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ANALYSIS OF ENGINEERED (cryIA(c)-BT TRANSGENIC) AND NATURAL RESISTANCE MECHANISMS IN POTATO (Solanum spp.) FOR THE CONTROL OF POTATO TUBER MOTH (Phthorimaea operculella Zeller.)

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ABSTRACT

ANALYSIS OF ENGINEERED [*cryIA(c)*-BT TRANSGENIC] AND NATURAL RESISTANCE MECHANISMS IN POTATO (*Solanum* spp.) FOR THE CONTROL OF POTATO TUBER MOTH (*Phthorimaea operculella* Zeller)

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Potato tuber moth (*Phthorimaea operculella* Zeller) is one of the major insect pests of cultivated potato (*Solanum tuberosum* L. [2n=4x=48]) in tropic and sub-tropic regions. Host plant resistance (HPR) is a key tool in an integrated pest management program to control potato tuber moth and has been found among the wild and cultivated *Solanum* germplasms. Genetic engineering offers the opportunity to introduce the *Bacillus thuringiensis* (B.t.) toxin gene into potato. In this study, two transgenic potato clones expressing a wild-type *cryIA(c)* B.t. gene were generated through *Agrobacterium*-mediated transformation. Gene integration was confirmed by polymerase chain reaction, Southern and northern analyses. One clone (FL 1607-A11) had two copies of the gene while the other clone (FL 1607-A30) had one copy. Detached leaf potato tuber moth bioassays, using first instars, were conducted on the transgenic lines, the untransformed control, and 12 other clones with putative host plant resistance mechanisms. Both transgenic lines, two leptine producing lines (USDA 8380-1 2x and 4x), and a wild species (*S. sparsipilum* PI 230502) exhibited resistance with 60-68% mortality of potato tuber moth larvae. Seven lines (Roslin Eburu, KWPTM 29 and 24, CIP 85-37.38, Cruza 148, TM-3), including one line with glandular trichomes (NYL 235-4), exhibited moderate resistance with 15-36% mortality. The untransformed control (FL 1607) and two other clones (Santa Catalina and CCC 1386.36) were not resistant (less than 13% mortality). B.t. expression and mortality were higher than previous reports using a similar wild-type gene specific to lepidoptera. Since B.t. can be expressed in any potato line, efforts should be made to introduce the B.t. gene into plants with natural HPR. This material could then be used to develop more durable HPR.

This thesis is dedicated to my wife,
Maria Estela Velasco Xocop de Hudy

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The cultivated potato (*Solanum tuberosum* subsp. *tuberosum*) is one of the world's most important food crops, ranking fourth in total production after wheat, maize, and rice. Its importance is best demonstrated in that it has the greatest rate of increase in production of any major food crop (International Potato Center 1984). It is a crop native to the Americas and was spread around the world after the Spanish arrival in the Americas. Over 35% of all potatoes are grown in developing countries, 40% in Europe and the former USSR, 15% in China, and 5% in North America (International Potato Center 1984).

One of the advantages that potatoes have is that they generate more food energy per unit area than most major crops. For example, they provide 75% more food energy per unit area than wheat and 58% more than rice. Potatoes also generate 54% more protein per unit area than wheat and 78% more than rice (Sieczka and Thorton 1993). The potato is a very nutrient-dense food providing a greater percentage of nutrients than its percent in the total diet. It can be a major provider of vitamin C in the diet as well as other essential vitamins and nutrients.

The potato is suited to a wide range of climates and cultural practices. It can be successfully grown from hot arid to cold environments at elevations of over 1000m above sea level (International Potato Center 1984). Because it is grown world-wide, it is subject to many pests which can cause severe yield and/or quality reductions. Major insect pests include the Colorado potato beetle (*Leptinotarsa decemlineata* Say), aphids (*Myzus persicae* Sulzer, *Macrosiphum euphorbiae* Thomas), potato leafhopper (*Empoasca fabae* Harris), flea beetle

(*Epitrix cucumeris* Harris), and potato tuber moth (Lepidoptera: Gelechiidae, *Phthorimaea operculella* Zeller). Other potato biotic stresses include early blight (*Alternaria solani* [Jones and Grout] Sarauer) and late blight (*Phytophthora infestans* [Mont.] de Bary), nematodes, and bacterial diseases.

THE POTATO TUBER MOTH

Morphology and Development

The potato tuber moth is the most damaging insect pest of cultivated potatoes in the subtopics and tropics (Ferguson 1989). Potato tuber moth is a pest of potatoes and other related crops under both field and storage conditions. It was originally reported to occur in Tasmania in 1855 and has since been found world-wide (Trivedi and Rajagopal 1992). The preferred host of potato tuber moth is cultivated potato (*Solanum tuberosum* L.); alternate hosts include tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill.), eggplant (*Solanum melongena* L.) and Cape gooseberry (*Physalis peruviana* L.), as well as members of the nightshade weed complex (*Solanum* spp.). Potato tuber moth can also attack and damage stored tomato and eggplant fruits, *Solanum melongena* L. (Ferguson 1989, Broza and Sneh 1994, Gilboa and Podoler 1994).

Potato tuber moth is a small, light colored moth. The adults have a wingspan of about 1.5 cm. There are 4 distinct life stages: egg (4-5 day), larva (12-14 day), pupa (6-9 day), and adult. Each female can produce 50-300 eggs, with the maximum number being produced at 28C. Under optimum conditions (26-28C), a new generation may be every 21 days (Raman 1980, Fenemore 1988, Ferguson 1989, Trivedi and Rajagopal 1992, Fuglie et al. 1993). The adults are relatively innocuous, feeding on nectar and pollen. They are considered weak flyers,

although they can fly considerable distances under laboratory conditions (Foley 1985). Indirect evidence suggests that the adults are attracted by certain host plant volatile compounds (Visser and Ave 1975). The female deposits the majority of her eggs on the soil adjacent to the plant and some on the foliage. Full fecundity is achieved only in the presence of suitable host plant material, especially tubers (Fenemore 1988).

The larvae cause the major damage to the potato crop by feeding on either the tubers or the foliage. The foliage feeding, is not a major source of crop damage but can result in weakened or broken stems and mined-out leaves. Larval attacks on the tubers cause irregular galleries that can completely riddle the tuber. The initial tuber infestations started in the field are carried to the storage where storage conditions allow the rapid cycling of the population (Langford 1934, von Arx et al. 1990, Ali 1993, Fuglie et al. 1993). This population growth and feeding on the tubers can make the crop unfit for human consumption or seed use. If the infestation proceeds unchecked, it can result in a loss of the entire stored crop. A loss of 70-90% is not uncommon if no preventative steps are taken (Raman 1980, Gilboa and Podoler 1994). Even if the entire crop is not infested, the infestations can lower the tuber quality and leave the tubers more susceptible to secondary infections that cause rot. This is even more true in the tropics where refrigerated storage is either unavailable or unaffordable (Trivedi and Rajagopal 1992).

Traditional controls

Several traditional controls are available which can help reduce damage caused by potato tuber moth. Many of these techniques reduce infestation levels in the field. If the field potato tuber moth population can be adequately controlled, it is easier to prevent storage losses (Fuglie et al. 1993).

Mechanical cultivation can provide earth barriers to make it difficult for first instars to penetrate through the soil and reach the tubers. The frequency of cultivation can also help control this pest. More frequent cultivation buries the eggs and mechanically damages them, preventing hatching (Trivedi and Rajagopal 1992, Ali 1993). Irrigation methods also play a role in controlling populations. Overhead sprinkler irrigation evenly moistens the whole field reducing the movement of first instars. Frequent application of irrigation maintains soil moisture and prevents the cracking of the soil as it dries. In wet soils, the lack of these cracks inhibits the spread of potato tuber moth larvae to the tubers. It has been found that first instars can penetrate through 12.5 cm of dry soil, whereas they can not penetrate wet soil (Trivedi and Rajagopal 1992).

Chemical controls are currently the method of choice because of their perceived efficacy (Raman et al. 1987, Fuglie et al. 1993, Broza and Sneh 1994). Nevertheless, some important factors need to be considered about the widespread use of insecticides--both natural and synthetic. Although it is true that in many cases chemical applications provide temporary control, the population dynamics of potato tuber moth have allowed it to develop resistance to a number of chemicals. The continued use of insecticides, which simultaneously places strong selection pressure on potato tuber moth and removes natural predators, suggests that potato tuber moth will be able to quickly develop resistance, resulting in the loss of those

insecticides (Raman et al. 1987). In Israel, this occurred in the processing tomato crop infested by potato tuber moth (Broza and Sneh 1994). Since 1987, the dichlorovos and methamidiphos were rendered ineffective for the control of lepidopteran pests, including potato tuber moth, because of the development of resistance (Broza and Sneh 1994). Additionally, potato tuber moth developed resistance to several major chemicals used in its control in other parts of the world over the past several years (Fuglie et al. 1993, Broza and Sneh 1994). An increase in broad-spectrum (non-specific) insecticide use may actually result in an increase in potato tuber moth damage. This may occur through the reduction in natural enemies of potato tuber moth (von Arx et al. 1990).

