T.R.
NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

DEVELOPMENT OF INSECT RESISTANT TRANSPLASTOMIC POTATO LINES AGAINST COLORADO POTATO BEETLE (*LEPTINOTERSA DECEMLINEATA*)

MD JAKIR HOSSAIN

JUNE 2021

M. J., HOSSAIN

PhD THESIS

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Supervisor

Asst. Prof. Dr. Allah BAKHSH

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The study entitled **"Development of insect resistant transplastomic potato lines against Colorado potato beetle (*Leptinotarsa decemlineata*)"** and presented by **Md Jakir HOSSAIN** under the supervision of **Asst. Prof. Dr. Allah BAKHSH** has been accepted as PhD thesis by the jury at the Department of Agricultural Genetic Engineering of Niğde Ömer Halisdemir University, Graduate School of Natural and Applied Sciences.

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THESIS DECLARATION

It is certified that I have written this thesis by myself. I further confirm that all information included in this thesis is scientific and is in accordance with the university rules and regulations. Any materials that I have used from external sources as well as help received and all sources used in finalizing this research work and preparing this thesis, all have been acknowledged in the thesis.

Md Jakir HOSSAIN

SUMMARY

DEVELOPMENT OF INSECT RESISTANT TRANSPLASTOMIC POTATO LINES AGAINST COLORADO POTATO BEETLE (*Leptinotarsa decemlineata*)

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Aimed to mitigate the CPB mediate crop losses, Potato chloroplast was transformed with biolistic gene gun mediated transformation protocol. Bombarded explants (Leaves and internode) were cultured on MS medium supplemented with BAP (2.0 mg L−1), NAA (0.2 mg L−1), TDZ (2.0 mg L−1) as well as GA3 (0.1 mg L−1) where spectinomycin 50 mg L-1 was used as a selection agent. Leaf explants of cultivar Kuroda induced highest (92%) number of calli where cultivar Sante produced the highest (85.7%) transplastomic shoots. Thiadiazuron was found more proficient (41%) for shoot regeneration. Cultivar Sante and Challenger showed 9.6% shoot regeneration efficiency (%) followed by cultivar Simply Red (8.8%). PCR amplification yielded 16 transplastomic plantlets out of 21 spectinomycin resistant shoots. Target gene integration was confirmed by PCR and Southern blot, whereas the RT-qPCR and ELISA was used to record the expression level. Localization of visual marker gene *gfp* was conducted by Laser scanning confocal microscopy. The transplastomic lines were capable to ensure high mortality (100%) to both larvae and adult CPB where transplastomic lines Sa7, SR5 and Ch3 showed more insecticidal effects. Foliage consumption and weight gain of CPB feed on transplastomic leaves were lower compared to their control plants.

Keywords: Insect pests, insect resistance, *bacillus thuringiensis*, transplastomic approach

ÖZET

KOLORADO PATATES BÖCEĞİNE (*Leptinotarsa decemlineata*) KARŞI BÖCEKLERE DAYANIKLI TRANSPLASTOMİK PATATES HATLARI GELIŞTIRILMESSI

HOSSAIN, Md Jakir
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Colorado Patates Böceğinden kaynaklanan ürün kaybının azaltılması amacıyla, patates kloroplastı, biyolistik gen tabancası aracılı transformasyon protokolüyle transforme edilmiştir. Bombalanan eksplantlar BAP (2.0 mg L-1), NAA (0.2 mg L-1), TDZ (2.0 mg L-1), GA3 (0,1 mg L-1) ve ayrıca seleksiyon için spektinomisin 50 mg L-1 içeren MS ortamında büyütülmüştür. Santae çeşidi en yüksek oranda (%85.7) transplastomik sürgün üretirken, Kuroda çeşidinin yaprak eksplantları en yüksek sayıda (%92) kallus indüklemiştir. Sürgün rejenerasyonu için Thiadiazuron daha yeterli (%41) bulunmuştur. Santae ve Challenger çeşitlerinin sürgün rejenerasyon etkililiğini (%9.6) Simply Red (%8.8) takip etmiştir. PCR amplifikasyonu 21 transplastomik filizin 16sı spektinomisine dayanıklı olarak sonuçlanmıştır. İlgili genlerin entegrasyonu, PCR ve Southern blot ile doğrulanırken, ekspresyon seviyesini kaydetmek için RT-qPCR ve ELISA kullanıldı. Görsel markör geni *gfp*'nin lokalizasyonu konfokal lazer taramalı mikroskop ile tespit edilmiştir. Transplastomik hatlar CPB'nin larva ve yetişkin aşamasında yüksek böcek ölümünü (%100) sağlamak için yeterli olmuştur. Sa7, SR5 ve Ch3 transplastomik hatları CPB'nin yetişkin ve larva instar aşamasında daha böcek öldürücü etki göstermiştir.

Anahtar Kelimeler: CPB, böcek direnci, *bacillus thuringiensis*, transplastomik yaklaşım

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SYMBOLS AND ABBREVIATIONS

Symbols	**Descriptions**
%	Percent
°C	Degree centigrade
g/ L	Gram per liter
mg/ L	Milligram per liter
ml	Milliliter
mM	Milli-molar
W	Watt
µl	Microliter
µM	Micro-molar

Abbreviations	**Descriptions**
BAP	6-Benzylaminopurine
bp	Base Pair
cm	Centimeter
ddH_2O	Double-Distilled Water
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Triacetic Acid
FAOSTAT	Food and Agriculture Organization Statistical Databases
GA_3	Gibberellic Acid
Kb	Kilobase
LB	Luria-Bertani Medium
MS medium	Murashige and Skoog Medium
NAA	1-Naphthaleneacetic Acid
NAA	1-Naphthaleneacetic Acid
ng	Nano Gram
OD	Optical Density
PCR	Polymerase Chain Reaction
p^H	Power of Hydrogen

RNA	Ribonucleic Acid
rpm	Rotate per Minute
RSM	Regeneration Selection Medium
TBE	Tris/Borate/EDTA
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is included in the largest genus *solanum* comprised of 1,500 to 2,0000 species, where the family Solanaceae contains 3,000-4,000 species from approximately 90 genera (Machida- Hirano, 2015). Because of genomic diversity (4,500 varieties and 98,285 accessions) of potato and the emergence of advanced genome research technologies potato has become a vital crop for genetic engineering since last few decades. Globally, Potato is a non-cereal staple food crop (Vincent et al. 2013) that ranks in 4th position in production (Hameed et al.,2018, FAOSTAT data, 2017) and plays a crucial role to ensure food security of the poor (Thiele et al., 2010, Bagri et al., 2018). It can mitigate the malnutrition and poverty status proficiently in more than forty countries (Brown, 2005, Caliskan et al., 2010). During 2019, global production of Potato was 376,826,967 tons (FAOSTAT, 2020). Turkey is one of the most popular potato supplying country among the world-famous 156 potato-growing countries (Göre, 2017) and cultivating an area of 172,000 ha with 4.8 million tons of output (Turkstat, 2017). Potato is an abundant source of carbohydrate, vitamins, protein, and minerals. Turkey stands for 17th position in terms of global potato production (FAOSTAT, 2019) that plays a vital role in Turkish economic growth (almost 3% of total GDP) (Çalışkan et al., 2010). In Turkey, Potato is the topmost vegetable (Rafique et al., 2004) and a good chunk of the population of the country directly or indirectly depends on the Potato for their nutritional uptake. Three potato crops per year are typically cultivated in the Mediterranean agro-climatic conditions as spring and autumn crops in plains and as summer crops in northern hilly regions of Turkey (Çalışkan et al., 2002). Because of very high inputs and excessive cost associated with the import of quality distress seeds, potato cultivation is remarkably expensive, and a slightest to moderate damage to potato yield can affect the farmers in the long run. Cultivation of vegetatively propagated Potato invades by various diseases causing agents and insect pest those are remaining prime biotic barriers for the potato production.

Several fungal (20 fungal pathogens), viral (11 viral pathogens), and bacterial pathogens (6 pathogens), 10 nematode species are most common and mostly responsible for the unwanted economic damages of potato crops (Raspor and Cingel, 2021, Kepenekci et al., 2013). Verticillium wilt by *Verticillium dahlia K.*, *Fusarium* mediated dry rot and wilt, early blight, and late blight by *Alternaria solani* and *Phytophthora infestans* respectively including black scurf by *Rhizoctinia solani K. are the common bacterial diseases.* At the same time, PVA, PVS, PLRV, PVX, and PVY viruses also cause remarkable loss to the potato crop yield. The common bacterial scab by Streptomyces scabies *L.* is also responsible for the unexpected economic damages. Insect pests from different inset order invade to potato crop during field condition and post-harvest period. Economic damages due to insect pests were estimated as 40% (FAOSTAT, 2020). Among those insect population, the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*), potato leafhopper *(Empoasca fabae* L.*)* attack foliage part, wireworm damage tubers and potato tuber moth *(Phthorimaea operculella* L.*)* and black cutworm *(Agrostis ipsilon* H.*)* attack both foliage and tubers and green peach aphids are importantly transmitting viral diseases. As a result of the more significant adverse effect of various biotic and abiotic factors, 40% of potato crops get losses during field and post-harvest storage condition (Oerke, 2006). Besides these, insect pests and diseases are also remaining a viable threat to global crop production; those contribute up to 40% crop losses (FAOSTAT, 2020).

Every growing season, notorious coleopteran insect pest CPB feed on the plant's foliage parts and can defoliate all potato plants in a field by mid-season. CPB is by far the most widely distributed destructive insect pest among the coleopteran (EPPO / CABI, 1997). As a big potato defoliator, CPB significantly damages the yield of potatoes across the northern hemisphere (Visser, 2005; Rondon 2010, Alyokhin et al. 2013). The Mexico as well as southeastern USA was native place for beetle (Casagrande et al., 2014) and spread rapidly from Kazakhstan throughout North America, Europe, and parts of Asia (Tower et al., 1906), and in the 1990s invaded China. The CPB has since spread eastward and distributed in most of northern China's Xinjiang Uygur Autonomous Region (Jiang et al., 2010). CPB was first reported in Turkey within the Asian countries in 1949 (CABI, 1992; Gürkan and Bosgelmez, 1984; EPPO, 2014; Gözel and Gözel, 2014). Adult CPB, as well as larval stages of CPB, feed on potato leaves, thereby significantly reducing yield and even killing plants.

CPB mediated damage to potato leaves is remaining a significant threat for the potato production in Turkey where CPB feeds only on potato crops (Zhou et al., 2012). Fast all the leaves of potato plants can be invaded by the beetles, which intensively affect the photosynthetic status of the plants. As a C3 plant, Calvin cycle occurs in the mesophyll cells of potato but due to extensive feeding of foliar part, leaf mesophyll cells get reduce heavily that interrupt the Calvin cycle and finally growth and development of potato plants.

With a high reproductive capacity, both adult and larval stages of CPB can severely defoliate the potato crops throughout the whole growing season (Hare, 1980), causing field damage and farmers financial loss (Hare et al., 1990 and Jacques et al., 1988). Heavily defoliated plants with uncontrolled CPB have reduced yields that can exceed 85 % (40–80 %) (Maharijaya and Vosman ., 2015), estimated at 2–2.5 billion USD in Russia (Skryabin, 2010, Maharijaya and Vosman ., 2015). In cases of severe CPB attack, potato yield losses of 100 % have been documented in Turkey (Orek 2006, Alyokhim, 2009). Therefore, the Solanaceous crops contains diverse types of secondary metabolites those enhance the detoxification mechanism of CPB and ensure fair co-evolution and survival of CPB against many natural and synthetic chemicals (Hussain et al. 2019; Naqqash et al. 2020). The beetles can develop resistance to newly introduced insecticides within short time by the dint of mysterious and unique biological make up (Mota-Sanchez and Wise 2017). CPB is today resistant to 56 different compounds belonging to all major classes of insecticides with various modes of action, especially capable of rapid growth and toxin resistance (Raspor and Cingel ., 2021, ISAAA., 2021).

Presently, the breeding of highly resistant potato varieties to combat potato insect pest is the unique challenge for the global breeder community, as no potato cultivar with built-in resistance to these insect pests has so far been reported. These insect pests are infamous for the development of short-term insecticide resistance. The various classes of rotational insecticides are recommended to enhance the development of resistance but there are still problems. Unfortunately, in some countries, farmers need to spray their potato fields more than 10 times throughout a single growing season to fight against these pests. Not only does it enormously increase input costs, but it also remains potential health hazards to farming community engaged in potato production (Tegg and Wilson, 2012).

With the advent of higher insecticide-resistant pests, pesticide applications are made ever more frequent and increasingly ineffective, thereby raising the danger to human health and the ecological status(Gould et al. 2018). As a control measure to this harmful pest, several methods including insecticides, biological control, crop rotation, traditional breeding, and space isolation have been utilized aimed to reduce the unwanted damages. Until now, exogenous spray of pesticides is remaining the most effective way to combat this insect pests because of environmental status, labor cost, and other hindrance factors. Solanaceous crops harbors diverse secondary metabolites those facilitates the detoxification mechanism of insects to survive against multiple and complex natural and synthetic chemicals. In these circumstances, it is crucial to explore a new alternate , sustainable and viable control measure against notorious insect pest CPB (Zhu et al., 2011).

In contrast, the CPB has durable adaptive capability and developed resistance against diverse chemical pesticides (Jiang et al., 2010 and Alyokhin et al., 2013). Besides, the third-instar larvae pupate as fourth-instar larvae in soil condition and back to the ground surface to complete their total life cycle. The insecticides, then do not prohibits the raging of CPB. Meanwhile, prolong exogenous insecticidal spray cause significant ecological damage (Casida et al., 1998 and Elbert et al., 2008). The spread of pesticides and insecticides through irrigation systems or field runoff into water supply contributes to water pollution that damages plants, beneficial insects, and livestock and it poses a serious threat to drinking water and water used for post-harvest activities. Concerns about the impacts on the environment and health have led to new technologies being developed and used to combat potato pests.

To grow potato plants which are more resistant to these notorious insect pests, it is, therefore, an urgent necessity to incorporate the knowledge gained from the new technologies into potato research. Thus, several agricultural methods have been used in fighting with CPB throughout the extended period include rotation in crop production, maintaining distance from previous potato fields, creation of barriers by physical means, thermal or electromagnetic aided protection, machinery-based elimination of insects. Since long past, physical as well as chemical CPB control measures of are remaining relatively unsuccessful that representing "125 years of mismanagement," while implementation of biological control measures can be more pest-specific and environment-friendly.

The second green revolution that took place in the early 1980s has led to the proposed use for CPB control of bio-rational insecticides and antifeedants, natural enemies, trap cropping and genetic modification of plants. Transgenic technology has been used over the past three decades to grow insect-resistant plants to minimize both yield losses and excessive use of pesticides.

Since ancient past, classical plant breeding is remaining a viable solution to develop plant traits of interest which requires years round careful and intensive greenhouse and field activities (Danida, 2002). Recently, many breeding practices have been implemented to develop insect resistance transgenic potato plants (Flanders et al., 1992; Raman and Palacios, 1982), but the progress regarding the production of transgenic especially transplastomic potato cultivar with optimum resistance levels against CPB expressing commercially valuable genes or traits is very less (Lagnaoui et al., 1999). Biotechnological advances instigated and added new technological aids to classical breeding to create a new horizon and alternative wings for insect resistance potato production. Crop plants introduced by the dint of biotechnological endeavors specially genetically modified (GM) crops can be more precious to unravel the study of insect resistance mechanisms. Narrow genetic base of potato is a potential barrier of conventional breading practices (Douches et al., 1996) and no CPB resistance genotype have been released with this technology (Roman, 2013).

Consequently, for the adoption of comprehensive and integrated pest management (IPM) strategies in potato transgenic technologies have become more precious and effective components (Kos et al., 2009). Although these transgenic technologies were initiated in 1996 for the first time, global agriculture has adopted it very rapidly and nearly 185.1 million hectares cropping areas have been cultivated for biotech crops especially for insect resistance (25 million hectares) and herbicide resistance (45 million hectares) crops (James, 2014). Among the above-mentioned transgenic crops, most of those were developed with insecticidal genes from *Bacillus thuriengenesis* (Bt) were constitutive promoter 35S was expressed in those plants (Carrière et al., 2016). There is no question that transgenic plants manifested with Bt toxins (Bt plants) are the best example which have achieved substantial economic and ecological success. The gradual integration and expression of target genes may impose metabolic abnormality to plant that can raise the potential environmental hazards of increase resistance in targeted insects.

Sustainability, capability of insect pests orders against transgenic crops have been reported frequently and remaining a burning question of the time (Zhang et al. 2011; Gassmann et al., 2011; Tabashnik et al., 2013; van den Berg et al., 2013).

Bt toxins proficiently kill the target pests and impose a negligible harm to the non-target organisms and regards as a critical biologically viable agent for CPB control (Acciarri et al., 2000 and Adang et al., 1993). Toxicity of Bt toxin depends on the toxic proteins (Vip proteins and parasporal crystalline protoxins) secrete during the plant growth (Palma et al., 2014; Ibrahim et al., 2014). Cry toxins were engineered successfully into transgenic plants as conventional sprayable bioinsecticides (Baum et al., 1996, and Gryspeirt et al., 2012). When consumed by susceptible CPB, Cry proteins get proteolytically triggered with the activation of intestinal proteases and the active toxin release and their toxicity may start by the receptor sites located on the brush border midgut of epithelium. Eventually gut epithelium gets broken down that leading to their death (Gómez et al. 2014). The adenylyl cyclase signaling pathway instigate by an alternative cell death cycle that is responsible for the pore-forming mechanism (Cheeke et al., 2013, and Li et al., 1991). Several genetic engineering-based methods were initiated to develop CPB resistance potato (Chougule et al., 2012 and Bravo et al., 2013) containing the cry3A gene and expressed in potatoes explicitly. Vegetables that express *cry3A* protein, which are resistant to CPB, have been commercially available in the USA since 1996. However, in 2001, those potato varieties containing *cry3A* gene had been withdrawn from the market due to low sales and buyer concerns on GMOs (Cooper et al., 2004). There are fewer reports in Turkey regarding the resistance of transgenic potato plants to CPB. Also, CPB’s propagation speed is remarkable in the province of Nigde.

There is a pressing need to develop new transplastomic potato varieties ensuring insect-resistant to reduce potential CPB damage. These newly introduced insect-resistant potatoes will be suitable enough for cultivation in the Nigde region and that the CPB will be lowly adapted to cry proteins. Concerns about the impacts on the environment and health have led to new technologies being developed and used to combat potato pests. To grow potato plants which are more resistant to these devastating insect pests, it is, therefore, an urgent necessity to incorporate the knowledge gained from the new technologies into potato research.

Insertion of target genes into the chloroplast genome successfully adopted to develop valuable agronomic traits and production of insect resistance transplastomic potato line is one of the remarkable achievements in this regard. Because of several lucrative alternatives to nuclear transformation, the plastid transformation has become a vital tool for the manipulation of the genome of some economically important crop plants (Wang et al. 2009). High ploidy number in plastid genome facilitate the high level of transgene expression (up to 1-40% of TSP) (Roudsari et al. 2009). As reported by Daniell *et al.* 2002 and Hou *et al.* 2003, protein expression in transplastomic plants was up to 18% while transgenic plants from nuclear transformation results in 0.5-3% of TSP. Transplastomic plant contains multiple copies of plastid transgene that leads to higher protein expression and affects by pre- or post-transcriptional silencing. According to Maliga et al. 2001, transplastomic plants also can ensure the multiple gene expression from polycistronic origin of mRNA, avoiding the epigenetic effects and gene silencing and it also offers enormous utility over the nuclear genome engineering.

This current research included the incorporation of genes of interest into the chloroplast genome, the selection and regeneration of transformants, the evaluation of transgene integration by modern molecular techniques and the bioassays of transplastomic lines. These transgenic lines are harboring high levels of functional protein in an environment friendly manner because transgenes are maternally inherited, and gene containment will be maintained in the next generation. The work generated transplastomic potato lines by expressing hybrid *SN-19* and single *cry3A* gene into the genome of chloroplast only in green sections of potato plants since chloroplasts are the plastids found only in green plant tissues (Gruissem 1989). This work addresses major GMO-related issues by confining foreign gene expression to green sections of plants alone. High levels of expression of target genes (*SN-19, cry3A*) in the form of an operon in the potato chloroplast genome to generate a functional chimeric protein resolved the emergence of resistance in insects due to low levels of toxin protein. The high levels of potato resistance expressing *SN-19* will help fight against CPB, leading to increased crop yield. Various reports denote that, crop losses because of this notorious insect pest have been estimated at 85 % (40–80%) (Maharijaya and Vosman ., 2015). Through the successful operation of the proposed study, crop damage controls up to that extent will be a remarkable achievement.

Our current work is original as it incorporates two different and novel ideas to grow insect-resistant transplastomic potatoes. First, it was a unique study to incorporate target genes (*SN-19*, *cry3A*) to ensure a higher level of foreign protein expression in potatoes to resist CPB. Secondly, our construct contained a green tissue-specific promoter (*psbA*) that confined the expression of genes only in the green parts of potato lines. Through this study, we developed insect resistance transplastomic potato lines expressing one single *Cry3A* and one hybrid *SN-19* gene into the chloroplast genome. Transplastomic lines developed from our current research initiative were resistance enough to CPB and ensured 100% mortality of larvae and adult CPB. Besides these, these lines minimized the economic damages in an eco-friendly manner.

1.1 Aim and Objectives of Present Research

The goal of this current study is to produce insect-resistant potato line by expression of green tissue-specific genes to fight against potato defoliating pest CPB. Although, modern genetic engineering technologies that play a pivotal role in implementing features of insect resistance in potatoes, scientists, environmentalists, farmers, and consumers have raised numerous concerns about genetically modified food crops. We conducted an exceptional, unique, compelling, and promising strategy to combat the problem of insect-pests in potato, taking into consideration the issues discussed. We developed transplastomic potato lines with the expression of green tissue specific promoter *psbA* having insecticidal proteins only in green sections of potato plants that solved the passive saying about the presence of insecticidal proteins in edible parts of plants. In addition to this, the expression of *Cry3A* and hybrid *SN-19* genes will be solved the possibility of emergence of insect resistance due to the low toxin protein accumulation in plant. It will be resolved in the form of an operon in the genome of potato chloroplast by the expression of our target genes.

Overall aim:

The overall objective of the current study is to develop insect-resistant transplastomic potato lines with confined insecticidal gene expression in only green parts of the plant. Specific goals to achieve the purpose mentioned above:

a. Genetic transformation of potato with the gene gun mediated Particle bombardment
b. *In-vitro* regeneration of transformed tissues with suitable selection agent (Spectinomycin)
c. Analysis and confirmation of transformed plants using modern molecular methods.
d. Localization of foreign chloroplast proteins.
e. Bioassays of transplastomic plants for insect resistance by stressing data on the insect mortality rate and foliage consumption area.

CHAPTER II

REVIEW OF LITERATURE

2.1 Importance of Potato

A solanaceous food crop, Potato (*Solanum tuberosum* L.) is an incredibly valuable crop worldwide that placed fourth after maize, rice, and wheat among the main staple crops. From its South American origins, short life span during cultivation and wide range of climatic adaptation have favored potatoes across various geographical barriers. Over 3,000 cultivars of potato are now cultivated extensively in more than 125 countries of the global farm market. These are mainly in the humid, sub-tropical and Tropical regions (Hameed et al., 2018). In contrast to other potential food crops, it can be an abundant source of carbohydrates, proteins, minerals, and vitamins per unit area of land (Zaheer and Akhtar, 2016). On average, 77% of water, 20% of carbohydrates and less than 3% of protein, dietary fiber, minerals, vitamins, and other compounds are present in potato tubers. Potato could replace other high-priced foods in low-income food shortage countries and can be used sustainably as a cheap food that provides ample calories (93 kcal/100 g tuber) to sustain an everyday life (Burlingame et al., 2009). Potato is primarily used in industry to produce processed food products, alcohol, starch, animal feed and the production of biofuel and being a raw marketable commodity (Hameed et al., 2018). According to the Food and Agriculture Organization of the United Nations (FAO STAT, 2020), it is the fourth most important food crop, after wheat, rice, and maize. According to a new United Nations study released today, the world population is predicted to rise by 2 billion people in the next 30 years, from 7.7 billion at present to 9.7 billion in 2050. Potatoes are eaten as a staple by more than 1 billion people, and crops play an increasingly important role in future global food security. Approximately 20 million hectares are cultivated globally, with an average yield of 17 tons per hectare, resulting in 370 million tons estimated at US$50 billion annually (FAO STAT, 2020). Potato tubers contain 17-20% starch, 1-2% protein, and enriched vitamins in the C, A, B, and K groups (Beals et al., 2019). Around 75% of achievable potato production will be lost to pests without crop safety (James, 2011). Oerke reported quantitative potato losses due to insect pests to be approximately 34% annually in 2006.

The global value of potatoes is unquestionable, and the United Nations celebrated 2008 as the "International Year of Potatoes" to commercialize its position in defeating food scarcity, hunger, and primarily malnutrition (Hameed et al., 2018).

2.2 Introduction to the Insect Pest CPB

Colorado potato beetle, CPB (*Leptinotarsa decemlineata*), is globally known harmful potato pests. Adults as well as larval instars consume the foliage portion of host the host plants, and complete defoliation occurs unless efficient control is ensured. Under favorable environmental conditions, populations are likely to grow considerably. Systematically CPB belongs to the order Coleoptera and the family Chrysomelidae. The CPB invaded potato in the Midwest region of the USA during the mid-1860s and spread out globally at the pace of 100 km every year (Johnson, 1967) affecting the West Coast of the USA around 20 years later. Currently, the CPB is widely distributed in North America, as well as Europe and Asia. The primary hosts of CPB are crops that belong to the Solanaceae family and weeds, but other plants such as ground cherry and climbing nightshades are also known to be targeted. Primary or secondary hosts can support the life cycle of CPB, where secondary hosts may play an essential role by providing a "green bridge" for CPB survival even in the absence of primary hosts. CPB has a considerable geographic variation in the original and expanded geographic areas (Weber and Ferro, 1994).

The complete life cycle of CPB comprises an egg stage, four larval stages, pupal stages, and an adult stage (Fig .1). The adult CPB start wintering in the soil for about 30 days (Capinera 2001). Adults migrate after emerging in the spring by walking and flying to their host (Boiteau et al., 2003). A polygamous insect pest CPB with numerous copulations of males and females with separate partners (Alyokhin et al. 2013) females begin to deposit yellow orange color masses of eggs on the lower foliage surfaces of host plants (Hare 1990). During the lifetime, a mating female lay more than 800 eggs and ensure three generations every year depending on the weather conditions (Ferro et al. 1985). After 4–12 days of oviposition, eggs start to hatch (Tauber et al. 1988) and newly born larvae immediately begin feeding. Adequately fed 4th instar larvae stop feeding in the soil and pupate. The life cycle consists of a pre-pupal quiet period before pupation (Hare 1990). Adults emerge after 5–7 days from pupation (Tauber et al., 1988;

Boiteau and LeBlanc, 1992). Adults can matt, migrate, stop feeding or enter diapause depending on temperature, photoperiod, and host plant condition (Voss and Ferro, 1990). Phenotypic diversity, plant visual and olfactory indicators (Fernandez and Hilker 2007) are essential factors that affect the host plant location by the CPB. Volatile chemical compounds harboring by the solanum species helps CPB to identify the host plants.

Many chemicals, including trans-2-Hexen-1-ol,1-Hexanol, cis-3-hexen-1-ol, trans-2-hexenal and linalool, methyl salicylate, and (Z)-3-hexenyl acetate were suggested to play a role in this relationship (Visser et al. 1979; Dickens 2002; Martel et al. 2005). Damaged leaves can attract CBP adults to the host plant (Bolter et al. 1997), as damaged leaves release the volatile compound(s). CPB's feeding behavior is highly influenced by stimulants released from potato leaves.

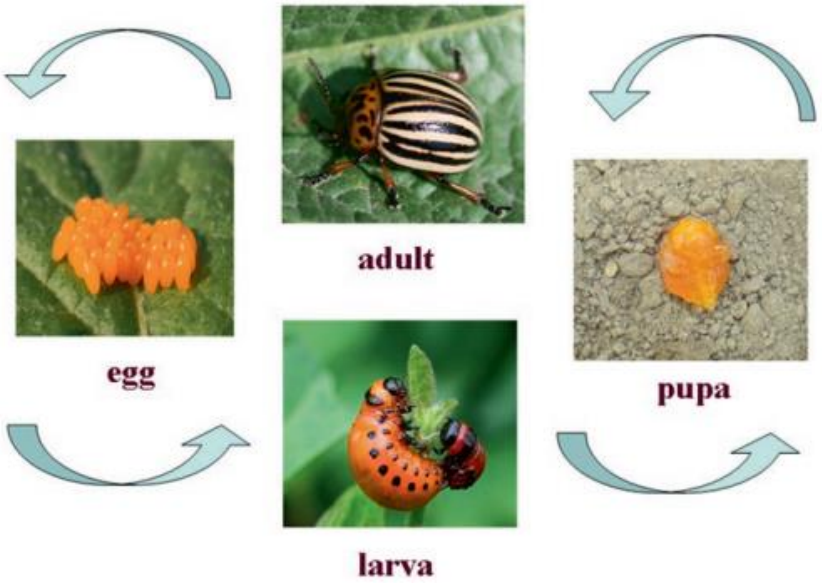

Figure 2.1. Diagram depicts the events of the life cycle of CPB. Adopted from Guo et al. (2017)

The fraction of sterols (cholesterol, β-sitosterol and stigmasterol) serves as a stimulant for feeding CBP larvae (Szafranek et al. 2008). Besides these, there are few more compounds present in potato leaves that act as feeding deterrents. A leaf extract from *S. berthaultii* enriched with methylene chloride and acetone, refrains adults CPB from feeding (Yencho et al., 1994). The CPB also has a protective impact on the leaves extracts from *S. tarijense* (Pelletier and Dutheil 2006).

Table 2.1. CPB mobility and nature of damage to primary host plants in their different life stage (The Australian Government, 2018)

Life stages	Invaded /Affected area of plants
Eggs	Oviposition is in cluster on the lower surface of leaves, sometimes on stalks of leaf and stems, and on soil on a rare basis.
1st instar larvae	Feed on the leaf surface and leaf edges that results in skeletonization. Cannibalism may occur during dry weather.
2nd instar larvae	Invade to growing tips
3rd instar larvae	Feeding occurs on leaf edges, petioles and stems that cause extensive defoliation
4th instar larvae	Feeding starts on leafy portion, petioles, and cause defoliation; Migrate to the soil after maturation and burrow to different depths.
Pre-pupa	in pupation chamber in the soil
Pupa	Remain into a pupation chamber in the soil.
Adult CPB	Emerge from soil and fly to nearby host plants, extensive feeding on leaves, occasionally tubers start; mating and oviposit occur. Winter diapause and hibernate stage throughout the winter start - and re-emerge in spring. Summer "aestival diapause" start and continue up to extended dry periods in hot regions.

