genes than in plants. These multiple A+T rich motifs resembling plant introns and ATTTA sequence (polyadenylation signal sequence) have been shown to destabilize mRNA in other systems. In addition, the presence of transcription predetermination signals in the *B. thuringiensis* system and the codon usage of native *Cry*-coding sequence, that is very unlike the preferred plant codon usage or rarely used in plants, are among the factors that restrict *Cry* gene expression (Steward et al. 1996, Van Aarssen et al. 1995, Regev et al. 1996, Perlak et al. 1991).

In addition, the problem appeared to be a result of either inefficiency in the synthesis of gene products, or rapid degradation of either the *B. thuringiensis* mRNA or protein following synthesis (Barton and Miller 1993). However, Aarssen et al. (1995) provided some evidences that the level of cytoplasmic import rather than mRNA instability is responsible for the inefficient cytoplasmic accumulation of Bt gene mRNA. He suggested that the increase in mRNA level in transgenic tobacco plants expressing *Cry* IA(b) with a replacement of aberrant native codons by preferred codons and increase in the G-C content, is not necessarily linked to an improved efficient and/or mRNA stability, but could be due to improved nuclear processing and/or transport.

The low expression of wild *Cry* gene in transgenic plants could be sufficient for an insecticidal effect to susceptible insects, but for adequate control of less susceptible insects in the field several approaches have been demonstrated to increase the expression

of these prokaryotic genes (Wunn et al. 1996, Blizzard et al 1991). Those strategies include the truncated form of the gene, modification of the coding sequence (codon-optimized synthetic Bt genes), and the use of strong promoters (Barton and Miller 1993, Steward et al. 1996, Wunn et al. 1991, Blizzard et al. 1991, Wong et al 1992, Carozzi et al. 1992).

Schnepf and Whiteley (1986) reported that a truncated form of the *Cry* gene, encoding only the amino-terminal portion of the protoxin, showed insecticidal activity. Transgenic tobacco and tomato plants carrying a truncated (1.8 kb) *Cry* I gene, expressing the 60 kDa toxin, produced slightly higher levels of Bt protein than transgenic tobacco carrying the full length (3.5kb) *Cry* I gene (Wong et al. 1992, Fishhoff et al. 1987). Transgenic tobacco carrying a truncated form of the *Cry* gene coding 645 amino acids showed levels of toxins generally at 0.001 % of the total cell protein or lower. The range of expression of these genes was typical of chimeric gene expression, with variation in levels of expression due to insertion into differing chromosomal locations (Barton and Miller 1993). The truncated version of Bt genes has a significant toxicity to insect; however, the levels of expression of these genes were too low to provide practical levels of resistance toward less sensitive insect pests such as *Spodoptera exigua* or *Heliothis virescens* (Barton and Miller 1993, Corozzi et al 1992).

Recognizing the high content of A+T in native Bt-*Cry* gene compared with typical plant genes, and the presence of ATTTA as a destabilizing element of mRNA, Perlak et al. (1991) proposed two approaches to enhance the expression of Bt genes (*Cry* IA(b) and *Cry* IA(c)) in transgenic plants of tobacco and tomato through modification of the coding sequence. These two approaches selectively removed DNA sequences from

the truncated wild *Cry* genes by site-directed mutagenesis. In the first, called partially modified synthetic gene (PM), it was done without changing the amino acid sequence. The other strategy, called fully modified synthetic gene (FM), was a more rigorous application of the principle used, taking codon usage in plants, potential secondary structure of mRNAs, and potential regulatory sequences under consideration. Both FM genes and PM genes were synthetic versions of the Bt-*Cry* I genes. The PM gene differs from a truncated wild type (WT) gene by 3% of the nucleotide, but this change was enough to increase more than 10-fold in the insecticidal protein levels in tobacco compared with the truncated WT gene. The FM genes encode proteins nearly identical in amino acid sequence to that of WT gene, eliminating ATTTA sequences, and almost all potential polyadenylylation sequences. In the transgenic plant, the FM genes appeared to be expressed 5- to 10 fold higher than the PM genes and up to 100-fold higher than the WT gene. The higher level of insect control protein in these transgenic plants was directly correlated to increased insecticidal activity (Perlak et al. 1991). Similar results were obtained using modified *Cry* III A (Perlak et al. 1993).

The other strategy to increase the expression of Bt genes is the use of the most efficient promoters. The cauliflower mosaic virus 35S promoter (CaMV 35S) has been a strong promoter in transgenic plants, shown to be active in most plant organs and is considered to be expressed throughout development (Carozzi et al. 1992, Fishhoff et al. 1987). The CaMV 35S promoter has been used in almost all examples of Bt transgenic plants. Carozzi et al. (1992) showed the levels of protein coded by a truncated native *Cry* IA(b) were higher in older than younger tissues, and speculated that flowering may induce higher levels of expression from the CaMV 35S promoter. The expression of this

truncated gene was unaffected by the different environmental conditions found between the field and greenhouse.

Wong et al. (1992) reported that in transgenic tobacco that the ats 1 A promoter with its transit peptide sequence fused to the truncated fully modified *Cry* IA(c) protein provided a 10- to 20-fold increase in *Cry* IA(c) mRNA and protein levels compared to gene constructs in which the cauliflower mosaic virus promoter with a duplication of the enhancer region (CaMV-En35S) was used to express the same Bt gene. The majority of the transgenic plants analyzed with the ats 1 A promoter and a modified transit peptide expressed the *Cry* IA(c) as at least 0.4 % of the total leaf protein (Wong et al. 1992).

So far, the truncated PM and FM genes have profound levels of insect control in transgenic plants (Perlak et al. 1991). However, it is difficult to directly compare the expression levels of these Bt genes in the mentioned reports without knowledge about when the samples were assayed because levels of *Cry* protein are related to the developmental phase of the plant and the age of the leaf tissue (Carozii et al. 1992).

RESISTANCE PROBLEMS

The use or high application of *Bacillus thuringiensis* in the laboratory or in the field has led to isolation of resistance colonies of various insect, such as *Plodia interpunctella* (Hubner), *Cadra cautella*, *Plutella xylostella* (L.), *Spodoptera exigua* (Hubner) and *Heliothis virescens* (F.).

The number of alternative ways to achieve resistance to Bt is as high as the given steps between inclusion indigestion and toxin insertion to the membrane, such as proteolysis of protoxins, activated toxin, binding to the plasma membrane and pore

formation (Perlak et al 1991, Williams et al. 1992, Moar et al. 1995). Changes in solubility and activation properties of crystal protein, as well as decreased affinity of receptor binding account for the development of resistance to Bt (de Maagd et al. 1996).

It has been clearly demonstrated that insects can develop resistance to a crystal protein by changing the binding characteristics of the midgut receptor. Therefore, the resistance to one crystal protein does not necessarily cause resistance to other crystal proteins (Peferoen 1992).

The use of transgenic plants that express Bt genes can increase the selection of Bt-resistant insects. However, some strategies such as combining Bt genes with other host resistance factors, pyramiding Bt with proteinase inhibitor transgenes, use of insect-friendly refuge in space and time may delay the time for insects to acquire resistance to Bt (Williams et al. 1992). Regained sensitivity of a resistant lepidopteran larval populations (resistance attributed to a loss of specific midgut receptors), lead to the strategy of maintaining a certain proportion of wild-type refuge plants or rotation with crops that do not express *Cry* proteins in field of transgenic Bt plants. In addition, co-applications of Bt and chitinase have significantly increased the insecticidal effect of Bt. Therefore, pyramiding Bt gene with some chitinase genes seems a good strategy to avoid resistance in transgenic Bt plants (Regev et al. 1996)

Another strategy to minimize the potential problems of resistance to Bt *Cry* protein is to identify novel *Cry* genes, with the aim of obtaining new proteins that bind alternative membrane receptors on the insect midgut cells. Increasing the diversity of genes available for transgenic plants should increase the efficiency of pest control and delay the emergence of resistance (Gleave et al. 1992).

POTY VIRUS Y (PVY)

Potato virus Y (PVY) is a type member of the family *Potyviridae*, which constitutes the largest known, economically. most important and the most widely distributed group of plant viruses (Thole et al. 1993, Dolja et al. 1994, Audy et al.1993, Dar et al. 1994, Van den Elzen et al.1989, Dinant et al. 1993, Singh et al. 1994, Singh et al. 1995). The Potyvirus group has three distinct genera (*Potyvirus, Bymovirus, Rymovirus)* and one possible genus (*Ipomovirus)*. Potato virus Y (PVY), which is a type species of the genus *Potyvirus*, has three distinct strains based on host range and symptomology. These strains are PVY^0 (common strain), PVY^n (tobacco venial necrosis strain) and PVY^c (stipple streak strain) (Singh et al. 1995 and Dhar et al. 1994).