The traditional lack of monitoring programs for potato tuber moth populations throughout the tropics and sub-tropics often leads to the excessive use of insecticides. This excessive use is neither cost effective nor desirable from a resistance management point-of-view (Raman et al. 1987). Lastly, the excessive use of insecticides is not desirable on potato tubers destined for human consumption (Raman et al. 1987, Ferguson 1989, Ali 1993).

Biological controls are also important for the control of potato tuber moth. There are many different naturally occurring biological control agents, including predators and parasitoids, granulosis viruses, fungi, bacteria and nematodes (Raman et al. 1987, Ferguson 1989, von Arx et al. 1990, von Arx and Gebhardt 1990, Trivedi and Rajagopal 1992). These are of variable efficacy for controlling potato tuber moth and no single treatment provides complete control, although granulosis viruses have received considerable acclaim as control agents (Falcon 1992). Additionally, potato tuber moth larvae are able to escape some of these pressures because of the protection afforded them by the tubers.

Potato tuber moth populations can cycle very rapidly. For effective control of the

tubers in storage, it is necessary to adequately control the field population (Fuglie et al. 1993). The most effective way to achieve this may be through a system of integrated pest management, including cultivation, irrigation, selective use of insecticides, and the use of varieties which express host plant resistance.

HOST PLANT RESISTANCE

Definition

Varieties that can be used to control insect populations all have some form of natural resistance to insect pests. This resistance, which allows the plants to grow and not be affected by insect pressure, is called host plant resistance (HPR). HPR may have either a genetic or ecological basis. The agent causing the resistance can vary and resistance may involve a combination of several factors. Genetic resistance may be categorized as antibiosis (some type of feeding deterrent or toxicity in the plant), antixenosis (causing a rejection of the host plant), or a combination of these (Kogan 1982).

In the potato and related species (*Solanum* spp.) there are two well-defined and quantified HPR systems, as well as other, more poorly defined systems. The two well-defined systems are glycoalkaloids and foliar trichomes. The other systems do not involve glycoalkaloids or trichomes.

Glandular Trichomes

Glandular trichomes are common in many wild species of potato such as *Solanum berthaultii*, *S. tarijense*, and *S. polyadenium* (Gibson 1971, Gibson 1976a, Gibson 1976b, Gibson 1976c, Gibson 1979, Tingey and Sinden 1982, Gregory et al. 1984, Tingey et al. 1984, Gregory et al. 1986, Avé et al. 1987, Tingey 1991, Flanders et al. 1992, Spooner and

Bamberg 1994). The resistance imparted by glandular trichomes is caused by many factors: physical entrapment, secretions which limit feeding ability, and plant avoidance strategies by the insects. The relative importance of each of these deterrent factors changes from insect to insect.

Most species possessing glandular trichomes have two distinct trichome types labelled type A and type B (Gibson 1976c). Type A trichomes are relatively small (120-210 mu) and bear a membrane bound, tetralobulate gland at their tips. Upon rupture, the gland releases a phenolic compound which hardens upon exposure to the air. This hardened exudate can, in some cases, physically trap certain insects. Even if the insect is not trapped, the hardened exudate can partially cover tarsi and mouthparts affecting development, feeding, and movement (França and Tingey 1994) The gland is not renewed upon removal (Gregory et al. 1986). Type A glands also volatile sesquiterpenes which act asrepellents for certain insect species (Tingey and Sinden 1982, Avé et al. 1987). Type B are the longer of the two (600-950 mu) and bear a drop of exudate their tips. This exudate is easily transferred to the insect upon contact and is renewed after removal, allowing the plant to replenish its defensive system (Gregory et al. 1986).

Many small insects, such as aphids and leafhoppers, experience significant mortality when trapped in the trichome exudate (Gibson 1971, Gibson 1974, Gibson 1976a, Gibson 1976b, Gregory et al. 1986). Upon landing on the leaf, the insect will make contact with one of the type B trichomes. Type B trichomes readily transfer their exudate to the insect, especially to the insect's tarsi and mouthparts. Increased movement by the insect to escape from the exudate will bring it into contact with, and cause it to rupture the gland on the type A trichome. This releases phenolic compounds which further entraps and coats the insect. If

the insect is small, the combination of the exudate from the type B and the hardened phenolics from the type A will render it trapped on the leaf surface unable to feed. Some insects are large enough to escape and will leave that plant, however, they will exhibit altered behavior patterns. These patterns are characterized, in part, by increased flying with less tendency to alight on other plants. Additionally, for some insects there is a decrease in host plant acceptance as well as a decrease in feeding time. Because of the increased mobility of the insects, they will be more likely to be subject to predation (Tingey 1991).

Glycoalkaloids

Glycoalkaloids are naturally occurring toxins present at some level in all potatoes. Many wild *Solanum* species have elevated levels of glycoalkaloids which may protect them from insects and other herbivores (Torka 1950, Gregory et al. 1981, Tingey et al. 1984, Sanford et al. 1984, Sinden 1987, Flanders et al. 1992, Sanford et al. 1992, Spooner and Bamberg 1994). However, at levels present in the tubers of commercial varieties (under 20 mg/100 g fresh tissue, or 20 mg %), glycoalkaloids are not known to produce harmful effects in human or animals (Tingey et al. 1984). Many distinct glycoalkaloids have been identified, the major ones being chaconine, solanine, demissine, solamargine, commersonine, and tomatine (Deahl et al. 1993). Most glycoalkaloids are present in all parts of the plant, however the highest concentrations are found in the foliage, flowers, and sprouts with little in the tubers (Deahl et al. 1993). The major exceptions to this are leptine and solamargine which are usually not found in the tubers. (Sinden 1987).

Leptines

Leptines are acetylated forms of the more common *Solanum* glycoalkaloids (Sinden et al. 1986b, Sinden 1987). They are relatively rare and have been found only in a few selectionsof *S. chacoense* Bitter (Sinden et al. 1986b). It is hypothesized that the production of leptines, via acetylization, is controlled by a single, or a very few, dominant gene(s) (Sinden et al. 1986a, Sanford et al. 1996) while the quantities that are synthesized are polygenetically controlled (Sanford et al. 1996). Leptines are very potent in conferring resistance against insects. At levels of 100 mg %, leptines can confer immunity to many insects (Sinden et al. 1986b), whereas the glycoalkaloids chaconine and solanine have only a partial deterrent.

Initially, the use of leptines as HPR agents looked promising due to their localization only in foliage, with no detectable amounts being found in the tubers (Sinden et al. 1986b, Sanford et al 1994, Sanford et al 1995, Sanford et al 1996). Further evidence for the specific localization of leptines is demonstrated by their increased synthesis in response to increased light intensity (Deahl et al. 1991) and the lack of wound inducible production (Sanford et al. 1996). However, leptines represent only a portion of the glycoalkaloids present in any potato species or variety. No lines have been identified which produce only leptines, other glycoalkaloids are always present, even if at low levels. Attempts to introgress the gene(s) responsible for leptine production have been only moderately successful (Sanford et al. 1996). While leptine synthesis was introgressed into a *S. tuberosum* background, levels of other, non-foliage specific, glycoalkaloids were increased. These increased levels of the other glycoalkaloids rendered the tubers unsafe for human consumption with glycoalkaloid levels above 30 mg %. Nevertheless, it appears that at some date, conventional breeding or genetic engineering for increased leptine synthesis may allow production of HPR lines which contain

only leptines as the primary insect deterrent (Sanford et al. 1996).

Other resistance mechanisms

Some plants express HPR which does not involve glandular trichomes or glycoalkaloids (Raman and Palacios 1982). High resistance has been found in *S. acroglossum* Juz. and *S. jamesii* Torr. (Hanneman and Bamberg 1986, Spooner and Bamberg 1994). At times it may be difficult to separate out the agents causing the resistance. An example of some unspecified resistance that does not involve either glycoalkaloids or trichomes is presented by Ojero and Mueke (1985). They suggest that the resistance mechanism might involve a reduced sugar content in the plant. This might reduce host plant preference and decrease the nutritive value of the tubers; thus, preventing the insects from completing their life cycle. França and Tingey (1994) also have demonstrated how poor nutritional quality of foliage may result in a decreased fecundity and fitness of insect pests. In most cases, these resistance levels are not very high but have a measurable effect.

Using *Bacillus thuringiensis* in HPR systems

The advent of genetic engineering has allowed plant breeders to circumvent the shortage of natural HPR mechanisms. Through these techniques, it is now possible to transfer the genes necessary for simple resistance mechanisms into target plants. The use of genetic engineering technology holds the promise to efficiently achieve HPR systems in potato cultivars that are adapted to local conditions and commercial standards.

One of the first resistance mechanisms to be transferred into potato was the δ-endotoxin genes from *Bacillus thuringiensis* (B.t.). The advantage of this system, which facilitated its use, include that it is under the control of a single gene, it gives very specific

insecticidal activity, and does not cause any known detrimental effects on mammals or birds (Martin 1994). To date, commercial production of B.t.-modified, transgenic potatoes (Stone and Feldman 1995), cotton (Stone and Feldman 1995), and corn (Stein and Lotstein 1995) has received conditional approval by the US Environmental Protection Agency and the US Department of Agriculture (Matten and Lewis 1995) depending on the implementation of resistance management plans. The use of B.t.-expressing transgenic plants could help overcome some of the stability problems associated with conventional, foliar, B.t. applications. Additionally, transgenic plants allow for control of pests that feed on plant parts that are difficult to treat by conventional methods (Martin 1994, Ebora and Sticklen 1994a).