Table 2.2 The pattern of damage and activity of CPB on potato plants. Other primary hosts are affected similarly, but damage to secondary host plants may vary from this (The Australian Government, 2018)

CPB invaded parts of plants	Nature of Attack	CPB life stages
Soil	N/A	4th larval instar burrowing; Pupae; Emerging and dispersing of Adult CPB by crawling.
Root	Yes	Adult CPB - Feed on tubers on an occasional basis.
Stem	Yes	Larvae and adults CPB- Black and sticky excreta deposited.
Leaves	Yes	Larvae and adults CPB – Extensive damages cause defoliation and black sticky excreta deposited.
Growing Tips	Yes	Larvae and adults CPB – Black sticky excreta deposited.
Buds	Yes	CPB larvae - heavy infestations cause damages.

CPB eggs on potato leaves

1st and 2nd instars larvae of CPB

2nd and 3rd instars larvae of CPB.

Adult CPB on potato plants

Massive Potato defoliation by CPB

Figure 2.2. Feeding and massive defoliation of potato crop by CPB (The Australian Government, 2018)

Phenotypic characters can also play a role in dissuading feeding of CPB. When it comes to *S. tarijense,* the CPB shows peculiar behaviour. *Tarijense* leaves, abundantly populated by trichomes, leave the plant after a few minutes by letting themselves fall to the ground. However, after the removal of trichome feeding of CPB is also limited, which suggest there are also another compounds in leaves or the leaf structure itself, apart from the glandular trichomes that reduce the CPB feeding (Pelletier and Dutheil 2006).

2.2.1 Pest status of CPB

2.2.1.1 Defoliation and economic damages by CPB

The CPB threatens the production of potatoes for many decades. Although, it was first identified as a potato pest in Nebraska in 1859, currently considered to be the most crucial potato insect defoliator (Radcliffe and Ligneous 2007; Vreugdenhil et al., 2011) globally. CPB population destroys the plant without distinguishing between the leaf tissues. Beetles start feeding on stems and exposed tuber once the vegetation is gone (Weber and Ferro 1993; Alyokhin 2009). Defoliation thresholds are reported as 25% before bulking tuber, 10% in the first half, four to six weeks and 25% after bulking. Damage to the wine results in a loss of yield due to foliage loss, which also supports tuber growth and malformation of tubers. The plant can be stunted by severe damage too. The CPB defoliates the potato plants that reduce tuber production significantly (Kennedy 2009; Alyokhin et al . 2013). Many major potato-producing countries in Eastern Europe have recorded high yield losses due to the CPB invasion (Radcliffe 1982; Heikkilä and Peltola 2004). The CPB is considered the most crucial defoliator insect pest of potatoes anywhere it is present. On average, a single beetle larval consumes 40 cm^2 of potato leaves, and an adult CPB consumes about 10 cm^2 of foliage every day (Ferro et al. 1985, Logan et al. 1985). When all the foliage portions of colonized plants are finished, beetles will feed on stems and tubers that are exposed. They are, however, a suboptimal diet relative to leaves, resulting in low larval growth and adult oviposition cessation. In the absence of control steps, it is not uncommon for beetles to kill potato crops entirely.

Nevertheless, potato plants can withstand a significant amount of defoliation without any reduction in tuber yield, mainly when damage occurs before or after the tuber bulking period. Without insecticides, CPB can cause yield losses of 40–80 per cent in potato crops and the total annual damages caused by the CPB are estimated to be USD 2–2.5 billion in Russia alone (Skryabin 2010). A loss of more than 75% of the foliage may cause total damage to crops (Hare 1980; Shields and Wyman 1984). Hare 1980, reported the effect of beetle feeding on remarkable crop losses. According to that report, 70% defoliation can result in around 20% yield losses and complete defoliation can reduce yield losses by nearly 64%.

Similarly, Cranshaw and Radcliffe 1980 reported that plants become fully capable of recovering up to 33% defoliation without any remarkable financial damage inflicted early in the season. But on the other hand, if the plants suffered from 67 % defoliation by the CPB invasion, it goes for only minor yield decreases. Wellik et al. 1981 also found that losing 29 % of the area of the leaf did not affect the yield of potato. Zehnder et al. 1995 established that 20% defoliation action thresholds from plant emergence to early bloom, 30% from first bloom to late bloom, and 60% from late bloom to harvest. During the two weeks immediately preceding vine kill, Ferro et al. (1983) and Zehnder and Evanylo (1989) found no effects of up to 100 per cent defoliation. Unfortunately, data from yield losses are often highly variable, and their analysis is often a challenge (Nault and Kennedy 1998). Commercial farmers are also extremely risk-averse and usually do not want to tolerate beetle infestations in their crops. Consequently, even non-damaging CPB infestation rates cause control steps, generally in the form of insecticidal sprays.

2.2.1.2 Tremendous adaptive malleability of CPB: from innocuous beetle to super-pest

The ten-striped and innocuous beetle CPB has turned into a prominent global pest and appears to be virtually "indestructible.' Different patterns of a life cycle, the plasticity of phenotypic aspects, an extreme adaptive norm to adverse environment status and the capacity to withstand toxins have favored it (Cingel et al. 2016). The global distribution of CPB from the Mexican highlands results from the evolution of various adaptations of different eco-physiological status (Figure 3). The outspreading adaptive success of CPB was primarily relying on the chronological changes of insect-plant interactions referring to host expansion. Besides, CPB populations possess ample genetic diversity in the characteristics of life cycles that is an essential component for the development of adaptive capabilities to various adverse environmental conditions. First, CPB contains leptinotarsin, a neurotoxin that protects against predation. However, some arthropod parasitoids and predators (*Coleomegilla maculata, Perillus bioculatus, Lebia grandis*) can significantly reduce CPB populations (Hazzard et al. 1991, Weber et al. 2006, and Biever et al. 1992).

Second, CPB is renowned for its rapid development of resistance to hundreds of insecticides and remains one of the potential challenges for the modern pesticide industry. Some chemicals like arsenicals, organochlorines, carbamates, organophosphates, or pyrethroids were unable to breach CPB 's adaptive requirements. Induction of detoxifying enzymes, decreased penetration of insecticides, increased excretion of pesticides develop insecticide insensitivity and these are some well-known CPB resistance mechanisms and responsible for creating resistance against more than 56 different active chemical ingredients from almost all insecticide groups (Whalon et al. 2012). It is remarkable to note that during the second, or even during the first year, some of the newly applied insecticides failed (Forgash et al. 1985). Even imidacloprid, a neonicotinoid that has been used successfully for almost 10 years, was starting to struggle in the early part of this century (Olson et al. 2000 and Zhao et al. 2000). Although several newly introduced insecticides are being tested against CPB (Scott et al. 2015) but there is no basis for dependence on any of them handling this voracious beetle successfully. Finally, there are several aspects of the life cycle, and its plasticity facilitates many benefits to CPB. Depending on the environmental condition, high fecundity, multivoltine , biology and prolonged diapause stage of female favor the genetic variation that provide high fitness with adaptive random mutations under new conditions. These characteristics can reduce the effectiveness of customized management strategies, enabling CPB to remain dormant and avoid adverse condition to restore population under favorable conditions. CPB has developed broad-spectrum physiological adaptations to its hosts, are shown in Figure 2. 3.

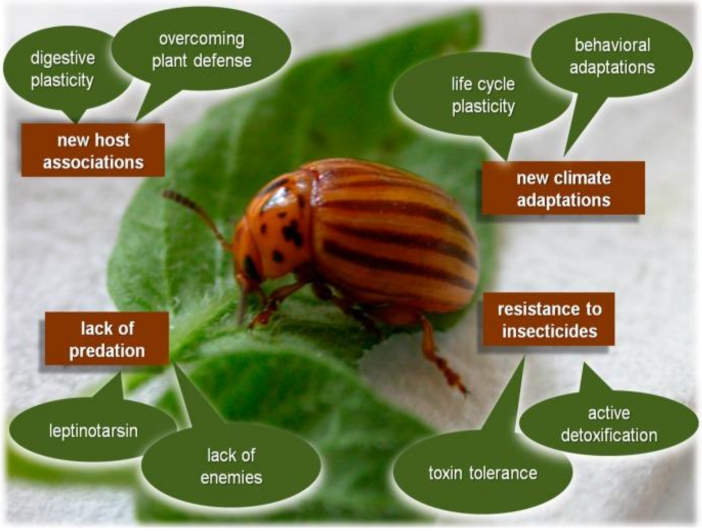

Figure 2.3. Various eco-physiological adaptations of CPB that render it as most invasive “super pest” worldwide (Cingel et al., 2016)

Depicted traits can also serve as pre-adaptations that allow CPB to become an "archetype" for the development of resistance to man-made control strategies (Alyokhin et al. 2015). Potato plants contain volatile organic compounds (VOCs), which serve as a source of positive anemones and attract CPB from its source even up to 50 m away (Schütz et al., 1997). These potato VOCs sensitivity and appeal increase insect hunger and sexual maturation (Dickens.,2002). CPB recognizes the contact chemoreception of plant compounds (Müller and Renwick., 2001) which inhale the decision on feeding. The male aggregation pheromone is also attractive to CPB and to the potatoes infested with male beetles (Dickens., 2006), probably due to volatile signals from both pheromones and plants, CPB colonizes potatoes for feeding and reproduction. As part of the constitutive defense against herbivory, the presence of abundant glycoalkaloids (solanine, chaconine, leptins, and demissine, shown in figure 4 in potato enhance constitutive defence against herbivory . However, CPB can tolerate (Armer et al. 2004) or detoxify allelochemicals of host plants (Zhu et al. 2016), without any growth and survival abnormalities.

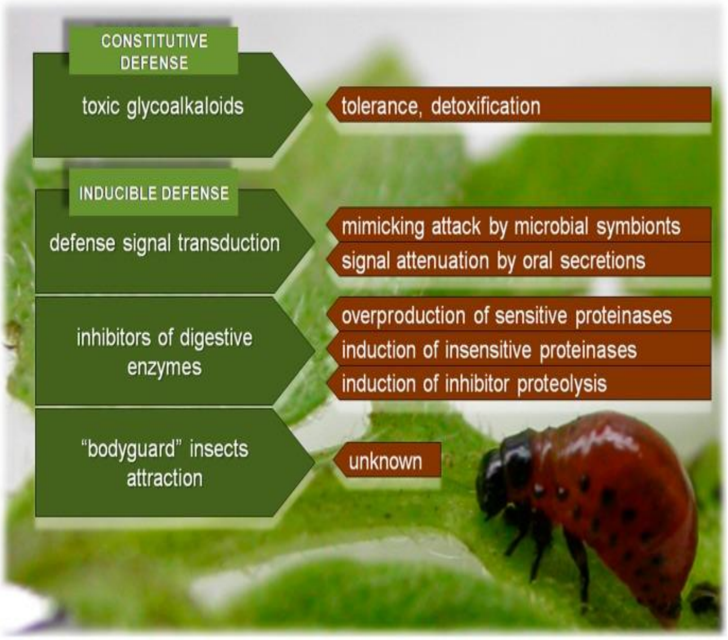

Figure 2.4. Defense strategies (shown by green boxes) of potato and the reciprocal CPB counter measures (indicated by red boxes) (Cingel et al., 2016)

Therefore, the potato must rely on the inducible one (Figure 2.4) caused by mechanical wounding as well as the components of CPB regurgitant and plants can distinguish between insect infestation (Heil et al. 2012) and regurgitant-responsive genes and wound-responsive genes.

Seventy-three potato genes have been triggered, while 54 genes have been repressed by applying CPB regurgitant to wounded leaves (Lawrence et al., 2008). Also, 29 leaf proteins and 16 of them were downregulated for CPB feeding, while only eight proteins alter expression in response to wounding (Duceppe et al., 2012). While the CPB attack initiates the potato defense machinery, and some of the genes are downregulated by feeding. For example, an isoform of potato aspartate PI, a wound-inducing aspartic proteinase inhibitor in CPB midguts, down-regulated by oral CPB secretions. In contrast, the second isoform is slightly repressed despite its up-regulation in wounded leaves (Lawrence et al. 2007). Despite CPB 's impairment of protective signal cataracts, large amounts of proteinase inhibitors (PIs) in response to the beetle attack are still synthesized in potato leaves (Gruden et al., 2003). However, CPB has various tactics in its fighting arsenal to conquer this line of potato defense. CPB increases the development of "standard" proteolytic enzymes to reach the high level of potato PIs and induces new isoforms (Gruden et al. 2003) and groups of digestive proteinases (Petek et al. 2012) that are resistant to potato inhibitors.

Besides, those that can cleave and thus inactivate the potato PIs are among CPB proteinases induced or upregulated as a response to potato PIs (Gruden et al. 2003). CPB larvae adjust their digestive proteolysis to be unaffected by potato PIs because of these reactions (Figure 4).

2.2.1.3 Extended resistance capability of CPB against control measures, chemical insecticides, and Bt endotoxins

Chemical therapy, biological regulation, and cultural traditions include CPB management and control methods. To date, in the long history of fighting CPB, several different agricultural methods have been used, such as rotation of crop production, maintaining distance with previous potato fields, physical barriers, and thermal regulation. In the early 1980s, the second green revolution contributed to the proposed use for CPB management of bio-rational insecticides and antifeedants, natural enemies, trap cultivation, and plant genetic modification. The incorporation of recombinant proteins having insecticidal properties into the potato genome has been recognized as a promising solution to the CPB regulation method. The widespread resistance of CPB to management techniques has increased as a new problem that is often most evident when insecticides in field conditions cannot control insects damage.

Although the critical past and current management strategies of CPB are based on pesticides (Zabel et al. 2000; Kennedy, 2008), the CPB has been able to establish resistance to most of the licensed insecticides by genetic adaptation (Sladan et al. 2012; Szendrei et al. 2012). An increased dosage can only provide short-term relief which enhances the risk of developing resistance significantly. CPB has developed resistance to all new insecticides that have been implemented to control the CPB and in a pest population, this resistance is due to a genetic alteration that results in repeated insecticide failure (IRAC, 2013).

Recently, CPB has developed insensitivity on 54 active chemical ingredients (Alyokhin et al. 2008, Whalon et al. 2012). Besides this, CPB also developed cross-resistance to organophosphates and carbamates and multiple resistances to organophosphates, carbamates, and pyrethroids.

In addition to synthetic insecticides, laboratory selection studies have revealed that CPB can evolve resistance to delta-endotoxin *Bacillus thuringiensis* subsp *tenebrionis*. The first CPB resistance to synthetic organic pesticides (DDT) was identified in 1952 and resistance to dieldrin, followed by other chlorinated hydrocarbons, was reported in 1958. In subsequent years, the beetle gained resistance to various organophosphates and carbamates, which are naturally resistant to a wide variety of insecticides, including arsenic, organochlorine, carbamate, organophosphate, and pyrethroid (Fig. 4). In some instances, within one year (e.g., endrin) or even within the first year of use (e.g., oxamyl), a newly implemented insecticide indicates failure.

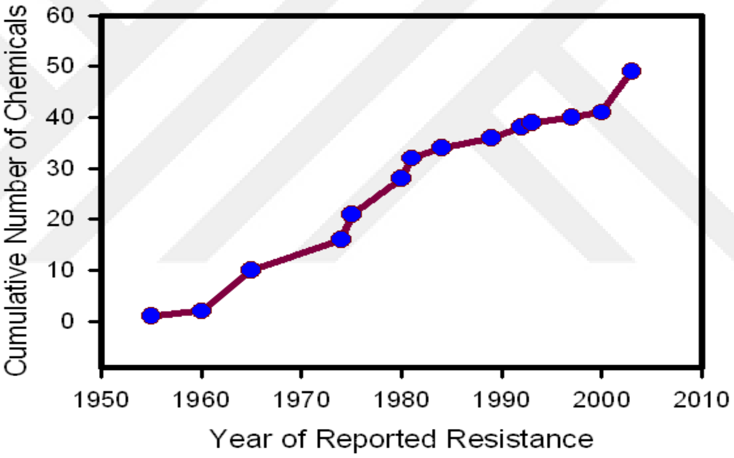

Figure 2.5. Cumulative number of insecticides to which resistance in the CPB has been reported (Data from Michigan State University)

The first evidence of imidacloprid resistance from the CPB have already been reported. At some of the resistant hotspots, Imidacloprid no longer suppresses CPB populations .

2.3 Transgenic Approach for CPB Resistance is a Viable Solution to the Chemical Control

To date, chemical control is remaining an extensive and hugely used method of CPB control globally although CPB has gained the capability to resist all chemical insecticides more or less. Besides this, chemical agents are more destructive to the natural environments and human health as well. Considering these issues, transgenic endeavors can be a viable solution to control the notorious insect pest CPB. Potato is included among the transgenic crops developed at the first efforts and basic intention was paved to initiate insect and disease resistance, improvement of nutritional values and vaccines delivery (An et al. 1986). As a part of CPB resistant plant development endeavors Bt protein from *Bacillus thuringiensis* and insect digestive enzyme inhibitors was utilized. The CPB proteinase inhibitor has shown significant inhibitory effects (Šmid et al., 2013) although CPB adapted quickly to the proteinase inhibitors (Zhu-Salzman and Zeng 2015). GMO potatoes were primarily produced with the incorporation of Cry3A toxin in 1995 (Thomas et al. 1997) and released with durable resistant against CPB that was in the USA market (Grafius and Douches 2008) between 1996-2000. Therefore, over the course of time, CPB was capable to develop resistant to Bt toxins (Alyokhin et al. 2008). Recently, CPB-resistant transplastomic potato was developed with dsRNA targeted against the *ACT* (Zhang et al., 2015) gene that encodes essential cytoskeletal protein β-actin and ensured 100% CPB mortality.

2.4 Introduction to *Bt* and *Bt* Mediated GM (Genetically Modified) Crops

Presently, Bt (*Bacillus thuringiensis*) is a hugely used agricultural and forestry bio-control agent over the world and found in a variety of distinct environments, from soil, to dessert, to tundra and was first discovered by the Japanese biologist Shigetane Ishiwatarias in 1901 (Milner 1994) and isolated and nomenclature was done in Thuringia, Germany by Ernst Berliner in 1911. The existence of a Bt crystal close to the endospore was reported by Berliner in 1995 but the activity of that crystal was not evolved clearly (Milner 1994). The main insecticidal activity of that crystal was found against lepidopteran insects in 1956 (Milner 1994). Commercialization of Bt crops was initiated in France (1938) and USA (1956) respectively and in the 1980s, the global utilization of Bt increased because of increased resistance of insect pests to the chemical insecticides (Milner 1994). The first CPB-resistant Bt crop lines were deregulated by Monsanto in the United States during 1995. This soil-borne gram-positive bacterium secretes many insecticidal proteins such as Sip (Secreted insecticidal proteins) and Vip (Vegetative insecticidal proteins). Beside these, Bt produce β-exotoxins, parasporal crystalline δ-endotoxins (kill cancer-causing cells), Cyt (cytolytic toxin) and Cry (Crystal toxin) during stationary phase of growth (Chattopadhyay and

Banerjee, 2018, Xu et al., 2014a, b). The colonization capability of Bt inside the insect gut makes it more suitable vector to kill the agricultural insect pests (Deist et al., 2014). Bt israelensis was reported as the first species that was proficiently capable to kill the dipteral larvae including black flies, mosquitoes, and chironomid midges. Although there are 84 strains of Bt, all the strains are not harmful for insects (NTB-88 strain) (Ibrahim et al., 2010), some are specific to host (Bt israelensis is only active against dipteran pests) (Land and Miljand, 2014) and few strains are only active against Lepidoptera and Coleopteran (Azizoglu et al., 2015).

Cry proteins are the key active ingredients in Bt-based microbial insecticides, which have been used as foliar sprays in agriculture for several decades (Perlak et al., 1993). Cry3A, a parasporal crystal protein produced by *Thuringiensis bacillus var. tenebrionis*, has insecticidal properties against CPB. This protein is distinguished by its high unit activity and specificity for certain coleopteran insect pests, such as CPB (Perlak et al., 1993). Bt insecticides have the advantage of not being harmful to humans, non-targeted animals, or beneficial arthropods. Bt is an important alternative to conventional chemical insecticides in many integrated pest management (IPM) programs due to its particular mode of action and selectivity. Bt sprays, on the other hand, provide only rudimentary plant defense since the toxins are photosensitive and degrade easily in contrast to most other chemical insecticides (Whalon and Wingerd et al., 2003). Furthermore, the use of Bt sprays for pest control poses concerns about the risk of accelerated Bt resistance (Sexson and Wyman., 2005., Christou et al., 2006).

Insect-resistant transgenic plants are often made using Cry genes derived from Bt (Fischhoff et al., 1996). The first genetically modified potato cultivars expressing the Cry3A toxin were published in 1995. (Thomas et al., 1997). Perlak et al 1993 injected the Cry3A protein into potato plants in one of the first trials. The addition of a Cry3A gene to Russet Burbank potato plants improved their resistance to insect attack and damage. The damage was significantly reduced by all insect phases in the laboratory and at several field locations, according to the findings. Further testing revealed that GM-potatoes were of the same quality as standard or non-GM Russet Burbank potatoes in terms of agronomic characteristics, including taste. The GM variety for human food was commercially available in the United States from 1996 to 2001, maintaining fair monitoring of the CPB during that period (Grafius and Douches, 2008). However, due to GM potato planting, new insecticide compounds, and public rejection, the GM potato did not last long on the market. The only commercially cultivated GM potato variety is 'Amflora,' which is only licensed for agricultural use and animal feed (James., 2011). Based on recent data, more than 700 identified Cry gens represent a precious means for the well-adopted strategy to integrate resistance in commercially important crops with high and selective toxicity against various insect taxa. The cloning, transfer, and expression of these genes have been well adopted in a greater scale aimed to control the insect pests. For instance, in USA about 82 percent of maize production is from Bt-maize while 88 per cent of cotton cultivated was from Bt-cotton in 2020 (Raspor and Cingel., 2021). Additionally, transgenic potato expressing cry1Ac or cry1Ia1 exhibit durable level of resistance to CPB and finally ensured 90% reduction of feeding followed by increased first instar larvae mortality. Moreover, transgenic potato was developed with the separate expression of *cry3A* , *cry1Ac9, cry9Aa2* and *cry1Ab* where gene expression was driven by light-inducible promoters. Such type of spatial expression of *cry* genes facilitates the high level of resistance against CPB with negligible or no toxin accumulation in the tubers (Raspor and Cingel ., 2021).

2.5 Global GMO Summary

The total cultivated area of GM crops was 1.7 million ha in 1997, and it steadily increased to 190 million ha in 2019 in 26 countries, 19 of which are developing countries and 7 of which are industrial countries. About 112-fold increase in GMO production was recorded in 2019 that added more than $225 billion economic growth (ISAAA., 2019). The GM crops primarily consist of five crops: two of them (corn and cotton) are insect-resistant alone or in combination with insecticides. Herbicide resistance is seen in the other three crops (soybean, canola, and sugar beet). In 2016, the area of GM crops in developing countries was 99.6 million ha (54%) compared to 85.5 million ha (46%) in industrialized countries. The United States developed 72.9 million hectares (or 39% of the world's total area), followed by Brazil (27%), Argentina (13%), Canada (6%), India (6%), Paraguay (2%), Pakistan (2%), China (2%), and South Africa (1%). In 2015, five European countries (Spain, Portugal, the Czech Republic, Slovakia, and Romania) planted approximately 117,000 ha, rising to 136,000 ha in 2016. Romania chose not to plant in 2016 due to the government's onerous requirements (James 2016).

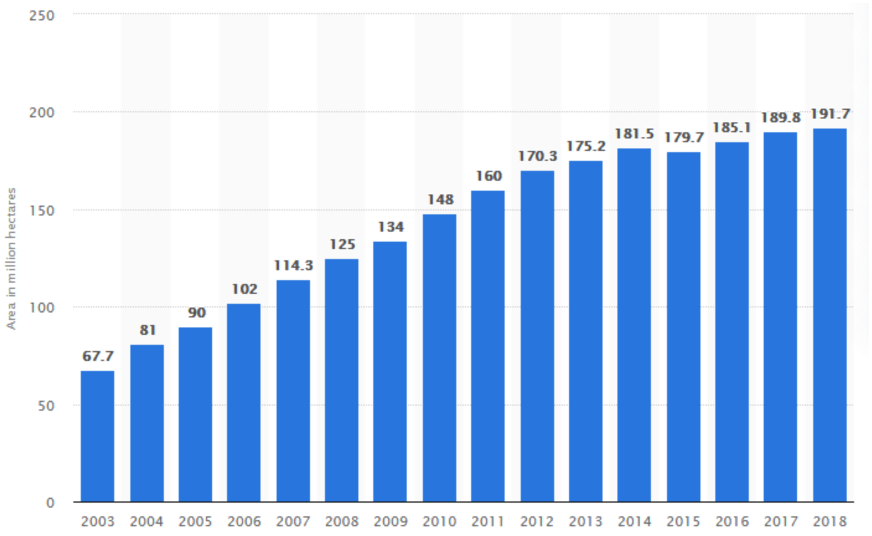

Figure 2.6. Global GMO production areas (million hectares, 2003 to 2018, based on Statista-2020 and statistics of ISAAA)

In 2018, the 22nd year of commercialization of GMO crops, up to 17 million farmers in 26 countries planted 191.7 million hectares of GMO crops. The 191.7 million hectares planted in 2018 represents a 113-fold increase from the initial planting of 1.7 million hectares in 1996, when the first biotech crop was commercialized (Figure-6). As a result, GMO crops are the most widely accepted crop technology in modern agriculture's history. The adoption of GM crops resistance to insect pests remarkably reduced the utilization of insecticides by 8.3% (775.4 million kg) and 18.5% reduction of chemical pesticide induced impact of synthetic chemicals on environmental where cutting the fuel usages and reduction of dependency on chemical spray were considered as vital factors. Besides these, greater impact of GM crops will remain viable solution for biosafety issues because of risks associated with gene flow, resistance development in insects as well as weeds, harmful impact on beneficial and non-target organisms, and toxic or allergic reaction to human health (Raspor and Cingel ., 2021).

2.6 Mechanisms of *Bacillus Thuringiensis* (Bt) for Insect Resistance

Bacillus thuringiensis (Bt) is a common gram positive, spore-forming, soil bacterium. When resources are limited, vegetative Bt cells undergo sporulation, synthesizing a protein crystal during spore formation. Proteins in these crystals are called Cry (from Crystal) endotoxins and have been known for decades to display insecticidal activity against specific insect groups. Three- domain Cry toxins from the bacterium Bt are increasingly used in agriculture to replace chemical insecticides in pest control as it is a bona fide pathogen, with many evolutionary adaptations to pathogenesis (Raymond, 2010). Examples include Cry1Ac active against certain Lepidoptera, Cry2Ab targetting some Lepidoptera and active on Diptera, and the coleopteran active Cry3Aa. These cry toxins are cytotoxic, not to all cells but specific insect species cells, killing insect cells by inserting pores into the membrane (Heckel, 2020). Current findings stated that there are several steps in the mode of action of 3- domain Cry toxins after ingestion by insect larvae (Bravo et al., 2007). The crystalline protein associated with the spore, or the plant's protein, dissolves in the insect midgut, releasing the protoxin, which may be as large as 135 kDa. The insect's digestive proteases cleave the protoxin down to a protease- resistant core, the active toxin of ~65 kDa. Benefits of this technology include high specificity and potency, reduction in chemical pesticide applications, and increased crop yield. Recently, how Cry toxins killed insects is an area of interest. Even though Cry toxins have been extensively used commercially, the specifics of their mode of action are still controversial. Most of what we know about how Cry toxins work is derived from Lepidoptera models. Considering that the 3-dimensional structure of Cry toxins is highly conserved (figure below), we would predict that the mode of action of Lepidopteran and Coleopteran-specific Cry toxins is very similar.

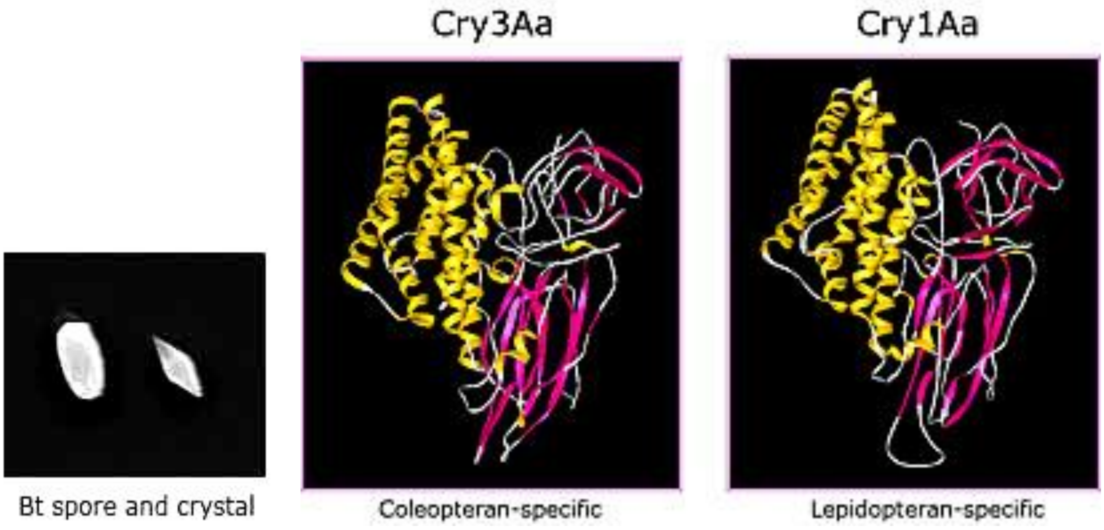

Figure 2.7. Models of the 3-Dimensional structure of Cry3Aa (Li et al., 1991) and Cry1Aa (Grochulski et al., 1995) toxins

However, not much is known about the mode of action, or the toxin receptors involved in Cry intoxication in Coleoptera. This common multi-step toxicity process (Figure below) includes Ingestion of the Cry protein by a susceptible insect, solubilization, and processing from a protoxin to an activated toxin core in the insect digestive fluid. (With the presence of alkaline PH, by the proteolytic activation). Active toxins consist of 3 structural domains: Domain-I (7α-helicase): Determine the toxicity and pore formation. Domain-I (3β-sheets): Receptor binding and specity and Domain III (2β-sheets): Receptor binding and protein processing. The toxin core travels across the peritrophic matrix and binds to specific receptors called cadherins (membrane- bound proteins) on the brush border membrane of the midgut epithelial cells. Toxin binding to cadherin proteins results in activation of an oncotic cell death pathway and/or formation of toxin oligomers that bind to GPI-anchored proteins and concentrate on regions of the cell membrane called lipid rafts. Membrane- spanning alpha- helix hairpins of the oligomers create a small pore (10–20 Å) in the membrane. The toxin induced pores formed in the columnar cells allow rapid flux of ions that lead swelling and osmotic lysis of the cells (colloid- osmotic lysis) (Knowles and Ellar., 1987). Lysis and bursting of midgut cells allow the midgut juices to pass into the hemolymph. Minor damage might be healed by the insect, but major damage destroys the midgut epithelium, resulting in rapid cessation of feeding and high blood PH that results starvation, septicemia, paralysis and eventually death (Heckel, 2020). Most chemical insecticides kill pest insects swiftly but are also toxic to beneficial insects and other species in the agroecosystem.