PVY can cause serious damage in several plant species belonging to the Solanaceae group, it can cause yield losses of up to 70% or 80% in sensitive potato cultivars (Vallejo et al. 1994, Pehu et al. 1995, Dinant et al. 1993). PVY^o causes various degrees of mosaic symptoms. However, among the three main groups of PVY, the tobacco venial necrosis strain of PVY (PVY^n) can cause the most devastating losses and because of these potential losses, PVY^n have been considered a quaranteen species in North America. However, in recent years, outbreaks of PVY^n in tobacco and in potato have been reported from North America (Van de Elzen et al. 1989). PVY^n first appeared in European seed potato growing areas in 1950s causing epidemics, but it probably originated in South America from where it was introduced to Europe, with wild potato species or native cultivars imported for breeding purposes (Van den Elzen et al. 1994).

The PVY genome is a single-stranded messenger-sense RNA molecule of approximately 10 kb with a 5'-terminal genome-linked protein (Vpg) and a poly(A) tract at the 3' end encapsidated into flexuous rods 680 to 900 nm in length and 12 to 15 nm in diameter. The viral RNA of potyviruses is translated into a large polyprotein precursor

(340-360 kDa) which is co- and post-translationally processed by at least three viral proteases (Thole et al. 1993, Dolja et al. 1994, Ohschima et al. 1993, Dougherty and Carrington 1988, Dinant et al. 1993). The PVY non-segmented positive-sense RNA strand is 9.7 kb in length and encodes a large protein, co- or post-translationally processed by viral-encoded proteases into at least eight mature functional proteins. The polyprotein contains the necessary protease activity to cleave itself into eight mature proteins (Van den Elzen et al 1989, Audy et al. 1993, Pehu et al. 1995).

PVY is transmitted by aphids in a non-persistent manner from an infected plant to a healthy plant giving a primary infected plant. At the end of the season the virus migrates down to the tubers. In the next season, seed tubers from the primary infected plant will be the source for secondary infections (Malnoe et al. 1994).

The severity of potyvirus epidemic is determined by the activity of the aphid population early in the season, the presence of primary infected plants serving as viral source and the presence of volunteer potato plants, other solanaceous crop and weeds (Valkonen et al. 1994, Malnoe et al. 1994).

The use of insecticides to eliminate the aphids has been inefficient to control PVY virus because the virus is transmitted in a non-persistent manner. In addition, classical breeding for virus resistant varieties is possible, but difficult because the cultivated potato varieties are tetraploid and highly heterozygous. Therefore, the introduction of coat protein gene in potato plant has improved the traditional methods for obtaining resistant plants against viral diseases (Malnoe et al. 1994).

COAT PROTEIN-MEDIATED RESISTANCE

Sanford and Johnston proposed the concept of pathogen-derived resistance (Audy et al. 1993) defined as the capacity of a host expressing particular parasite sequences to overcome a disease. This concept was applied in plant virology by Powell and collegues who reported that transgenic tobacco plants expressing tobacco mosaic virus (TMV) coat protein showed resistance to TMV infection. The use of pathogen genes is based on the principle that in any given set of host-pathogen interactions, if one of any pathogen-specific functions is disrupted by expressing a pathogen gene at the wrong time, in the wrong amount or in a counterfunctional form, the pathogen process should be stopped (Grumet 1990). However, the underlying mechanism(s) of virus resistance in transgenic plants expressing viral genes is not yet completely understood, and it is likely that there are different mechanisms of resistance in different host/virus combinations and that there may be more than one mechanism in a given system (Reimann-Phlipp and Beachy 1993).

Genetically engineering virus resistance has two potential advantages over the use of host-derived resistance genes; these are:

1. the source of resistance genes would not be limited, as the genome of each pathogen would provide resistance genes, and
2. the smaller genomes and shorter life cycles of a pathogen than those of hosts make the isolation of such genes much simpler (Grumet 1990).

Genetically engineering virus resistance or pathogen-derived resistance has been reported in several plant systems (Grumet 1990). This resistance approach is constituted by several strategies that use viral coat protein gene, movement protein gene, satellite sequence of viruses, viral antisense RNAs, replicase sequence and the complete viral

genome of a mild strain (Grumet 1990, Audy et al 1993, Reimann-Philipp and Beachy 1993). So far, among these distinct strategies the coat-protein-mediated resistance has been generally applicable and is a successful strategy (Grumet 1990). Several groups have demonstrated that expression of viral capsid genes by the host results in either prevented, delayed, or reduced symptoms and systemic spread of infection (Grumet 1990, Grumet and Fang 1993).

Coat protein-mediated resistance has been termed as genetically engineered cross protection, in reference to the hypothesis that the protection conferred by a mild virus strain against subsequent infection by a more severe strain is due to the presence of coat protein form from the mild strain. This role of coat protein in cross protection is supported by the fact that classical cross protection can be overcome by inoculating with viral RNA, and the protection of coat protein transgenic plants is also overcome by inoculating with viral RNA. However, the mechanism of cross protection is not well understood, and some evidence using coat protein mutants, transencapsidated viruses and viroids (which do not have a coat protein) have shown that coat protein is not necessary to confer cross protection. In addition, the classical cross protection as well as the resistances of transgenic plants expressing coat protein of TMV, AIMV and PVX, but not CMV were overcome by increasing the amount of inoculum (Grumet 1990).

MECHANISM OF PROTECTION IN COAT PROTEIN TRANSGENIC PLANTS

Since the initial demonstration that the expression of the coat protein (cp) gene of tobacco mosaic virus (TMV) in transgenic plants could provide effective protection in transgenic plants by Powell et al. (1986), the use of viral coat protein (cp) genes has been

the most widely used strategy for genetically engineered virus resistance to date (Farinelli and Malnoe 1993, Grumet 1994). This strategy for engineering virus resistance has been reported in at least 20 different RNA viruses which vary in particle morphology, genome organization, and mode of transmission (Kavanagh and Spilane 1995).

The mechanism(s) of virus resistance in transgenic plants is not yet completely understood; however, several mechanisms have been proposed, including prevention of uncoating of the incoming virus, interference with viral translation and/or replication, and interference with cell-to-cell and/or long distance movement (Grumet 1994, Reimann-Philipp and Beachy 1993). So far, it is clear that there are different mechanisms of resistance in different host/cp/virus combinations and that there is more than one mechanism in a given system (Reimann-Philipp and Beachy 1993).

These mechanisms could operate in the initial interaction between cp, plant and the challenge virus, an early event in infection prior to replication of the incoming virus ("inhibited-uncoating" hypothesis), or a latter stage during viral movement which can delay disease development and systemic spread (Hackland et al. 1994).

Viral infection starts upon the introduction of plant viruses into its host with release of the nucleic acid for translation by host ribosome. In the first hypothesis, it is believed that coat protein gene expression interferes with the disassembly of the virus (Hackland et al. 1994). Register et al. (1989) postulated two models whereby cp could inhibit uncoating of virions, in one he specified that a site for virus uncoating within the cell would be blocked by endogenous cp. The second model states that virus disassembly is inhibited by shifting the disassembly-assembly reaction in favor of assembly, thereby impeding virus infection. If this were limited to the exchange of cp subunit of the virion

with the endogenous cp in the transgenic cell, then the endogenous cp would be required to mediate this resistance (Clark et al. 1995).

These models are valid in the case of tomato mosaic virus (TMV), rice stripe virus (RSV), alfalfa mosaic virus (AIMV), potato virus X (PVX) and tomato yellow leaf curl virus (TYLCV). The strength of resistance correlated positively with the levels of coat protein in transgenic plants, and plants that accumulated only coat protein transcripts and non-detectable coat proteins were not resistant (Grumet 1995, Grumet 1994, Kavanagh and Spillane 1995). In addition, some studies with TMV, AIMV and TSV showed that resistance was overcome by inoculation with viral RNA. This proposes that cp interferes with the initial uncoating of the virus (Grumet1994, Hackland et al. 1994).

However, for other studies with viruses such as PVX (potato virus X), PVS (carlavirus), and ArMV and GCMV (nepoviruses), the cp-mediated protection is effective against viral RNA as well as whole virions. This suggests that cp affects some other step in addition to uncoating (Grumet 1994). In the case of potato virus Y (PVY) and potato leafroll (PLRV), resistance correlated with the levels of coat protein transcripts and not with levels of coat protein which was undetectable for some virus resistant transgenic lines. But the mechanism of resistance is more complex in the case of TEV and TSWV, virus resistant plants were obtained when an intact nucleocapsid protein transgene was rendered untranslatable through removal of the initiating ATG codon (Kavanagh and Spillane 1995).