Bacillus thuringiensis is a gram positive, soil-living bacteria that is found world-wide. Upon sporulation, the bacteria produce a crystalline protein that has insecticidal properties. The insecticidal crystalline proteins (called Cry proteins) have very specific toxicity to certain insect families and do not affect non-target insects or animals. The specific insecticidal activity, structure, and unique nucleotide sequences have been used to group the different proteins into classes (DeWald 1995, McGaughey and Whalon 1992).

B.t. has a very selective action. Most classes of Cry proteins must undergo solubilization and activation before they becomes toxic although the CryIIIA class does not need this step (DeWald 1995). These processes take place in the insect midgut where high pH solubilizes the protein releasing protoxins. Following this, the protein is proteolytically activated, being cleaved by midgut enzymes into smaller, toxic polypeptides. These processes will take place in all insects with high gut pHs, however, the protein will only bind to the receptors on midgut epithial cells of susceptible insects (Bravo et al. 1992, Escriche et al. 1994). This binding to the specific receptors is responsible for the specific toxicity of the

protein. The protein causes pores to be formed in the microvilli which leads to their swelling and disruption. Eventually, the cytoplasm of the microvilli is released into the midgut region and the cell dies, with the process spreading until a pore forms in the epithial membrane. This affects the insects ability to regulate osmotic pressure, causing the insect to die due to massive water uptake (DeWald 1995). Non-target insects lack the necessary receptors and therefore are not affected by these proteins. Because of this, it is possible to use B.t. to very selectively control only the target group without affecting predacious insects (Bravo et al. 1992, McGaughey and Whalon 1992, DeWald 1995). Cry proteins have been divided into different groups based on their insect specificity. CryI proteins have specific activity against lepidopteran larvae, cryII against lepidopteran and dipteran larvae, cryIII against coleopteran larvae and adults, cryIV against dipteran larvae, cryV against lepidopteran and coleopteran larvae, with other cry classes being identified and that have different insect activities (DeWald 1995).

The mode of action of B.t. was thought to make it difficult for insects to develop resistance (Boman 1981). It was argued that the action was too complex for resistance to develop, involving multiple toxins and multiple target sites, or that there was a wide range of available proteins so that even if resistance did develop to a few specific ones, there would still be others available to allow continued insect control. This hypothesis was based on the premise that B.t. formulations would be prepared only from whole, natural pathogens (i.e., intact bacteria). Conventional applications and production of B.t. insecticides conformed to the assumptions of Boman's hypothesis (Gawron-Burke and Johnson 1994, Carlton 1995). Indeed, only few examples of high levels of field resistance have been found when conventional B.t. pesticides were applied without consideration of strategic deployment to

allow for long-term duration of the resistance mechanism (McGaughey and Whalon 1992).

The development of transgenic plants expressing the genes for the B.t. δ-endotoxins raises entirely different biological questions. With this technology, several of the assumptions of Boman are violated: the use of multi-functional agents; multiple targeting of toxins; and a short time of exposure to the pesticide. The currently accepted point of is that without proper management of the available proteins, resistance will develop (McGaughey and Whalon 1992, Gould et al. 1994, Gould 1995). Laboratory tests seem to confirm that resistance can be quickly achieved by the target insects (Whalon et al. 1994, Gould 1995). Additional tests suggest that once an insect obtains resistance to any one protein, it can quickly acquire cross-resistance to other similar proteins (Gould 1995). Thus, it appears that the advantages of B.t. could be lost through improper management.

Characteristics of HPR Systems

Maxwell (1984) categorized the following advantages to using HPR as a control mechanism as opposed to other, external effects: 1) specificity to the target organism, usually without affecting the rest of the natural checks and balances effecting the pest; 2) cumulative effectiveness in which case high resistance levels are not necessary to help control the pest; low, constant levels can be effective because the resistance mechanism is working on all life stages; 3) persistence in the environment without the need to reapply the treatment; 4) harmony with nature avoids the problem of contaminating the natural system or endangering man or wildlife; 5) ease of adoption in that there is usually no, or very little, additional cost associated with the switch to the use of this technique with no new technology is required; and 6) compatibility since it can be easily and effectively combined with other management

techniques.

Of the above advantages, maybe the most noteworthy is the cumulative effect of reducing the pest fitness, even in small increments. It has been hypothesized that this reduction will allow for a greater natural control by subjecting the pest population to the additional pressures of biocontrol agents including parasitiods and predators.

The primary disadvantages are as follows: 1) there is a genetic limitation on the resistance mechanisms available. Lack of sufficient resistance mechanisms could make the development of these HPR lines impossible; and 2) the development time often will be extremely long due to the need to identify resistance mechanisms and transfer them into commercially viable lines.

In potato, both of the two major HPR systems exhibit this second problem. With the trichomes, the problem is in obtaining progeny that produce a sufficiently high trichome density. Crosses between *S. tuberosum* and *S. berthaultii* to produce commercial, trichome-bearing lines have failed to produce plants that maintain high trichome densities to be effective against insect pests (Kalazich and Plaisted 1991, Lentini et al. 1990, Surikov and Zhitlova 1985). Likewise, it has been found that it is difficult to transfer leptine synthesis into cultivated *S. tuberosum*. The progeny that are produced exhibit a large variability for leptine biosynthesis and for total glycoalkaloid production (Sanford et al. 1994, Sanford et al. 1996).

GENETIC ENGINEERING

Biotechnology offers the opportunity to transfer genes among and across species, genera, and even kingdoms without the limitations presented by traditional sexual crossing. It is now possible to create new cultivars that express different traits that were difficult, if not

impossible, to obtain previously (Fischhoff et al. 1987, Perlak et al. 1990, Fujimoto et al. 1993, Koziel et al. 1993, Li et al. 1998, Tao et al. 1997). Plants that have been transformed include tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum L.*), cotton (*Gossypium* spp.), and maize (*Zea mays)*. Examples of transformation in potatoes include development of viral, bacterial, and fungal resistance, resistance to insects and nematode pests (Martin 1994), as well as quality traits such as high starch expressing lines.

In potato, the transformation method of choice is that of *Agrobacterium tumefaciens* (Ebora and Sticklen 1994b, Martin 1994). This method is preferred because of its low cost and ease of transformation and regeneration with minimal induction of somaclonal variation. Additional transformation systems include particle bombardment, microinjection, electroporation and vacuum infiltration (Martin 1994, White 1993). The range of possible transformation procedures reflects the genotypic effect on regeneration and transformation of the available germplasm.

Wild-type *A. tumefaciens* causes the crown gall disease which is induced by the action of a few genes contained on a large plasmid within the bacteria. During the tumor-forming process, a small discrete portion of the plasmid (called T-DNA) is transferred to the host plant where it is inserted into the genome. Specific genes, called *vir* genes, control this process. Three of the genes on the plasmid control the synthesis of two plant growth regulators and their synthesis alters the physiological development of the transformed plant tissue (White 1993). Other genes control the production of opines, which are used by the bacteria as the preferred substrate for growth.

Genetic engineering allows alteration of the wild-type *A. tumefaciens* to make it

amenable to transferring genes of interest in the T-DNA. Such "disarmed" *A. tumefaciens* strains have the genes responsible for crown gall disease removed and replaced with a new T-DNA segment containing the desired genes. In this way, the bacterium inserts the foreign genes in the same manner that the wild-type *A. tumefaciens* transfers its tumor-inducing genes into the plant (White 1993, Martin 1994, Ebora and Sticklen 1994b). Once the genes are inserted, they function under the control of their promotor, usually the cauliflower mosaic virus promoter (CaMV 35S), although other promoters, such as patatin, mannopine synthase, octopine synthase, or a wound inducible promotor, allow for tissue specific gene activity and may greatly increase the transcription of the gene (Martin 1994, Ni et al. 1995).

There are two different ways in which the *A. tumefaciens* vectors function: cointegrated plasmids have the *vir* genes and the genes of interest on the same plasmid. Binary vector have the *vir* genes on one plasmid and the genes of interest (T-DNA) on another. The use of binary vectors allows for a greater ease of genetic manipulation of the T-DNA. Additionally, since the plasmid containing the T-DNA does not contain the *vir* genes, a larger-sized piece of T-DNA can be carried on the plasmid and inserted into the host plant.

RESISTANCE MANAGEMENT

The need to reduce the development of insect adaptation to insecticides is central to allow long-term, strategic deployment of resistance mechanisms. Many times this concept is poorly understood or accepted by the people who develop the resistance mechanisms into the commercial plant lines. Nevertheless, several management strategies have been put forth as possible alternatives to current practices. These are summarized by Gould (1995) as follows: 1) constitutive expression of high levels of a single toxin in all plants; 2) constitutive

expression of high levels of two or more toxins in all plants; 3) low levels of expression of single toxins interacting with the pests' natural enemies; 4) targeted B.t. gene expression; and 5) spatial or temporal mixtures of plants with high levels of constitutive expression of one or more toxins with other plants with no toxin expression. All of these strategies aim to maximize the time before resistance is acquired by the insect pest.