Cry toxins enjoy the advantages of high selectivity and the possibility of the application by sprays or transgenic plants. However, these benefits are offset by the limited host range and the evolution of resistance to Bt toxins by insect pests.

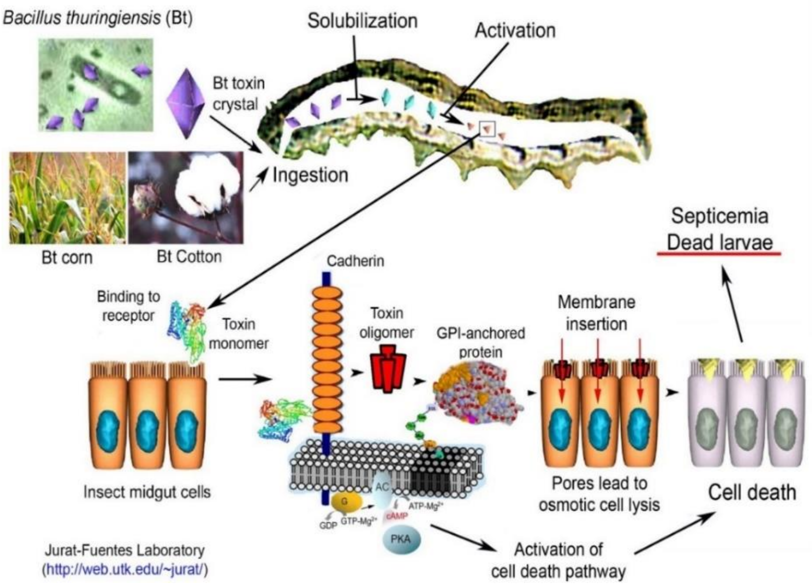

Figure 2.8. Entire process of mode of action of cry protein to kill the insect pest (Jurat-Fuentes Laboratory)

2.7 Genes Used in This Current Study: Brief Description

2.7.1 *Cry3A* gene

Source and structure: The CPB and other coleopteran species are poisoned by the *Cry3A* toxin produced by Bt (*Bacillus thuringiensis*) var. *tenebrionis* and other strains (Mahadeva Swamy et al., 2013). The *Cry3A* toxin's comparatively simple structure, which is surprisingly similar to *Cry1Aa*, makes it an excellent model for studying the structure-function relationship between ligand and receptor. Three functional domains make up the three-dimensional structure of the toxins: (i) a cluster of seven K-helices predicted to be involved in membrane interaction; (ii) three antiparallel L-sheets involved in receptor binding; and (iii) an L-sandwich implicated in receptor binding and ion channel activation in associated Cry toxins (Chen et al., 2005).

Protein size: The *cry3Aa* gene encodes a 68 kDa protein that is proteolytically converted to a 65 kDa polypeptide during or after crystal formation, resulting in the Cry3A protein being visible as 1 or 2 protein bands on SDS–PAGE (Sekar et al., 1987).

Gene Size: 1.836 kb.

Regulation: The Bt *cry3A* gene is regulated in a different way than the majority of other cry genes. Because of its non-sporulation-dependent cry3A promoter, its expression starts during late-exponential growth rather than sporulation, as with the other cry gene groups. Many studies have shown that the *cry3A* promoter can improve other cry genes' expression and increase the output of those proteins significantly (Oppert et al, 2011).

Product: Cry3A delta-endotoxin

Toxicity: High toxic against CPB and cause midgut lining but no evidence has been recorded regarding toxicity to human and mammals.

Efficacy: 1st transgenic potato line was produced in potato with the incorporation and expression of *Cry3A* and registered by the Environmental protection Agency **(**EPA) in 1995. Presently more than 30 events of engineering transgenic potato with Bt gene *Cry3A* has been registered in different countries and companies (ISAAA, 2017). These potato lines were capable to show sustainable level of resistance against CPB and other coleopteran pests. Cry3A delta-endotoxin(source: *B. thuringiensis* var. *tenebrionis*) mediated transgenic potato lines ensured remarkably elevated resistance to the CPB where incorporation of Cry3A toxin caused 100% mortality of neonate larvae within two days and 99% adult mortality within two 14 days (Perlak et al, 1993). 100% mortality of *Agelastica alani* (alder leaf beetle). About 90% mortality of *Amphimallon solstitiale* (European June beetle) was ensured and Reduction of the biomass accumulation and foliage consumption of transgenic plant lines were recorded.

2.7.2 *SN-19* (synthetic/hybrid insecticidal gene)

Source: *Bacillus thuringiensis* delta endotoxin, constructed by Naimov et al., 2003. Encodes proteins consisting of domains I and III of cry1Ba and domain II of cry1Ia (Naimov et al. 2001, 2003).

Gene Size: Hybrid gene measuring 1.981kbp consisting of cry1Ba and cry1Ia.

Protein size: 73kDa

Toxicity: Highly toxic against tomato leaf miner and CPB in both immature larvae and adult and ensured 100% mortality (Ahmed et.al, 2017).

Transgenic potato developed by Naimov et al., 2003 showed adequate resistance against CPB. Naimov et al., 2006 reported *SN-19* as broad spectrum and potential source of pest control endeavors that proved greater resistance against both adult and larvae of CPB.

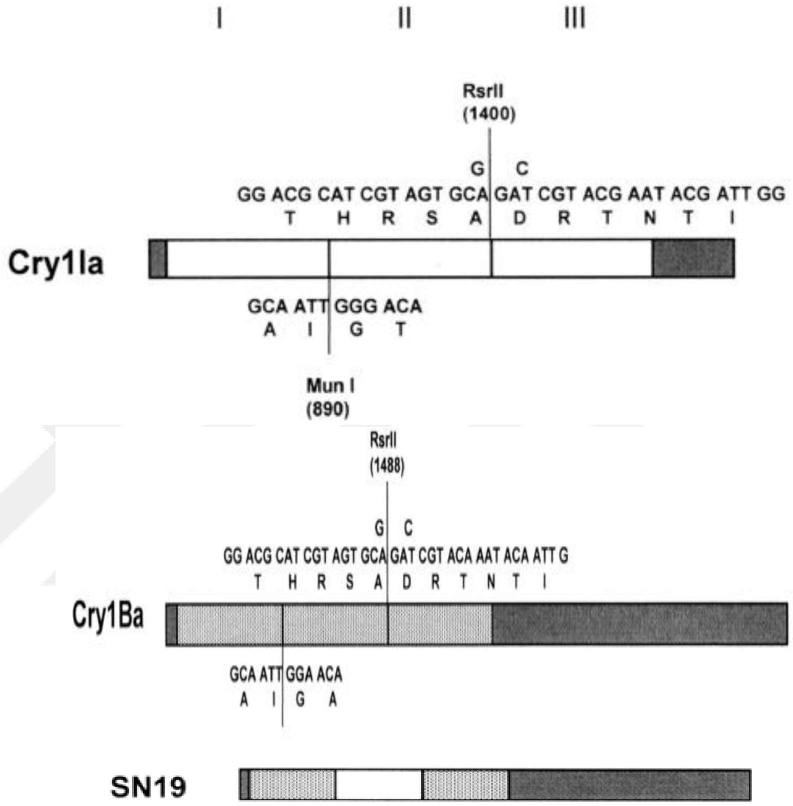

Figure 2.9. Hybrid insecticidal gene (*SN-19*) used in the present study, developed by Naimov et al., 2003

Above mentioned results with both genes confirmed toxicity potential of them to combat notorious insect pests and detailed utilization of these genes in the last decades and their outcomes have been presented in table number 2.3.

2.8 Development of Resistance and Cross-Resistance Against Bt Crops

Because of over reliance on chemical pesticides and uniform crop cultivation practices instigates the insect resistance against insecticides and by 2020 it has recorded 17,000 cases of insecticide resistance among 612 insect species (ISAAA, 2021). Although the

Bt-crops were capable enough to provide strong and uniform selection pressure on insect populations, due to two decades long commercialization , field-evolved resistance in 16 out of 33 major crop pest have been reported in 2017 (Tabashnik and Carrière., 2017). Development of Bt-resistance is a complex and diverse process, comprises with two major mechanisms are: alteration of midgut proteases involved in processing of Cry proteins in the insect midgut; and modification of binding sites for Bt-toxins. Besides these, retention of Bt-toxin by the midgut peritrophic membrane, aggregation of toxin proteins by the midgut esterase, elevated melanization activity of the hemolymph and midgut cells, increased rate of repair or replacement of affected epithelial cells, and increased antioxidant activity may include as other resistance mechanisms (Wu ., 2014, Raspor and Cingel ., 2021). Additionally, life cycle plasticity of CPB, lifelong greater genetic variations and capability to effectively cope with naturally occurring host plant toxins or as well as to every chemical insecticide, CPB can develop resistance to Bt-potato (Shelton et al., 2002).

The broad-spectrum application of Bt biopesticides bears the risk of choosing insect biotypes that are tolerant or resistant to Bt toxins and planting millions of hectares of Bt plants to protect crops from pests. The presence of resistance may be due to changes to their mode of action at any point, from solubilization and activation steps to pore formation capacity (Jurat-Fuentes and Crickmore., 2017). The lack of solubilization, especially the pH, is favored by the midgut fluids' physicochemical conditions. The acidic midgut of the coleopteran insects seemed to be a limiting factor in the solubilization, such as Cry1B and Cry7Aa (Bradly et al., 1995), although recent reports seem to suggest that more factors are involved as Cry7Aa proteins are dissolved in *L. decemlineata* and *H vigintioctomaculata* midgut fluids (Domínguez-Arrizabalaga et al., 2019). Protoxins are proteolytically cleaved into activated toxins until the Cry toxin in the midgut is solubilized. This toxin processing depends on the presence in the host midgut fluid of the correct digestive enzymes. For an instant, it was observed in *D. virgifera* larvae that the Cry3Aa protein has been poorly processed by its proteases, resulting in low Cry3Aa activity against rootworms (Walters et al., 2008).

The introduction of a recognition site for chymotrypsin/cathepsin in Domain I of Cry3A has been shown to increase this toxin's bioactivity against the larvae of the western corn rootworm (Walters et al., 2008).

Molecularly, the basis of insect resistance is the alteration or loss of particular receptors or mediators of the midgut cell membrane, which prevents or decreases the toxin's ability to activate a lethal pathway (Ferré et al., 2008). Cross-resistance between Cry toxins is frequently correlated with sequence similarities linked to unique protein binding in domains II and III (Carrière et al., 2015). Populations of *L. decemlineata* and *C. scripta* under laboratory conditions resistant to Cry3Aa (Bauer., 1995) have been identified. To date, despite the widespread use of products based on the same protein, the appearance of field resistance is still relatively poor, which increases the possibility of resistance growth.

Conversely, tolerance to all proteins used in transgenic corn has been produced by rootworm populations. The ongoing exposure of insects to Bt toxins has increased pest resistance due to extreme selection pressure. *Diabrotica* has gained resistance to Cry3Aa and Cry34/35Ab binary protein after the first case of resistance to Cry3Bb1 Bt-maize in 2009. New techniques, including the combined use of multiple proteins in the same Bt plant, are introduced to try to delay resistance (Yu et al., 2016). Pyramidization of two Bt proteins will delay tolerance to these proteins because most of them would still be susceptible to the other toxin when insects become resistant to one toxin (Gassmann et al., 2016). However, there is already evidence of cross-resistance to Cry3 and even Cry34/35 proteins, which could make the use of all these proteins invalid in the long run (Zukoff et al., 2016).

2.9 Transplastomic Technology Alternative to Nuclear Transformation

Plastid genome engineering to produce viable transplastomic plants remains a blessing for the trait development endeavors of crop plants. Plastids seem to be an appropriate compartment for the accumulation of certain proteins or their biosynthetic (fatty acid and amino acid) products and photosynthesis with thousands of copies of the genome measuring about 155-kb where every plant cell harbor 10 to 100 plastids (Yu et al., 2017). The first effective chloroplast transformation was recorded in *Chlamydomonas* (Boynto et al., 1988) and this technique is routine and stable only in tobacco (*Nicotiana tabacum*; Svab and Maliga, 1993). Hopefully, reproducible plastid transformation protocols have also been identified in tomatoes (*Solanum lycopersicum*; Ruf et al., 2001), potatoes (*Solanum tuberosum*; Valkov et al., 2011), lettuce (*Lactuca sativa*;

Ruhlman et al., 2010), Arabidopsis (*Arabidopsis thaliana*; Yu et al., 2017; Ruf et al., 2019) and soybean (*Glycine max*; Dufourmantel et al., 2004). Engineering plastid genome to produce transplastomic plants facilitates high rates of protein expression, the capability of expressing multiple proteins from polycistronic mRNAs, transgene stacking in operons, and the lack of epigenetic effects allowing stable transgenic expression and gene containment due to lack of pollen transmission (Wani et al., 2015; Hossain and Bakhsh, 2020). Transplastomic plants have been widely used to manufacture therapeutics, vaccines, antigens, and industrial enzymes, and for the development of various agronomic characteristics, including biotic and abiotic stress tolerance. Except tobacco, low transformation efficiencies have been reported for several species (Skarjinskaia et al., 2003; Zubko et al., 2004; Nguyen et al., 2005), and multiple reasons are attributed to this low regeneration of transplastomic shoots. Relatively lack homologous recombination system, non-optimal homology and length of flanking sequence, the promoter that drives the expression of the selectable marker gene, the type and nature of explants, or the regeneration protocol is the mentionable causes those are basic hindrance for the sufficient regeneration of transplastomic plants. Additionally, technical factors restricting this approach include the lack of efficient in vitro regeneration and cereal transformation protocols, reduced transgene expression in non-green plastids, a limited number of selection markers, a restricted range of transformable plants and low access to inducible gene expression (Valkov et al., 2009, Ahmad et al., 2016).

Despite recent developments in many species, potato plastid transformation is still constrained by low frequencies of transformation and low expression of transgenes (Nguyen et al., 2005; Gargano, 2005). Transgene expression in potato with the plastid rrn and the bacterial trc promoters ranged from 1% to 20%, but due to its low efficacy, *gfp* concentration achievable with the prokaryotic promoter was lower than with rrn one (Nguyen et al., 2005). Generation of the transplastomic line is a long and complex process involving organelle sorting to accomplish homoplasmic shoots during repeated cell divisions in-vitro (Bock, 2001; Maliga, 2004). Therefore, the implementation of an appropriate method for transplastomic potato selection and regeneration is essential. Besides, the transformation of potato plastid has been attempted to date with vectors designed for tobacco. However, vectors with homologous potato flanking sequences could be constructed and tested to analyze the impact of increasing homology on the efficiency of plastid transformation as the full potato ptDNA sequence is available (Gargano et al., 2005).

The regeneration procedure of transplastomic plants (organogenesis versus somatic embryogenesis) is a crucial bottlenecks of efficient plastid transformation. In dicot crops, in-*vitro* regeneration of transplastomic plants from leaves on culture medium via organogenesis yielded high success. In comparison, the somatic embryogenetic pathway is usually followed by most of the cereal crops for *in-vitro* regeneration that is basically limiting the transplastomic endeavors in significant food crops (Bansal and Saha., 2012). In several crops plants including carrot, cotton, soybean, and rice, species-specific plastid transformation vector is useful and transplastomic crop development through somatic embryogenesis has been established. With somatic embryogenesis, achieving homoplasmic monocot cereal crops experienced complicated and remains a significant challenge that needs to be revealed (Clarke and Daniell., 2011). The challenge of engineering non-green plastids (amyloplasts, chromoplasts, elaioplasts, leucoplasts and proplasts) found in fruits, tubers, roots, and grains regards another major barrier for the expansion of transplastomic technology in crop plants. Due to the downregulation of most genes encoded in plastids other than chloroplasts (Zhou et al., 2008), transgene expression is significantly reduced. Therefore, besides the above-mentioned limitations, the necessity of species-specific vector and regulatory sequences for stable protein expression are the key obstacles faced by the implementation of plastid transformation in crop plants, as well as food crops.

With several lucrative alternatives to nuclear transformation, plastid transformation technology has become an important tool for the manipulation of plastome pattern of some economically important crop plants (Wang et.al 2009). Plastid genome with enrich ploidy number allows the improved protein or transgene expression (ranges between 1-40% of TSP) (Roudsari et.al 2009). As described by Daniell et al. 2002 and Hou et al.2003, the level of protein expression by plastid transgene is remarkably higher (up to 18%) than nuclear transformation (0.5-3% of TSP). Every plant harbor multiple copies of plastid transgene that affected by pre- or post-transcriptional silencing and leads to greater production of expressed proteins. According to Maliga, 2001, Plastid transformation technology is capable to ensure the expression of multiple genes from polycistronic mRNA, avoid the epigenetic effects and it also offers huge utility over the nuclear genome. Besides these, the transplastomic strategy offers unique advantages such as high and durable protein production, biological transgene containment, lack of gene silencing and position effects. Implementation of transplastomic technology in crop improvement promote the production of insecticidal, antibacterial, and antifungal compounds to increase the quality and production of crops.

On the contrary, paternal inheritance in nuclear transformation cannot ensure gene containment in nature and reveal a low copy number of a gene, its low manifestation as well as lower accumulation of foreign proteins. One promoter is not enough to continue the manifestation of respective sub-units and lack of the primitive type of gene expression machinery inhibits simultaneous gene expression, random integration of gene cause position effect and gene silencing, lethal effects exert from impure toxic proteins deposition in the cytosol.

2.10 *Bacillus thuringiensis (Bt)* Mediated Transplastomic Endeavors to Treat the Baleful Insect Pests Including CPB.

Although the current IPM (integrated pest management) practices aimed at sustainable potato production includes the combination of cultural, biological, and chemical approaches, conventional insect pest control strategies largely depended on the extensive and unjust application of chemical agents (Maharijaya and Vosman, 2015). Only in 2001, the annual global consumption of chemical pesticides cost was recorded near about $9 billion. Through this practice, besides the economic damages, environmental and ecological status along with human health was being threatened. We are losing many eco-friendly and beneficial insects from the crop field. Considering the above-mentioned consequences, the introduction of alternative methods to the conventional chemical-based approach is crucially important and demand of the time. Genetically modified (GM) crop plants aimed to produce functional insecticides can be an alternative to the spraying (several times a single growing season) of costly and hazardous chemical pesticides. Typically produced in plants, the biologic pesticides are highly specific and non-hazardous to humans and other higher livestock. Most chloroplastic resistors to date are based on transgenes used successfully for nuclear transformation. Glyphosate, glufosinate, imidazolinone, sulfonylurea and pyrimidinyl carboxylate, and d-amino acid were, for instance, resistant to herbicides; sulcotrione and isoxaflutole (Bock, 2015).

Transgenic approaches devoted to controlling insect pests is one of the viable strategies, which involves the expression of the insecticidal crystalline protein (CRY genes) originated from soil- born bacteria *Bacillus thuringiensis* . Several successful attempts were taken with Bt proteins for insect pest control as an alternative to existing chemical insecticides. Cry-toxin genes are generally implemented as a strategy of incorporation of resistance in commercially significant crop plants through the cloning, transfer, and expression in transgenic plants.

For instance, 73% of total cotton production is made from Bt-cotton in the US, whereas Bt-maize accounts for 63% of all crops (NASS, 2015). Insecticidal potentiality of transplastomic tobacco produced with the incorporation of Bt cry1A(c) insecticidal protein was established for the first time in 1995 (McBride et al., 1995). When the coding region of the bacterial Bt genes express into the nuclear genome, its expression usually less because of premature transcription termination, aberrant mRNA splicing, mRNA instability, and inefficient use of codon. On the other hand, the expression of Bt protein from a synthetic coding area having increased GC content revealed significant protein yield ranges between 0.2% to 0.3% of TSP. Besides this, when the incorporation and expression of the unmodified bacterial coding region into the plastid genome, the mRNA was stable and protein accumulation was recorded as 3% to 5% of TSP and this protein accumulation was regarded as outstanding. Therefore, different Bt proteins were expressed into the plastome yielded the same high ranges of protein accumulation from the bacterial coding sequence. The foreign protein accumulation in tobacco and cabbage was recorded as 10% and when two ORF (open reading frames) was included into the upstream of the cry2Aa2 operon (consist of ORF1 and ORF2) then the protein accumulation was raised to 45% of TSP (De Cosa et al., 2001). Because of the unique potentiality of 2 ORF, transplastomic tobacco expressing Cry2Aa2 proteins ensured up to 46% of TSP and these transplastomic tobacco plants revealed 100% mortality of tobacco budworm, cotton bollworm, and beet armyworm (De Cosa et al., 2001). Transplastomic tobacco harboring Cry2Aa2 resulted from elevated level of protein accumulation and avoided the possibility of resistance development, finally these transplastomic tobacco leaves ensured 100% mortality of tobacco budworm (*Heliothis* *virescens*), cotton bollworm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*) (Kota et al., 1999).

The capability of these transplastomic tobacco plants to kill the insect pests showed 40,000 times higher insecticidal activities compared to the usual one and provided complete resistance to insects that feed on transgenic plants (Kota et al., 1999). However, *Cry1Ab* and *Cry1Ac* are also commercially used cry proteins to encode resistance against the target pests of corn and cotton. But due to the 'codon bias' effects of these prokaryotic types of genes (AT-rich) into the eukaryotic nucleus (GC rich) remarkably reduce the expression level of these genes that open a possibility to create insect resistance against these cry protein derived crop plants.

Consequently, ecological hazard based on transgene escape through pollen due to the absence of transgene containment and injustice application of Cry genes in nuclear transformation is a burning question in the transgenic technologies that needs to be taken under consideration. As an alternative to the above-mentioned consequences, incorporation of Bt originated Cry1Ac gene into the tobacco plastome driven by 16S rrn promoter with rbcL chimeric ribosome binding site and 3′ UTR of rps16 gene demonstrated high protein accumulation in transplastomic leaves (3–5% of TSP) (McBride et al. 1995).

Subsequently, a high level of expression (> 10 % TSP) of cry protein cry9Aa2 was obtained in tobacco (Chakrabarti et al. 2006) and the expression of cry1Ab gene in cabbage also recorded (Liu et al. 2008). Recently, Jin et al. (Jin et al. 2011), recorded an elevated level of β-glucosidase (Bgl-1) expression into tobacco plastid that encoded broad spectrum resistance against aphids and whiteflies with increased growth in biomass and density of trichomes.

Interestingly, transplastomic plants express a wide range of resistance not only to different pests and diseases but also to many abiotic stressors in both the leaves and root plastids. These results intensify the comprehensive and still unexplored dynamic properties of agricultural breeding of transplastomic plants and draw our attention to the enhanced mechanisms of abiotic stress tolerance. The main advantage of plastid genome resistance is that there is an increased transgenic containment of maternal chloroplast inheritance, in addition to the often very high protein accumulation. Extreme levels of expression have been achieved, for instance in tobacco chloroplasts with Bt toxin genes (De Cosa et.al. 2001).

In one reported case, however, high Bt protein accumulation in transgenic plastid resulted in a phenotype of growth (Chakrabarti et.al. 2006), suggesting the need for carefully optimized expression of the resistance gene, so as to provide adequate protection without a yield penalty.

Table 2.3. Transplastomic plants developed to improve resistance towards different insect pests

Plant Species	Transgenes	Expression Level (% of TSP) and efficacy	Pests /Pathogens affected (Bioassays)	References
Solanum tuberosum	Cry3A	Resistant enough for 100% mortality.	*Leptinotarsa decemlineta*	Zhou et.al., 2012
	Cry3A	Season long resistance	*Leptinotarsa decemlineta*	Duncun et.al., 2002
	Hybrid gene SN-19 (Domain I and III of Cry1Ba, Domain II from Cry1Ia)	Resistant enough.	*Leptinotarsa decemlineta, Pthorimaea operculella and Ostrinia nubilalis (Hübner)*	Naimov et.al., 2003
	Hybrid gene SN-19	Resistant enough for 60 % mortality.	*Tecia solanivora*	Lopez-Pazos et.al., 2009
	Hybrid gene *SN-19*	Resistant enough for 60% mortality.	*Tecia solanivora*	Lopez-Pazos et.al., 2010
	Hairpin dsRNA to β-actin gene of CPB	Approximately 0.4 % of total cellular RNA.	*Leptinotarsa decemlineta*	Zhang et al., 2015
Nicotiana tabacum	Partially modified Cry1Ab	10-fold higher expression with 100% mortality	*Manduca sexta*	Perlak et.al., 1990
	Bt cry 1A(c)	3-5%	*Helicoverpa zea, Heliothis virescens, Spodoptera exigua*	McBride et al., 1995
	Bt cry2Aa2	2-3%	*Helicoverpa zea, Heliothis virescens, Spodoptera exigua*	Kota et al., 1999
	Bt cry1A(c)	a	*Helicoverpa armigera*	Zhang et al. 2009
	Bt cry 2Aa2 Operon	45.3-46.1%	*Helicoverpa zea, Heliothis virescens, Spodoptera exigua*	De Cosa et al., 2001
	Bt cry 1Ia5	Approximately 3%	*Heliocoverpa armigera*	Reddy et al.,2002
	Btcry1C	1.1-4%	*Spodoptera litura*	Lin et al., 2003
	Bt cry9Aa2	Approximately 10%	*Pthorimaea operculella*	Chakrabati et al., 2006
	Bt *cry1Ab*	a	Not tested	Jabeen et al. 2010
	bgl-1 (β-glucosidase)	a	*Bemisia tabaci, Myzus persicae*	Jin et al., 2011

	Pta (Agglutinin)	5.2-9.3%	*Bemisia tabacci, Helicoverpa zea, Heliothis virescens, Spodoptera exigua, Myzus persicae*	Jin et al., 2012
	Bt cry 1Ab	45-46 ng mg-1 fresh weight	Not tested	Mirza and Khan 2013
Nicotiana benthamiana	sporamin, CeCPI, chitinase	0.85–1 % for each protein	*Spodoptera exigua, Spodoptera litura*	Chen et al., 2014
Brassica napus	Bt cry1Aa10	a	*Plutella xylostella*	Hou et al., 2003
Brassica oleracea var. capitata (cabbage)	Bt cry1Ab	4.8–11.1 %	*Plutella xylostella*	Liu et al. 2008
Gossypium herbaceum	Cry1Ac and Cry2A	Cry1Ac (0.673 ng) and Cry2A (0.454 ng), 100% mortality	American bollworm	Muzaffar et at., 2015
Glycine max	Bt cry1Ab	a	*Anticarsia gemmatalis*	Dufourmantel et at., 2005
Alfalfa	Cry1C	Resistant enough for 100% mortality	*Spodoptera littoralis*	Strizhov et.al., 1996
Oryza sativa	Truncated Cry1Ab	Resistant enough for 100% mortality	*Scirpophaga incertulas*	Datta et.al., 1998
	Cry1Ab+Cry1Ac	Highly toxic with 97-100% mortality	*Scirpophaga incertulas*	Nayak et.al., 1997
	Translationally fused Cry gene (Cry1Ab-1B)	Highly toxic with 100% mortality	*Scirpophaga incertulas*	Ho et.al., 2006

Bt- *Bacillus thuriengensis,*
a indicates observed adequate gene expression, but no quantification
b represent *Erwinia cartovora* is the former name of Pectobacterium carotovorum subsp.

2.11 Utilities Offered by Transplastomic Lines

Transplastomic technology offers many applications in both applied and basic research (Fuentes et al, 2018, Ruf et al, 2021) and also instigate remarkable development in synthetic biology (Boehm & Bock, 2019; Scharff & Bock, 2014). Since the integration of the transgenes into the plastome occurs proficiently by homologous recombination, transplastomic endeavors remains a lucrative method for functional genomics by reverse genetics. The knockout of ORFs and the incorporation of specific point mutations into chloroplast genes are straightforward. This technology also ensures the study of all aspects of plastid gene expression in vivo (transcription and transcriptional regulation; RNA processing and turnover; and translation and translational regulation.) Finally, the technology can be used for the introduction of foreign genes into the plastome, including reporter genes and biotechnologically relevant transgenes (Ruf et al, 2021). Besides these, integration of foreign genes into the chloroplast genome offers the following utilities:

2.11.1 Introduction of broad-spectrum strategy for insect pest control

The incorporation of the target insecticidal gene into the plastome ensures the high level (3-5% of TSP) of toxin accumulation although nuclear transformation yields less (> 0.2% of TSP) accumulation of protein (McBride et al., 1995). Although rather than tobacco (*Nicotiana tabacum*), durable and robust transplastomic plant development was challenging and difficult (Gatehouse et al., 2008) but transplastomic cabbage with the incorporation of *cry1Ab* gene manifested with toxic protein ranges between 4.8 to 11.1% of TSP. These transplastomic lines were capable to ensure sustainable mortality of insect pests while fed on these leaves.

2.11.2 Containment of natural genes

During field condition, normal transgenic plants can suffer from crossing and dissemination of their genetic materials into wild species and to their relative species (Stewart et al., 2003). The genetic dispersal of canola (*Brassica napus L.*) from nuclear transformation is an ideal example. But transplastomic plants can harbor gene containment due to maternal type of inheritance, major angiosperm plants can show this characteristic (Jaffe et al., 2008, Greinner et al., 2015). Few events of diffusion of transgenic plastids have been recorded for tobacco pollen (0.00024-0.008%) (Svab and Maliga, 2007) and also for *Arabidopdis thalian*a (0.0039%, Azhagiri and Maliga, 2007). To mitigate this research gap, *in-vitro* regeneration of transplastomic efforts were launched that instigated the transgene containment within the plastid genome (Gilbert, 2013).

2.11.3 Site-specific integration of extra-chromosomal DNA into the chromosome facilitate random integration of genes

If the target gene confirm the site-specific incorporation into the pre-determined site of the plastid genome, it confers the avoidance of gene silencing and unwanted mutation by the random integration of transgenes. During transplastomic event homologous recombination has been noticed where in cases of *Agrobacterium* mediated nuclear transformation the transgene leads to the random integration (Vieler et al., 2012, Kohil et al., 2010) and there is a possibility of active interaction of foreign genes with native nuclear genes that actually because of the non-allelic interaction that can hide the functions of native genes (Scheid et al., 1991). Still, controversy is remaining regarding the robustness of the nuclear transformation when the genes are incorporated into the plastid genomes and transplastomic technology is a prominent way to investigate the usefulness of PEP protein by the dint of the knockout creation and site-specific mutants (Qin et al., 2003, Bock, 2011, 2015).