Nejidat and Beachy (1989) examined the levels of TMV cp in transgenic tobacco plants under different temperature conditions and concluded that increase in continuous temperature resulted in a decline in cp accumulation, but not in the accumulation of cp

mRNA, and reduction in the protection against TMV. This involves the cp and not the mRNA in this protection mechanism. However, in transgenic tomato under the same condition, protection did not decrease. This indicates that the mechanisms of protection can differ with the different plant host (Nejidat and Beachy 1989, Hackland et al. 1994).

To test the inhibiting uncoating hypothesis, Osburn et al. (1989) encapsidated the chimeric reporter mRNA encoding β-glucuronidase (GUS) in TMV cp to form "pseudovirus" particles. When the TMV-like particles were introduced into protoplasts they uncoated and expressed their non-replicating "seudo-genomes" transiently. In this way, the level of expression was a direct measure of the extent of pseudovirus disassembly. The fact that GUS particles were expressed 100-fold less efficiently in cp(+) transgenic protoplasts and unencapsidated GUS mRNA was expressed only 2.8-fold less efficiently showed that the endogenous cp must interfere with the inhibition of the (GUS) nucleocapside disassembly and a later stage of infection involving the viral RNA (Hackland et al. 1994).

Another manifestation of cp-mediated protection is the delay of the spread of virus (known as interference of later events of infection) from inoculated leaves to upper leaves. In a systemic viral infection in plants, viruses replicate and move short distances from cell to cell through the plasmodesmata requiring viral encoded movement proteins (MP). For long distance movement, viruses move through the vascular system, and viral genes and their products seem to be required for it. Therefore, several groups reported a delay in the development of systemic disease symptoms after inoculation of cp(+) transgenic plants with the respective viruses. This is evidence that the cp interferes with: 1) the spread of viruses from cell to cell in the inoculated tissue; 2) the movement of

viruses from inoculated leaf into the vascular tissue; 3) the movement through the vascular tissue, and 4) the movement into upper or lower non-inoculated leaves.

To determine whether long distance movement was reduced in cp(+) tissue, Wisniewski et al. (1990) initiated several grafting experiments. Using non-transgenic tissue as rootstock, and apical section from cp(+) plants with and without leaves, and cp(-) plants, he reported that the long distance movement of TMV was reduced and the symptoms were fewer in grafted plants that contained a cp(+) stem section with leaves compared with those with the cp(-) stem section. However, grafted plants with cp(+) apical section without leaves showed no effect on TMV spread. It was suggested that the leaf in cp(+) tissue acted as a virus sink. After entering these leaves, these viruses may not be able to establish an infection, reducing the virus titer and disabling further spread.

Wisniewski et al. (1990) also reported that the spread of TMV to closely adjacent tissues (1-3 mm) was similar in cp(+) and cp(-) sections, but spread to more distant tissues (5-10 mm) was significally reduced. Since the cp-mediated resistance can be overcome with RNA in the case of TMV, the adjacent cells should become infected. This evidence suggests that the mechanisms of preventing initial infection in cp-mediated resistance differs from those of preventing long distance movement of infection within the plant.

Clark et al. (1990) compared plant lines expressing similar levels of TMV cp from rbcS and CaMV 35S promoters and found that those whole plants with the CaMV 35S:cp constructs exhibited higher resistance than those with the rbcS:cp constructs. This result suggests that whatever the transport form, it is likely that the presence of cp in the phloem and associated cells interferes with viral long-distance spread, since the CaMV

35S promoter is highly active in phloem-associated cells. Therefore, there is more than one mechanism in cp(+) plants, and these proposed mechanisms active during early events in virus infection could provide further protection against virus multiplication; therefore, no rule can be made for the spectrum of resistance delivered by cp genes (Hackland et al. 1994).

On the other hand, protection in transgenic plants given by viral coat protein can extend to related viruses. It has been reported that trangenic plants expressing the coat protein (cp) gene of a given virus were protected against infection by that virus (homologous virus) and this protection was extended to related strain or viruses (heterologous virus) (Grumet and Fang 1993). For example, transgenic tobacco expressing the coat protein gene of soybean mosaic virus (SMV) to which tobacco is a non-host species showed protection to PVY and tobacco etch virus (TEV). Similar results have been reported with viruses which share approximately 60 % or greater amino acid sequence homology with the TMV coat protein (Kavanagh and Spillane 1995).

Dinant et al. (1993) reported that transgenic tobacco plants, *Nicotiana tabacum* cv. Xanthi, containing a modified coat protein gene of LMV showed heterologous resistance to five different strains of potato virus Y. This group observed different phenotypes, including lines with complete resistance, delay and attenuation of symptoms in some lines, and delay in symptoms with no modification of symptoms in one line. In the two latter cases, the accumulation of PVY and symptoms were reduced. The PVY and LMV cp's are clearly distinct, with a 66% amino acid sequence homology; however such high resistance was achieved (Dinant et al. 1993).

CONSTRAINTS IN THE USE OF CP-MEDIATED PROTECTION

The scientific community and the public in general have expressed a number of concerns about the release of genetically engineering organisms for crop production. Field testing has followed strict USDA-APHIS guidelines, and these field trials try to assess the possible impact of these engineered plants on agricultural ecosystems (Grumet 1994).

These experiments have focused on the spread of transgenes into related species (mainly by pollen), and on the transfer of the transgenes to microorganisms. However, the possible appearance of new viral strains and diseases by heterologous encapsidation or by template switching (recombination) are other concerns in the case of cp-mediated protection also need to be taken into consideration (Grumet 1994, Farinelli et al. 1992).

Transencapsidation or heterologous encapsidation refers to the potential for altered vector-specificity due to the encapsidation of an incoming virus in the coat protein being produced by the transgenic plant (Grumet 1994). Heterologous encapsidation is a natural process that takes place when two virus strains infect the same plant and the genomic RNA of one strain becomes totally (transencapsidation) or partially encapsidated (mixed encapsidation or phenotypic mixing) by cp's of the other strain. The transcapsidated particle can have altered ways of transmission (Farinelli et al. 1992).

There are several examples of transencapsidation. The RNA from cp minus mutant TMV was inoculated onto transgenic TMV-cp expressing plants, the progeny viruses were capable of spreading further within the transgenic plants than within inoculated control plants. From the transgenic plants, but not the control, encapsidated viral particles were isolated and visible by electron microscope (Grumet 1994).

In another study, Farinelli et al. (1992) infected PVY^n-cp transgenic tobacco plants that were completely or partially resistant to PVY^n with PVY^o. Electron microscopy of virus particles isolated from transgenic plants showed a mixture of viral particles with the two cp types.

The potential risk associated with this phenomenon must be evaluated carefully. However, the transencapsidation is a natural process that seems far more likely occur in non-transgenic plants infected with several viruses than in transgenic plants. In fact, infected non-transgenic plants have higher levels of cp than cp-transgenic plants (Grumet 1994).

To circumvent transencapsidation, cp-mediated resistance could use mutant cp genes from non-transmissible virus strains or use truncated cp genes lacking transmission-important domains (Grumet 1994).

The other potential risk in the cp-mediated resistance is termed template switching, copy choice or recombination. By this mechanism, the transgenic RNA could become incorporated into viral genome of the incoming virus. The incorporation of cp sequences into the genome of RNA virus could in theory lead to appearance of new viral strains with altered virulence, host range or transmission (Grumet 1994, Farinelli et al. 1992).

It is believed that polymerase begins on one strand of RNA and then moves to another. Several groups have isolated recombinant viruses from transgenic plants inoculated with a virus mixture under strong selection (Grumet 1994).

However, to test for recombination in non-selective conditions could reflect the real situation of transgenic plants. Under this consideration, Angenent et al. (1989) failed

to detect recombinants using tobaviruses. This suggests that the strong selection pressure favors recombination.

Recognizing that recombination is the incorporation of transgenic RNA into viral RNA in transgenic plants, the potential risk could be even greater in viral mixed-infected non-transgenic plants, because they contain higher viral RNA levels than those observed in transgenic plants (Grumet 1994).

OBJECTIVES

The main goal of this research was to study the inheritance of the PVY^O coat protein and the truncated wild type Bt-*Cry IA(c)* transgenes, in tetrasomic potato lines.