All of the above concepts are based on certain premises that may not be valid under certain conditions. For example, strategies 1 and 2 assume that the insect pest will not have the genetic plasticity to overcome the insecticides, however past experience has shown otherwise. Strategy 3 could be very effective, but it is too difficult to adequately gauge the effect on the target population. Strategy 4 is valid, but may be difficult to engineer. Lastly, strategy 5 is based on the assumptions that there will not be a genetically dominant gene that could confer resistance to the population, i.e., that the plants expressing B.t. will always have a high enough level of expression to kill all heterozygous individuals in the insect population, and that the refuges will be close enough to the toxin expressing plants and the insects mobile enough to allow a free crossing of susceptible and resistant individuals (Gould et al. 1994, Gould 1995).

Despite all the problems associated with strategy number 5, it is the one that has been most embraced by the companies that are currently marketing B.t. expressing plants. For example, Monsanto is requiring its potato growers using its NewLeaf potatoes to grow 25 acres of conventionally treated potatoes (or 4 acres of potatoes without any pesticide use) for every 100 acres of NewLeaf potatoes. Similar requirements have been mandated by the EPA for transgenic cotton (Bullock and Sollad 1995). It is hoped that the use of refuges will keep the engineered gene system viable for an extended period of time (Stone and Feldman 1995).

OBJECTIVES

The purpose of this research was to determine the effectiveness of natural and synthetic HPR systems on the control of potato tuber moth under laboratory conditions. This information would subsequently help develop breeding strategies that utilize and combine natural and genetically engineered resistance mechanisms.

To achieve the objective, the study focused on these areas:

1) Produce transgenic plants expressing the gene that codes for the synthesis of the Cry IA(c) δ-endotoxin of *Bacillus thuringiensis*.

2) Confirm the transgenic nature of plants via molecular analysis involving Southern and northern analyses.

3) Conduct insect bioassays, on the effectiveness of different host plant resistance systems and B.t.-transgenic potatoes to control the potato tuber moth.

MATERIAL & METHODS

PLANT MATERIAL

The plant lines shown in Table 1 were obtained to determine the ranges of potato tuber moth resistance.

TRANSFORMATION

A wild-type *cryIA(c)* Bt endotoxin gene was graciously obtained from Dr. Wayne Barnes (University of Washington, St. Louis). The construct consisted of a CaMV 35S promoter joined to a *cryIA(c)/nptII* gene fusion. It was transformed into *Agrobacterium tumefaciens* strain LBA4404 and exhibited resistance to kanamycin (25 mgL^{-1}).

Potato lines were maintained under standard propagation conditions. Lines FL1607 and NYL 235-4 were selected for transformation. The standard propagation conditions used modified Murashigie and Skoog basal salts and vitamins obtained from Sigma (St. Louis, MO), supplemented with 3% sucrose, 0.25 mgL^{-1} gibberellic acid (GA_3), 1 mgL^{-1} D-pantothenic acid, 1 mgL^{-1} thiamine-HCl, 8 gL^{-1} agar, pH 5.6. Plants were cultured and maintained under a 16h photoperiod under cool-white fluorescent lights (30$uEm^{-2}s^{-1}$) at 23C in GA-7 Magenta vessels (Magenta Corp., Chicago, IL) each containing 25 mL of medium.

The transformation protocol was a modified version of Horvenkamp-Hermelink et. al. (1987) and involved a three-step process with one medium for pretreatment, one for co-cultivation, and one for shoot regeneration.

Table 1. Potato lines used in potato tuber moth bioassays.

Line identification	Description	Source/Reference
85-37.38	An advanced cultivar provided by CIP. It is a cool-tropical cultivar.	provided by CIP
CCC1386-26:	A selection of *S. phureja.* A short-day line from the cool tropics.	CIP 800249
Cruza 148	Provided by the International Potato Center (CIP) in Peru. An early maturing, late blight resistant, tropical variety originally from Mexico.	CIP 720118
FL1607	Selection from Frito Lay breeding program. Reported to be easily transformable.	Wenzler et. al. 1989
KWPTM 29	Selected for tuber resistance to potato tuber moth by Dr. Kazuo Wantanabe, Cornell University.	CIP, Personal communication
KWPTM 24	Selected for tuber resistance to potato tuber moth by Dr. Kazuo Wantanabe, Cornell University.	CIP, Personal communication
NYL 235-4	A release from Cornell University selected for type A trichomes. Selected from a cross of *S. tuberosum* var. *Hudson* X *S. berthaultii* (PI 310925) followed by backcrosses to *S. tuberosum*. Has field resistance to Colorado potato beetle, potato leafhopper, and potato flea beetle, *Globodera* spp. (nematode), PVY and PVX.	Plaisted et. al. 1992
PI 230502	A selection of *S. sparsipilum* ssp. *sparsipilum* (Bitter) Juz. and Buk. with moderate resistance to potato leafhopper and resistance to nematodes *(Globodera* spp.).	Hanneman and Bamberg 1986
Roslin Eburu:	A variety, originally from Kenya. Has a late maturity and is from a short-day tropical region. It is used as differential line for late blight resistance.	provided by CIP

Table 1 (cont'd)

Table 1 (cont'd)

Santa Catalina	Late blight resistant tetraploid clone	provided by CIP
TM-3	An advanced cultivar provided by CIP. Originally identified to be source for potato tuber moth (tuber) resistance. It is a cool-tropical cultivar.	CIP 382433.9
USDA 8380-1 (4x)	From USDA 8380-1 (2x) but with a chromosome number doubled by colchicine treatment	Sinden, personal communication
USDA 8380-1 (2x)	A clone selected from *S. chacoence* (PI 458310) for high levels of leptines. Field tested for high levels of resistance to Colorado potato beetle	Sinden et. al. 1986a, 1986b

The pretreatment medium was modified MS salts and vitamins, 80 mgL^{-1} NH_4NO_3, 147 mgL^{-1} $CaCl_2$, 1% sucrose, 10 mgL^{-1} naphthaleacetic acid (NAA), 9 mg L^{-1} benzylaminopurine (BAP), pH 5.6.

The co-cultivation medium was MS salts and vitamins with 1% sucrose, 4% mannitol, 0.175 mgL^{-1} indolacetic acid (IAA), 2.25 mgL^{-1} BAP, 6 gL^{-1} agar, pH 5.6.

The regeneration medium was MS salts and vitamins with 1.5% sucrose, 2.25 mgL^{-1} BAP, 4.85 mgL^{-1} GA_3, 8 gL^{-1} agar, pH 5.6.

Agrobacterium tumefaciens strain LBA4404 was cultured in 5mL of LB medium (Maniatis et. al. 1982) containing 25 $ugmL^{-1}$ kanamycin at 28C on a rotary shaker at 250 rpm.

All growth regulators were added by filter-sterilization. Plants were pre-cultured 2d and then co-cultivated by placing them in an overnight-grown *Agrobacterium tumefaciens* solution for 20 minutes, lightly blotting them dry, and placing them abaxial side down on the co-cultivation medium for 3d. The plates were sealed with Micropore gas permeable surgical tape (3-M Company, St. Paul, MN) and cultured at 22°C with 16h photoperiod at 6 $uEm^{-2}s^{-1}$. After co-cultivation, the explants were washed in 100 mgL^{-1} of Timentin (SmithKline Beecham, Philadelphia, PA) and placed on fresh co-cultivation plates containing the co-cultivation medium with the addition of 100 mgL^{-1} Timentin for 4d. Finally, they were transferred to the regeneration medium which was supplemented with 100 mgL^{-1} Timentin and 25 mgL^{-1} kanamycin sulfate. The explants were transferred to fresh regeneration medium every two weeks until shoots emerged.

After transfer to regeneration medium, shoots that arose from the explants were transferred for rooting to individual 25X100 mm test tubes each containing 15 mL of standard propagation medium supplemented with 100 mgL^{-1} Timentin and 25 mgL^{-1} kanamycin sulfate.

Only one shoot was selected per explant so as to avoid shoots derived from the same callus.

Individual plants which rooted in the kanamycin-containing standard propagation medium were selected for potato tuber moth bioassays and molecular characterization. After selection, these plants were maintained in tissue culture on standard propagation medium without kanamycin or Timentin. Tissue culture-grown plants were removed from culture, acclimitazied to laboratory conditions for 2 d, and then moved to the greenhouse for growth. Plants that were 6-10 weeks old were used for potato tuber moth bioassays and other analyses.

MOLECULAR ANALYSIS

Polymerase Chain Reaction Assay

Two 2.5 mm leaf discs from selected putative Bt-transgenic lines of of greenhouse-grown plants were collected by punching out the discs with the lid of a 1.5 mL Eppendorf tube. DNA was isolated from the leaf discs by CTAB extraction (Edwards et al. 1991). Into each tube was added 400 uL of CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 0.2 M EDTA, 0.1 M Tris-HCl, 1% beta-mercaptoethanol) and the tissue was then macerated in the extraction buffer with a pestle. The DNA was quantified with Hoeschst 33258 dye in a mini-fluorometer (model TKO100, Hoeffer Scientific, San Fernando, CA). Amplification reactions (100 uL) were set up with final concentrations of 1X buffer, 100 nM $MgCl_2$, 140 nM primers, 0.2 mM dNTPs, 2.5 U Taq polymerase (Gibco BRL, Gaithersburg, MD) and 100 ng of template DNA. Two primer sets were used, one specific for the *cryIA(c)* gene and the other specific for the *nptII* gene.