2.11.4 Improved level of foreign protein expression

Due to the high ploidy number, transplastomic events ensure a high level of foreign protein expression. For instance, the incorporation of enterotoxigenic *E. Coli* β-subunit into the nucleus of tobacco yielded 0.01% of TSP (Haq et al., 1995), where the integration of that same *E. Coli* β-subunit into the plastome yielded 410-fold increased expression (Haq et al., 1995, Daniell et. al., 2001). Besides this, a proof of high expression of the foreign gene 70 % of TSP of tobacco can be mentioned. Transplastomic event also is lifesaving aspects of plants as it was recorded in tobacco cytosol where the existence of a very minute amount of (0.3% TSP) cholera β-toxin arrested the normal physiological functions (Arakawa et al., 1997) but as an outcome of transplastomic events 14-fold higher level of expression was recorded without having any adverse effects (Daniell et al., 2001). In some cases, the extensive and high level of protein expression may cause deformity of plant functions and physical fitness (Scotti and cardi, 2014) that was enlisted for 25% of TSP of C type tetanus toxin fragment expression (Tregoning et al., 2003). Identical findings were recorded in tobacco was ~ 7% TSP in glutathione –S- transferase expression affects the cytoplasmic male sterility but in cases of low expression no remarkable effects were detected. Through the sensible utilization of regulatory elements, the procurement of foreign proteins can be affected positively or negatively, and the chloroplast gene expression machinery is remarkably controlled during transcriptional and translational levels (Maliga, 2003, Whitney et al., 2008). Considering the above-mentioned consequences, the expression level of the resistance gene needs to be optimized carefully to provide sufficient protection without incurring a yield penalty (Bock., 2015).

2.11.5 Engineering of important metabolic pathways

Several nutritionally essential biochemical pathways (biosynthesis of carotenoid and fatty acids) have been adopted successfully for the metabolic engineering of transplastomic plants (Apel, W. and Bock, R., 2009, Craig et al, 2008). The possible utilization of transgene stacking mechanisms in synthetic operons have opened the greatest attraction of the transplastomic plants for metabolic pathway engineering, limited expression of native bacterial operons in plastid was not too successful and plastome was found an efficient way to reengineered them for the optimization for efficient expression (Scharff and Bock., 2014). For the introduction of traits of interests in plants, simultaneous activation of different enzymes and metabolic pathways are crucial for the escape of biotic as well as abiotic stresses, filialness of nutritional stuffs, production of industrially important metabolites for the crop improvement . For example, nitrogen-fixing bacteria *klebsiella pneumonia* 24kb in size and contains 20 genes, on the contrary, Streptococcus is about 25kb in length and harbor 16 genes responsible for the polysaccharide formation (Cieslewics et al, 2001). Recently, many important and lucrative metabolic pathways have been implemented successfully into transplastomic plants those have paved a new horizon for the different biological characteristics, includes Polyhydroxy butyrate Production (Lossl et al.,2003, 2005), β-carotene content enhancement (Apel and Bock 2009), higher yield of Vitamin-E in

Tobacco (Lu et al., 2013), Production of a Triterpene named Squalene (Pasoreck et al., 2016) etc.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Materials

Sterilized leaves and internodal explants from four potato cultivars (Kuroda, Simply Red, Challenger and Sante) were used as plant materials for the transformation experiment. Internodes and leaves from mentioned cultivars were treated with 70% ethanol for 1 min and then washed into autoclaved distilled water for 2-3 times. After that, intermodal segments were treated with 0.1% $HgCl_2$ and 2-3 drop Tween-20 mixture for 2 minutes and leaves were treated with 10% bleach and 2-3 drop Tween-20 mixture for 5 minutes with some modification as described by Çimen (2020) for citrus. Both explants were washed 2-3 times with sterilized distilled water and dried on sterilize filter paper.

3.2 Brief Description of Transformation Vector

The final potato chloroplast transformation vector was constructed with the combination of two insecticidal cry genes named *SN-19* and *cry3A* under the control of green tissue-specific promoter *psbA* to grow Colorado potato beetle and potato tuber moth resistant transplastomic potato lines. The basic features of the chloroplast transformation vector are as under: We amplified trnA-trnI region of potato plastome and ligated it into cloning vector. There is a small intergenic region between trnA and trnI where we cloned our gene of interest and marker gene along with their UTRs. Once, vector construction (cloning) was completed, we coated that vector onto gold particles and bombarded them onto potato explants. Once this vector is inside the chloroplast, there occurs homologous recombination between trnA-trnI (in chloroplast genome) and *trnA*-GOI-Marker-*trnI* (in vector). It results in the integration of our GOI and marker gene into the chloroplast genome. Such integration is predetermined and site-specific. Hence, there is no chance of wrong integration. Potato chloroplast transformation vector has been constructed by PCR amplification and subsequent ligation of potato chloroplast genome (plastome) sequences to be used as flanks in the final transformation vector. A DNA fragment of more than 900 bps from trnA region of potato chloroplast genome was used as flanking sequence on one side. In contrast, DNA fragment of almost same size from *trnI* region of potato chloroplast genome was used as a flanking sequence on the other side of the potato chloroplast transformation vector. Initially, trnA gene was PCR amplified using gene specific primer (RFF: 5'-GGT ACC CCT TTT TTA CGT CCC -3' (Kpn1), RFR: 5'- AAG CTT CTG GGC CAT CCT GGA -3' (HindIII)) and PCR product of around 0.92 Kbps was ligated into pBlusescript vector at Kpn1/HindIII sites. Size of pBlusescript vector is around 2.96 Kbps. Later, a marker gene, along with promoter and terminator was obtained through restriction digestion by HindIII and EcoR1 from plasmid pMSK49 already available in our lab (Daniell and Khan, 1999). The restriction sites HindIII and EcoR1 were unique sites in pMSK49 and the eluted fragment of around 2.0 Kbps was ligated into the above-mentioned intermediate vector at HindIII/EcoRI sites. Then, trnI gene was PCR amplified using primer set (LFF: 5'-GCA TGC GCC AGG GAA AAG A -3' (Sph1), LFR: 5'- TCT AGA CAC GAG TTG GAG -3' (Xba1)) and the PCR product of around 0.94 Kbps was ligated into above vector at Xba1/EcoR1 sites. An additional Sph1 site after EcoR1 site was intentionally engineered in the forward primer used for amplification of trnI region to help cloning of the gene of interest at Sph1/EcoR1 sites. A synthetic DNA fragment of around 2.4 Kbps (comprised of the promoter, fused *SN-19* + *Cry3A* gene and terminator) was ligated in the above vector at Sph1/EcoR1 sites. Then, the final construct was confirmed using restriction digestion and construction of potato chloroplast transformation vector (PCTV) was completed. For this purpose, two regions of potato chloroplast genome (*trnA* and *trnI*) were cloned as left and right flanking regions, respectively. Its size is around 3 Kbps. Its MCS is shown in figure which will help to understand the process of vector construction described in methodology section of this report.

‘FLARE-S’ was used as a selectable marker gene that stands for fluorescent antibiotic resistance enzyme and confers resistance against spectinomycin and streptomycin, respectively. ‘FLARE-S’ is a system of marker gene for plastid transformation and obtained by translationally fusing aminoglycoside-3-adenyl-transferase with the *Aequorea victoria* green fluorescent protein from rice chloroplast transformation vector pMSK49. pMSK49 harbored antibiotic and visual selection marker which helped in screening and selection of transformants owing to resistance against spectinomycin and streptomycin. This method facilitates the extension of plastid transformation to non-green plastids in embryogenic cells of cereal crops. Cry genes (fused *SN-19* and *Cry3A*) were cloned under green tissue-specific plastid promoter, which will help in restricting the expression of Cry genes only to green tissues.

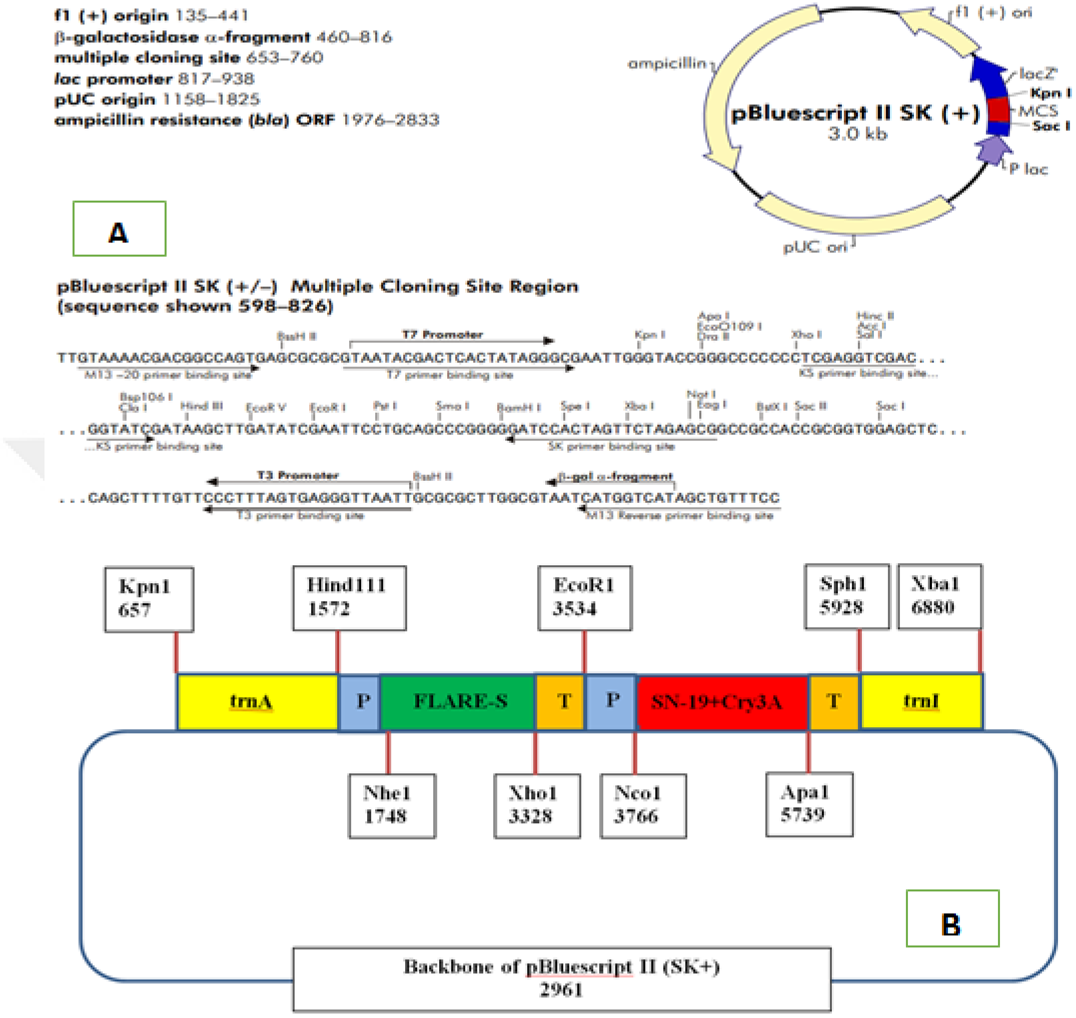

Figure 3.1. Map of pBluescript II SK (+) vector (a) and Schematic representation of Potato Chloroplast Transformation Vector (PCTV). Vector contains *FLARE-S* (derived by the fusion of *aadA* and *gfp* genes to give dual selection on plant regeneration medium as described by Khan and Maliga 1999) as selectable marker under the control of prrn promoter. *SN-19* gene was cloned under the control of *psbA* promoter. *trnA* and *trnI* were used as flanking sequences from potato plastome (b)

3.3 Genetic Transformation of Potato With Prominent Gene Transfer Method

For the transformation of our gene of interest into the chloroplast genome, in this research, we used a prominent gene transformation strategy commonly known as biolistic method of gene transfer or gene gun.

3.3.1 Equipment setup for gene gun

The bombardment of microparticles was conducted following existing user guidance. Conditions of configuration underlying: A distance of about 8 mm has been maintained between the high-pressure gas chamber and the carrying membrane containing microparticle-coated coated DNA. About 13 mm distance between the conveyor membrane and the retention screen was ensured , and 80 mm was set from the retention and bombardment material. 27 inches Hg was held to the vacuum pressure while 1, 200 p.s.i was maintained to the gas pressure from helium.

3.3.2 Preparation of gold particle for gene gun

Entire contents of 250 mg gold particles (0.6 μm in size) were diluted with 1 ml molecular grade 100% ethanol, vertexes properly for 2 mins and centrifuged for few seconds at highest rpm. All supernatants were discarded, and gold particles adhered on the falcon wall was removed carefully by pipette and mixed with pellets. After that, three times successive wash was done by sterilized D/W. During 1st and 2nd washing, the supernatant was completely discarded, but during 3rd wash, 11 ml of supernatant was kept that ensured 500 μl of gold particles in each aliquot. In cases of every washing, gold particles adhered on the falcon wall was collected carefully by pipette and mixed with pellets. Entire washed particles were divided into 22 aliquots where every aliquot contained 500 μl of gold particles. This 500 μl of gold particles were usable for 20 bombardments or 20 plates. So, for every five plates required 125 μl and every single plate required 25 μl of gold particles.

3.3.3 Preparation of microparticle-coated DNA

All necessary ingredients – DNA, $CaCl_2$ and Spermidine was kept in ice before use. After that, approximate 10 μl DNA+ 50 μl 2.5M $CaCl_2$+ 20 μl 0.1M Spermidine was added together with 152.5 μl of gold particles. The mixture was adequately vortexed for 30 mins, and 40°C temperature was maintained during vertex. Centrifuge was done for a short period of time, and the supernatant was discarded. Then, wash was done by 100% ethanol, and the supernatant was discarded. Three times successive wash was done by sterilized D/W, and the supernatant was discarded. After 3rd washing, total pellet was dissolved into 30 µl ethanol. After the final centrifugation step, the gold particles were resuspended in 1 ml of sterile 50% vol /vol glycerol and store at - 20°C until ready to use.

3.3.4 Chemicals and hormonal stock preparation

NAA, 6-BAP, GA_3, Transzeatine, Transzeatine riboside and Thiadiazuron hormone powder was weighted (0.05gm) and dissolved by 1N NaOH, Alcohol or water depending on hormonal nature. Then the volume was completed up to 50 ml with distilled water. Sterilization of these hormones was done by autoclaving or filter sterilization according to the hormonal nature. $CaCl_2$ 2.5 M: Autoclaved and distributed aliquots into 200-µl micro centrifuge tubes. Plasmid DNA: Higher level purity of the DNA sample was ensured. Pure preparation of plasmid DNA contained OD_{260}: OD_{280} value of 1.8 (Sambrook et.al, 1987). Spermidine 0.1 M: The entire contents of a bottle (1g) were dissolved into 68.8 ml of distilled water. Sterilization was done by filtration (in 0.22-µm filter). Aliquots were distributed into 200-µl micro centrifuge tubes. Osmotic media (OM) was consisting of MS salts plus vitamins, Sorbitol: 0.1 M, mannitol: 0.1 M and Sucrose: 3% (w/v).

3.3.5 Gene gun mediated plastid transformation procedure

For biolistic transformation, explants from above-mentioned potato cultivars were arranged on sterilized whattman no.1 filter paper and placed on regeneration medium. All activities were conducted inside the Laminar Air Flow Cabinet. Before bombardment, fresh potato explants were treated on an osmotic medium supplimented with MS salts and vitamins, 0.1 M sorbitol, 0.1 M mannitol, 3% sucrose. During this short treatment, abaxial side of explants kept on touch of medium and dark condition was ensured. After a 24-h treatment, the gold particles (0.6 µm in diameter, Aldrich) were coated with chloroplast transformation vector PCTV and were transferred into the plastid genome by biolistic gun PDS-1000/He (Bio-Rad Laboratories, Hercules, and CA) as described by Khan and Maliga, 1999.To facilitates thetransformation, 1100 psi rupture disk and a helium cylinder (to ensure sufficient pressure) was used. A macro-projectile accelerated with air pressure and shot into plant tissue. A perforated plate helped to stop the macro-projectile that allows the microprojectiles to pass through to the cells then the transgenes were released from the particle surface and may incorporate into the desired organelle, especially in chloroplast.

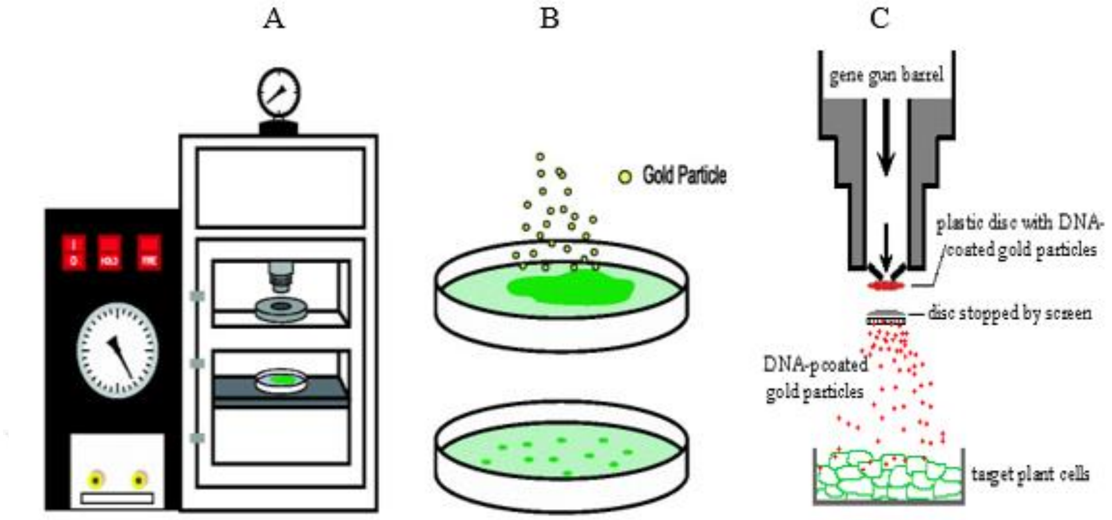

Figure 3.2. Particle gene gun mediated integration of foreign gene / DNA into the potato chloroplast genome. Helium gas accelerates particles towards potato explants (a), Gold or tungsten particles are coated with a plasmid carrying the genes of interests and acceleration pressure penetrates the explants placed on the selection medium having appropriate selection antibiotic (b) and Diagrammatic illustration of Gene Gun mediated gene delivery method (c) (as described by Khan and Maliga, 1999)

All operations were conducted under laminar cabinet and cabinet was sterilized by UV and Ethanol spray. Macro carrier, stop screen, Rupture disc, Holder and essential equipment’s were sterilized with 100% pure alcohol and dried properly. Micro carrier or gold particle solution was labeled on macro carrier and waited up to completely drying. Rupture disc was placed and fixed properly by a screwdriver on a holder that is upwards of the gene gun. Then macro carriers along with micro carrier or dried gold particles were placed on holder in opposite direction. Vacuum was created by pressing the button in lower direction and holed the button how long the vacuum was raised up to -25 approximately. After fixing the adequate vacuum, fire button was pressed to run the helium pressure. When the macro carrier got pressure from upside by rupturing the rupture disc, then the macro carrier was stopped /controlled by stop screen and the micro carrier or dried gold particles were transferred into our explants. Selectable markers were used to identify the cells that take up the transgene. Plates containing bombarded explants were placed at 27±1°C .

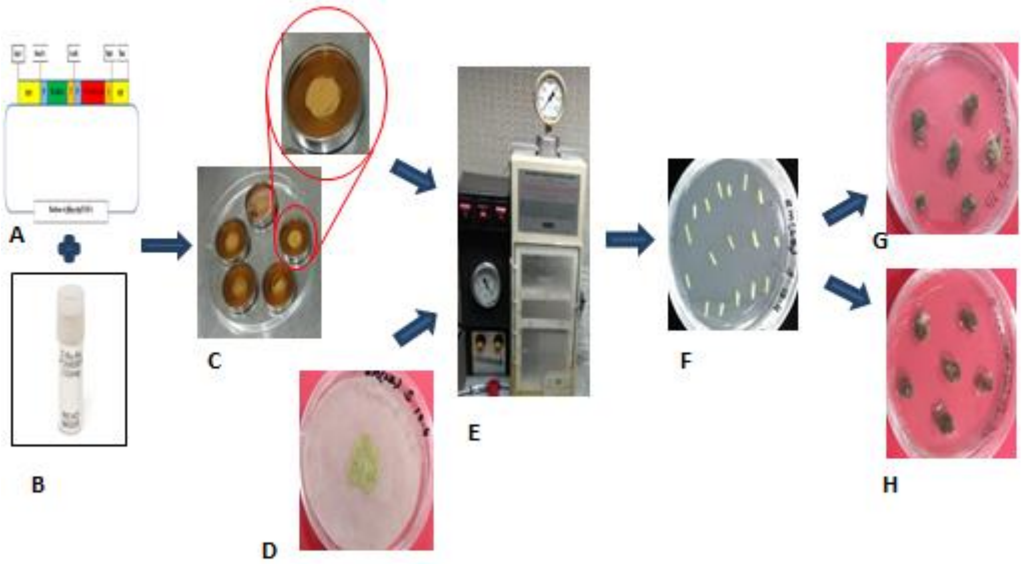

Figure 3.3. Pictorial overview of various steps of biolistic transformation of potato. Midi-prep of plasmid DNA (Final Potato Chloroplast Transformation Vector) (a), gold particle (microcarriers) of 0.6 µm size (b), plasmid DNA of Final Potato Chloroplast Transformation Vector was coated on the surface of microcarriers and the microcarriers were spread on the surface of microcarrier discs. Closer view of one disc shows gold particle dried on its surface (c), explants ready for being bombarded with plasmid DNA coated gold particles (d), plasmid DNA coated gold particles were bombarded onto explants in this Biolistic PDS-1000/He Microprojectile Delivery System (gene gun) (e), after bombardment, explants were shifted onto regeneration medium containing spectinomycin as selection agent (f), the plate shows that most of the explant tissues became dead except one shoot emerged from an explant which is a putative transgenic shoot. It was propagated on more stringent selection media and then subjected to various molecular analyses (g) and no explant survived showing no regeneration of putative transgenic shoots (h)

3.4 In Vitro Regeneration of Bombarded Potato Tissues With Appropriate Selection Agent

3.4.1 Primary culture of bombarded explants for callogenesis

Forty-eight hours after the bombardments, explants were placed on regeneration medium (bombarded side in contact with the medium) for the first round of selection to facilitate the preferential replication of plastids containing transformed ptDNA copies. Regeneration medium (RM1) was supplemented with BAP 2 mg/L, NAA 0.2 mg/L, GA3 0.1 mg/L, and Transzeatine riboside (TR) 2 mg/L and antibiotic (Spectinomycin-50 mg/L for selection of transformed cells) that induced the division of cells of explants and the formation of embryogenic callus. Un-bombarded explants were cultured on the same media with and without antibiotics as negative and positive controls, respectively. Petri dishes were placed under white, fluorescent lamps in the culture room (1,900 lux) with a dark period of 16h/8h at 26 ± 2°C. After 4-8 weeks of incubation, bombarded explants on regeneration and selection medium, the Calli were formed. The percentage of callus induction from the total number of bombarded explants was calculated as given below.

$$Percentage\ (\%)\ of\ callus\ induction = \frac{Number\ of\ calli\ formed}{Total\ number\ of\ explants\ bombarded} 100 \quad (3.1)$$

3.4.2 Microscopic detection of transformation events in well-developed Calli

Laboratories of faculty of Agricultural sciences and technologies, Nigde Omer Halisdemir university are furnished with the inverted microscope Leica dmil led that is comprised with a CCD camera and *gfp* detection system. This microscope was used for the analysis of transgenic plastids in well-developed calli tissues. The efficiency of the newly developed protocols in plant transformation technology is assessed by visible reporter genes. The fundamental idea is to distinguish between transformed regenerants and heterogeneous population. While the delivery of transformation vector into the chloroplast genome was done, *gfp* was used as a visual selection marker gene. Transplastomic segments in the callus tissues were visually recognized and thus the time and attempts to achieve genetically stable plants were significantly reduced.

With the presence of this reporter gene, the *gfp* expression produced early fluorescent calluses which helped in the early identification of transgenic cells Transformed Calli multiplying on selection medium have often been checked for the fluorescence of *gfp* using a handheld long-wave UV light. A stereomicroscope for *gfp* detection system confirmed the transformation. Fluorescent sectors varied according to the separation of the transformed callus cells from wild tissues in different heterozygous transgenic Calli. The system consisted of gfp filters on detector channels ranges between 488 and 568 nm. Channels for green fluorescence images can be adapted. Images were observed from several fields in normal white fields and fluorescent light, and images from *gfp* fluorescence using led inverted microscopy were carefully recorded. These results also verify the presence in transplastomic potato Calli of a fluorescent protein. Therefore, transgenic Calli have been regenerated by visually detecting fluorescence in various stages of development, and the homozygous and heterozygous tissues have also been identified. There has been no toxic effect of *gfp*, and the typical callus phenotypes have kept their toxicity status unchanged. The 0.5 x 0.5 cm section of callus tissue was cut using sharp blades and put in a glass diaper as part of the procedure, added 1-2 droplets of water and then covered with a transparent cover glass. On the attached computer screen, the images developed by the microscope were viewed. Leica dmil led inverted microscope was tested for subcellular localization of the fluorescent protein. The excitation wavelength was adjusted from 488 to 568 nm for *gfp* fluorescence. In a computer screen attached to the microscope, the images generated by *gfp* fluorescent were analyzed.

3.4.3 Transfer to shoot formation medium and selection

Well-developed transformed calli were transferred to a shoot formation medium supplemented with MS salt, BAP 1 mg/L, GA3 0,1 mg/L, Spectinomycin 50 mg/L. Five to six weeks later, shoot primordia started to emerge from the well-developed calli and the numbers of putative transgenic shoots were recorded . Percentage (%) of shoot proliferation was calculated as given below.

$$Percentage\ (\%)\ of\ shoot\ proliferation = \frac{Number\ of\ shoots\ regenrated}{Total\ number\ of\ calli} 100 \qquad (3.2)$$

To evaluate the performance of different cytokinines (Transzeatine, Transzeatine riboside and Thiadiazuron) on shoot induction especially for the axillary and adventitious shoot proliferation and somatic embryogenesis, two more media RM2 and RM3 were prepared where Transzeatine riboside (TR) was replaced with 2 mg/L of Transzeatine (TZ) and Thiadiazuron (TDZ) respectively. Besides this, we evaluated the efficacy of TDZ, TZ and TR for the axillary, and adventitious shoot proliferation.

3.5 Screening of Putative Transgenic Potato Shoots Using Standard Molecular Techniques

For the confirmation of gene integration and their protein formation in the chloroplast genome and to explore the gene expression profile, advanced molecular techniques were adopted proficiently. Putative transgenic shoots for the precision integration of transgenes into the genome of chloroplast were analyzed by PCR and Southern blot. Transgene expression analysis was conducted by qRT-PCR. The amount of total soluble protein was quantified by Bradford assay and presence of target pteotein in transplastomic lines were quantified by ELISA. SDS-PAGE confirmed the presence of target protein from the complex protein mixure. Microscopic localization of *gfp* in transplastomic shoots were performed by advanced computer aided microscopic operation (Laser Scanning Confocal Microscopy).

3.5.1 Genomic DNA extraction from putative transplastomic plants

To conduct molecular techniques for the screening of putative transgenic, genomic DNA isolation is a fundamental prerequisite before the second round of selection. Entire DNA extraction procedure from 4 transplastomic potato cultivars was conducted by the method described by Zhang et al., 2000 with some modifications. Extraction buffers were used as described below. According to the number of explants, the amount of buffer was determined. The same DNA isolation procedure was implemented for every cultivar and non-transgenic control plants.

Table 3.1. Composition of plant DNA extraction buffer: (For 1 ml)

Components	Amount
10% CTAB	0.2 ml
1M Tris-Cl	0.1 ml
0.5 M EDTA	0.1 ml
5 M NaCl	0.28 ml
10% Na-Sulphite	0.1 ml
10% PVP	0.2 ml
H_2O	0.02 ml
Total	1 ml

About 0.5 g fresh leaf samples were grinded into powder in a pre-cooled mortar and pestle by the addition of liquid nitrogen, and then the powder was placed into 2 mL Eppendorf tubes. 500 µL of DNA extraction buffer (mentioned in the table above) was added into the tube mixture and mixed properly. Samples were kept at 65°C for 1 hour with vigorous shaking and tubes were inverted in every 10-15 minutes. After that, centrifuged for 10 minutes at 14800 rpm at 4 degrees. 500 µL of bottom part of Phenol: Chloroform: Isoamyl alcohol (25:24:1), or 500 µL of Chloroform: Isoamyl alcohol (24:1) was added to the mixture. Again, the mixture was centrifuged for 10 minutes at 14800 rpm at 4 degrees. Supernatant was separated into another new 1.5 mL Eppendorf tube. 0.6 volumes isopropanol (0.6 ml Isopropanol × Amount of Supernatant) was mixed with supernatant. Then, supernatant - isopropanol mixture was incubated overnight at -20°C. The incubated mixture was centrifuged for 10 minutes at 14800 rpm at 4 degrees. After having the pellet by centrifuging, the supernatant was removed, and the pellet was slightly dried on tissue paper without disturbing the pellet. The dried pellet was washed carefully with 70% Ethanol (adding 500 µL). The supernatant was removed, and the pellet was let to dry appropriately. Then 50-100 µL of TE buffer (Tris-EDTA) was added to pellet to dissolve it. TE buffer and pellet was appropriately mixed and let them dissolve completely. Two µL of RNAse enzyme was added and mixed by inverting or tapping. That enzyme and pellet mixture was incubated at 37°C temperature for 45 minutes to 1 hour with slight shaking. Again, that mixture was incubated at 65°C temperature with slight shaking for 5 minutes to dissolve the pellet completely. Then the mixture was centrifuged for 10 minutes at 14800 rpm at 4 degrees. Finally, the supernatant was separated into a fresh 1.5 mL Eppendorf tube. This procedure yields˜ 20–30 mg of DNA that stored at -20°C. After the extraction, genomic DNA was quantified by nanodrop and agarose gel electrophoresis (0.8% agarose, under UV light). When the quality and quantity of DNA was measured, they were diluted to desirable concentrations. The genomic DNA was then subjected to several molecular techniques.

3.5.2 Screening of putative transgenic potato by PCR

For the confirmation of transgene integration into the potato chloroplast genome, putative spectinomycin resistance shoots were analyzed by PCR. Before conducting the PCR, genomic DNA from putative transplastomic plants was isolated by the method described by Zhang *et al.* (2000) with some modifications (Described in 3.1). The genomic DNA was then subjected to PCR. PCR analysis of transgenic plantlets was carried out following Saiki et al., (1990). The PCR analysis was conducted using gene-specific partial primer pairs for the *SN-19, cry3A, aadA* and *gfp* genes (Table 3.2).