The specific objectives of this approach were:

- Confirm the incorporation of these foreign genes, PVY^O coat protein gene and the truncated *Cry IA(c)*, in potato clones (cv. Atlantic and FL-1607) through PCR and Southern blot analysis.
- Determine if the segregation of *Bacillus thuringiensis*_(*Cry IA(c)*) gene carried by FL1607 potato lines, and PVY coat protein genes carried by Atlantic, follow tetrasomic ratios.
- Determine if crosses between transgenic plants carrying Bt genes and plants carrying the PVY coat protein gene and cultivated varieties yield progeny carrying these genes.

MATERIALS AND METHODS

Plant materials

All potato clones were obtained from the MSU Potato Breeding and Genetics Program. The transgenic plants contain either a truncated wild type version of Bt-*Cry IA(c)* gene (FL1607-A30) or one or more copies of PVY^{O} coat protein genes (ATL-PVY5). Figures 1 and 2 show the two constructs carried by the Bt-transgenic plants and the PVY cp transgenic plants, respectively.

The plants were grown *in vitro* in GA-7 Boxes (Magenta Corporation) before transplanting, and six plants of each transgenic clone were transplanted to planting medium in the greenhouse. After one month, 4 plants per clone were selected and each of these plants was transplanted to a 4 liter plastic pot.

Controlled crosses and self-pollinations were made between Bt-transgenic plants (FL1607-A30), PVY CP transgenic plants (ATL-PVY5), and the non-transgenic plants (Lemhi Russet and MS702-80). The fruits were harvested, seeds were collected, cleaned and planted in the greenhouse. The progeny plants were used for segregation analysis of the transgenes (Table 1). Leaf tissue from the transgenic plants was also used for DNA isolation.

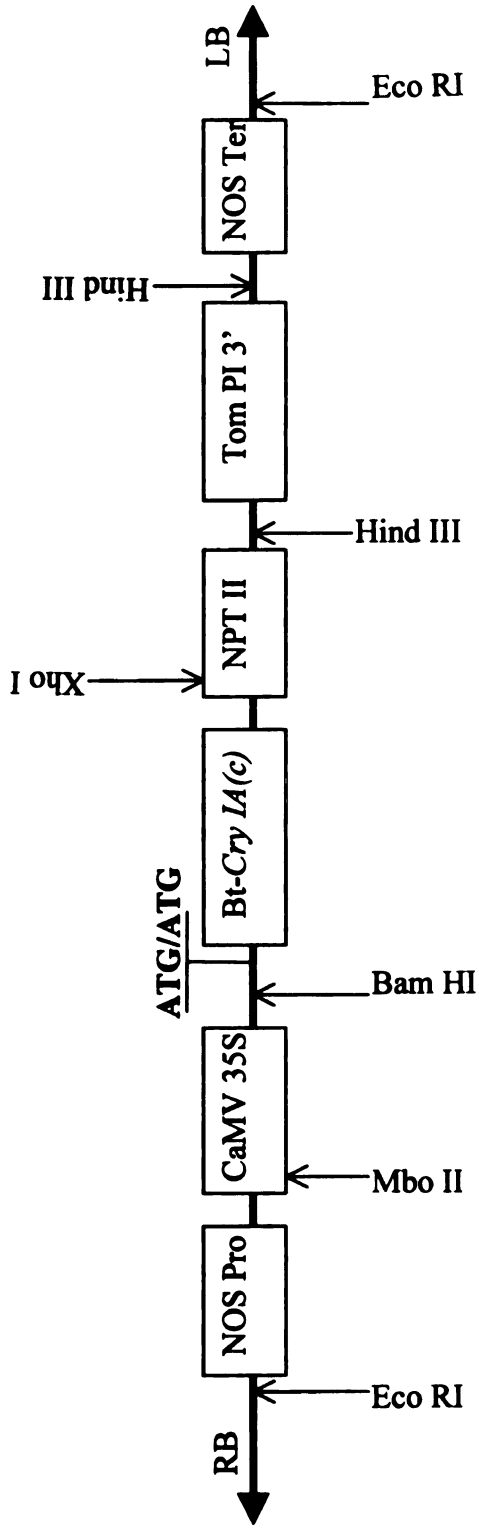

Figure 1. Diagram of the plasmid pWB139 Bt/Kan (not to scale).
The construct was obtained from Dr. Wayne M. Barnes of Washington University, St. Lois, MO.
This plasmid is based on the pRK252.
NOS Pro: Nopaline synthase promoter.
NOS ter: Nopaline synthase terminator.
CaMV 35S: Cauliflower mosaic virus promoter.
Bt-*Cry IA(c)*: Truncated version of the gene which codes the 68 kDa B.t. toxin.
NPT II: Neomycin phosphotransferase II from Tn5.
Tom PI 3': Tomato protease inhibitor I 3' end.

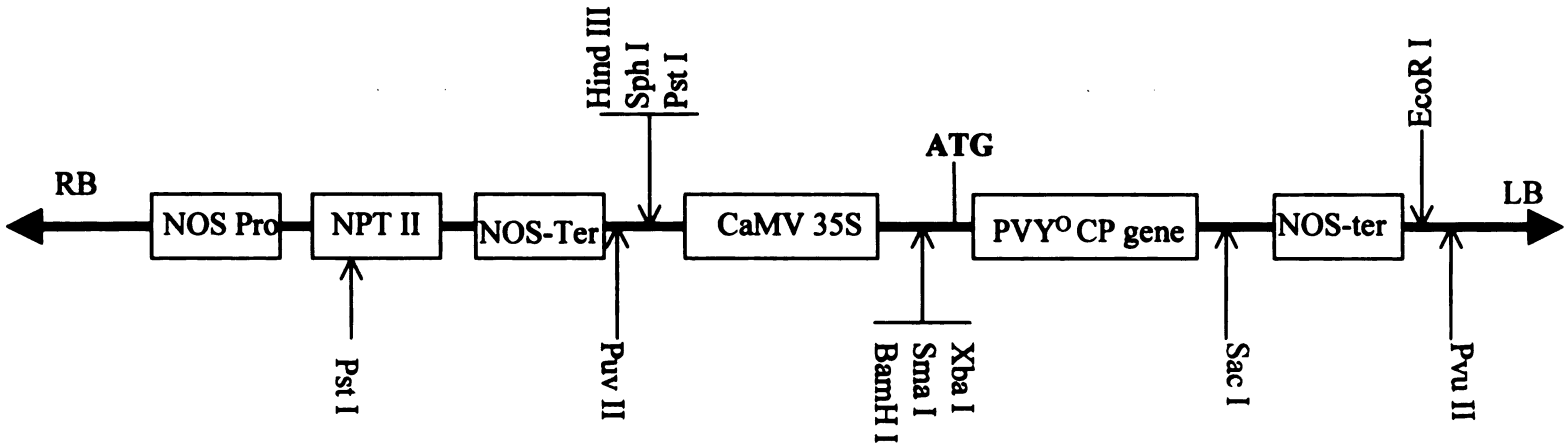

Figure 2. Diagram of the plasmid PVY/PBI 121(not to scale).
The construct was obtained from Dr. T. German, Department of Plant Pathology, University of Wisconsin at Madison.
The PVY CP gene was inserted in place of the GUS gene in the pBI 121.
NOS Pro: Nopaline synthase promoter.
NOS ter: Nopaline synthase terminator.
NPT II: Neomycin phosphotransferase II from Tn5.
CaMV 35S: Cauliflower mosaic virus promoter.
PVY^O CP gene: Mutated coat protein gene of PVY^O that have a Sma I restriction site at the 5'end, a Sac I restriction site at the 3'end and a change from glycine to glutamate at the amino acid triplet that confers aphid transmissibility.

Table 1. Crosses used in PCR analysis

Crosses (female x male)	Segregating transgenes	Number of progeny
FL1607-A30 x ATL-PVY5	Bt	67
	PVY CP	80
	NPT II	66
ATL-PVY5 x FL1607-A30	Bt	74
	PVY CP	69
	NPT II	0
ATL-PVY5 x MS702-80	PVY CP	66
	NPT II	66
ATL-PVY5 x Lemhi Russet	PVY CP	31
	NPT II	31
Lemhi Russet x ATL-PVY5	PVY CP	35
	NPT II	35
ATL-PVY5 (x)	PVY CP	35
	NPT II	31

Southern blot analysis:

Genomic DNA was extracted by the CTAB method (Saghai-Maroof *et al.* 1984) modified by adding 2% beta-mercaptoethanol to the extraction buffer. DNA (20 μg) from ATL-PVY5 clone was digested with *BamHI* and *EcoRI* to cut the PVY gene and copy number was analyzed by digesting with just *BamHI* .