The Bt primers were designed using the revised OLIGO program to be specific to the

cryIA(c) gene. The program analyzed potential primers based on their free energy of binding, hybridization temperature, ability to form stable duplexes, specificity, and lack of formation of deleterious secondary structures such as dimer formation and self complementation which would lead to hairpin loop formation. Two 25-base primers were identified that were similar with regard to complementation and free energy of binding and were produced by Genosys Biotechnologies, Inc. (Woodlands, TX). The primer complementary to the transcribed strand of the *cryIA(c)* gene from base pair 802 had a sequence of 5' AGT GCC CTT ACA ACC GCT ATT CCT C 3'. The primer complementary to the non-transcribed strand of the gene from base pair 1243 had a sequence of 5' TAC TTC TTT CTA TGC CCT GAG CCG A 3'. The size of the expected fragment derived through PCR amplification with these primers was 456 base pairs.

The NPTII primers were identified with the same program and produced by the Macromolecular Structure Facility, Michigan State University. The 26-base primer complementary to the transcribed strand of the *nptII* gene had a sequence of 5' CGC AGG TTC TCC GGC CGC TTG GGT GG 3'. The 25-base primer complementary to the non-transcribed strand of the gene had a sequence of 5' AGC AGC CAG TCC CTT CCC GCT TCA G 3'. The size of the expected fragment derived through PCR amplification with these primers was 255 bp

Amplification reaction conditions were 4 min at 94°C, followed by 40 cycles of 1 min at 94°, 1 min at 58°, and 1.5 min at 72°. Completed reactions were stored at 4° until the products could be analyzed by agarose gel electrophoresis. Electrophoresis conditions were in a 2% (w/v) agarose LE gel (Boeringer Mannheim, Indianapolis, IN) containing 0.005% w/v ethidium bromide in 1X Tris-acetate/EDTA (TAE) buffer, pH 8.0 (Sambrook et al. 1989).

Gels were run overnight at 25 mV or until the bromophenol blue running buffer was within 1 cm of the end of the gel. The gels were visualized with a UV transilluminator (Gel Print 2000I, Biophotonics).

PCR amplification was also used to produce the probe for Southern analysis. For this process, the PCR procedure was as above except that plasmid DNA from pWB139 was isolated from BSII/SK and was used as the template DNA following linearization with *HindIII*. After the PCR amplification with the B.t. specific primers, the probe was purified by electrophoresis through a 1% low melting temperature agarose gel that contained 0.005% w/v ethidium bromide in 1X TAE buffer. The probe was recovered using ELUTIP purification (Schleicher and Schuell, Keene, NH) as per the protocol. From this, 3 ug of probe DNA was subject to random priming with digoxigenin (DIG)-labeled dUTP (Boeringer Mannheim, Indianapolis, IN) and the labeled probe was then used for Southern analysis.

Southern Analysis

Genomic DNA was extracted from 2-3 grams of fresh leaf tissue using the CTAB method (Saghai-Maroof et al. 1984), modified by adding 2% beta-mercaptoethanol in the extraction buffer. For each plant, total genomic DNA was digested with either *HindIII* or *BamHI* in reaction volumes of 50 uL containing 37.5 units of enzyme added in two equal additions, 1/10 volume BSA (5 $mgmL^{-1}$), 1X enzyme-specific digestion buffer, and 24 ug of the DNA of interest. The digests were placed at 37°C with ½ of the restriction enzyme added at the start and the other ½ added after 1 hour. The digests were left for a total of 4h at which point the reactions were stopped by adding a 5X bromophenol blue loading buffer containing 50% glycerol, 0.25% bromophenol blue, and 1 mM EDTA pH 7.0. The DNA fragments were separated in a 0.8% agarose gel containing 0.005% (w/v) ethidium bromide in 1X TAE

buffer. For each sample, 10 ug of digested DNA with the running buffer was heated to 65°C for 5 minutes, quickly chilled on ice and added to the appropriate lane. Digested plasmid (1 ug) and DIG-labeled molecular weight markers were used as standards. The fragments were separated by electrophoresis at 25 mV for 16h then capillary transferred (Maniatis et. al. 1982) to nylon membrane (Hybond N, Amersham, Buckinghamshire, England) via Southern transfer. Following the transfer, the DNA was bound to the membrane by UV cross-linking (Stratalinker) at 1200 Joules on the DNA side and 200 Joules on the other side. Prehybridization was for 2 hours at 42°C in a rotary hybridizaiton oven (Model 301, Robbins Scientific, Sunnyvale, CA) in a solution containing 5X SSC, 1% skim milk, 0.1% N-lauroylsarcosine, 0.02% SDS, 50% formamide and 125 ugmL^{-1} sheared salmon sperm DNA. Hybridization was performed overnight at 42°C in fresh solution containing the denatured DIG-labeled DNA probe (465 bp fragment of the *cryIA(c)* coding region) was added. Stringency washes and development were according to the Genius system (Boeringer Mannheim, Indianapolis, IN) for chemiluminescence detection. The image of the Southern blot was obtained by exposing the blot to Kodak X-omat AR film for 1 to 3h as needed.

Northern Analysis

RNA was obtained from greenhouse-grown plants using the Qiagen RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, CA). All labware was previously baked or washed with 1N NaOH followed by 0.1% diethyl pyrocarbonate (DEPC) treated distilled H_20 to remove extraneous RNases. From each plant line, 100 mg of tissue was subject to extraction and RNA was quantified by spectrophotometric analysis.

An RNA probe was transcribed from the pWB139 plasmid contained in BSII S/K. The plasmid was purified by a Wizard Miniprep (Promega, Madison, WI) and linearized by *BamHI* digestion. The RNA probe was produced by transcribing the linearized pWB139 plasmid with a T7 RNA polymerase which incorporated DIG-labeled uridine triphosphate units into the mRNA probe and was quantified compared to DIG-labeled RNA standards.

Samples of 15 ug of total plant RNA were loaded onto a 1% formaldehyde-agarose gel in a 1X MOPS buffer (20 mM morpholinopropansulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7). Prior to loading, the samples were prepared by adding an equal volume of 2X sample buffer (250 uL formamide, 83 uL formaldehyde, 50 uL 10X MOPS, and 0.5 uL ethidium bromide 10 $mgmL^{-1}$) and 1/5 volume of a 5X running buffer (50% glycerol, 0.25% w/v bromophenol blue, 1 mM EDTA pH 8.0). The gel was run overnight at 23 mV and then held at 5 mV until the transfer was prepared. The RNA was transferred to a Hybond-N+ (Amersham, Buckinghamshire, England) membrane by capillary transfer and was UV cross-linked to the membrane at 200 Joules. Pre-hybridization, hybridization, and detection conditions were as described for the DNA analysis except that the pre-hybridizaiton and hybridization were at 52°C and the DIG-labeled RNA probe was used.

POTATO TUBER MOTH BIOASSAYS

All bioassays were conducted using the University of California, Berkeley population of potato tuber moth, provided by Dr. Lowell Etzel, at 25-30C, 60 to 75% relative humidity, and dim lights.

To initiate adult populations, approximately 200 pupae were placed in a 3.8 L glass Mason jars with honey/water (1:1) as a food source. The mouth of the jar was covered with a screen (36 squares/cm^2) and a 10 cm diameter #1 Whatman filter paper was placed on top as a substrate upon which the female potato tuber moth could oviposit.

To maintain the culture, the filter paper containing an even-aged cohort of eggs was placed on non-transformed, field-grown potato tubers. The larvae that hatched were allowed to feed freely on these tubers. Corrugated cardboard cubes measuring 5 X 5 X 2.5 cm were provided as chambers into which the mature larvae could pupate. Full pupae chambers were then used to start the new adult cultures.

For bioassays, larvae were located using a dissecting stereoscope at 6 X magnification and removed by gently sweeping them with a moistened, fine-point paint brush. Only first instars (neonates) or, if necessary, fully mature and unhatched eggs, were selected. By preference, neonatal larvae were used instead of eggs.

Detached leaves of greenhouse-grown plants were used for bioassays. Individual leaves were detached by cutting them across the petiole with a single-edged razor blade while the petiole was submerged in water. The petioles of the leaves were then wrapped in a pre-moistened sponge and the petiole/sponge arrangement placed in a 3.5 mL shell vial of water. The leaf/vial arrangement was placed in a 150 X 20 mm Petri dish on moistened Whatman

#1 filter paper.

For each bioassay, 10 neonate potato tuber moth larvae or eggs were used. Larvae and/or eggs were dispersed evenly over all the leaflets to minimize competition. The Petri dishes were closed, but not otherwise sealed. They were placed in the culture room and maintained at 25-30C.

After 48 h, the number of surviving larvae was counted. Surviving larvae were identified based on their reaction to the sharpened tip of a dissecting needle. Any of the original larvae that could not be found were counted as dead because of an assumed lack of preference for the leaf material.