Table 3.2. Primer's name, sequences, product size and annealing temperature information used for the PCR

Primer	**Sequence (5'-3 ')**	**Product size(bp)**	**Annealing Temperature (^{o}C)**
cry3A*F- par	TGAGGGGATACGGGACAACA	509	55
***cry3A*R- par**	TCCTTGCATCAGGAAGCACAT		
***SN-19*F**	TGCATTGCTGAGGGTAACAAC	480	55
***SN19*R**	AGCATCACGAAGCAAAAGCAA		
***aadA*F**	GGCTCCGCAGTGGATGGCGGCCTG	540	55.8
***aadA*R**	GGGCTGATACTGGGCCGGCAGG		
***gfp*-F**	AGCAGATCATATGAAGCGGCACG	398	56.3
***gfp*-R**	CGTAAGGGCAGATTGTGTGGAGA		
***SN-19*F$_1$(RT-qPCR)**	ATGCACCATCCTTTTCTGCT	140	55
***SN-19*F$_1$(RT-qPCR)**	TCCAACTTATGACCTCCCCA		
***SN-19*F$_2$(RT-qPCR)**	TGGACAGTTGGCATCATTCT	160	55
***SN-19*R$_2$(RT-qPCR)**	ACCCAAACCTTGAAGACGTG		
***gfp*- (RT-qPCR)**	AGGAGGACGGAAACATCC	145	55
***gfp*- (RT-qPCR)**	GATCAGCGAGTTGCACGC		

*Par: Partial length primer, RT: Primer for qRT-PCR

A total of 20 µl PCR reaction mixtures in a 0.2 ml PCR tube was prepared. DNA from untransformed leaf and plasmid DNA of chloroplast transformation vector were used as negative and positive controls, respectively. All the reactions were made using polymerase Taq DNA (Fermentas, USA).

A reaction volume of 20 µl contained: (i) Reaction buffer (ii) dNTPs (100 µM) (iii) primers (30 pmol) (iv) 50 mM $MgCl_2$ for Taq polymerase (v) suitable volume of template DNA solution containing 50-100 ng of template DNA (vi) suitable volume of sterile DNAase / RNAase free water to generate a total of 20 µl. The PCR profile was as follows: Denaturation at 95°C for 5 minutes, followed by different annealing temperature for studied genes for 15 seconds. About 55.8°C annealing temperature was ensured for *aadA* gene whereas 56.3°C was maintained for *gfp* gene. For the annealing of both *SN-19* and *Cry3A* 55 °C annealing temperature was maintained accordingly. The extension was repeated at 72°C for 30 seconds. The final extension was performed for 7 minutes at 72°C. PCR amplification was conducted using Master cycler Gradient PCR (Eppendorf AG 22331, Hamburg, Germany). Once the plants were confirmed for transgene integration by PCR, they were subjected to further rounds of selection.

3.5.3 Calculation of transformation efficiency

Transformation efficiency for transplastomic potato cultivars was calculated based on the data recorded during different cultural conditions and results obtained from the PCR. It revealed information regarding the incorporation of desired genes into the chloroplast genome. Percentage of transformation efficacy (% MTE) was calculated as stated below:

$$Percentage\ (\%)\ of\ transformation\ efficiency = \frac{Number\ of\ transplatomic\ plants\ regenrated}{Number\ of\ explants\ bombarded} 100 \quad (3.3)$$

3.5.4 Selection rounds (2nd and 3rd)

Once the plants were confirmed for transgene integration by PCR, they were subjected to further 2nd rounds of selection. For the 2nd round of selection, 2 mm^2 pieces of leaves were cut from PCR-positive leaves and placed them on selection medium. Those leaf sections were grown in culture room under white, fluorescent lamps (1,900 lux) with 16 h light/8 h dark cycle at 26°C. After every 15 days culture was transferred into a new fresh media with the same combination of hormones and selection agent. These leaf sections produced transgenic shoots within 3-4 weeks. Regeneration data from 2nd round of selection was recorded carefully and compared with 1st round of selection. Regenerated shoots were cut again, and these shoots were retransferred to selection medium containing spectinomycin. This step has been termed the third round of selection.

3.5.5 Transfer to root formation medium and selection

Heteroplastomic spectinomycin resistant shoots were grown on selective media and regenerated shoots were transferred to rooting medium (supplemented with NAA 0.1 mg/L and Spectinomycin 50 mg/L) for rooting. After successive culture for 3-4 weeks rooting was occurred.

3.5.6 Acclimatization and growth of putative transplastomic plants

Then after having adequate root, transgenic homoplasmic plants (approximately 3-6 cm long) were transferred into the growth chamber for acclimatization. Plantlets having aqeduate roots were shifted to plastic culture vessels containing peat moss and perlite (2:1) and placed into a controlled growth chamber for acclimatization under 25 ± 2°C under 16 h light photoperiod. in a fully controlled growth cabinet (60% relative humidity, 47 µmol m-2s-1 irradiance and 58 W fluorescent light). After ten days, the plant pots were uncovered for a short time each day to adapt to the environment. Two weeks later, the plants were placed in a well-equipped greenhouse located on the land of Niğde Ömer Halisdemir University, Ayhan Şahenk Faculty of Agricultural Sciences and Technologies.

3.6 Screening of Putative Transgenic Potato Plantlets After Acclimatization

When the plants were acclimatized and growing in soil, total DNA isolation was performed (according to 3.5.1) and gene integration by PCR analysis (according to 3.5.2) and expression level analyzes were performed using qRT-PCR (according to 3.9). In addition, the expression of *gfp* in the shoots of the plants was analyzed using a Leica dmil led inverted microscope. Therefore, ELISA was conducted to detect the *cry3A* gene expression and SDS-PAGE was done to detect the presence of genes (*Cry3A* and *SN-19*) of interest up to protein level.

3.6.1 Microscopic localization of *gfp* in transplastomic shoots

In order to determine the localization of *gfp* in the leaves of plants grown in the soil, a transplastomic plant (Sante 6) and a non-transgenic wild-type potato leaf were imaged with Laser Scanning Confocal Microscope (Zeiss LSM 510) in METU Central Laboratory (sample registration no: 1953). Sections taken from leaf samples during the analysis were visualized with a 20X objective using excitation at 488 nm/514 nm/543 nm wavelengths and emission parameters at BP:505-550 nm / LP: 585 nm wavelengths. For the microscopic operation, leaf tissues were sectioned measuring about 0.5 x 0.5 cm were placed on a glass slide; 1-2 drops of water were added and then covered with a transparent cover slip. The images developed by the microscope was viewed on attached computer screen. Sub-cellular localization of fluorescent protein was verified by confocal laser scanning biological microscopy (Laser Scanning Confocal Microscopy). The images developed by *gfp* fluorescence using confocal laser scanning microscopy was recorded carefully. These results also verified the presence of fluorescent protein in the transplastomic potato cells.

3.6.2 DNA dot blot and southern blot analysis

Southern DNA dot blot analysis for the assessment of gene integration in transplastomic plants was performed according to the protocol described by Skarnes (2000) with minor modifications. Total genomic DNA of transplastomic plants (K3, Ch3, SR4, SR5, Sa6 and Sa7) was first denatured by heating at 95°C for 5 minutes, then 150 µl of 0.5 M NaOH was added and 30 minutes incubation was done at 37°C. 1 µg of DNA was dotted onto the Hybond-N+ nylon membrane (Amersham). The *SN-19* fragment amplified by PCR was labeled with biotin, thus creating the *SN-19* probe. The membrane was first soaked in pre-hybridization buffer and then hybridized with the generated *SN-19* probe at 55°C overnight. Identification of transgene DNA as a result of hybridization was carried out following the protocol of the commercial detection kit (Fermentas Biotin Chromogenic Detection Kit; Cat# K0661). The biotic labeled *SN-19* fragment was dotted on the membrane as a positive control. The DNA of the non-transgenic plant was dotted onto the membrane as a negative control.

Following this analysis, Southern blot analysis of genomic DNA samples isolated from positive transgenic plant leaves was performed according to the method specified by Southern (1975) to determine the copy number of integration into the chloroplast genome. Genomic DNA was extracted using CTAB mediated DNA extraction method from both PCR positive and nontransgenic plant leaves (Chapter 3.5.1). About 20 µL of transgenic plant DNA was digested with HindII/EcoRI to release the fragment from plasmid. Digested DNA sample was run in 1% agarose gel at 40 Volts for four hours and then then 12 volts over night.

After the electrophoresis, depurination of the gel was done by the 0.25M HCl for 10 minutes on a digital rocker with slight shaking at 50 rpm. Next, the gel was treated with 1.5M NaCl and 0.5M NaOH for denaturation for 30 minutes and 0.5 Tris-HCl and 1.5M NaCl were applied to neutralize the gel, which ensures 7.5 P^H. Then, the digested DNA samples were transferred to the HybondTM-N+ Nylon membrane (Amersham, Cat lo no: RPN119B) by the capillary action with 10X SSC buffer solution. By placing the membrane under UV light for 6 minutes, DNA was fixed on the membrane. After the DNA was fixed, membrane and a buffer solution for pre-hybridization were then transferred to the hybridizing tube and treated at 55°C for 2 hours. The probe was hybridized for 24 hours. The user manual of the Fermentas Chromogenic Detection Kit was used for detection (Cat lo no: K0661). The 1 X (10ml) wash/blocking buffer was applied to wash the membrane with moderate shaking at 50 rpm. The membrane was then immersed for 30 minutes in 10 ml of blocking solution and a moderate shaking was provided during this operation. The blocking solution was removed and the membrane saturation was performed with 10 ml of diluted streptavidin AP conjugate and washing was done by 1X block/wash buffer. Wash buffer was removed and 10 ml detection buffer was add incubated for 30 minutes. As a final operation, 1X NBT/BCIP was prepared and dissolved into detection buffer to carry out enzymatic reaction which requires incubation of membrane in 20 ml of 1X NBT/BCIP for 30 minutes.

To detect the gene fragment (*SN-19*), probe labeling was done by using Thermo Scientific Biotin Deca Label DNA labeling kit (Cat.No: K0651). According to manufacturer instructions, 13 µl of template (100ng), 5µl of decanucleotides in the 5X reaction buffer along with nuclease free water up to 44 µl was added in the eppendof tube. Then the tube was vortexed for 3-5 minutes to spin down. Then the tube was placed into boiling water bath for 5-10 minutes and it was chilled down quickly in ice. After the spinning, the mixure was transferred to eppendrof tube. 5µl of Biotin labeling mix with 1 µl Klenew fragment was added into the eppendrof tube.

Then the tube was spun down with all added contents and incubation at 37°C for 20 hours was confirmed. After this incubation, 1µl of 0.5M EDTA (PH: 8.0) was added to stop the reaction. The labeled probe (DNA) was estimated by spotting technique that on the nitrocellulose membrane along with the control DNA labeled as control in consecutive (Serial) dilutions and finally was detected using Biotin Chromogenic Detection kit (Cat lo no: K0661).

3.6.3 Quantitative real time PCR

Quantitative Real time PCR was conducted to find out the expression levels of introduced genes in primary transformants. Relative expression of target genes was measured whwre relative expression relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. This quantification described the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study. Total RNA isolation was performed from PCR positive transgenic plants following the instructions as provided in total RNA isolation kit (RiboZol™ RNA Extraction Reagent). Any genomic DNA contamination was eliminated with the help of DNAseI treatment. RNA integrity and concentration was measured in 1% agarose gel electrophoresis and nano-drop as well. Furthermore, 1µg of RNA was used to convert into cDNA following protocol as described in Fermentas cDNA synthesis kit (Cat. No. 1621). RT-qPCR assay was performed following protocol as described by Hussain et al. 2019. qRT-PCR was performed in a 20 ul volume using SYBR Green master Mix and data was analyzed on the Real-Time PCR Detection System. Endogenous plant genes (eF1α, B-tubilin and actin) was used as control (Nicot et al. 2005). mRNA expression levels of both genes gene was revealed. QRT-PCR contents were included total Syber green master mix (2X), F Primer (1 µM), R Primer (1 µM), RNase-free water and diluted cDNA as template (1:10). qRT-PCR temperature cycle was set up as 95°C for 15 min, 40 cycles at 95°C for 10 sec, 55°C for 15 sec, 72°C for 20 sec and the melting curve analysis at 70°C to 99°C with an increment of 1.0 °C/min. For normalization, elongation factor 1-α (ef1α) was selected as reference gene for purpose of quantifying the expression of genes (Nicot et al. 2005). The threshold values of samples in target gene expression analysis was analyzed by Software of Rotor-Gene Q (QIAGEN) RT-PCR instrument. According to qRT-PCR analysis results, the standard deviations of Ct values of the samples were calculated by using Microsoft Excel program and the expression level of the genes were determined according to the $2^{-}\Delta\Delta$Ct proportional calculation method (Livak and Schmittgen, 2001).

3.6.4 Enzyme-linked immunosorbent assays (ELISA)

Although the presence of transgene can be determined by PCR, its level of expression is determined by evaluating the product of the gene with ELISA. The protein (*Cry3A*) quantities was determined by direct ELISA using respective antibodies. For quantification of trans-proteins, fresh leaf samples from each transgenic plant along with respective control was harvested and grinded with liquid nitrogen. Grinded tissue (0.5mg) was transferred into a 2ml eppendof tube and protein extraction buffer (500 µl) was added into each tube without any delay. Then the tubes with entire contents was centrifuged (with 14000 rpm, 15 mitues under 4^0C) and only supernatant was transferred into a 1.5 ml eppendrof tubes. After the extraction of protein, ELISA was determined according to the manufacturer's method by direct ELISA using the relevant antibody by Agdia qualiplate Kit (Cat. No: PSA05900). The protein in each tube was diluted 10 times with EB2 extraction buffer from the above mentioned kit. Each ELISA plate well was supplemented with 50 µL of cry enzyme conjugate and incubated at 37 °C for 1 hour. Chloroplast enriched protein and cytosolic extract (5 µg) were added to the respective wells. The contents of the wells were then shaken into a sink vigorously. Following three items of washing with 1X of PBS, the well was then mixed and incubated with 100 µL of substrate for 30 minutes at 37 °C. Following incubation, each well was added to 100 µL of stop solution (1 N HCl) and thoroughly mixed. The contents of the well-turned yellow. On ELISA-reader (SPECTRAmax plus 384) the plate was retrieved and relevant data were recorded. The crude-leaf protein was extracted to detect recombinant transgenic expression in each transgenic potato line for ELISA's analysis of future generations.

3.6.5 SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis)

A BioRad Mini gel apparatus was used to conduct SDS-PAGE following operating procedure instructed by Laemmli (1970). 50-100 µg of total soluble protein was separated on 12% SDS- polyacrylamide gel . 0.2 gm of potato leaves from each transplastomic lines were utilized for the extraction of total soluble protein (TSP) where leaves were ground in liquid nitrogen to form a fine powder. Soluble proteins were extracted using 1ml of extraction buffer (For 5 ml: Glycerol- 0.5mL, 0.5M EDTA- 0.4 mL with pH 7.5, 5M NaCl- 0.15 mL, 1M Tris-Cl 0.05 mL having pH 7.5 , NH4Cl - 26.7 mg, DTT- 15 mg and PMSF - 2 mM). Cellular debris was removed by several rounds of centrifugation (in 14000 rpm, for 20 min at 4 ˚C) and the supernatant was used for SDS-PAGE. Optical density (OD) of extracted proteins were measured with spectrophotometer and spectrophotometer was warmed up before use. Same amount of protein level was ensured in every samples with required dilution and also based on spectrophotometer OD of those samples. Protein samples were prepared with protein loading dye (Trans, contains: 10% SDS, 10mM Dithiothreaitol / DTT, 20% Glycerol, 0.2 M Tris-HCl, pH 6.8 and 0.05% Bromophenol blue) with 5 minutes incubation at 90°C and stored at -20 up to running in the gel. After that, 12% separating gel (Water 2.8mL Acrylamide (30%) 3.2mL 4xTris-SDS 2.5mL (pH8.8) APS (10 %) 26.7µL TEMED 5.3µL) and 4% stacking gel (Water 2.5mL Acrylamide (30%) 0.533mL 4xTris-SDS 1mL (pH6.8) APS (10 %) 31.5µL TEMED 6.3µL) was prepared and 45 -60 minutes was allowed for both gels to solidify. 5 µl protein ladder and 10 µl sample was run into each well and electrophoresis was done for 1 hours with 100V . 1X running buffer (30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O) was used for electrophoresis. The gel was stained with Coomassie Blue Staining (0.1 % Coomassie Blue in 40 % ethanol, 10 % acetic acid) and destaining was done with a solution containing 10 % ethanol and 7.5 % acetic acid. Visualization of destained gel with Gel DOC^{TM} XR^{+} Imaging System of Bio-Rad.

3.7 Leaf Bio-Toxicity Assays of Primary Transformants Against Targeted Insect Pests

3.7.1 Insect culture and foliar feeding experiment

The efficacy of introduced insecticidal genes in transplastomic potato lines to combat CPB was evaluated by subjecting transplastomic potato plants to leaf bioassays under laboratory condition. Transplastomic plants from first round of selection were acclimatized and bioassay was conducted with first and second instar CPB larvae and mortality rate was recorded up to 48 hours. After that, another bioassay experiment was done with the second-round selection availed transplastomic lines on which both larvae (first and second instar) and adult CPB was released for feeding. From this bioassay experiment, we recorded lethal effects (day required) of transplastomic lines on mentioned CPB population. We have already a well-developed insect rearing laboratory in sister department in our faculty with required temperature (25 ± 1°C), light condition (16:8 h light–dark photoperiod) and relative humidity (60-65%). Sensitive and resistant potato beetle (CPB) colonies prepared for the use in foliar feeding (bioassay) experiments and its continuity was ensured (Hussain et al., 2019; Naqqash et al., 2020; Salim et al., 2021).

3.7.2 Determination of mortality rate of CPB feed on transplastomic lines

To feed the insect pests on transplastomic potato leaves, the freshness and watery content of leaves were maintained carefully, and petiole of leaves were placed into 1.5 ml Eppendorf tube containing water and wrapped. Leaf trifoliate from each transplastomic and control lines were placed on cleaned filter paper in 100 × 20mm diameter petri plates and 10 neonate pre starved CPB larvae were released on each leaf for feeding to check the efficacy of transplastomic lines. Three replication of each treatment was conducted at different occasions for each stage and cultivars along with their controls. Efficacy of transplastomic lines against adults CPB also tested individually using same experimental treatments. Leaves were replaced with fresh one at 12 hrs. intervals. The toxicity potential of transplastomic potato lines as well as control plants were evaluated against the larvae and adults CPB and recorded properly.

3.7.3 Calculation of foliage consumption area

Photo of consumed portion of each leaf were taken using a flat background with contrast to the potato leaves with no flash. Leaf region was automatically identified by the software and the image did not impose any reflection on the leaf surface, by default, the first circle was checked. Furthermore, the BioLeaf software (Machado et al., 2016, Salim et al., 2021), is capable to remove dirt particles, sand grains and pieces of leaves or noise around the leaf (mentioned by red arrow, fig-1B). To avoid uneven illumination with reflection on the leaf surface (fig-1C: a. with reflection), second circle was checked (fig-1C: c. shown by red-hand cursor). Then the result of the segmentation by fine tuning with the slider was adjusted and assessed visually. If only internal portion of the leaves were consumed (fig-D), we calculated defoliation result, but in cases of border consumption, it was required to reconstruct them by touching the screen. For the measurement of the defoliation rate with precision, we zoomed in and zoomed out the region of the leaf opening and closing two fingers (hand cursor -2 in figure fig-E. a) to set the control points of the curve. We set total 3 points, first two in the endings of the region and finally the mid-point (hand cursors in figure fig-E. b and c). Three replications of this experiment at different occasions for each stages were confirmed.

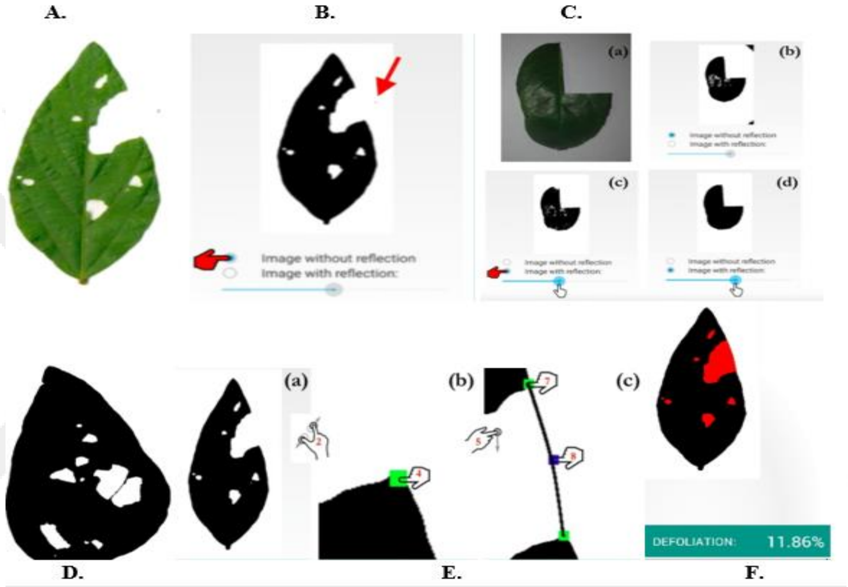

Figure 3.4. Principles and procedure of leaf area consumption measurement by BioLeaf software (Machado et al., 2016)

3.7.4 Determination of net weight changes of CPB larvae feeding on transplastomic potato leaves

To check the effect of transplastomic potato leaves harboring insecticidal gene on the growth and development of CPB, 1st and 2nd instar larvae were feed on the transplastomic potato leaves as well as wild type/control leaves for 72 hours. Weight of insect larvae were taken before and after of feeding the leaves according to Swift (1997). The increase in weight of the larvae was recorded. For each treatment, 10 larvae were used. The experiment was repeated at three different occasions for each stages and cultivars.

3.8 Statistical Analysis

In-vitro regeneration data of studied cultivars were recorded and calculated with standard statistical tools. Statistically significant differences between the means of the data were calculated by ANOVA (5%). To measure whether the means are significantly different from each other or not Tukey HSD test was applied . Mean and Standard error (Mean ± S.E) of data were calculated from 3 replications (n = 3). All statistical analysis was done by Statistix 8.1 software.

CHAPTER IV

RESULTS

4.1 Selection and Regeneration of Bombarded Tissues on Selection Medium

Around 100 explants (internodes and leaf) from every potato cultivar (Kuroda, Simply Red, Challenger and Sante) were bombarded with plastid transformation vector (PCTV1) bearing FLARE-S as a marker gene. Forty-eight hours after the bombardments, explants were shifted to the regeneration medium supplemented with suitable growth regulators and antibiotics for the selection of transformed cells. Both callus induction and plant regeneration from explants required the appropriate combinations and concentrations of plant growth regulators in the culture media. Callus induction was observed after 4-6 weeks, and mean percentage of callus induction was calculated. The un-bombarded explants (used as control) and bombarded explants which were not transformed, due to selection pressure; those were turned to dark color and were found to get dead within two weeks of culture due to selection pressure (Figure-1, B). No callus and shoots were developed from such non-transformed explants. Selection with spectinomycin was continued greening was achieved, because spectinomycin prevents wild cells from greening, only visible green cell clusters were established by spectinomycin-resistant cells. Among the bombarded potato cultivars, Kuroda revealed the highest callus induction percentage from internodes (89.0%) and leaves (92.0%) (Table-1). Callus induction proficiency of cultivar Kuroda depicted higher in number compared with cultivar Simply Red (internodes - 87.0%, Leaf explants - 82.0%). Lower callus induction proficiency from the leaf (74.0%) and internodes (71.66%) explants of cultivar Challenger was recorded compared with cultivar Kuroda and Simply Red (Table-6). Among the four potato cultivars, internodal explants of cultivar simply Red induced more calli in number than leaf explants that were recorded as an exception because all cultivars induced more calli from their leaf explants. Callus color and callus morphology data were recorded where calli were manifested several colors from studied cultivars. More prevalence of light brown (LB) color of calli was recorded during the experiments.

Figure 4.1. Different step of generation of transplastomic potato (1st rounds of selection, callus induction, regeneration, and plantlet production) on spectinomycin containing regeneration medium. Biolistic gene gun mediated bombarded explants on regeneration selection medium (a), selection of primary transformants: non-transformed explant denotes its swollen and greatly bleached because of inhibition of plastid protein biosynthesis (b), selection of transformed tissues where non transformed tissues were died due to the inhibition of plastid protein biosynthesis (c), fully developed mature calli (d), emergence of micro-shoot from calli under selection pressure (e), putative transplastomic plantlets that is resistant to spectinomycin (f), multiplication and culture of micro shoot with presence of selective agent on which pseudo-transplastomics were unstable (g+h), 2nd round of selection of micro shoots under spectinomycin to purify the transplastomic line to homoplasmy (ı), rooting of stable transfomants (j), transplastomic plant on soil pot. (k) and transplastomic potato tubers (l).

Table 4.1. *In-vitro* regeneration of transplastomic potato with different hormonal combinations along with selection pressure

Potato cultivars		Callogenesis						Total number of calli	Total Shoot Regenerated from different cultivars	Shoot regeneration (%)	Number of Sp^r shoots	% of Sp^r shoots	Number of PCR Positive shoots	% of PCR Positive Shoots	Total PCR positive shoots
		Induction (%)		Color		Morphology									
		Internodes	Leaf	Internodes	Leaf	Internodes	Leaf								
Kuroda	PGR : BAP 2 mgl^{-1}, NAA 0.2 mg l^{-1}, GA3 0.1 mgl^{-1}TDZ 2 mgl^{-1}	89.0 ± 0.57a	92.0 ± 0.57a	LB	YG	C	G	181.0	11 ± 1.15a	6.0 ± 1.52a	3.0 ± 0.57a	27.2 ± 1.09b	2.0 ± 0.33b	66 .6 ± 1.52b	16 ± 0.57
Simply Red		87.0 ± 1.15a	82.0 ± 1.15b	B	G	C	F	169.0	15 ± 1.52a	8.8 ± 0.23a	5.0 ± 0.30a	33.3 ± 1.15b	4.0 ±0.57a	80 ± 1.15a	
Challenger		71.6 ± 0.33c	74.0 ± 1.15c	LG	DG	G	F	145.6	14 ± 0.57a	9.6 ± 0.51a	6.0 ± 0.83a	42.8 ± 1.10a	4.0 ± 1.00a	66 ± 1.52b	
Santee		80.0 ± 0.57b	86.0 ± 1.15b	LB	G	C	G	166.0	16 ± 1.73a	9.6 ± 0.75a	7.0 ± 0.95a	43.7 ± 1.45a	6.0 ± 0.83a	85.7 ± 1.83a	

Spectinomycin resistant: Spr. Every regeneration selection media was supplemented with 50 mg/L Spectin0mycin. Yellowish Green: YG, Light Green: LG, Green: G, Dark Green: DG, Light Brown: LB, Brown: B, Compact: (C) Granular: (G) Friable: (F). TZ: Transzeatine, TR: Transzeatine riboside and TDZ: Thiadiazuron. Mean of different values shown by different letters in the same columns are statistically different determined using Tukey's HSD test at 0.05 level of significance. Results and Standard error (Mean ± S.E) were calculated from 3 replications (n =3).

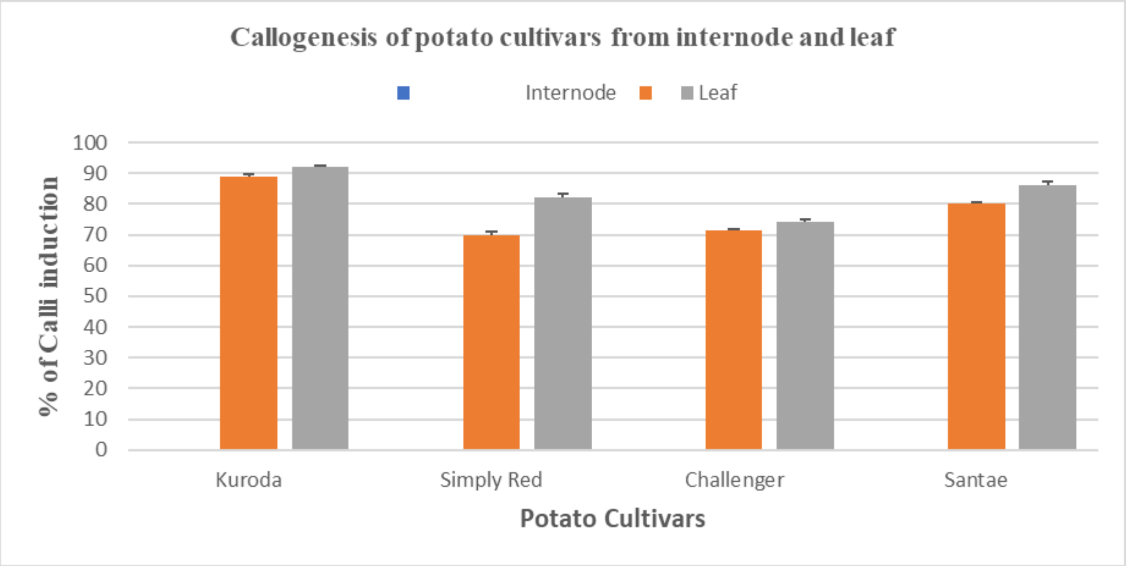

Figure 4.2. Callogenesis of bombarded explants from four potato cultivars under *in-vitro* condition

Besides this, Yellowish Green (YG), Light Green (LG), Green (G), Dark Green (DG), and Brown (B) color calli were found from studied potato cultivars. Internodal explants from all cultivars induced light brown (LB) color calli, and leaf explants induced Greener (G) color calli. From internodal explants, cultivar Kuroda and Sante induced relatively light brown (LB) Calli whereas cultivar challenger and Simply Red induced Brown (B) and Light Green (LG) calli. Contrarily, from leaf explants, cultivar Kuroda and Challenger induced Yellow Green (YG) and Dark Green (DG) calli respectively and cultivar Simply Red and Sante induced Green (G) color of calli. As for as callus morphology is concerned, Compact (C), Granular (G), and Friable (F) type of calli were induced and recorded properly during this current experiment. Explants from potato internodal segments induced more compact and granular type calli. Cultivar Kuroda and Simply Red produced compact type calli and cultivar Challenger and Sante induced granular and compact type calli, respectively. When leaf tissues were used as explants, Cultivar Kuroda and Sante induced granular calli whereas cultivar Simply Red and Challenger induced friable type of calli. In sum, from internodal and leaf tissues, Kuroda induced a total of 181.0 calli that was the highest number among all cultivars studied. From cultivar Simply Red and Sante, 169.0 and 166.0 calli were induced, respectively. Therefore, cultivar Challenger induced the lowest number (145.6) of calli.