The fragments were separated by electrophoresis through a 1.0% agarose gel, eluted onto a nylon membrane (Hybond N, Amersham, England) using a capillary transfer procedure (Sambrook et. al.1989). Nucleic acids were fixed to the membrane by auto-cross-linking.

Prehybridization was conducted for 2 h at 42°C in the solution containing 5X SSC (0.75 M of NaCl, 0.075 M of NaCitrate, at pH 7), 1% skim milk, 0.1% N-lauroylsarcosine, 0.02% SDS, 50% formamide and 125 μg/ml sheared salmon sperm DNA. Hybridization was performed at 42°C overnight in fresh solution with a non-radioactive random primed DIG-labeled probe (1.2kb SmaI\SacI fragment of the PVY coding region was inserted into a Bluescript SK+ vector [Stratagene Inc. La Jolla CA] amplified by PCR using the T7\T3 primers) according to manufacture's instructions (BMB, Indianapolis, IN). Following hybridization, the membrane was washed twice in 2X SSC, 0.1% SDS for 15 min at room temperature, twice in 0.5 X SSC, 0.1% SDS for 20 min at 65°C. CSPD (BMB, Indianapolis, IN) was used for chemiluminescence detection by following the manufacturer's instructions. The membrane was then exposed to X-ray film (Kodak X-OMAT) for 15-30 min.

Isolation of DNA for PCR analysis

DNA was extracted from fresh potato leaves following the quickprep isolation procedure, a modified version of Edwards et al. (1991). Two or three disks of leaf tissue

were punched out with the lid of a sterile 1.5 ml Eppendorf tube. CTAB (400 µl) extraction buffer (0.1M Tris HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA and 2% cetyltrimethylammonium bromide) and 1% of beta-mercaptoethanol were added. The tissue was macerated with a sterilized pestle, and then incubated at 65 °C for 20-30 minutes. Sample DNAs were extracted adding 400 µl of chloroform:isoamyl alcohol (24:1) to each tube and mixing the two layers for 1 min. Then, the tubes were centrifuged in a microfuge (14,000 rpm) for 5 minutes. The top (aqueous) layer was transferred to a new sterile Eppendorf tube, and the nucleic acid was precipitated by mixing the aqueous phase with 400 µl of cold isopropanol. The nucleic acids were recovered by centrifugation in a microfuge (14,000 rpm) for 5 minutes, the supernatant was discarded and the small white pellet at the bottom of the tube was dried in a Speed-vac for 10 minutes and resuspended in 100 µl of $T_{10}E_{0.1}$(10 mM tris-acetate and 0.1 mM EDTA) . Single-strand RNA was digested with 4 µl of Rnase A from bovine pancreas (10 mg/ml stock solution) for 15 minutes at room temperature. The DNA is then precipitated with 300 µl of cold 95% ethanol, mixing gently the tubes by inversion, and the tubes were centrifuged in a microfuge (14,000 rpm) for 10 minutes. The supernatant was discarded and the pellet was dried in the Speed-vac for 10 minutes and resuspended in 50 □l of $T_{10}E_{0.1}$. The samples were maintained in a freezer at -20 °C .

Quantification of DNA

The amount of DNA in each sample was measured by the flourometric method, using a Hoefer Scientific Miniflourometer Model TKO100 (Hoefer Scientific, San Francisco, California).

The 10X TNE Buffer (100 mM of Tris, 10 Mm of EDTA-Na_2, 1.0 M of NaCl and pH to 7.4 with conc. HCl) was diluted to 1 X and 10 µl of fluorescence dye, Hoeschst 33258 (0.1 µg/ml stock solution) was added to form the dye solution. The sample readings were taken adding 2 µl of sample DNA to the 2 ml of dye solution. The cuvette was rinsed with filtered (0.22 µm) water between each reading. The extracted DNA samples were standardized to a uniform concentration of 100 µg/µl or to 50 µg/µl otherwise.

Polymerase Chain Reaction (PCR)

The presence of the transgenes was verified in the parental material through PCR and Southern analyses. In addition, potato plants within each segregating population were chosen at random for PCR-based analysis of segregation of the three transgenes (NPT II, Bt-*Cry IA(c)* and PVY coat protein genes).

PCR reaction was performed in a volume of 25 µl, amplifying each transgene separately, following the recommendation for amplification with Taq DNA polymerase (Gibco BRL, Life Technologies Inc, MD, USA). For Bt-*Cry IA(c)* gene, the 25µl of PCR reaction contained 1X PCR buffer (20 mM of Tris-HCl pH 8.0 and 50 mM of KCl), 0.2 mM of dNTP mix (0.2 mM of each dNTP), 1.5 mM of $MgCl_2$, 0.625 unit of Taq DNA polymerase, 0.6729 µM of Btk-802 primer, 0.6688 µM of Btk-1634 primer, 150 ηg of sample DNA template. The primer sequences (5'-3') are shown in Table 2.

For the PVY coat protein gene, the PCR reaction had 1X buffer buffer (20 mM of

Table 2. Primers used in the PCR analysis

Gene	Name of the Primer	Primer Direction	Sequence (5' to 3')	Nucleotide Bases	Amplification Product (bp)
Bt-*Cry I A(c)*	Btk 802	Forward	AGTGCCCTTACAACCGCTATTCTTC	25	450
	Btk 1634	Reverse	TACTTCTTTCTATGCCCTGAGCCGA	25	
PVY CP	DAD 31	Forward	CTCGGGCAACTCAATCATAGTTT	23	500
	DAD 30	Reverse	TCGGTGGTGTGCCTCTCTGTGTTC	24	
NPT II	DAD 29	Forward	CGCACCTTCTCCGGCCGCTTGGGTGG	26	255
	DAD 28	Reverse	AGCAGCCAGTCCCTTCCCGCTTCAG	25	

Tris-HCl pH 8.0 and 50 mM of KCl), 0.2 mM of dNTP mix (0.2 mM of each dNTP), 1.5 mM of $MgCl_2$, 0.625 unit of Taq DNA polymerase, 0.5058 μM of DAD 30 (formerly DAD 19) primer, 0.5066 μM of DAD 31 (formerly DAD 18), 150 ηg of sample DNA template. The primer sequences (5'-3') are shown in Table 2.

For NPT II gene, the 25 μl of PCR reaction contained 1X buffer (20 mM of Tris-HCl pH 8.0 and 50 mM of KCl), 0.2 mM of dNTP mix (0.2 mM of each dNTP), 1.5 mM of $MgCl_2$, 0.625 unit of Taq DNA polymerase, 0.5004 μM of DAD 28 (formerly DAD 16) primer, 0.5064 μM of DAD 29 (formerly DAD 17) primer, 100 ηg of sample DNA template. The primer sequences (5'-3') are shown in Table 2.

The PCR components were added to a sterile 0.5 ml microfuge tube, then briefly centrifuged and overlaid with a 50 μl layer of sterile mineral oil. The reaction conditions for the three transgenes was: 30 cycles of 1 minute at 94 ^{o}C (denaturation); 1 min. at 58 ^{o}C (annealing); and 1.5 minutes at 72 ^{o}C (extension). Once completed, the tubes were held at 4 ^{o}C until loading on a gel.

The amplifications of DNA template were performed in a Perkin Elmer Cetus DNA Thermal Cycler 480. Two negative controls (one without DNA template and the other with non-transgenic plant DNA) and one positive control (transgenic parental plant DNA) were place in the machine for each set of reactions.

Reaction products were resolved by electrophoresis in a 1% (v/w) agarose gel at 80 V for 2 to 3 hours at room temperature. The 300 ml Tris-acetate (0.04 M of Tris-acetate, 0.001 M of EDTA) gel was stained with a 10 μl solution of ethidium bromide (10 mg/ml stock concentration) at a final concentration of 0.33 μg of ethidium bromide/ml of

gel. Before loading the PCR products into the gel, 2.5 µl of 10X stop buffer (10mM of Tris-HCl [Tris(hydroximethyl)aminomethan-HCl] pH 7.5, 1 mM of EDTA pH 8.0, and 0.025% bromophenol and 50% of glycerol) were added to each tube.

The PCR fragments were observed and photographed on a UV transilluminator (Gel print 2000I, Biophotonics). The expected amplification fragment sizes were 225 bp, 450 and 500 for NPT II, Bt and PVY, respectively (Figure 3). The presence or absence of these bands (visualized using UV light) was interpreted as presence or absence of the transgenes.