Because of the difficulty of simultaneously obtaining sufficient leaf material of all lines and enough potato tuber moth larvae, no single study involved all potato lines. Instead, multiple studies were conducted over time with each study involving 6 to 12 different lines. Most lines were analyzed between 4 and 8 times, resulting in data on mortalities of 40 to 80 individual insects per line. Because the data that were collected reflected the mortality of the potato tuber moth larvae where all larvae had an identical probability of mortality in any single study, the effects of sampling were balanced across all lines, and the insects were spaced to such that they did not exert an influence on the actions of other insects, they fit a binomial distribution. The data for the individual lines were ranked according to percent mortality and then differences between lines were determined by pairwise comparison. Additional differences were found by making comparisons between groups of data. The comparisons were analyzed statistically by using X^2 tests at $p<0.05$ and one degree of freedom.

RESULTS

Agrobacterium tumefaciens-mediated transformations conducted with the *cryIA(c)* construct resulted in 30 putative transgenic shoots derived from FL1607 and 5 lines from NYL 235-4 that had the ability to root on propagation medium containing 25 mgL^{-1} of kanamycin sulfate.

PCR analysis using *cryIA(c)* specific primers identified 2 of the above 30 FL1607 lines that contained the B.t. gene (Figure 1). PCR analysis using NPTII specific primers yielded the same results (Figure 2).

Southern analysis following *HindIII* digest confirmed the presence of the *cryIA(c)* gene in these 2 lines (Figure 3). Southern analysis following *BamHI* digest indicated that one line (FL 1607-A11) contained two copies of the gene as identified by bands at 5,100 and 4,900 bp while the other line (FL 1607-A30) contained a single copy of the gene as shown by a single 18 Kb fragment (Figure 4). Northern analysis confirmed gene transcription by the presence of a 2.6 kb mRNA fragment in both transgenic lines but not in the untransformed FL 1607 control (Figure 5).

Data from the detatched leaf bioassays are presented in Table 2. Statistical analysis allowed the lines to be divided into four categories of resistant, moderately resistant, susceptible and highly susceptible with a confidence of >95%. Both Bt-transgenic lines were grouped into the resistant category while the untransformed control (FL1607) was in the susceptible category.

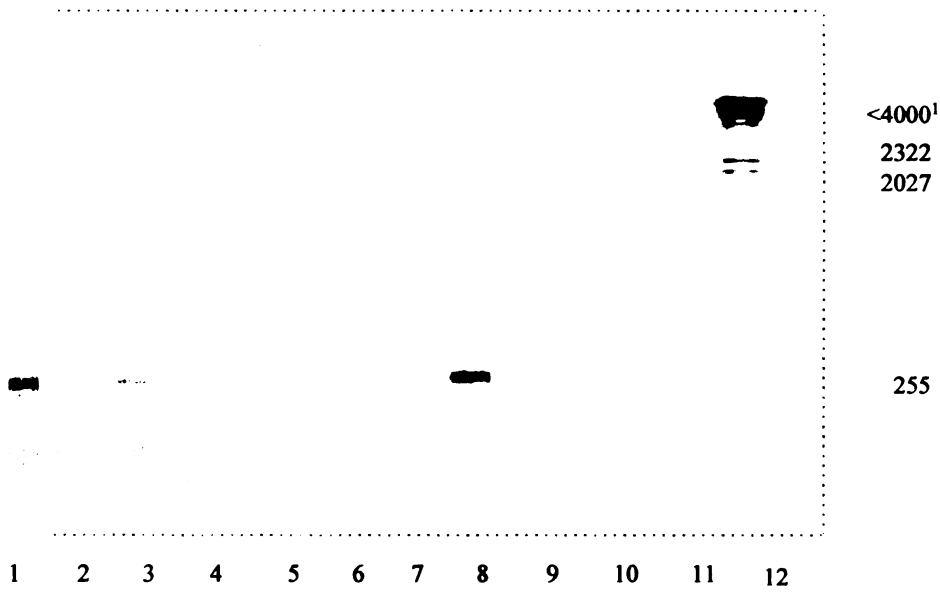

Figure 2. Polymerase chain reaction assay of total potato genomic DNA using *nptII* specific primers. A fragment of 255 bp corresponding to the *cryIA(c)/nptII* gene fusion is expected in transformed lines. Lane 1 - plasmid positive control from same reaction, different gel; Lane 3 - FL1607-A30; Lanes 2, 4-7, 9, 10 - untransformed regenerates of FL1607; Lane 8 - FL1607-A11; Lane 11 - untransformed FL1607 control;. Lane 12 - *Lambda* DNA digested with *HindIII* to yield molecular weight markers.

[1]All numbers are base pairs.

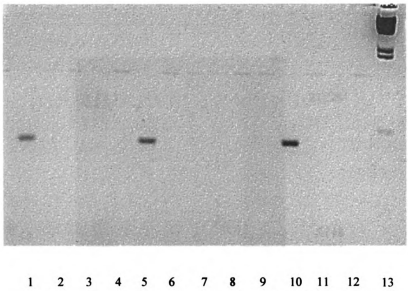

Figure 1. Polymerase chain reaction assay of total potato genomic DNA using B.t. specific 25mer primers. A fragment of 456 bp corresponding to the *cryIA(c)* gene is expected in transformed lines.
Lanes 1- plamsid positve control; Lanes 2-4, 6-9, 11 - untransformed regenerates of FL1607; Lane 5 - FL1607-A30; Lane 10 - FL1607-A11;
Lane 11 - untransformed FL1607 control; Lane 13 -*Lambda* DNA digested with *HindIII* to yield molecular weight markers.

[1]All numbers are base pairs.

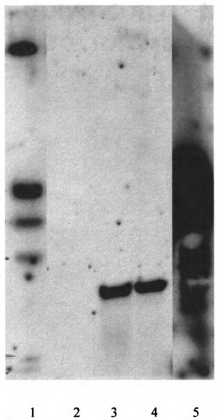

Figure 3. *HindIII* digest of total potato genomic DNA probed with a 256 bp fragment from the *cryIA(c)* gene. A fragment of 2.6 kb corresponding to the gene is expected in transformed lines. Lane 1 -*Lambda* DNA digested with *HindIII* and *EcoRI* to yield molecular weight markers. Lane 2 - untransformed FL1607 control; Lane 3 - FL1607-A11; Lane 4 - FL1607-A30; Lane 5 - plasmid positive control.

[1]All numbers are base pairs.

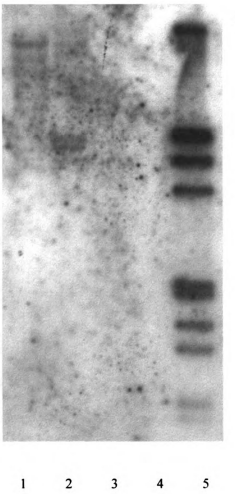

Figure 3. *BamHI* digest of total potato genomic DNA probed with a 256 bp fragment from the *cryIA(c)* gene. Lanes 1 - FL1607-A30; Lane 2 - FL1607-A11; Lane 3 - untransformed FL1607 control; Lane 4 - blank; Lane 5 *Lambda* DNA digested with *HindIII* and *EcoRI* to yield molecular weight markers.

[1]All numbers are base pairs.

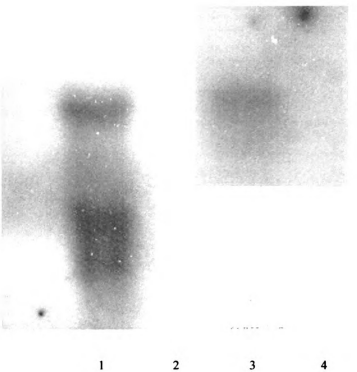

Figure 5. Northern analysis of total potato RNA probed with a 256 bp mRNA fragment from the *cryIA(c)* gene. Lanes 2, 4- blank.
Lane 1 - FL1607-A11; Lane 3 - FL1607-A30;
Lane 5 - untransformed FL1607 control.

Table 2. Insect Bioassay Results.
χ^2 Tests for homogeneity to identify resistance categories. Individual tests were conducted to identify resistance catagories within the bioassay data. The tests listed below are those that were used to form each of the four resistance catagories.

Resistant

Test 1: H_0: $P_1=P_2=P_3=P_4=P_5$
H_1: not all equal, $p>>0.05$ (n.s.)

Test 2: H_0: $P_1=P_2=P_3=P_4=P_5=P_6$
H_1: not all equal, $p<<0.05$*

Moderately resistant

Test 3: H_0: $P_6=P_7=P_8=P_9=P_{10}=P_{11}=P_{12}=P_{13}$
H_1: not all equal, $p>0.05$ (n.s.)

Test 4: H_0: $P_6=P_7=P_8=P_9=P_{10}=P_{11}=P_{12}=P_{13}=P_{14}$
H_1: not all equal, $p<0.01$**

Susceptible

Test 5: H_0: $P_{10}=P_{11}=P_{12}=P_{13}=P_{14}$
H_1: not all equal, $p>0.05$ (n.s.)