4.1.1 Microscopic monitoring of the *gfp* expression in transformed calli

Well-developed, mature calli from four potato cultivars were chosen to check the integration and expression of the visual marker green fluorescent protein *gfp* gene. The expression of *gfp* protein can be monitored from the early developmental stage of the callus in the callus-induced transformation (CIT) process. After two weeks of development in the selection medium, the formation of the *gfp* signal in the calli can be detected microscopically (Figure 4.3). In CIT strategy, a callus is induced first and then a shoot develops from this callus, was also applied in our study. This method was created by changing the callus-mediated shoot formation method (Lee et al., 2004). Although the CIT method works well, there are some problems are remaining. For example, the selection efficiency of obtaining a positive shoot from the transferred calli is low. Shoots are easily grown directly from the cut tissue of the cotyledons, and these direct shoots give false positive results in most cases. After these shoots have grown, they can show resistance even at high concentrations of selection agents. In contrast, indirect shoots grown from callus have high transformation efficiency (Lee et al., 2004). Therefore, questions may arise about how to distinguish an inherited callus from a non-transferred callus and how to obtain an indirectly grown transgenic shoot from a transformed callus. Detection of an inherited callus and shoot at an early stage of transformation is very important for the genetic transformation of the potato. To avoid these problems, the use of *gfp* as a visual marker facilitates the confirmation of transformation at early developmental stage (Ghorbel et al., 1999; Elliot et al., 1999; Chung et al., 2000; Vain et al., 1998; Stewart 2001; Parveez 2007) .

Four different potato cultivars were used to control the induction and transformation levels of calli using the *gfp* gene. As a result of the comparison of panels B and D in Figure 3.3, it is obvious that there is a clear difference between transplastomic calli and control calli. The highest rate of gfp protein accumulation (based on the level of luminosity in green) was recorded in Kuroda cultivar. Therefore, the image of the Kuroda cultivar is given in Figure 16 as an example to show the *gfp* localization in the calli. Calli with observed fluorescence expression from *gfp* were selected, transferred to a new medium, and subcultured. By this means, rapid selection of positive calli was achieved.

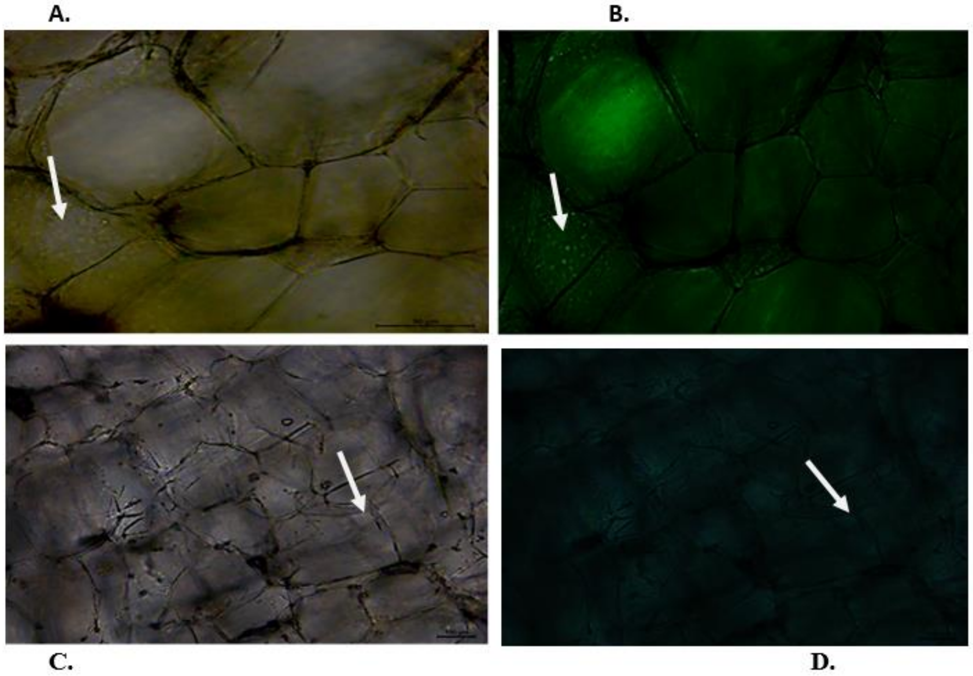

Figure 4.3. Microscopic visualization of *gfp* expression in transplastomic potato calli. Transplastomic calli under white field (a), transplastomic calli under *gfp* filter (b), control calli under white field (c) and control calli under gfp filter (Top: Arrows pointing to the region of *gfp* expression in the transplastomic callus in the figure. Bottom: The control plant does not show any *gfp* expression, but slight luminescence is observed due to chlorophyll autofluorescence (d)

4.1.2 Selection for shoot regeneration on selection media

After having the confirmation of *gfp* gene integration and expression, *gfp* positive calli were cultured on the fresh regeneration media with same hormonal combination. Later, embryogenic growth was observed in developed calli after 2-3 weeks of callus formation. The, shoot formation was noticed from those embryogenic cells, and the number of transplastomic candidate shoots were recorded. Three cytokinin’s (TDZ, TR and TZ) showed various responses in cases of shoot regeneration proficiencies and days required. Concerning shoot formation, TDZ produced the highest percentage of shoot formation (41%) requiring 49 days, whereas TR resulted in 40.3% shoot formation within the same number of days. TZ's shoot production ranges were recorded as 17% to 39.6% within 49 days (Figure 4.4).

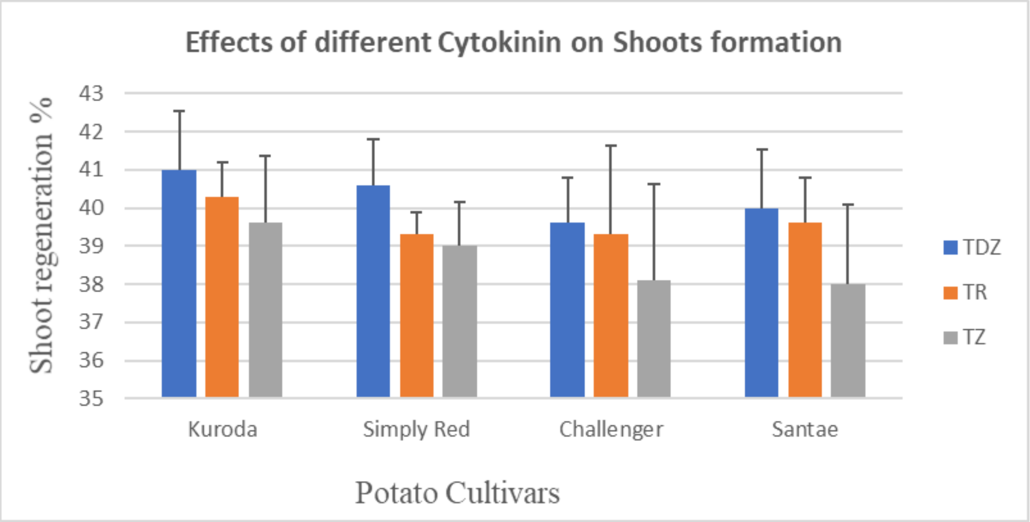

Figure 4.4. Effects of 3 different Cytokinines on shoot regeneration

Shoot production proficiencies also varied depending on cultivar types. Cultivar Kuroda and Sante produced a higher number of shoots, whereas cultivar Kuroda and Challenger respectively yielded the lowest number of shoots. The transformation efficiency was calculated accordingly. The bombarded explants of different cultivars showed varying responses on regeneration media containing selection agent. After that, the total number of shoots produced by every cultivar was calculated and recorded. Cultivar Sante and Challenger showed 9.6% shoot regeneration efficiency followed by cultivar Simply Red (8.8%). On the other hand, cultivar Kuroda produced the lowest (6%) shoot regeneration efficiency (Figure 4.5).

Proliferated shoots were sub-cultured for further rounds of selection to achieve homoplasmic condition on MS media supplemented with 50 mg/l spectinomycin. After successive selection, cultivar Sante produced the highest number of Spectinomycin resistant shoots (43.7%) followed by cultivar Challenger (42.8%). Cultivar Simply Red and Kuroda produced 33.3% and 28.0% Spectinomycin resistant shoots, respectively (Figure 4.5).

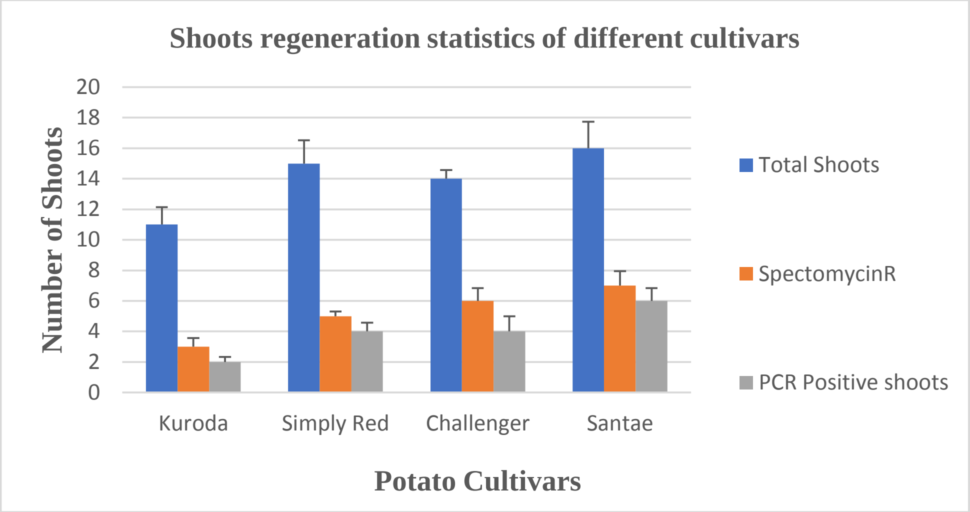

Figure 4.5. Shoot regeneration statistics of 4 different potato cultivars

4.2 Screening of Spectinomycin Resistant Shoots by PCR

We screened our putative spectinomycin resistant shoots by PCR with gene specific primers to check the primary integration of genes of interests (*SN-19* and *Cry3A*) and bi-functional visual (*gfp*) and selectable marker FLARE-S' (*aadA*) into the chloroplast genome. Standard PCR revealed a required band of 480 bp for *SN-19* gene (Figure-3.6) and 398 bp *gfp* gene (Figure 3.7) was also confirmed by standard PCR. All findings of PCR were enlisted in table -7.

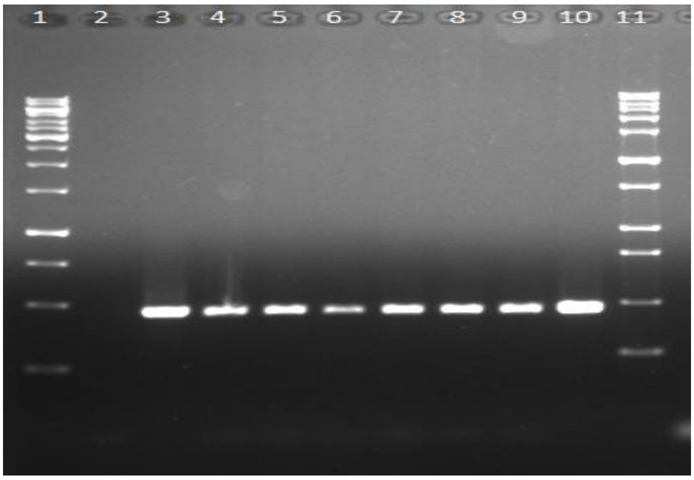

Figure 4.6. Molecular evaluation of transplastomic shoots with the *SN-19* gene. PCR analysis shows amplification of the inner fragment (480 bp) of *SN-19*. Lane 1 and 11: 1kb gene ruler mix (Thermo scientific), Lane 2: Negative control, Lane 3: PCTV plasmid used as positive control, Lane 4 to 10: representative Spectinomycin resistant shoots from potato cultivars

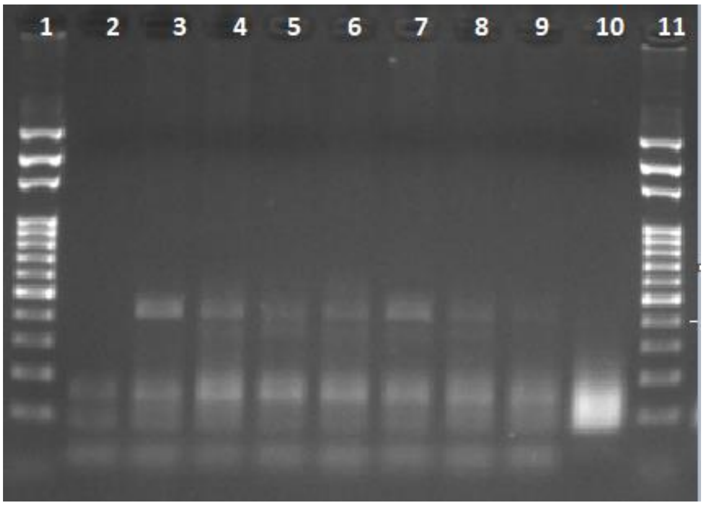

Figure 4.7. PCR assay to amplify Green fluorescent protein gene (*gfp*) from transplastomic shoots. Lane 1 and 11: 100 bp ladder (Thermo scientific), Lane 2: Negative control, Lane 3: plasmid DNA PCTV as positive control, Lane 4-10: representative transplastomic shoots from cultivars

As PCR findings, highest number of PCR positive shoots were recorded from cultivar Santee (85.7%) and cultivar Kuroda produced lowest number of PCR positive shoots (66.6%) accordingly.

Although the cultivar Kuroda produced a smaller number of spectinomycin resistant shoots, its percentage of PCR positive shoots (66.6%) were followed by the cultivar challenger (66.0%).

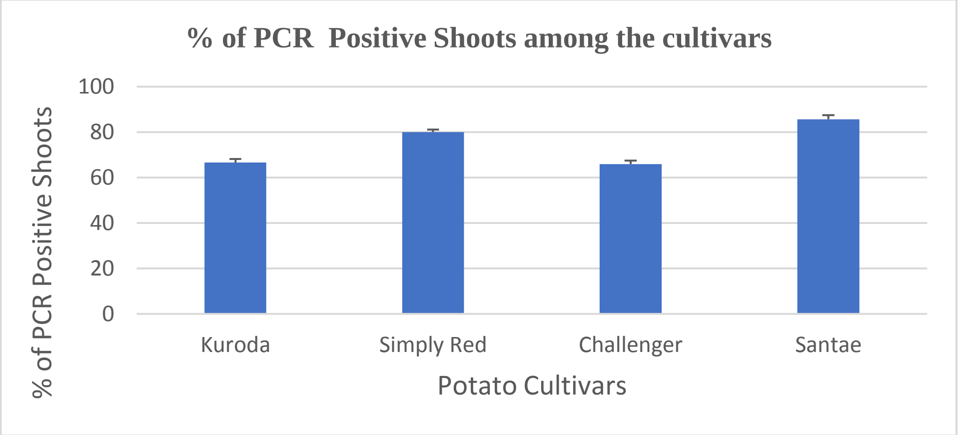

Figure 4.8. Percentages (%) of PCR Positive shoots among the potato cultivars

Cultivar simply red and challenger showed intermediate and equal number of PCR positive shoots although their spectinomycin resistant shoots were different in numbers and their PCR positive shoots percentages also 80% and 66 % respectively. Finally, from four cultivars, we got a total of 16 PCR positive shoots and those shoots were used for further analysis (Figure 4.8).

Table 4.2. Detailed results of PCR analyses conducted with spectinomycin resistant shoots (under *in-vitro* condition)

Serial No.	Plant name		Analysis Results		
			PCR analysis		
	Allowed name	Original name	SN-19	gfp	qRT-PCR
01	K1	Kuroda	+	+	+
02	K2	Kuroda	-	-	-
03	K3	Kuroda	+	+	+
04	K4	Kuroda	+	+	+
05	Kc	Kuroda Control	-	-	-
06	SR1	Simply Red	+	+	+
07	SR2	Simply Red	+	+	+
08	SR3	Simply Red	-	-	-
09	SR4	Simply Red	+	+	+
10	SR5	Simply Red	+	+	+
11	SRc	Simply Red Control	-	-	-
12	Ch1	Challenger	-	-	-
13	Ch 2	Challenger	-	-	-
14	Ch 3	Challenger	+	+	+
15	Ch 4	Challenger	+	+	+
16	Ch 5	Challenger	-	-	-
17	Ch 6	Challenger	+	-	+
18	Chc	Challenger Control	-	-	-
19	Sa1	Sante	+	+	+
20	Sa 2	Sante	+	+	+
21	Sa 3	Sante	+	+	+
22	Sa 4	Sante	-	-	-
23	Sa 5	Sante	+	+	+
24	Sa 6	Sante	+	+	+
25	Sa 7	Sante	+	+	+
26	Sac	Sante control	-	-	-

4.3 Quantitative Real-Time PCR (qRT-PCR) Analysis to Check the Relative Expression of Target Gene

Quantitative Real-Time PCR (qRT-PCR) was used to determine the expression level of the *SN-19* gene in selected putative shoots in spectinomycin-containing medium. Accordingly, *SN-19* gene expression in transplastomic shoots was significantly higher in some transgenic plants when compared between plants (Figure 4.9). In this study, since the transgene was not found in the genome of non-transgenic wild-type potato plants, these plants were not used in gene expression analysis. Instead, the expression levels in the others were determined based on the plant showing the lowest expression level among the transgenic plants. Accordingly, since the lowest expression level was recorded in Ch3 (Challenger), this plant was used as the reference group for normalization and the transgene expression level in the remaining cultivars was determined compared to Ch3. K1 (Kuroda) resistant shoot showed the highest expression (42X), followed by K3. Sa6 (Sante) showed 25-fold higher expression levels compared to the non-transgenic control, followed by SR5. Then comes Simply Red and Challenger.

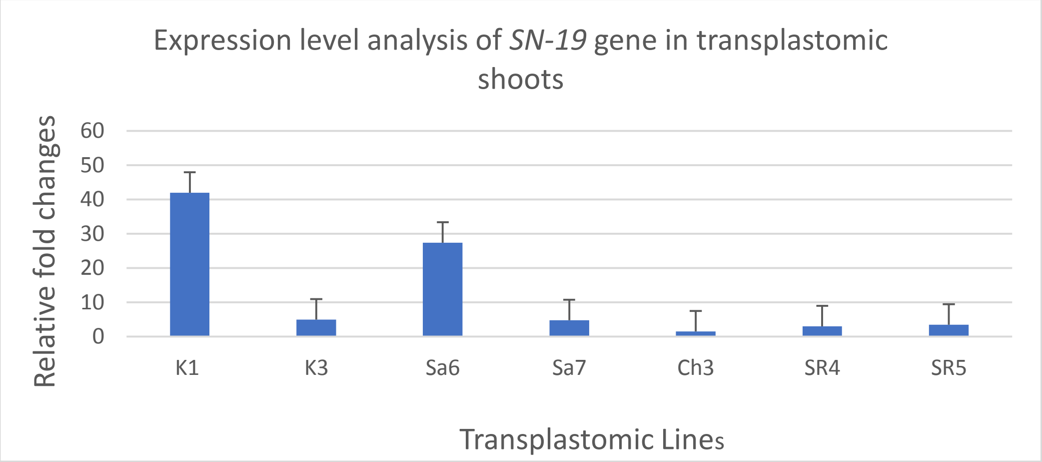

Figure 4.9. Expression level analysis of the *SN-19* gene in transplastomic shoots by qRT-PCR. Since there was no expression of these genes in control plants, the lowest expression level was used to determine the transgenic expression level. Accordingly, since the lowest expression level was recorded in Ch3 (Challenger), this plant was used as the control group and the transgene expression level in the remaining cultivars was determined compared to Ch3. K: Kuroda, Sa: Sante, Ch: Challenger, SR. Simply Red

4.4 Second Round of Selection

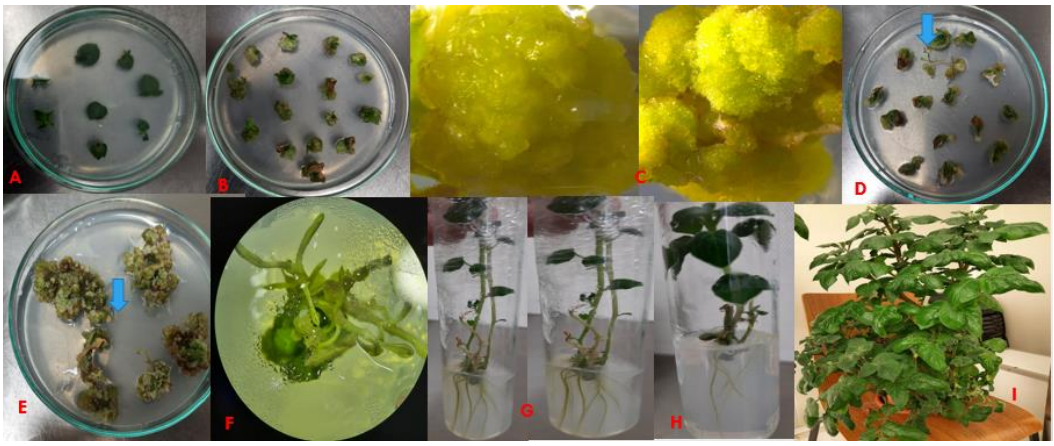

Figure 4.10. Successive steps of 2nd round of selection. Leaves from PCR positive transplastomic lines on selection media regenerates from previous selection round (a), Calli after 4 weeks of incubation (b, c), micro shoots from well-developed calli (d, e), microscopic view of micro shoots (f), growth of spectinomycin resistant micro shoots on MS media supplemented with Spectinomycin 50 mg /L^{-1} (g), rooting of micro shoots under selection (h) and transplastomic plants in soil condition (ı)

4.5 Molecular Evaluation of Transplastomic Plants After Acclimatization

Spectinomycin resistant transgenic potato lines grown in *in-vitro* culture conditions were acclimatized in the growth chamber (Figure 4.10, A, B, C) and subjected to various molecular assays to confirm their expression by gene integration. Total DNA was isolated from soil growing transplastomic plants Kuroda (K3, K4), Sante (Sa6 and Sa7), Simply Red (SR4, SR5) and Challenger (Ch3, Ch4) genotypes . Next, spectinomycin-resistant shoots were screened by PCRs using gene-specific primers to check for genome integration of the genes of interest (*SN-19* and *cry3A*), the bifunctional visual marker (*gfp*), and the selectable FLARE-S' (*aadA*) marker . The required bands of 480 bp for the *SN-19* gene (Figure 4.11) and 509 bp for the *cry3A* gene (Figure 4.12) were obtained by standard PCR.

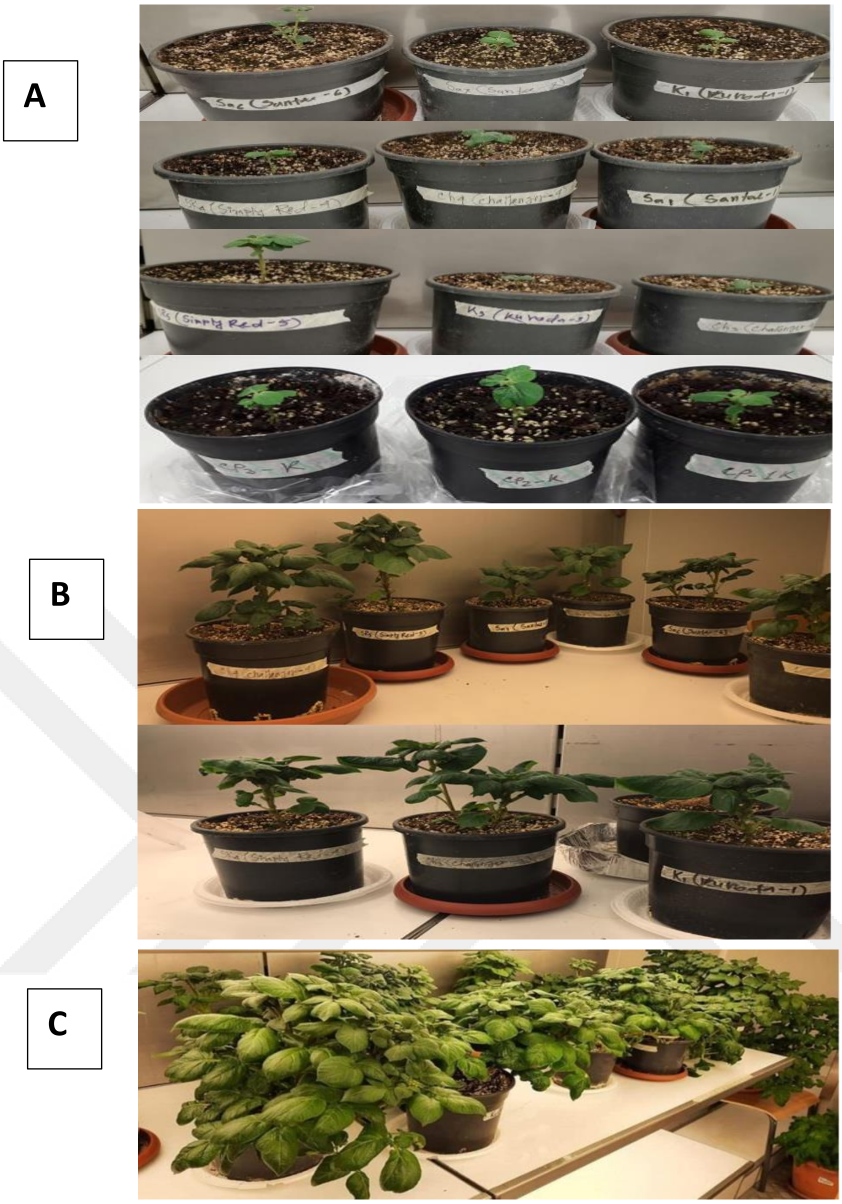

Figure 4.11. Image of PCR positive transplastomic plants on soil pot (peat-perlite mixture). Primary growth stage of plants on soil, photographed on 15.08.2020 (a), secondary growth stage of plants on soil (photographed on 22.09.2020) (b) and mature growth stage of plants on soil (c) (photographed in December 2020) (K: Kuroda, Sa: Sante, Ch: Challenger, SR. Simply Red)

In addition, the 540 bp *aadA* gene (Figure 4.13) and the 398 bp *gfp* gene (Figure 4.14) were confirmed by standard PCR. DNA from non-transgenic wild-type plants was used as negative control in PCR analyses. All findings of PCR results are summarized in Table 4.3.

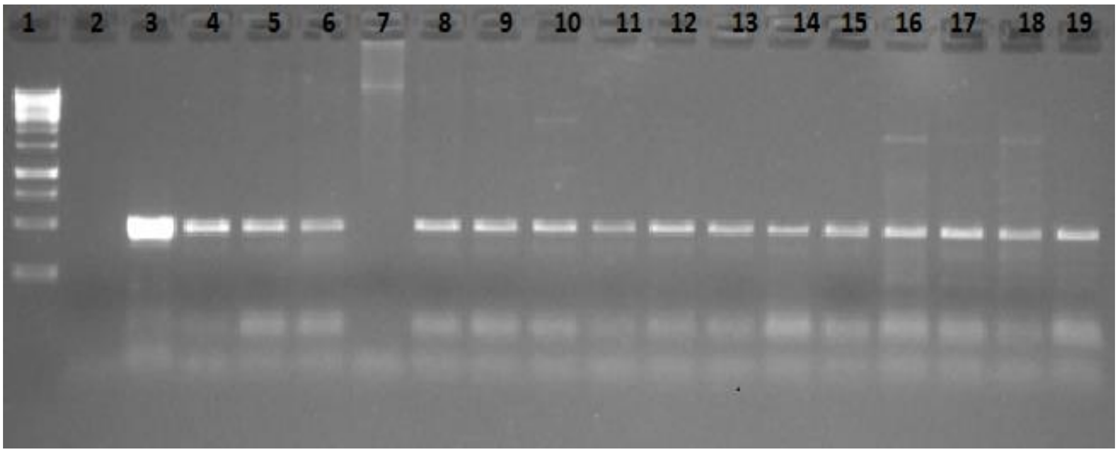

Figure 4.12. *SN-19* PCR analysis of transplastomic plants. Amplification of the fragment (480 bp) of the *SN-19* gene is shown. Lane 1: 1 kb DNA marker (Thermo Scientific), Lane 2 and 7: Negative control, Lane 3: Positive control plasmid pCTV DNA, 4-6 and 8-19: Results from transplastomic shoots of different potato cultivars (4-6 Kuroda, 8-10 Simply Red, 11-14 Challenger, 15-19 Sante)

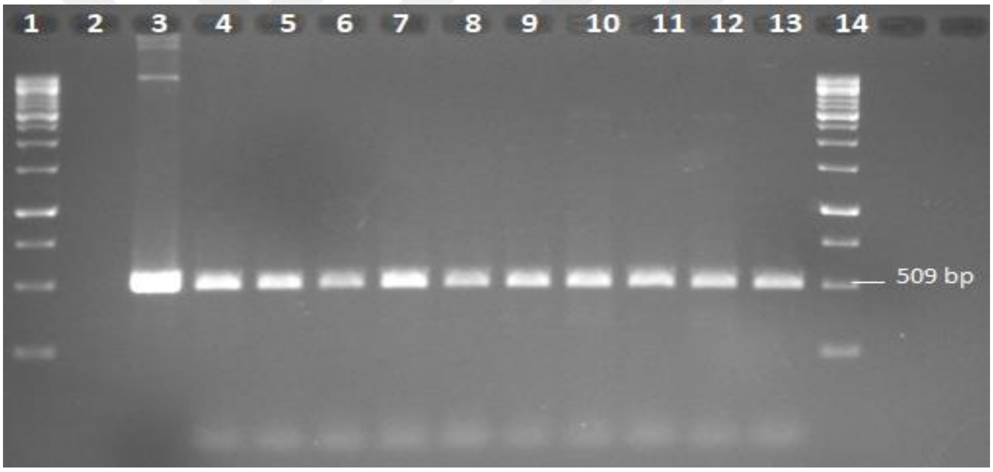

Figure 4.13: *Cry3A* PCR analysis of transplastomic plants. The amplification of the fragment (509 bp) of the *cry3A* gene is shown. Lane 1 and 14: 1 Kb DNA marker (Thermo Scientific), Lane 2: Negative control, Lane 3: Positive control plasmid pCTV DNA, Lane 4-13: Results from transplastomic shoots of different potato cultivars (4-5: Kuroda) , 6-8: Simply Red, 9-10, Challenger, 11-13: Santae)

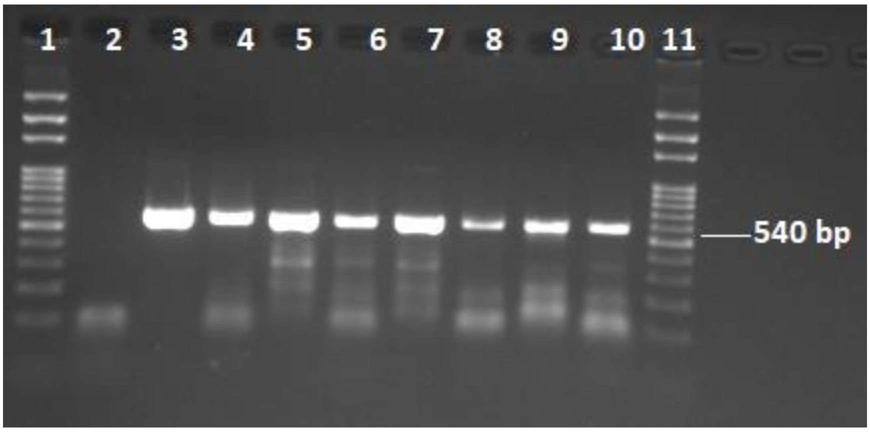

Figure 4.14. *aadA* PCR analysis of transplastomic plants. The amplification of the fragment (540 bp) of the *aadA* gene is shown. Lane 1 and 11: 1 Kb DNA marker (Thermo Scientific), Lane 2: Negative control, Lane 3: Positive control plasmid pCTV DNA, Lane 4-10: Results from transplastomic shoots of different potato cultivars (4: Kuroda, 5 -6: Simply Red, 7: Challenger, 8-10 Sante)

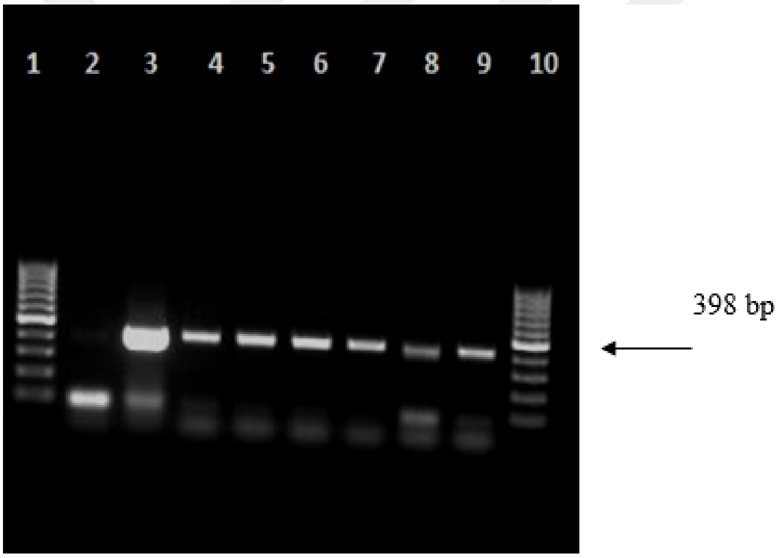

Figure 4.15. PCR analysis showing amplification of the green fluorescent protein (*gfp*) gene. Lane 1 and 10: 100 bp marker (Thermo Scientific), Lane 2: Negative control, Lane 3: Positive control plasmid pCTV DNA, Lane 4-9: Results from transplastomic shoots of different potato cultivars (4: Kuroda, 5- 6: Simply Red, 7-8: Challenger, 9: Sante)

Table 4.3. Detailed results of PCR analyze with transplastomic lines (soil condition)

Plant names		**Analysis Results**			
Allowed name	**Original name**	**PCR analysis**			
		SN-19	*cry3*	*gfp*	*aadA*
K1	Kuroda	+	+	+	+
K3	Kuroda	+	+	+	+
K4	Kuroda	+	+	+	+
SR1	Simply Red	+	+	+	+
SR2	Simply Red	+	+	+	+
SR4	Simply Red	+	+	+	+
SR5	Simply Red	+	+	+	+
Ch 3	Challenger	+	+	+	+
Ch 4	Challenger	+	+	+	+
Ch 6	Challenger	+	+	+	+
Sa1	Sante	+	+	+	+
Sa 2	Sante	+	+	+	+
Sa 3	Sante	+	+	+	+
Sa 5	Sante	+	+	+	+
Sa 6	Sante	+	+	+	+
Sa 7	Sante	+	+	+	+

4.5.1 Confirmation of *gfp* expression in transplastomic plants after acclimatization

In order to confirm the expression of gfp in transplastomic plants acclimatized into the growth chamber, a leaf of Sante 6 plant was taken and cut into thin pieces and the expression of *gfp* in leaf cells was visualized under fluorescent light microscope (Figure 4.15). Accordingly, *gfp* expression was observed in many leaf cells obtained from the transplastomic plant. Variations in *gfp* expression were observed due to Heteroplasmy in plants in this step and collectively shown in Figure 4.15.