Kanamycin assay

Kanamycin resistance of transgenic potato plants in the segregating populations was determined through seed germination on a solidified general tissue culture propagation medium enriched with 100 mg/l of kanamycin. One liter of this media contained 4.4 gm of Murashige and Skoog basal salt mixture (Sigma, M5519), 30 gm of sucrose, 1 ml of Ca-panthothenic acid (2.0 mg/ml) 1.0 ml of gibberellic acid (0.25 mg/ml) and 8 gm of Bacto-agar, pH 5.6. Four ml of kanamycin stock solution (5mg/ml) was added to 200 ml of media through a single use 0.45 µm filter unit (Millex-HA Millipore), after autoclaving for 30 minutes. Approximately 50 ml of the kanamycin medium was poured in each Petri dish (100 x 20 mm) under sterile conditions.

One hundred twenty five seeds per each cross were selected at random and placed in Petri dishes (25 seeds per Petri dish). Before planting, the seeds were sterilized with a Clorox solution that contained 10 % of commercial Clorox (Sodium hypoclorite 5.25 %) and 3 drops of Tween-20 for 10-15 minutes. Only one hundred seeds were scored for the

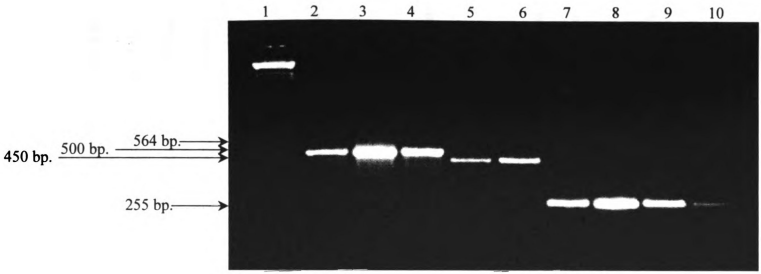

Figure 3. PCR amplification products.

Lanes:

1.- λDNA digested with Hind III
2.- Amplification of PVY CP gene from ATL-PVY5
3.- Amplification of PVY CP gene from the plasmid pBS-PVY-Amp^r
4.- Amplification of PVY CP gene from the plasmid pBI-PVY
5.- Amplification of Bt-*Cry IA(c)* gene from FL1607-A30
6.- Amplification of Bt-*Cry IA(c)* gene from an individual in the FL1607-A30 x Lemhi Russet progeny
7.- Amplification of npt II gene from the plasmid pBS-PVY-Amp^r
8.- Amplification of npt II gene from the plasmid pBI-PVY
9.- Amplification of npt II gene from FL1607-A30
10.- Amplification of npt II gene from ATL-PVY5

number of germinated and non-germinated seeds, dead plants, and resistance plants after 6 weeks.

Statistical Analysis

Chi-square and goodness-of-fit for binomial analysis were used to test the segregating generation to tetrasomic ratios. The non-significant null hypotheses were not rejected when the probability was equal to or greater than 0.05.

RESULTS

The presence of Bt-*Cry IA(c)* in the clone FL1607-A30 was confirmed by Southern blot analysis. The labeled *Cry IA(c)* probe hybridized one fragment of genomic plant DNA digested with *Hind III* or with *BamHI*. This indicated the presence of one copy of the Bt-*Cry IA(c)* gene in FL1607-A30 (Figure 4 and 5).

The Southern blot analysis of ATL-PVY5 showed 2 copies of the PVY coat protein gene. When the DNA from ATL-PVY5 was digested with *BamHI* and *EcoRI*, the labeled probe hybridized only one restriction fragment. Therefore, the presence of one band on the revealed X-ray film indicated that these two restriction sites of the enzymes flank the PVY cp gene. However, the presence of two revealed bands, when only *BamHI* was used, suggested the insertion of two copies in the ATL-PVY5 clone (Figures 6 and 7).

The potato clone ATL-PVY5 was positive for the NPT II gene and the PVY cp

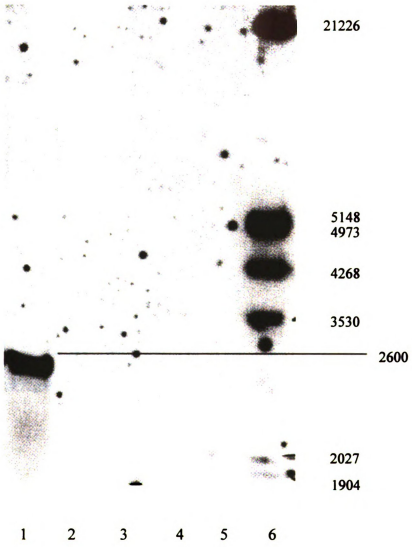

Figure 4. Southern blot for Bt-*Cry IA(c)* using *HindIII* .
The labeled probe, a 256 bp fragment from the *Cry IA(c)* gene hybridized a fragment of 2.6 kb in the transformed FL1607-A30

.

Lane 1: FL1607-A30.
Lane 2 to 5: Untransformed FL1607.
Lane 6: *Lambda* DNA digested with *HindIII* and *EcoRI* to yield a molecular weight marker.

All numbers to the right are base pairs.

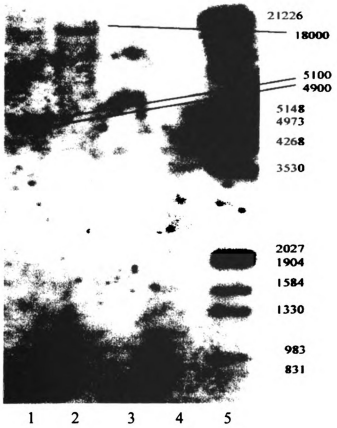

Figure 5. Southern blot for Bt-*Cry IA(c)* using *BamHI*. The labeled 256 bp fragment from *CryIA(c)* gene (the probe) hybridized a fragment of 18000 bp in the FL1607-A30 line and Two fragments in the line FL1607-A11.
Lane 1: FL1605-A11
Lane 2: FL1607-A30
Lane 3: Un-transformed FL1607
Lane 4: Blank.
Lane 5: Molecular weight marker: *Lambda* DNA digested with *BamHI*

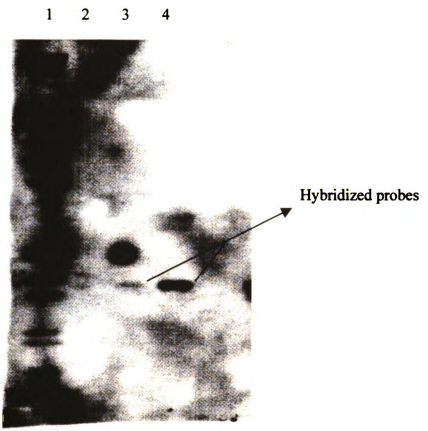

Figure 6. Southern blot for PVY cp gene using *BamHI* and *EcoRI*.
The labeled probe hybridized a fragment of 1584 bp in the ATL-PVY5 and Desiree-PVY5
Lane 1: Molecular weight marker: *Lambda* DNA digested with .....
Lane 2: Non-transgenic potato cv."Atlantic"
Lane 3: ATL-PVY5
Lane 4: Desiree-PVY5

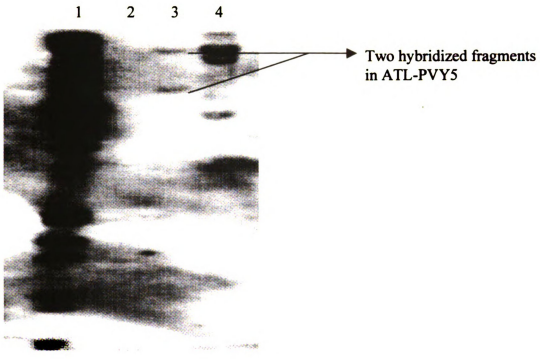

Figure 7. Southern blot revealed film for PVY cp gene using *BamHI*.

Lane 1: Molecular weight marker: *Lambda* DNA digested with……
Lane 2: Non-transgenic potato cv."Atlantic"
Lane3: ATL-PVY5
Lane 4: Desiree-PVY5

gene, while the clone FL1607-A30 was positive for the NPT II gene and the Bt-*Cry IA(c)* gene based upon separate PCR amplification reactions. The non-transgenic potato cv. Lemhi Russet was negative for both genes (Figure 8, 9 and 10).