Test 6: H_0: $P_9=P_{10}=P_{11}=P_{12}=P_{13}=P_{14}$
H_1: not all equal, $p<0.05$*

Highly Susceptible

Test 7: H_1: $P_{15}=P_{16}$
H_1: not all equal $p>0.05$ (n.s.)

Test 8: H_0: $P_{14}=P_{15}=P_{16}$
H_1: not all equal, $p<<0.001$**

n.s. = not significant
* = significant, $p<0.05$
** = highly significant, $p<0.01$

Table 2. Insect Bioassay Results.

Line	Resistance Factor	Total Number of potato tuber moth Tested	Mortality (%)[1]
FL 1607-A11	*cryIA(c)* transgenic	108	68.5a
FL 1607-A30	*cryIA(c)* transgenic	76	60.5a
FL 1607 (control)	none	150	12.7c[2]
USDA 8380-1 (2x)	leptines	88	67.0a
USDA 8380-1 (4x)	leptines	80	63.8a
PI 230502	undetermined	38	63.2a
Roslin Eburu	undetermined	60	36.7b
KWPTM 29	undetermined	119	30.3b
KWPTM 24	undetermined	150	30.0b
NYL 235-4S[3]	glandular trichomes	74	28.4b
85-37.38	undetermined	27	22.2bc
Cruza 148	undetermined	40	20.0bc
TM-3	unknown, tuber	37	16.2bc
NYL 235-4[4]	glandular trichomes	39	15.4bc
Santa Catalina	none known	20	5.0d
CCC 1386.36	none known	38	2.6d

[1]Separation based on χ^2 test for homogeneity

[2]Average of 15 trials. Mortality ranged from 0-20% in individual trials.

[3]Untransformed regenerate of NYL 235-4.

[4]Control parent. Not subject to regeneration.

Mortality of potato tuber moth larvae on the transgenic lines was significantly higher than on the untransformed control line ($p<<0.001$). Three HPR lines also were grouped into the resistant category: USDA 8380-1 (2x), USDA 8380-1 (4x), and PI 230502. The moderate resistant category included of Roslin Eburu, KWPTM 29, KWPTM 24, and an untransformed regenerant of NYL 235-4. Four lines could not be differentiated between the moderate resistant and susceptible categories: 85-37.38, CIP 720118, TM-3 and NYL 235-4. FL 1607 (untransformed control), was also in the susceptible catagory. The highly susceptible clones were Santa Catalina, and CCC 1386.36.

DISCUSSION

TRANSFORMATION

Two of 35 putative transgenic lines were confirmed to contain the *cryIA(c)* gene. This efficiency of transformation was lower than expected. Transformation frequencies can vary considerably depending on the regeneration protocol used. Previous transformation protocols on potato yielded frequencies of 50% to 100% (De Block 1988, Hulme et al. 1992, Wenzler et al. 1989) when $50mgL^{-1}$ of kanamycin was used for selection. Here, lower stringency conditions contributed to the high percentage of escapes produced.

The pWB139 plasmid used in these trials contains the *nptII* gene which confers resistance to the selectable agentr, kanamycin. The construct has both the *cryIA(c)* and *nptII* genes fused and functioning under the control of a single promoter. With this arrangement, kanamycin resistance was conferred at 25 mgL^{-1} (0.0429 mM). Wenzler et al. (1989) demonstrated that at a selection pressure of 25 mgL^{-1} of kanamycin sulfate, less than 10% of regenerated shoots expressed the transgene, while with a selection pressure of 50 mgL^{-1}, greater than 65% of all shoots expressed the transgene. Cheng et al. (1992), also using the same pWB139 construct used here, also observed low transformation frequency (0.4%). It appears that the low kanamycin-resistance expression of this construct limits its potential for transfomation of potato.

High expression of the antibiotic resistance is necessary to allow effective selection of transformed plants. The pWB139 construct transcribed the *cryIA(c)/nptII* gene fusion directed by a single CaMV 35S promoter. Other promoters can offer much greater levels of expression. Ni et al. (1995) reported that constructs containing the chimeric promoter

sequences of mannopine and octopine synthase genes with a trimer of octopine synthase activators directed 156-fold more GUS expression than a CaMV 35S promoter alone and 26-fold more expression than a doubled CaMV 35S promoter sequence. It would be expected that such "super-promoters" could prove to be beneficial even when working with unmodified "wild-type" genes.

Additionally, most constructs have separate promoters for the transgene and selectable marker (Li et al. 1998, Chen et al. 1995). This allows each protein to be made separately and function independently. With the pWB139 construct, there is a single gene product that is produced that must direct both the B.t. and NptII activities.

MOLECULAR ANALYSIS

The transformation of the two lines was confirmed by PCR amplification of the *cryIA(c)* and *nptII* genes (Figures 1 and 2), Southern and northern analysis of the *cryIA(c)* gene and its mRNA copy (Figures 3, 4, and 5), and 1st instar-potato tuber moth feeding studies for the effect of the B.t. protein (Table 2). Two different primer sets were used for the PCR assays: one specific for the *cryIA(c)* gene and the other for the *nptII* gene.

The use of PCR amplification of the gene(s) as a quick screen for transformation was confirmed by subsequent Southern analysis of the untransformed control and four putative transformed lines, which included the two PCR positive plants and two PCR negative plants. Southern confirmation of both gene insertion and copy number was necessary since DNA uptake is possible without stable integration (White 1993). Cheng et al. (1992), working with the same pWB139 construct used in these studies, found that 4 of 7 PCR positive plants did not give positive Southern assays. They hypothesized that this was due to the gene not being

inserted into the plant genome. In our studies, only those lines that were PCR positive gave positive Southern results. Since all putative transgenic lines were screened with primers specific for both *cryIA(c)* and *nptII*, we were also able to determine if either gene was inserted without the other, as if the construct were not fully integrated. This was not the case. Thus, the escapes were true escapes and did not represent regenerants possessing one gene without the other.

CryIA(c) EXPRESSION

Northern analysis demonstrated RNA transcription of the transgene in the 2 B.t. lines. Translation of the cryIA(c) gene was demonstrated by potato tuber moth bioassays of potato foliage which showed a significant difference in resistance between the transformed and untransformed plants. However, the lack of additional transgenic lines limits the conclusions that can be derived from the data. More transgenic lines would have given a better understanding of the range of resistance and expression presented by an unmodified, wild-type *cryIA(c)* gene.

A codon-modified *cryIA(c)* gene may have resulted in even better potato tuber moth control. Perlak et al. (1991, 1993) reported that codon modification increased the transcriptional/translational efficiency (i.e. protein production) of *cryI* and *cryIII* B.t. Depending on the degree of modification, effects of 100- to 500-fold protein expression can be detected. Li et al. (1998) were able to obtain complete mortality of potato tuber moth in tuber bioassays using a codon-modified *cryV* gene. Tao et al. (1997) obtained very high resistance levels (96-99%) using a codon-modified *cryIA(c)* gene in Japanese Persimmon against a lepidopteran pest, *Monema flavescens* Walker. Synthetic *cryIA(b)* genes have been

transformed into cotton and corn (Perlak et al. 1990, Koziel et al. 1993) and such modification has been found to offer strong resistance (up to 100%) to lepidopteran insect pests, while a truncated version of the same class of *cryIA(b)* genes in tomato only offered tolerance (Fischhoff et al. 1987). Fujimoto et al. (1993), also working with a similar codon-modified *cryIA(b)* gene in rice was able to achieve significant control (40-50%) of two major rice insect pests.

Previous research has suggested that it is necessary to evaluate large numbers of transgenic lines due to the wide range in transgene expression. Perlak et al. (1993) screened 308 *cryIIIA* transgenic-Russet Burbank potato lines to identify 55 with sufficiently high expression for resistance to Colorado potato beetle larvae and, of those, only 23 expressed the CryIIIA protein at levels of 0.1% or higher (total protein). Cheng et al. (1992) screened 243 regenerated shoots to identify 1 plant with sufficient *cryIA(c)* expression for feeding studies with European corn borer, although there was only a 10% difference in resistance between the control and transgenic line. Other authors have reported over a 20-fold variation in gene activity for identical regenerants of a common transformation trial (Stiekema et al. 1988).

Some of the differential response may be due to the number of genes inserted, as well as their location in the genome. Wenzler et al. (1989) reported that 2 of 2 transgenic lines contained multiple copies. De Block (1988) had 3 of 9 lines with 2 or more copies. Douches et al. (1998) reported that copy number in their population of 25 lines varied from 1 to 3. Nevertheless, none of these authors attempted to compare gene expression to copy number.

Since the *cryIA(c)* gene in these studies was under the control of a CaMV 35S promoter, it was expected that the B.t. should have been expressed constitutively throughout

the plant in all tissues. The results from potato tuber moth foliar bioassays demonstrated that there was foliar expression. Recently, Douches, Pett, and Grafius (unpublished data) analyzed tubers and foliar tissue of FL 1607-A11 in field trials in Egypt and found that, under those conditions, there was no difference in foliar damage compared to the control but there was a decrease in tuber mining and damage (60% damaged in FL 1607-A11 vs. 80% FL 1607 control).