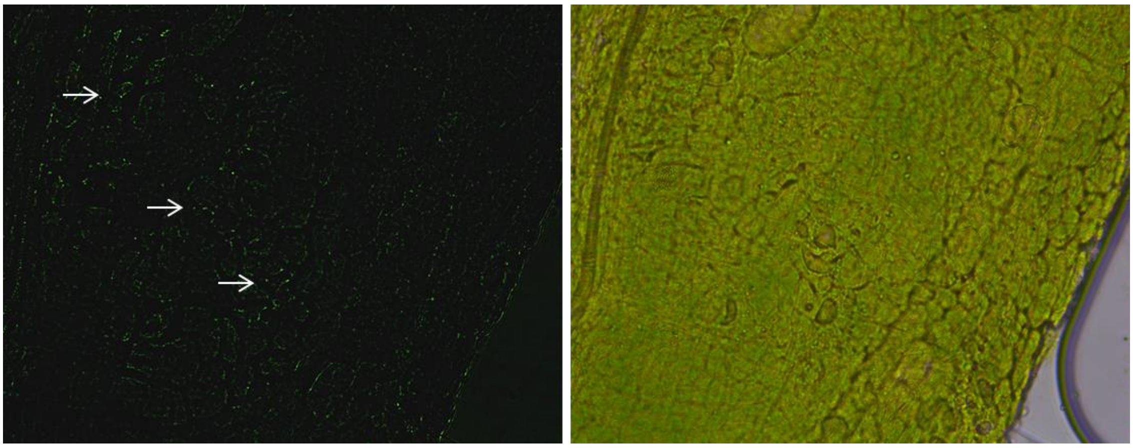

Figure 4.16. Microscopic visualization of *gfp* expression in transgenic plant shoots. As an example, expression of *gfp* in leaf section of Sante 6 transplastomic plant was demonstrated under fluorescent light microscopy. White arrows indicate the *gfp* localization within the cell. The left figure is the *gfp* expression under UV light, the right figure is the leaf image under white light

Although the level of *gfp* expression in the leaves of the acclimatized plants was determined under a fluorescent light microscope in our faculty, due to the low reliability of these data, a transplastomic plant with positive PCR results and leaf samples of non-transgenic wild type (control) plants were analyzed in the Middle East Technical University Central Laboratory to monitor *gfp* expression. It was analyzed with the Laser Scanning Confocal Microscope (LSCM, Registration No. 1953). 488 nm/514 nm/543 nm excitation, BP: 505-550 nm/LP: 585 nm emission and 20X objective depth were used in LSCM analysis. Accordingly, no *gfp* expression was obtained from the leaf sample of the control plants, as expected, whereas *gfp* expression was observed on a cell basis in the leaf sample obtained from the transplastomic plant (Figure 4.170). The analysis was repeated three times on the same plants and the same amount of *gfp* expression was determined each time.

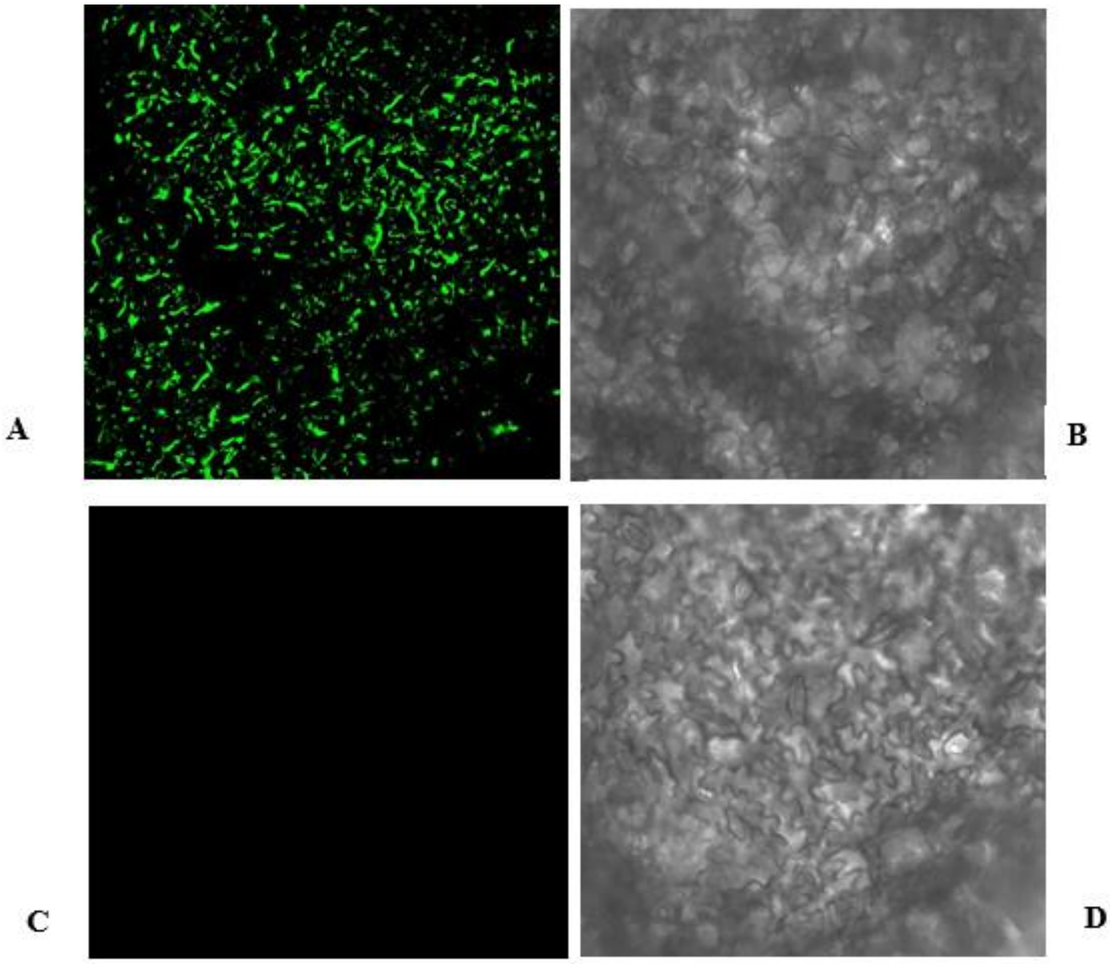

Figure 4.17. Microscopic visualization of *gfp* expression of transplastomic and control plants with Laser Scanning Confocal Microscope. *gfp* image under FITC (Fluorescein isothiocyanate) detector at 488-514 nm wavelength (a, c) and leaf samples under white light (b, d)

4.5.2 Southern DNA dot blot and southern blot analysis

Integration of the fusion gene into the plant genome was confirmed by Southern dot blot analysis. As seen in Figure 4.18, the spots on the membrane of DNA samples from transgenic plants showed the expected hybridization signal when subjected to hybridization with the PCR-amplified biotin-labeled *SN-19* probe. This presence of the signal indicates the integration of the relevant gene into the genome of the transgenic plants studied. The intensity of the signal is higher in K3, Ch3, Sa6 and Sa7 plants. It is followed by SR4 and SR5. No signal was detected in the non-transgenic plant.

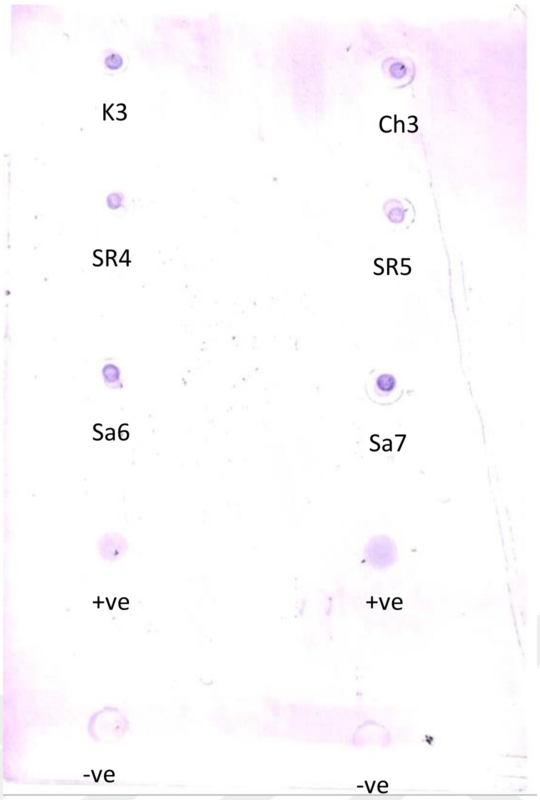

Figure 4.18. Southern DNA dot blot analysis of transplastomic plants. Genomic DNA was denatured and dotted on the Hybond-N+ membrane. After UV fixation, hybridization was performed using the PCR-labeled SN-19 probe. SN-19 fragment amplified by PCR was used as positive control (+ve), and DNA obtained from non-transgenic plant was used as negative control (-ve)

Southern blot analysis was performed with genomic DNA extracted from 6 PCR positive transplastomic lines (from Kurado, Simply Red, Sante cultivars, two lines each) to confirm the integration of the target gene into the chloroplast genome. The extracted DNAs were first cut with SphII and EcoR1 restriction enzymes, and the bands were separated by running in 1% agarose gel electrophoresis. The separated DNAs were transferred onto positively charged nylon membranes with an overnight capillary impregnation approach. A biotin labelled probe was designed. As a result of Southern blot analysis, specific bands showing the *SN-19* gene were determined in the cut DNA samples transferred onto the nylon membrane (Figure 4.19). In Southern blot analysis, a single band of approximately 1.981 kb and consistent with the signal obtained from the plasmid DNA was obtained from all potential transplastomic lines screened with the probe specific for the *SN-19* gene. The analysis results are compatible with the integration of the plasmid into DNA as a single copy. When the prepared transformation vector enters the chloroplast, homologous recombination occurs between the *trnA-trnI* regions in the chloroplast genome and the trnA-GOI-Marker-trnI region in the vector. This integration is pre-designed and region specific. That is, the fusion transgene is targeted to be effectively inserted as a single copy into the chloroplast genome. Southern blot analysis results also support this goal.

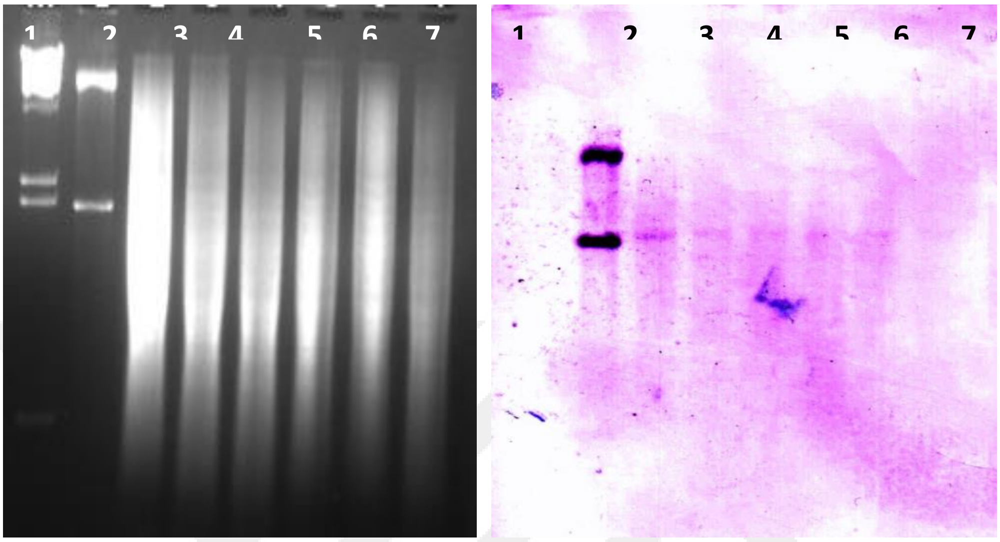

Figure 4.19. Southern blot analysis of transplastomic plants. Lane 1: Lambda HindIII marker (marker not visible on membrane), Lane 2: pCTV plasmid used as positive control, Lane 3-4: Kuroda, Lane 5-6: Sante, Lane 7: Challenger, Lane 8: Simply Red

4.5.3 Determination of gene expression levels in leaves by qRT-PCR analysis

To determine the expression levels of transgenes transferred in leaves of acclimatized plants, expression levels of *SN-19* and *gfp* genes were determined following total RNA isolation and cDNA synthesis. Our results in well-stabilized transplastomic plants revealed high expression of the *SN-19* gene. While the expression level of the gene increased approximately 2.5 times in Sante (Sa6) line, this increase was found between 1.5 and 2 times in Simply Red1 (SR1) and SR5 (Figure 4.20). In addition, high levels of expression of the *gfp* gene were also detected in the same plants. Accordingly, the expression level of *gfp* was approximately 4 times higher in Sa6 and Sa7 lines compared to SR5, while this rate was determined as 2-2.5 times in K3 and SR4 (Figure 4.21). It is pertinent to note that the transplastomic plant with the least expression was considered 1 for calculating fold expression since the *gfp* genes as well as *SN-19* are heterologically expressed in potato lines.

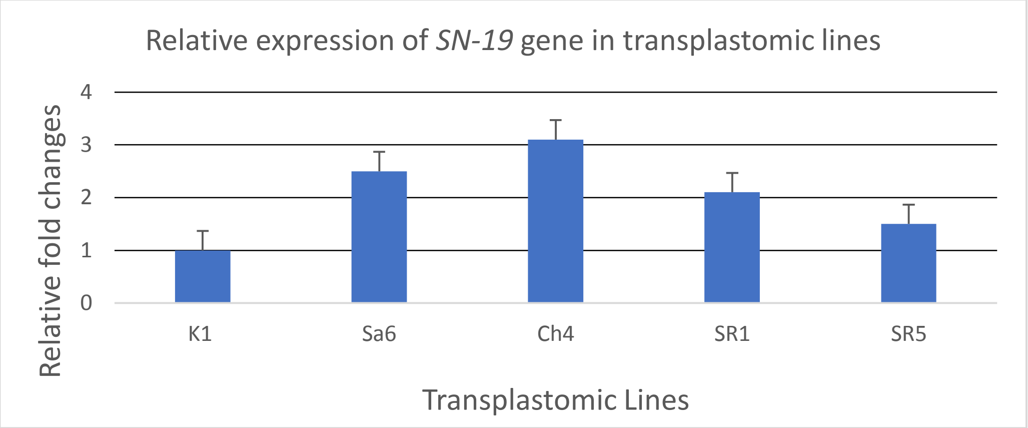

Figure 4.20. qRT-PCR analysis of *SN-19* gene in soil-grown transplastomic plants. Coefficients of variation of *SN-19* mRNA compared to control plants. An increase in expression level was observed in other plants compared to K1 (control). K: Kuroda, Sa: Sante, SR. Simply Red

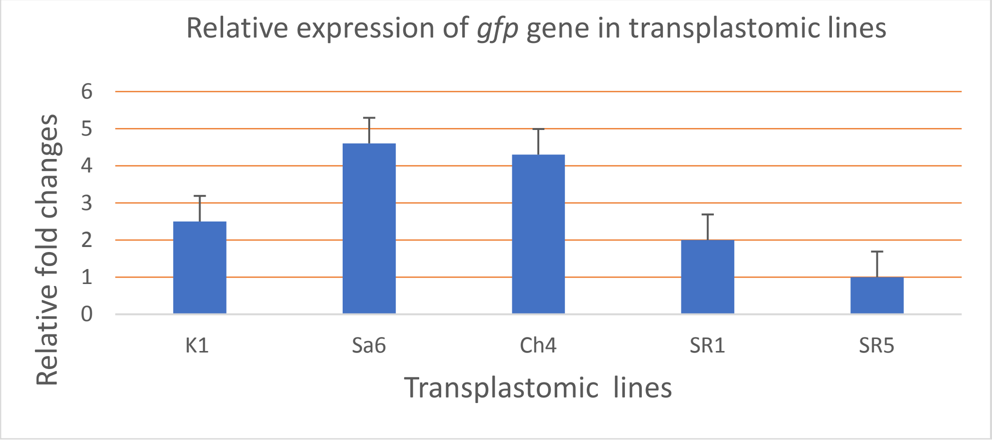

Figure 4.21. *gfp* expression level in soil-grown transplastomic plants. An increase in *gfp* expression level was observed in other plants compared to SR5 (control). K: Kuroda, Sa: Sante, SR. Simply Red

4.5.4 Enzyme-linked immunosorbent assays (ELISA) of transplastomic plants to characterize the expression of Cry3A protein

To confirm *Cry3A* protein accumulation in transplastomic leaves, ELISA was performed using PCR positive transplastomic lines. Monoclonal antibody coated wells were incubated with total soluble protein from selected transplastomic lines. The Cry3A protein was used as the antibody, confirming the precise nature of the recombinant protein. Commercial kit (Cat. No. PSA 05900/0480 Agdia) was used as positive control. The absorbance or OD of the samples tested was measured using a Microplate Reader

(SpectraMax 190) at 450 nm and Cry3A protein levels were calculated. All tested transplastomic lines showed distinct signals relative to the positive control. The level of Cry3A protein in the different transplastomic lines was assessed by ELISA and the observed protein levels were recorded accordingly.

Table 4.4. Evaluation of Cry3A protein level in transplastomic lines by ELISA

Transplastomic plants	Intensity of optical density (OD) at 450nm wavelength	Remarksa
Ch3 (Challenger-3)	0.525	+++
SR5 (Simply Red-5)	0.659	+++
Sa2 (Sante-2)	0.416	+++
Sa6 (Sante-6)	0.499	+++
Sa7 (Sante-7)	0.619	+++
Sa1 (Sante-1)	0.310	++
K1 (Kuroda-1)	0.302	++
SR1 (Simply Red-1)	0.399	++
Ch1 (Challenger-1)	0.606	+++
Ch6 (Challenger-6)	0.162	+
SR2 (Simply Red-2)	0.410	+++
Ch4 (Challenger-4)	0.277	+
SR4 (Simply Red-4)	0.295	+
K3 (Kuroda-3)	0.309	++
Negative Control	0.150	-
Positive Control	0.370	+++

a*: +++ (High Expression), ++ (Medium Expression), + (Low Expression), - (No Expression)

Depending on the results obtained, Cry3A protein levels in transplastomic lines were divided into three different groups as high, medium, and low (Table 4.4). Differences in the density of the Cry3A protein in transplastomic lines indicate varying expression levels. Transplastomic lines Ch3 (Challenger-3), SR5 (Simply Red-5), Sa6 (Sante-6), Sa7 (Sante-7) and Sa2 (Sante-2) show the highest Cry3A protein density, while transplastomic lines K1 (Kuroda-2) 1), SR1 (Simply Red-1) and Ch1 (Challenger-1) show moderate expression of the Cry3A protein. In contrast, the transplastomic lines K3 (Kuroda-3), SR4 (Simply Red-4), Ch4 (Challenger-4), Sa1 (Sante-1), Ch6 (Challenger-6) and SR2 (Simply Red-2) It stands out as the lines with the lowest expression (Fig. 4.22).

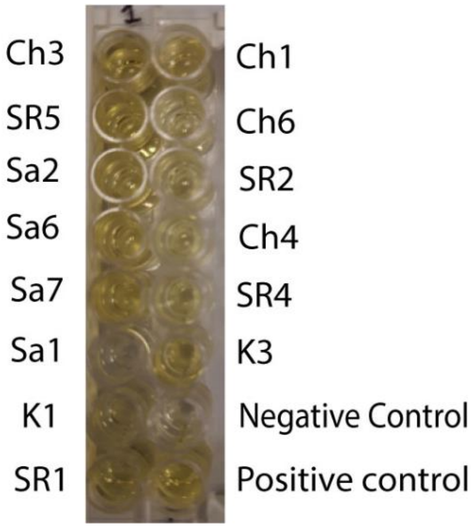

Figure 4.22. Analysis of different transplastomic lines representing the Cry3A protein by developing color from the antigen-antibody reaction (ELISA). The color intensity indicates the amount of Cry3A protein in the corresponding well

4.5.5 Determination of SN-19 and Cry3A protein presence in transplastomic plants by SDS-PAGE

Since there is no commercial ELISA kit specific to SN-19 protein that can be used, the presence of this protein and Cry3A protein in transplastomic lines was also evaluated by SDS-PAGE method. The expected 68 kDa band for the Cry3A protein and the 73 kDa band expected for the SN-19 protein were detected in the transplastomic lines (Figure 4.23). In order to test the accuracy of the findings, the pET22b plasmid containing the cry3A gene was induced with IPTG in E. coli to produce the Cry3A protein. Then, Cry3A protein was isolated from bacteria and used as a positive control in SDS-PAGE analysis. As shown in Figure 39, the expected 68 kDa band for Cry3A protein was determined in the positive control as well. The results obtained confirm that the *cry3A* and *SN-19* genes are produced not only at the gene expression level, but also at the protein level.

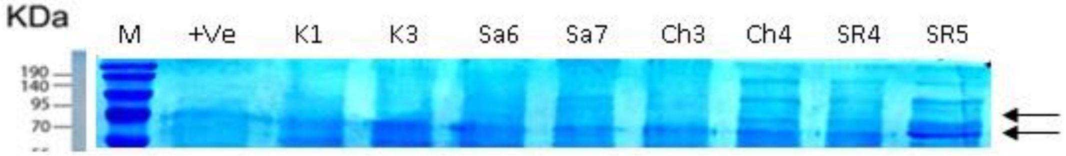

Figure 4.23. Determination of the presence of SN-19 and Cry3A protein in transplastomic plants by SDS-PAGE. M: Molecular protein marker (Blue Plus® V Protein Marker, DM141-01, 10-190kDa), +Ve: Total protein isolated from E. coli with plasmid pET22b containing the IPTG-induced *cry3A* gene

4.6 Insect Resistance and Toxicity Potential of Transplastomic Potato Lines

First and second instar CPB larvae were allowed to feed on PCR positive transplastomic plant leaves to determine the insecticidal potentiality mortality. The results did not show any mortality of larvae fed on non-transgenic leaves, whereas larvae fed on transplastomic plants showed remarkable mortality during the incubation period (Fig. 4.24- 4.25). About 100% mortality was recorded in 1st instar larvae compared to 2nd instar CPB larvae. After 48 hours of incubation, K3, Sa6 and Sa7 showed maximum mortality (100%) followed by cultivar Simply Red and Challenger plants. No mortality was recorded during the first 24 hours of incubation in 1st instar CPB larvae fed these plants (Fig. 4.24. A). Similarly, mortality rates of approximately 65% were recorded from 2nd instar larvae at Sa6 and Sa7 following 24 hours of incubation.

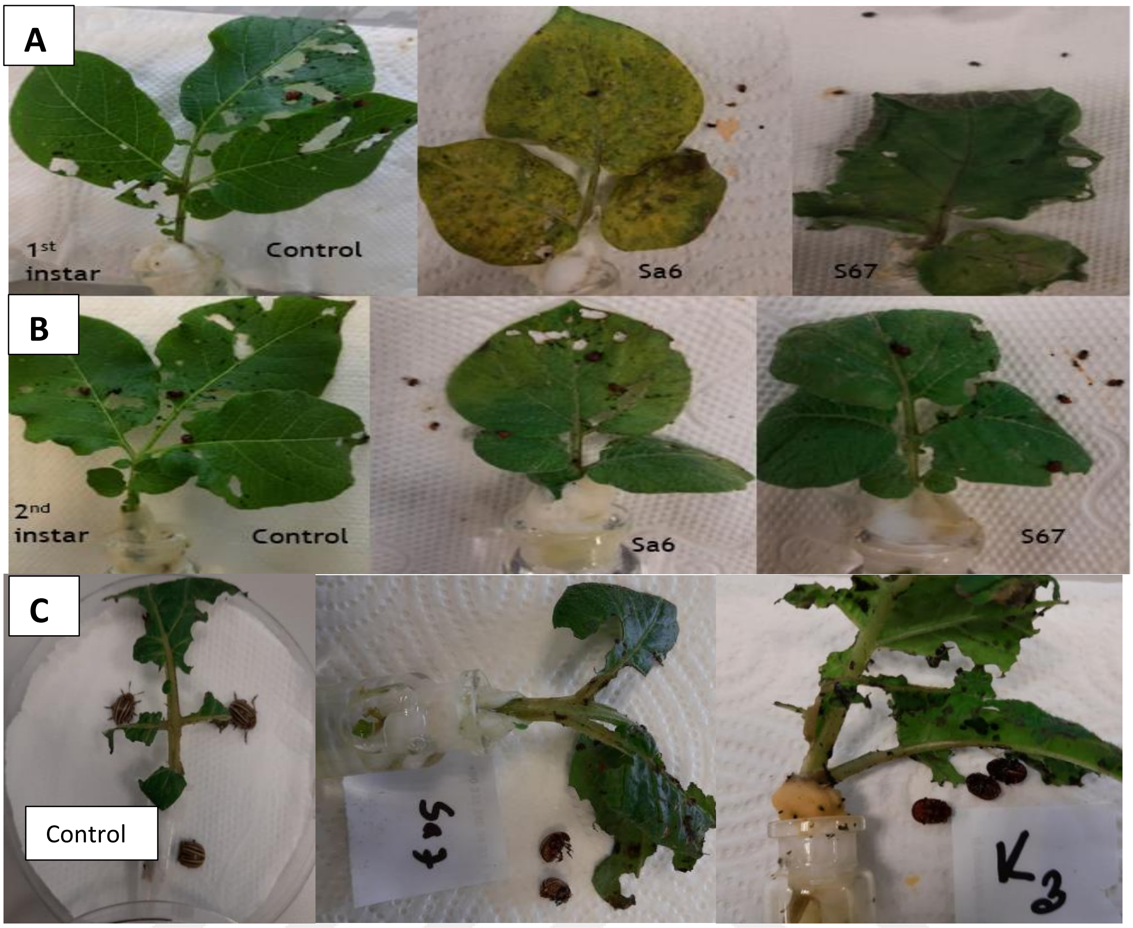

Figure 4.24. Representative images of the leaf biotoxicity analysis of CPB fed on transplastomic lines. 1st instar CPB larvae (a), 2nd instar CPB larvae (b) and adult CPB (c). K3: Kuroda 3, SR5: Simply Red 5, Sa6: Santae 6, Sa7: Santae 7

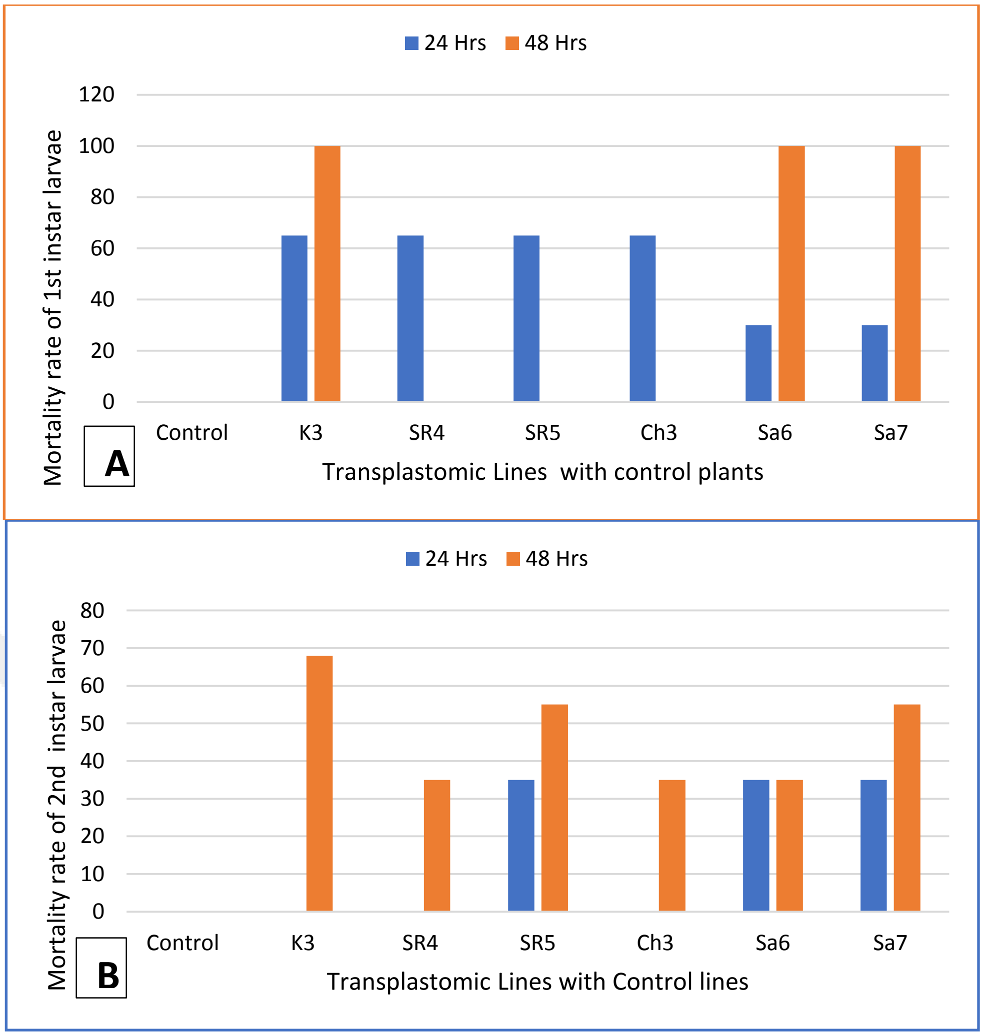

Figure 4.25. Mortality rates of CPB larvae fed on transplastomic plants. CPB mortality rates were calculated as a result of leaf biotoxicity tests against first (A) and second (B) instar CPB larvae. No mortality or morbidity was observed in the control plants. Ch: Challenger, K: Kuroda, Sa: Sante, SR. Simply Red

Following the development of the acclimatized plants, bioassay experiments were carried out with more detailed entomological parameters after having sufficient leaf samples and CPB . For this purpose, mortality rate of 1st and 2nd instar larvae and adult CPBs fed with transplastomic line leaves and wild-type control leaves were checked and recorded (10 days for larvae and 25 days for adults).