The cultivated potato is considered to be an autotetraploid with a genome of 2n=4x=48. Traits inherited in a tetrasomic manner from autotetraploids are much more complex than traits inherited in a disomic manner from diploids (Poehlman and Sleper 1995). In a two allele model, there are five possible genotypes at a locus in an autotetraploid. These can be defined on the basis of the number of dominant or recessive alleles present as nulliplex (no dominant alleles: aaaa), simplex (one dominant allele: Aaaa), duplex (two dominant allele: Aaaa), triplex (three dominant alleles: AAAa) and quadriplex (only dominant alleles: AAAA). If there are four alleles at a locus, the different combinations are termed nulliplex (aaaa), simplex (aaab), duplex (aabb), trigenic (aabc) and tetragenic (abcd). The gametes obtained from a tetraploid are diploid (2x), and the different gametes depend on the parental genotype. For example: only aa gametes are expected from nulliplex; aa and Aa gametes in a ratio of 1:1 are expected from a simplex; aa, Aa and AA gametes in a ratio of 1:4:1 are expected from a duplex; and so on.

The segregation pattern is even more complex in transgenic potato plants that have multiple transgenes that segregate independently. One copy of a transgene should behave as a single allele in a specific locus (simplex), and two copies of a transgene should behave as two alleles at two different loci (several simplex loci), if there is no linkage between them. In this way, for one copy of any transgene, the expected segregation ratio would be 1:1 (non-transgenic:transgenic) in a progeny from the cross

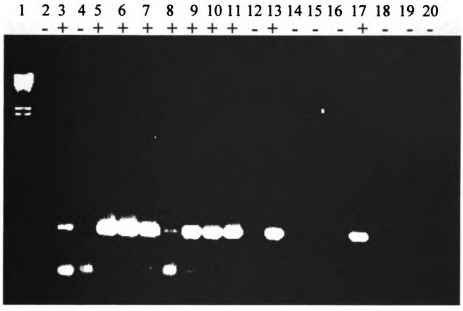

Figure 8. PCR amplification for NPT II gene.

Lanes:
1.- λDNA digested by Hind III
2.- reaction without DNA template
3.- Transgenic ATL-PVY5
4.- Non-transgenic potato cv "Atlantic"
5.- Transgenic FL1607-A30
6 to 20.- Progeny from FL1607-A30xATL-PVY5

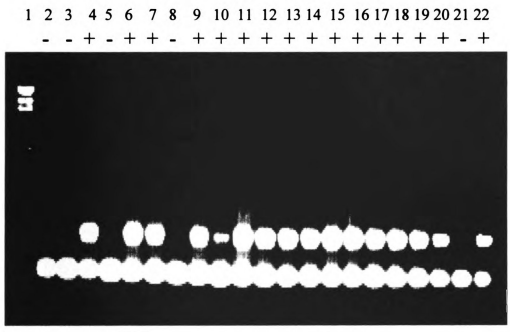

Figure 9. PCR amplification for Bt-*Cry IA(c)* gene.

Lanes:
1.- λDNA digested by Hind III
2.- Transgenic ATL-PVY5
3.- Non-transgenic potato cv "Atlantic"
4.- Transgenic FL1607-A30
5.- Reaction without DNA template
6 to 22.- Progeny from FL1607-A30xATL-PVY5

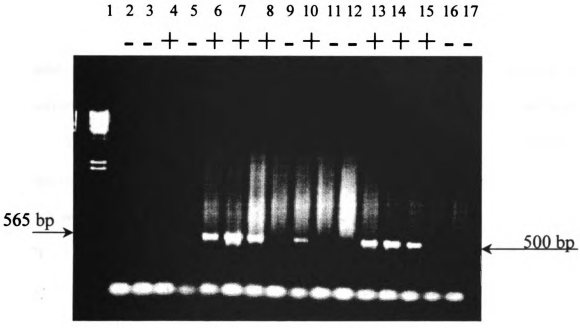

Figure 10. PCR amplification for PVY Coat protein gene.

Lanes:
1.- λDNA digested by Hind III
2.- Non-transgenic potato cv "Atlantic"
3.- Transgenic FL1607-A30
4.- Transgenic ATL-PVY5
5.- Reaction without DNA template
6 to 17.- Progeny from ATL-PVY5 x Lemhi Russet

between transgenic and non-transgenic tetraploid potato plants, 1:3 (non-transgenic:transgenic) in a progeny from a self-pollinated transgenic tetraploid potato plants. To determine the expected ratio of any segregating population for any copy number, we need to consider each copy as an independent locus and the probability of a genotype in the offspring will be the product of the individual probabilities of each copy.

To verify the presence of one copy of the Bt gene in FL1607-A30 clones, the progenies of ATL-PVY5 x FL1607-A30 and of the reciprocal cross FL1607-A30 x ATL-PVY5 were scored for the amplification of the Bt gene by PCR. The progeny of ATL-PVY5 x FL1607-A30 segregated in a ratio of 1:1 for presence:absence of the amplified band of the Bt gene. However, progeny of the reciprocal cross had a ratio that deviated from the expected 1:1 ratio for one copy in the donor parent but not from the 3:1 ratio (Table 3).

These two reciprocal cross families were scored also for the presence of the PCR amplification band for the PVY cp gene. The ratio for presence:absence of the PCR amplification band of the PVY cp gene in the ATL-PVY5 x FL1607-A30 progeny, and the reciprocal cross progeny (FL1607-A30 x ATL-PVY5) fit the 1:1 ratio. However, both segregating populations had ratios that deviated significantly from the Mendelian ratio of 3:1 expected for two independent copies of the PVY cp gene in the donor parent (Table 3).

The segregation of NPT II gene should be 3:1 (presence:absence) for progenies in which both parents have one copy of the gene or 7:1 for progenies in which one parent has one copy and the other has two copies. The FL1607-A30 x ATL-PVY5 deviated significantly from the expected ratio (7:1) but fit the ratio 3:1 (Table 3). This indicated

Table 3. Test of Goodness of fit for binomials in the segregating populations

Segregating Population (female x male)	Transgene	Observed Ratio (presence: absence)	Copy Number	Expected Ratio (pres.:abs.)	Goodness of Fit for Binomials (X^2cc)	$X^2_{0.05,[1]}$
FL1607-A30 x ATL-PVY55	Bt	46:21	1	1:1	8.597 **	3.8415
			2	3:1	1.119 N.S.	
			3	7:1	20.062 **	
	PVY CP	37:43	1	1:1	0.1286 N.S.	
			2	3:1	17.1428 **	
	NPT II	46:20	1 from each parent	3:1	0.7272 N.S.	
			1 from one parent and 2 from the other	7:1	17.5324 **	
ATL-PVY5 x FL1607-A30	Bt	43:31	1	1:1	1.6351 N.S.	
			2	3:1	10.3784 **	
	PVY CP	37:34	1	1:1	0.0563 N.S.	
			2	3:1	18.6338 **	
ATL-PVY5 x MS702-80	PVY CP	34:32	1	1:1	0.0151 N.S.	
			2	3:1	18.1818 **	
	NPT II	37:29	1	1:1	0.7424 N.S.	
			2	3:1	11.6363 **	
ATL-PVY5 x Lemhi Russet	PVY CP	15:16	1	1:1	0.00 N.S.	
			2	3:1	10.4734 **	
	NPT II	16:15	1	1:1	0.00 N.S.	
			2	3:1	7.9641 **	

Table 3 (cont'd) Test of Goodness of fit for binomials in the segregating populations

Lemhi Russet x ATL-PVY5	PVY CP	21:14	1	1:1	1.0285 N.S.	3.8415
			2	3:1	3.4380 N.S.	
	NPT II	15:20	1	1:1	0.4571 N.S.	
			2	3:1	17.6095 **	
ATL-PVY5 x ATL-PVY5	PVY CP	23:12	N/A	1:1	1.1496 N.S.	
			1	3:1	42.27 **	
	NPT II	16:15	N/A	1:1	0.00 N.S.	
			1	3:1	7.9641 **	

Goodness of Fitness for Binomials $X^2cc=\Sigma(([obs.-exp.]-0.5)^2)/exp.$

N.S: No significant difference from the null hypothesis. Where $X^2cc< X^20.05[1]$

*: Significant difference from the null hypothesis. Where $X^2cc> X^20.05[1]$

**: Highly significant difference from the null hypothesis. Where $X^2cc> X^20.01[1]$

the presence of one copy of the gene or a possible linkage between copies.

The proportion of individuals that had both PVY cp and Bt transgenes in ATL-PVY5 x FL1607-A30 progeny fit in the 25% of the total segregating population expected for one copy of PVY coat protein gene or a linkage between the copies of the cp gene and one copy of the Bt-*Cry IA(c)* gene, and it also fit in the 37.5% expected from one copy of one gene and two copies of the other. However, the proportion (26 out of 67) of the reciprocal progeny with the two genes differs from the 25% proportion expected if there are one copy of Bt-*Cry I A(c)* gene and one copy of PVY cp gene, but not from the 37.5% (Table 4).