LEAF BIOASSAYS

Potato tuber moth feeding assays were conducted for all plant material over a period of several weeks. No-choice bioassays proved to be a reliable method to determine actual plant resistance to potato tuber moth. Similar no-choice studies, with both leaves and tubers, have been used to determine potato tuber moth resistance levels in potato species and cultivars (Chavez et al. 1988, Ojero and Mueke 1985, Raman and Palacios 1982, Westedt et al. 1998, Li et al. 1998).

The potato tuber moth leaf bioassay studies were conducted at random over time as the material permitted. This was done to spread environmental effects randomly over all lines and reduce the effect of error on any single line. Ideally, it would have been possible to measure the effects across time by fully sampling all lines for each individual trial. Since this was not possible with our design, the control data were studied to detect a normal distribution. The average percent mortality in susceptible lines varied from 0-20% (avg=12.7%, std. dev.=8.8).

Abbott (1925) presented a method to analyze mortality data and achieve a more realistic percent control for various treatments taking into consideration the mortalities present in controls. This approach is based upon removal of the mortality expressed in the control population with the treatment effect adjusted as follows, where S = % survival:

$$100 \times \frac{S_{treatment} - S_{control}}{S_{control}} = \%\ mortality\ due\ treatment$$

This method allows comparisons of results of different treatments where different controls were used. In such a way, equal importance can be placed on the adjusted effects of all treatments compared to respective controls. Our data were collected using a single control, the susceptible, untransformed, cultivar FL 1607. Since there were not multiple, independent experiments, each with separate controls, the method advocated by Abbott was not used.

In these studies, insects which could not be found, or "escapes", were counted as dead. The first reason that this was done was due to unobserved mortality; when first instar potato tuber moth larva die and desiccate, it can be difficult to identify them. The second reason, was to reflect host plant resistance mechanisms which functioned through host plant avoidance rather than through direct toxicity; both mechanisms could be beneficial, our system was designed to note effects of either although neither could be measured individually.

No tuber feeding studies were conducted. Douches, Pett and Grafius (unpublished data) analyzed the tubers of these Bt-transgenic lines versus the control and found that the *cryIA(c)* reduced potato tuber moth damage from 80% for the untransformed control to about 60% for the transgenic lines with no reduction in yield.

STATISTICAL ANALYSIS

For statistical analysis, three assumptions were made about the data: 1) the effect of time of sampling was balanced across all lines; 2) individual insects within trials did not influence the action of other insects; and 3) all insects had an equal probability of mortality in any single study so that the mortality could be analyzed through a binomial distribution. Each of these will be discussed below.

1) Effects of sampling balanced across all lines. Since the samples were conducted at random times, it is important that all effects be distributed without bias on any single line. Although there may have been such an effect, by the sampling method, this effect was evenly distributed.

2) Insects within a study did not influence the action of other insects. We can be relatively confident of this because of the design of the feeding study. All ten insects were dispersed over the available leaf surface. In almost all cases, the leaves consisted of either three or five leaflets. When three leaflets were present, three larvae were placed on each of the two smaller leaves and four on the largest. When five leaflets were present, the larvae were distributed two to each leaflet. Additionally, the larvae consumed very little leaf tissue, $\leq 10\%$ of the total leaf surface of the controls. Thus, given the distribution of the larvae and the lack of intense mining of all available leaf, we can assume that there was no significant competition between larvae.

3) All insects had an equal probability of mortality. It is assumed that there was no resistance that developed in the population and that all insects started with the same initial health. One area of concern would deal with the method employed to move the larvae from

the rearing facilities to the leaves. For that, a moistened paint brush was used to gently sweep the larvae and then carry them to the leaves where they would be gently rolled off of the brush onto the leaves. For all the feeding studies, larvae were used almost exclusively except in those few cases when only eggs were available. It can thus be argued that since only larvae were used and all larvae were treated equally, any effect of this movement technique would be equally spread over all lines and studies.

The experimental procedure allowed all three assumptions to be met and allowed for statistical analysis through a χ^2 test for homogeneity of the data. The test depended upon the data fitting a binomial distribution which is defined as having a fixed number of trials, recording the data as either 0 or 1 (dead or alive in this case) where all trials are independent. This test had the ability to test between any pair of the ranked data and also allows the development of groups (categories) when there was no clear difference between adjacent data.

Transgenic Bioassay Results

Since only 2 *cryIA(c)* transgenic lines were produced, it was difficult to draw conclusions about how best to incorporate this form of resistance. It was noteworthy that both lines exhibited high resistance to potato tuber moth with over 60% average mortality (up to 100% in some trials). The *cryIA(c)* gene that was used was a truncated wild-type gene, otherwise unmodified to increase activity. Nevertheless, it did control the potato tuber moth after only 48 h exposure. We did not measure the long-term effects of exposure, but it has been noted that there can be a cumulative effect over time including a lengthening of the life cycle and a decrease in fecundity in insects that are able to survive on B.t. treated plants (Perlak et al. 1993).

An additional area that would have been interesting to explore involved the effect of multiple gene insertions on insect mortality. One of the transgenic lines had 2 copies of the *cryIA(c)* gene (FL 1607-A11) while the other line had a single copy of the gene (FL 1607-A30). Additionally, FL 1607-A11 exhibited 68.5% average mortality versus the 60.5% mortality of FL 1607-A30. However, the difference was not statistically different and both were grouped into the high mortality group. Li et al. (1998) suggested that with cryV transgenic potato lines, higher copy number may contribute to higher mRNA production and higher resistance levels. In these studies, it would have been interesting to test additional lines with single and multiple copies of the gene to determine if multiple copies will result in higher resistance to potato tuber moth.

Host Plant Resistance Bioassays

Of the host plant resistance lines tested, 3 lines were identified that provided high resistance to potato tuber moth: USDA 8380-1 (2x and 4x) and PI 230502. Both the 2x and 4x lines of USDA 8380-1 primarily use leptine glycoalkaloids as their basis of resistance (Sinden et al. 1986b, Deahl et al. 1991, Sanford et al. 1996). The line PI 230502 is a selection of *S. sparsipilum* spp. *sparsipilum* and possesses resistance to potato leaf hopper and nematodes but has not been reported to confer resistance to potato tuber moth (Hanneman and Bamberg 1986).

Leptines can confer strong host plant resistance against certain insects (Sinden et al. 1980, Sinden et al. 1986b, Deahl et al. 1991). Similar results have been observed with Colorado potato beetle larvae (*Leptinotarsa decemlineata* Say) with these two lines (Pett, personal communication). Our data also indicate that high levels of resistance to potato tuber moth are present. The lack of true "immunity" seen in these bioassays may be due more to

the design of the studies than is indicated. The bioassays were only conducted over a 48 h time period after which time the surviving insects were counted. Observations indicated that most potato tuber moth larvae that were able to survive 48h on these three lines did not develop as quickly nor feed as much compared to the potato tuber moth larvae on untransformed FL 1607. It was expected that most of the surviving larvae on these three host plant resistance lines would also die within the next 24-48 h if the assays would have been continued. Thus, the true mortalities could have increased to 100% in feeding assays of longer duration.

All other host plant resistance lines exhibited moderate resistance, while the two cultivars, Santa Catalina and CCC 1386.36 were highly susceptible to potato tuber moth foliar feeding. This may be due to the definition of host plant resistance that previous sources have used, as well as our methods of determining resistance.

One fault that some previous reports had about host plant resistance with potato tuber moth was a lack of sufficient controls and a reliable method to quantify the resistance (Ojero and Mueke 1985). A common method for previous screens for resistance was simply to leave open bags of potatoes in a room into which adult potato tuber moth were introduced. After a period of time, feeding damage of potato tuber moth larvae on those potatoes was measured and resistance was determined. However, such a system cannot separate of host plant preference effects from true resistance (Kogan 1982). When the insect would be faced with a "no-choice" option, as in a farmer's field, such "resistance" could either not be durable or would offer no protection from insect pressure (Ali 1993, Trivedi and Rajagopal 1992, von Arx et al 1990b). Indeed, our analysis of TM-3 and other lines with reported resistance indicates that their resistance does not hold up under no-choice conditions.

Although convenient, mortality may not be the best method to gauge host plant resistance. França et al. (1994) note that *Solanum berthaultii*, a wild species which contains glandular trichomes and from which NYL 235-4 is derived, can limit ovarian development and fecundity of Colorado potato beetle females, although it does not cause outright death in adults. There may also exist conditions in which the host plant avoidance plays a larger role in insect control than host plant toxicity would. As a resistance strategy, insects would not be faced with a forced evolution to accommodate themselves to their food source. They would, instead, seek alternate sources of food and switch their behavioral characteristics accordingly. Therefore, to further understand the host plant resistance mechanisms that function against potato tuber moth, other bioassays must be conducted in the laboratory, greenhouse, and field, including tuber tests, choice tests, and field trials.

B.t. can be expressed in any potato line though genetic engineering. With this in mind, research efforts should be made to introduce the B.t. gene into plants with natural host plant resistance. The pyramiding of multiple insect resistance mechanisms may allow for a more durable HPR. Insect pests would be selected against at different levels, including natural host plant resistance, host plant avoidance and transgenic resistance. Such a durable host plant resistance would contribute to an IPM program to control potato tuber moth.

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