Progeny from crosses between ATL-PVY5 and non-transgenic parents (clone MS702-80 and cv. Lemhi Russet) were used to verify if the Mendelian 1:1 ratios (presence:absence) expected for one copy or the linkage between copies of the PVY cp gene in the donor parent appears in the segregation of the PVY cp and NPT II genes. The observed ratios (presence:absence) in the segregation of PVY cp gene were 15:16 for ATL-PVY5 x Lemhi Russet progeny, 21:14 for Lemhi Russet x ATL-PVY5 progeny and 34:32 for ATL-PVY5 x MS702-80 progeny. All these segregations fit a 1:1 ratio expected for one copy. Similar results were observed in the segregation of NPT II. The segregations of this gene were 16:15 for ATL-PVY5 x Lemhi Russet progeny, 15:20 for Lemhi Russet x ATL-PVY5 progeny and 37:39 for ATL-PVY5 x MS702-80 progeny. In addition, the segregation of the PVY cp gene in the self-pollinated progeny supports the above results. The observed ratio of the PVY cp was 23:12 and fits in the ratio 3:1 expected for the segregation of one copy of PVY cp gene or co-segregation of these copies or linkage between copies in the selfed progeny of ATL-PVY5 (Table 3).

Table 4. Chi-Square analysis to test the individual probabilities of Bt-*Cry IA(c)* and PVY coat protein genes

Segregating Population (fem.xmale)	Total number of individuals	Null Hypothesis (Copy Number)	Individual probabilities of these genes	Observed Individuals with Both PVYCp and Bt genes	Expected proportion	Expected Individuals with Both PVYCp and Bt genes	Goodness of Fitness for Binomials
ATL-PVY5 x FL1607-A30	71	1 copy of each gene	p=0.5 q=0.5	21	25.00%	17.75	0.57416 N.S.
		1 copy of			37.50%	26.625	1.57858 N.S.
		2 copies of the other	q=0.75				
		2 copies of each gene	p=0.75 q=0.75		56.25%	39.9375	19.4688 **
FL1607-A30 x ATL-PVY5	67	1 copy of each gene	p=0.5 q=0.5	27	25.00%	16.75	7.56716 **
		1 copy of one	p=0.5		37.50%	25.125	0.12039 N.S.
		2 copies of the other	q=0.75				
		2 copies of each gene	p=0.75 q=0.75		56.25%	37.6875	6.2944 *

Goodness of Fitness for Binomials: $X'^2cc=\Sigma(([obs.-exp.]-0.5)'2)/exp.$

N.S: No significant difference from the null hypothesis. Where $X'^2cc< X'^20.05[1]$

*: Significant difference from the null hypothesis. Where $X'^2cc> X'^20.05[1]$

**: Highly significant difference from the null hypothesis. Where $X'^2cc> X'^20.01[1]$

Some progenies showed resistance to the antibiotic kanamycin. Therefore, the kanamycin assay indicated stable integration and expression of the introduced NPT II gene in the segregating progeny of these transgenic clones (FL1607-A30 and ATL-PVY5). The kanamycin assay corroborated the segregating pattern of NPT II observed by PCR analyses. The ratios for presence:absence of resistance plants fit the ratios of 3:1 in the ATL-PVY5 x FL1607-A30 progeny, of 1:1 in the ATL-PVY5 x MS702-80 and ATL-PVY5 x Lemhi Russet progenies and of 1:1 in the selfed progeny of ATL-PVY5. Except for the selfed progeny, these ratios suggested the presence of one copy of the NPT II gene or a tight linkage between the two inserted copies (Table 5).

Discussion

The production of transgenic plants has increased the diversity of germplasm sources that can be used by the breeder. However, such transgenic plants only will be of value if their phenotype is faithfully transmitted in a predictable manner through subsequent generations (Finnegan and MacElroy 1994).

The segregating ratios for Bt, PVY cp and NPT II genes were assessed in several offspring from crosses using transgenic lines (ATL-PVY5 and FL1607-A30) and non-transgenic lines (MS702-80 and cv. Lemhi Russet). These ratios can be used to explain how the transgenic loci act.

In the case of the Bt gene in FL1607-A30, the observed PCR segregation pattern of the ATL-PVY5 x FL1607-A30 progeny confirmed the data from the Southern blot analysis. Therefore, the gathered data support the contention that there is one copy of the

Table 5. Kanamycin assay results

Goodness of Fitness for Binomials $X^2cc=\Sigma(([obs.-exp.]-0.5)^2)/exp.$

N.S: No significant difference from the null hypothesis. Where $X^2cc< X^20.05[1]$

*: Significant difference from the null hypothesis. Where $X^2cc> X^20.05[1]$

**: Highly significant difference from the null hypothesis. Where $X^2cc> X^20.01[1]$

pres.:abs.= presence:absence

Table 5. Kanamicyn assay results

Segregating Population (femalexmale)	Number of Germinated Seed per plate	Total number of Germinated Seeds	Number of transgenic plants per plate	Total number of transgenic plants	Number of dead plants per plate	Total number of dead plants	Tested ratio (pres.:abs.)	Goodness of Fitness for Binomials (X^2cc)
ATL-PVY5xFL1607-A30	22	92	15	65	7	27	3:1	0.6879528 N.S.
	22		17		5		7:1	22.36024 **
	24		18		6			
	24		15		9			
ATL-PVY5xMS702-80	21	88	11	27	10	44	1:1	0.0113636 N.S.
	24		10		14		3:1	28.015 **
	23		13		10			
	20		10		10			
ATL-PVY5xLemhi Russet	24	95	18	49	6	46	1:1	0.04210 N.S.
	24		11		13		3:1	26.5578 **
	23		10		13			
	24		10		14			
ATL-PVY5xATL-PVY5	24	48	15	27	9	21	1:1	0.52083 N.S.
	24		12		12		3:1	26.5578 **
Cv. Chaleur (non-transgenic control)	22	87	N/A	N/A	22	87	N/A	N/A
	24				24			
	16				16			
	25				25			

Bt gene in the clone FL1607-A30. However, the FL1607-A30 x ATL-PVY5 progeny showed segregation distortions from the expected Mendelian segregation ratio. This distortion could be due to chance, linkage between the gene of interest and other genes that affect gametic or zygotic selection, or linkage to lethal alleles (Aron et al. 1997).

The observed inheritance patterns for the PVY cp gene of the progenies in which ATL-PVY5 clone was involved, are incongruent with the Southern blot analysis. One way to explain the observed patterns is that the two copies are linked in coupling. This conclusion is also verified by the segregation pattern for the NPT II gene in the same progenies.

The selectable marker gene used, neomycin phosphotransferase II (NPT II) detoxifies neomycin, kanamycin and G418 by phosphorylation. Our selection medium was enriched with the antibiotic kanamycin, which is the most widely used selection agents in dicot transformation systems (Webb and Morris 1992). The kanamycin assay indicated stable integration and expression of NPT II gene, and provided evidence that the inserted NPT II gene is inheritance as a single dominant alleles. It also corroborates the segregation pattern found in the ATL-PVY5 x FL1607-A30, ATL-PVY5 x MS702-80, ATL-PVY5 x Lemhi Russet progenies using PCR. However, the selfed progeny showed a ratio of 1:1 both in the kanamycin assay and the PCR analysis that deviated from the Mendelian ratios expected either for one or two inserted copies of the gene. This distortion from the Mendelian ratios may be explained for bias due to the low number of samples used, or a linkage of these copies to factor involved in self-incompatibility. Since the same ratio appears in the PCR analysis and in the Kanamycin assay and this distortion from the Mendelian ratio appears only in the selfed progeny of ATL-PVY5 and not in

other progenies with ATL-PVY5 as parent, the second hypothesis is more likely.

Currently, the selectable marker is used to distinguish the transgenic genotypes after the transformation event. Since the selectable marker (NPT II) and PVY cp gene from ATL-PVY5 seem not to segregate independently, a strategy to produce plants with multiple resistance using this clone could be selection in kanamycin medium of germinating seed. In this way, we assure to discard those plants that do not have the gene(s) of interest, shortening the selection process.

In this study, we also produced plants that carry the combined genes for resistance against potato tuber moth and PVY virus, the proportion of these plants can be determined by the combination of the individual probabilities of these genes, when no distortion was found. A total of 47 individuals with both resistant genes were obtained. Further research is needed to determine the value of this multiple resistance, since the loss of transgene expression does not always correlate with the loss of the transgene, but rather with its inactivation (Finnegan and McElroy 1994). Therefore, greenhouse and field studies are needed to evaluate the level of resistance to potato tuber moth and virus.

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