Thirty percent and 50% mortality for 1st instar larvae of CPB fed transplastomic potato lines were observed between 1.67 (Sa7) to 2.67(Ch3) days and 2.17 (Sa7) to 3.67 (SR4) days , respectively. All of the leaf-feeding larvae from the control plants survived the entire feeding period (Figure 3.29). While 80% insect mortality was seen between 3.83

(Sa7) and 5.83 (Ch4) days among transplastomic lines, the days required for the highest 100% mortality were between 5.33 (Sa7) and 7.66 (SR4). According to these findings, CBPs fed with the Sa7 line died earlier. Therefore, it can be said that the Sa7 line is a more effective transplastomic line with high resistance potential.

Also, 30% mortality for second instar larvae ranged from 2.5 (Sa7) to 3.5 (Ch3) days, while larvae fed on control plants survived the entire feeding period (Figure 3.29). While 30% mortality was recorded as 2.17 (Sa7) and 3.67 (SR4) for these larvae, 3.5 (Sa7) and 5.67 (SR4) days were found to be sufficient for the 80% mortality. The 100% mortality of the 2nd instar larvae feeding on the leaves of the Sa7 and SR4 lines were determined as 6.33 and 8.67, respectively. Based on these results, it can be said that the 1st instar larvae feeding on the leaves of the Sa7 line are more aggravated than the 2nd instar larvae, and therefore the Sa7 has higher insecticidal activity against the 1st instar CPB larvae.

Similarly, adult CPBs fed on the Sa7 line showed earlier death (10.17 days) compared to adults fed control plants. In contrast, adults fed control plants during this time remained vigorous and active. The days required for the 50% mortality ranged from 11.5 (Sa7) to 12.67 (SR4). The transplastomic line Sa7 showed 50% mortality in a shorter time. On the contrary, a longer day length was needed for the same level of mortality observed in adults fed on SR4. The Sa7 transplastomic line showed 80% and 100% mortality on adult CPB at 14.17 and 22.33 days, respectively, while the SR4 transplastomic line showed the same mortality between 16.33 and 24.67 days .

4.6.1 Weight changes of 1st and 2nd instar larvae of CPB

While feeding on transplastomic and control leaves, the larvae were gained weight. Therefore, the CPB larvae were weighed after leaf bioassays to determine the inhibitory effects of insecticidal target genes in insect feeding on transplastomic leaves. There was a significant difference in the increase of weight in different larval instars when fed on transplastomic plants compared to the control plants (Figure 4.26 and 4.2). In the first instar CPB larvae, a significant increase of up to 6.13 mg in weight was observed in insects feeding on control plants (Fig. 4.26). On the other hand, the insects feeding on SR4 and Ch3 lines showed 3.30 mg and 2.53 mg of higher body weight, respectively.

Transplastomic lines SR5, Ch3, and Sa6 did not show any significant statistical differences among their weight gain. Besides these, transplastomic lines K1, K3, Ch4 and Sa7 showed the same level of weight gain that was ranges between 1.70 mg to 2.10 mg.

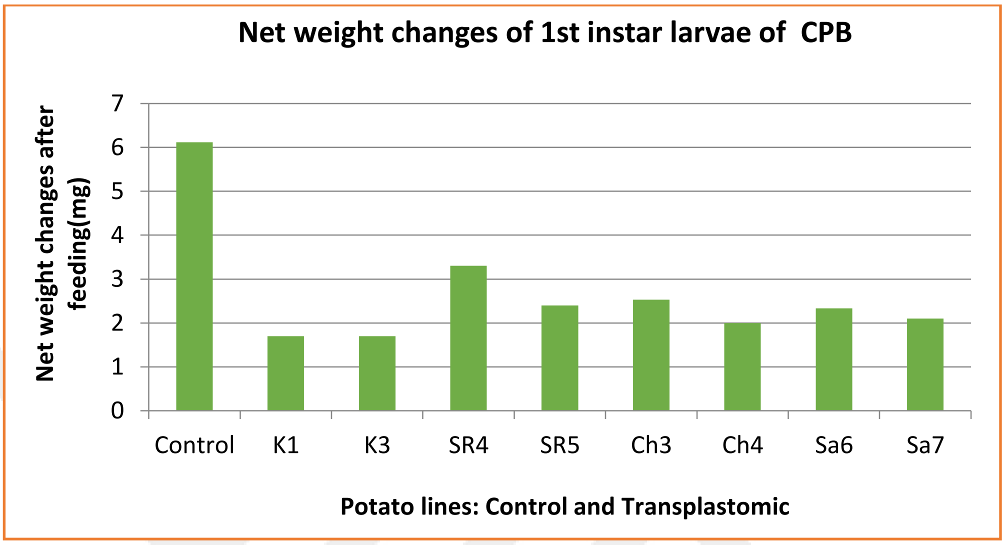

Figure 4.26. Net weight changes of first instar CPB larvae feeding on transplastomic and control potato leaves for 10 days expressing insecticidal Cry genes (*Cry3A+ SN-19*). Net weight increase values consist of mean from 3 replications

Similarly, 2nd instar larvae feeding on transplastomic line SR4 showed 3.60 mg of increase in weight, whereas insects feeding on control plants gained 6.87 mg more weight (Figure 4.27). Transplastomic lines Ch3, Sa6 and Sa7 showed the same level of weight gain without having any significant differences and ranges between 3.33 gm to 3.56 gm.

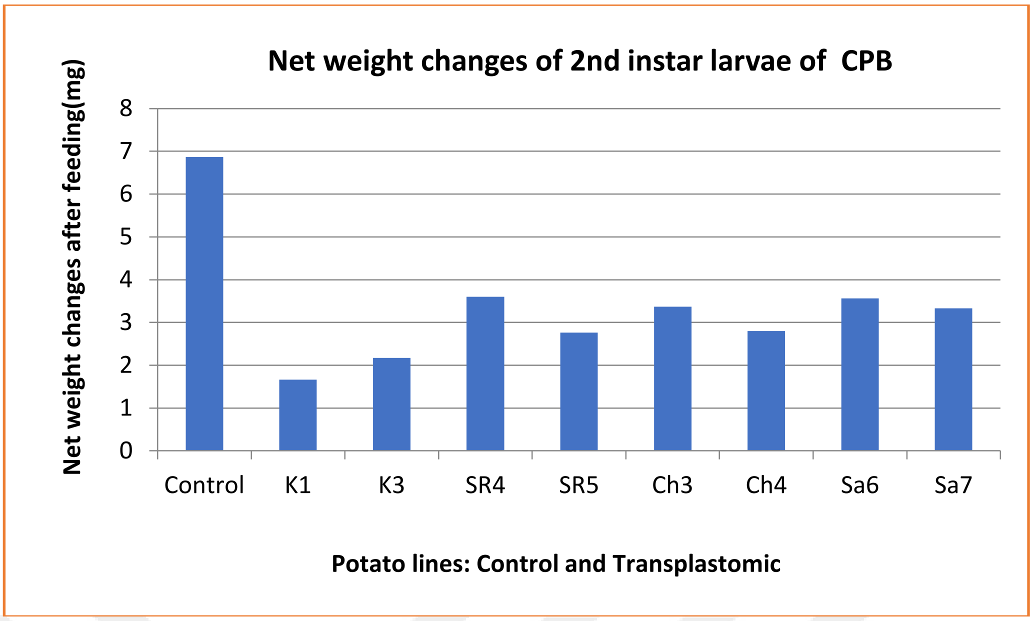

Figure 4.27. Net weight changes of 2nd instar larvae of CPB fed on transplastomic and control potato lines transferred with our desired construct. Net weight increase values consist of mean from 3 replications

These results suggest that the insects feeding on control plants gained more weight while the ones fed on transgenic plants expressing the target gene gained less weight. Taken together, results of leaf-feeding bioassays indicated that potato transformants expressing target cry genes were more resistant to CPB and the level of resistance is related directly to the expression level of cry gene in plant leaves.

4.6.2 Foliage consumption of CPB on transplastomic potato Lines

The foliage parts of transplastomic and control plants were allowed to feed by the 1st and 2nd instar larva and adult CPB for a specific period (24 hours, 72 hours). Consumed parts/area of transplastomic leaves were measured by mentioned software and compared with respective control leaves.

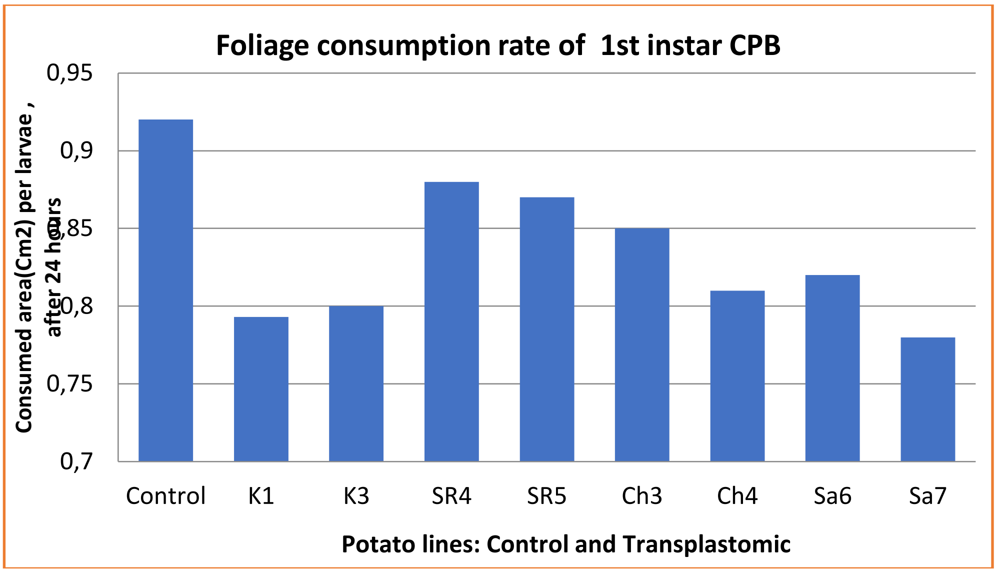

Figure 4.28. Foliage consumption by individual 1^{st} instar CPB fed on transplastomic and control potato lines. Consumption area was measured 72 hours after treatment. Leaf consumption values consist of means from 3 replications

Consumption of foliage part by individual 1st instar CPB larvae feed on different transplastomic lines transformed with a gene of interest, and relevant control are shown in figure 4.28. The data showed significant differences in the feeding behavior of 1st instar CPB larvae that were allowed to feed on different transplastomic potato lines as compared to non-transgenic control plants. Significantly higher leaf consumption was recorded in control plant (0.92 cm^2) whereas transplastomic line Sa7 consumed lowest (0.78 cm2) area of leaves. Among the transplastomic lines, SR4 and SR5 showed highest consumption rate (0.88 and 0.87 cm^2) those are less than the control line. Remarkably, no one of these rates is statistically significant ($P \leq 0.05$) from each other. In terms of foliage consumption, after 24 hours of feeding, transplastomic lines did not show any statistically significant differences among them.

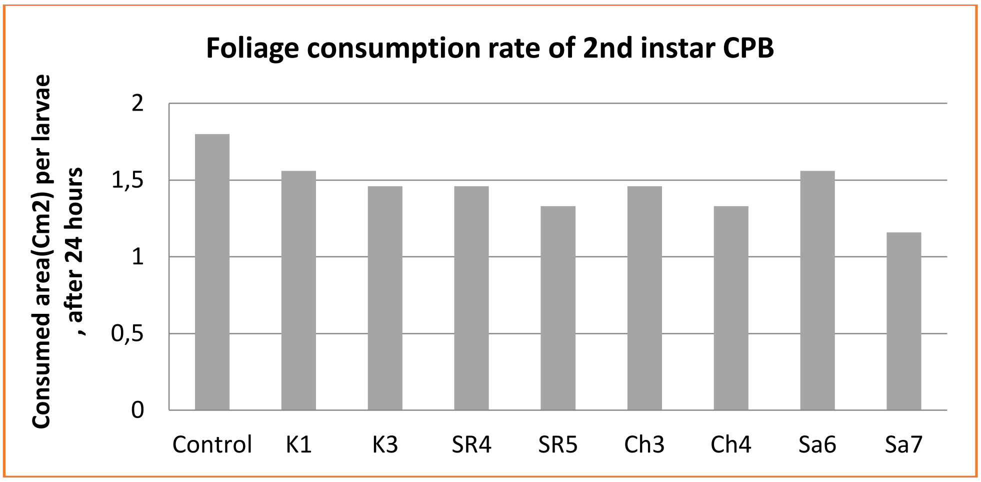

Figure 4.29 Foliage consumption by individual 2nd instar CPB fed on transplastomic and control potato lines. Consumption area was measured 72 hours after treatment. Leaf consumption values consist of means from 3 replications

Foliage consumption rate (area) by 2nd instar CPB larvae feed on transplastomic and control potato lines for 24 hours have been presented in figure-13. Based on the recorded data, compared with transplastomic plants, control plants showed more consumption rate (1.8 cm^2). Transplastomic lines K1 (1.56 cm^2) and Sa6 (1.56 cm^2) depicted the highest level of consumption rate among the transplastomic lines. Transplastomic lines K3 (1.46 cm^2), SR4 (1.46 cm^2), SR5 (1.33 cm^2) and Ch3 (1.46cm^2) showed an equal level of consumption rate without having any statistically significant differences. Transplastomic line Sa7 revealed the lowest foliage consumption rate among all potato lines embedded to this experiment.

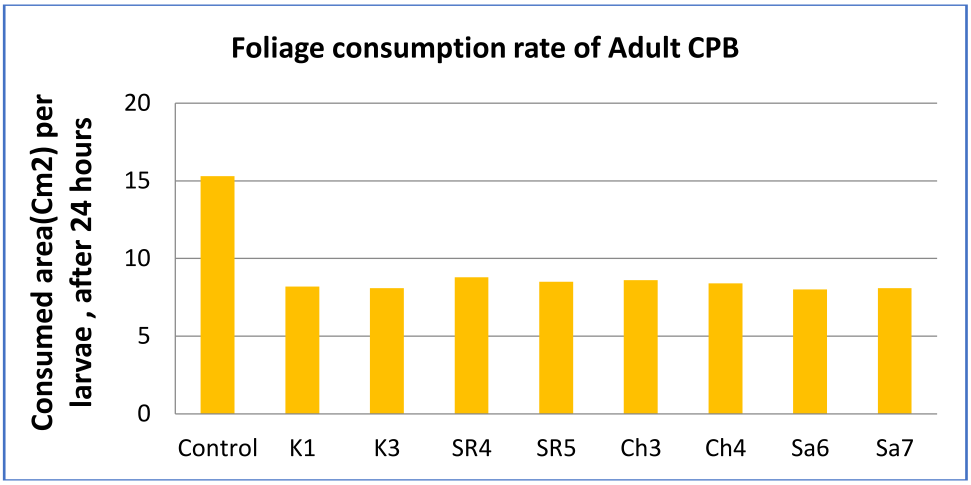

Figure 4.30. Foliage consumption by individual adult CPB fed on transplastomic and control potato lines. Consumption area was measured 72 hours after treatment. Leaf consumption values consist of means from 3 replications

Foliage consumption by individual adult CPB fed on transplastomic and control potato lines have been presented in figure 3.34 on which control plant demonstrated the highest level of foliage consumption was recorded for control plant (15.3 cm^2). Although among the transplastomic lines SR4 showed more foliage consumption rate (8.8 cm^2), compared with rest of transplastomic lines it was not statistically significant, and concerning foliage consumption rate, all transplastomic lines showed the same level of consumption rate.

CHAPTER V

DISCUSSION

Transformation of crop plants with target gene to develop the trait of interests conduct with several genes where Bt genes remain a prominent means of transformation since the last few decades. In our current endeavors, chloroplast of four potato cultivars were transformed with one single cry gene (*Cry3A*) along with one insecticidal hybrid gene (*SN-19*: encodes proteins consisting of domains I and III of *cry1Ba* and domain II of *cry1Ia*). For the chloroplast transformation, gold particles of around 0.6 μm diameter were coated with $pPCTV_1$ following Joyia, 2012 and biolistic transformation was carried out with those DNA coated gold particles under a partial vacuum (25 mmHg) with a Dupont PDS-1000/He Biolistic gun following methodology described by Khan and Maliga, 1999. An efficient protocol for the selection of transplastomic cells and *in-vitro* regeneration after particle bombardment is a prerequisite for implementing the plastid transformation strategy in potato. Generating viable transplastomic potato lines in laboratory conditions is quite challenging. Many factors are primarily responsible for poor *in vitro* regeneration of plant cells after particle bombardment, especially when chloroplast transformation is attempted. One of the several reasons is the relative sizes of chloroplasts/plastids and gold particles (microcarriers). The average size of a mature chloroplast is 4-5 μm and the smallest available size of gold particles 0.6 μm. The size of immature/undifferentiated plastids in calli is even smaller ranging 20 mμ-1.5 μm which is merely double of the size of smallest gold particles. Then, the particles are accelerated to very high velocity for bombardment. Such a high momentum of metal particles causes substantial damage to the chloroplasts after particle bombardment on explants (Russel et al., 1992; Mookkan, 2018).

In this study, we contemplated establishing reliable and effective potato plastome engineering protocol through the incorporation of the target genes into the potato plastome. We recovered plastid transformants with the two-step regeneration protocol utilized by Nguyen et al., 2005 and this requirement for a callus phase for recovery of transplastomic potato is similar to that reported for tomato by Ruf et al., 2001. However, according to Nguyen et al., 2005, only one month on callus induction medium was required for potato compared to about six months for tomato before shoot regeneration whereas in our experiment well-developed calli were formed within induction 3-5 weeks.

A good number of calli was induced from bombarded explants (181.0 calli from 200 explants), where cultivar Kuroda induced a high level of calli followed by the cultivar Santae (169.0 calli from 200 explants). Leaf explants were found more proficient at inducing calli than internodal explants. Among the induced calli, prevalence of light brown (LB) color calli was recorded, and Yellowish Green (YG), Light Green (LG), Green (G), Dark Green (DG), and Brown (B) color calli also were enlisted. Internodal explants induced light brown (LB) color calli, whereas leaf explants induced Greener (G) color calli.

The proficiency of three Cytokinins were evaluated where TDZ produced the highest percentage of shoot (41%), followed by TR (40.3%) and TZ (39.6%). Cultivar Kuroda and Santae produced a higher number of shoots, whereas cultivar Simply Red and Challenger yielded the lowest shoots. Although cultivar Santae and Challenger produced 9.6% shoots, cultivar Santae produced the highest number of Spectinomycin resistant shoots (43.7%). On the other hand, PCR screening revealed that cultivar santaee produced highest number of PCR positive shoots (85.7%). Expression of visual marker gene gfp in calli and shoots were visualized by inverted microscopy (Leica dmil led inverted microscope). gfp expression level among potato cultivars did not show statistically significant differences (ranges between 93% to 95%). The potato cultivars' differences were noted concerning the gfp expression in leaves and probably because of heteroplastomic status.

In comparison, Sidorov et al., 1999, documented potato plastid transformation with a single step procedure on which they used a non-commercial genotype (FL1607 breeding line) based on its responsiveness in *in-vitro*. They reported 6.67% (1 transformant out of 15 shots) and 2.85% (1from 35 shots) regeneration performance from two vectors (pZS197 vector and vector targeting the RPS12-RRN16 intergenic region, respectively). These findings are much more lesser than the regeneration proficiencies (ranges between 66.00% to 85.7%) of four potato cultivars used in our study. On the other hand, Nguyen et al., 2005 used Désirée, a commercially important cultivar and a two-step process tissue culture procedure was adopted to produce transplastomic potato on which they recorded 5.5 % (1 out of 18 shots) and 4.0% (1 out of 25 shoots) from pZS197 and pMSK18 vectors, respectively. However, Nguyen et al., 2005 reported integration of *aadA* and *gfp* in pMSK18 transplastomic potato plants by PCR as a proof of transplastomic event. Adopting the same genotype and underlying regeneration protocol previously published by Nguyen et al., 2005, Valkov et al., 2011 roughly doubled (11 shoot per 100 bombardments / one shoot every nine bombardments) the efficiency of transformation compared to previous results from Sidorov et al., 1999 and Nguyen et al., 2005. This finding obtained by Valkov et al., 2011 is similar to our current findings regarding the cultivars Challenger and Sante.

During *in-vitro* regeneration in *Arabidopsis*, Yu et al., 2017 used leaf tissue as explants and obtained eight events on five bombarded plates in the *acc2-1* mutant background and four events on four bombarded plates in the Slavice accession (approximately 100-fold enhanced). This transformation efficiency is comparable to the transformation efficiency obtained in tobacco by Maliga and Tungsuchat-Huang, 2014 where four to five transplastomic events per bombardment was achieved. Khan et al., 2015 conducted an experiment to produce transplastomic tobacco to confer enhanced tolerance to salinity and drought in plants on which nine transgenic clones were recovered from 35 bombardments on selection and regeneration medium. When homologous potato sequences replaced tobacco-flanking sequences in transformation vectors, a drastic increase in transformation efficiency was reported to be about one shoot per bombardment. The biolistic approach was employed with species-specific vectors that allowed up to 1–2 shoots per bombardment to boost the frequencies of plastid transformation.

On the contrary, when the recombination of flanking sequences originating from the petunia plastome was used, the tobacco transformation efficiency decreased by more than tenfold (DeGray et al., 2001). Using homologous flanking sequences in a transformation event is an essential factor that can improve the frequency of transformation, and Scotti et al., 2011 ensured this in potato. Another study by Valkov et al., 2014 on potatoes reported one transplastomic shoot per bombardment. This efficiency is comparable to that commonly obtained in tobacco production. Nonetheless, our decreased efficiency compared to Valkov et al., 2014 may be due to the disparity in vector size, flanking sequences, and genetic variation between the cultivars under analysis (Valkov et al., 2011). Svab & Maliga, 1993 and Langbecker et al., 2004 got higher lines of transplastomic tobacco. Svab and Maliga conducted high-frequency plastid transformation and regeneration in tobacco, 1993 on that experiment they achieved 84 spectinomycin resistant clones from 79 bombarded leaf cultures. DNA gel blot analysis of 50 clones confirmed 40 (80%) *aadA* integration into the plastid genome. This finding (85.7 %) correlates our present findings. On the other hand, plastid transformation was much lower than tobacco in Arabidopsis, potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*). For instance, one transplastomic line was achieved from 40 (2.5%) or 151 (0.66%) bombardments in *Arabidopsis*, from 35 bombardments (2.85%) in potato, from 25 (4.0%) bombardments in oilseed rape, from 20 (5.0 %) bombardments in tomato (Sidorov et al., 1999; Ruf et al., 2001).

It is essential for the regeneration strategy of transplastomic plants to identify transplastomic events. It took 5 to 12 weeks for the RLD (Reduced Number of Long Days) ecotype to regenerate in 1998 (Sikdar et al., 1998). The use of spectinomycin-sensitive knockout lines *acc2* and the dicistronic operon vector pATV1 shortened the period for transplastomic event detection to 3 to 5 weeks. The use of the *acc2* knockout lines shortened scoring because spectinomycin effectively prevented the proliferation of non-transformed cells, allowing the identification of green cell clusters resistance to spectinomycin. Spectinomycin resistance may be due to the plastid genome integration of *aadA*, nuclear genome integration of *aadA*, and fortuitous expression of an upstream promoter or spontaneous *rrn16* gene mutation (Svab and Maliga, 1993). *gfp*, encoded in the second ORF, is expressed only in chloroplasts, allowing confocal microscopy to rapidly classify transplastomic clones in a limited number of heteroplasmic cells (Yu et al., 2017).

Our current research also coincides with these current studies, and we hope our spectinomycin resistant shoots are likely due to the plastome incorporation of *aadA*, which decreased the transplastomic event detection to 5 to 7 weeks only. Although transplastomic technology offers considerable advances, it has not yet entered the market. Integration and transmission of chloroplast expression vectors into the chloroplast genome is a relatively straightforward, yet lengthy method. Besides, the recovery of homoplasmic plant lines is another big obstacle for this technology (Ahmad et al., 2016). Current attempts to streamline this process include introducing high throughput cloning methods for chloroplast expression vector construction (Gottschamel et al., 2013, Vafaee et al., 2014) and finding new selectable markers (Bellucci et al., 2015). Nevertheless, it has proven extremely difficult to extend the transplastomic technology to field crops.

Integration and expression of target genes into the chloroplast genome of transplastomic plants were confirmed by PCR, southern blotting, qRT-PCR, ELISA, and SDS-PAGE . Localization of our visual marker *gfp* gene was also confirmed by Laser scanning confocal microscopy (LSCM). PCR analysis of transplastomic plants with gene specific primers confirmed the presence of selectable marker gene *aadA* (Svab and Maliga, 1993., Langbecker et al., 2004). Our findings are in line with the findings with several researchers including Sidorov et al., 1999, Ruf et al., 2001, Nguyen et al., 2005, Valkov et al., 2011, 2014 and Yu et al., 2017.

Southern blot analysis of PCR positive transplastomic plants were conducted to ensure the separation of gene of interest from complex DNA mixtures. The Nylon membrane showed a recognition of about 1.981kb gene that confirmed that our plants were successfully transformed with our gene constructs. Similar findings with expected hybridization signal were also reported by Wu et al., 2019 , He et al., 2020 , Xu et al., 2020 and Salim et al., 2021.

Real-time quantitative PCR (qRT-PCR) widely adopted as the transcriptomic tool of choice because of its superiority in terms of speed, sensitivity, and reproducibility, as well as the large range of commercially available instruments and reagents (Kheirelseid et al., 2010). It has now emerged as standard molecular technique to determine relative expression of target gene (Maqbool et al., 2010; Rao et al., 2011). In addition to these excellent benefits, qPCR gene expression differs from time to time, since the degree of expression representing variations in cellular input, RNA content, and reverse transcription efficiency should be normalized to that of an invariably expressed internal control gene (de Kok et al., 2005). qRT-PCR analysis of transplastomic lines were performed to diagnose expression levels of *SN-19* and *gfp* genes. PCR positive plants were subjected to the qRT-PCR analysis and as result, a remarkably high expression of *SN-19* and *gfp* genes were noticed in transplastomic plants. Expression level of gene was different in various transplastomic plants (Figure 22, 33 and 34). Our results agree with previous studies of Li et al., 2017 and Occhialini et al., 2020. According to the authors, expression level of any gene can vary in different transgenic lines. It has become increasingly evident that there is no mRNA with a constant level of expression and that the amount of mRNA of the housekeeping gene will differ quantitatively in response to multiple factors, such as developmental stage and cell cycle, and various experimental conditions (Peng et al., 2012).

Enzyme-linked immunosorbent assays (ELISA) represents various level of target gene existence in transplastomic lines by the antigen-antibody reaction mediated color development. Lower to higher level of transgenes presence were recorded based on the color and respective OD of the samples. ELISA based evaluation of transgene screening was established by many researchers (Salehian et al., 2021, Sufyan Tahir et al., 2021, Karthik., 2021 Salim et al., 2021, Zhong et al, 2018, Albright, 2015, Wang et al., 2007) .

After successfully confirming the integration and expression of target genes into the chloroplast genome by molecular screening, leaf bioassays with transplastomic and control plants were conducted. The main achievement of our current study is that all studied cultivars were successfully transformed with two gene constructs and resulted in more excellent resistance to all CPB (100% mortality was recorded) stages compared to non-transgenic control plants. Good insecticidal potentiality of transplastomic leaves to kill the target pest was also established. To check the insecticidal efficacy, 1st, and 2nd instar larvae and adult CPB was allowed to feed the transplastomic and control leaves. After 12 hours of feeding, the adverse effect of cry protein on insect pest was manifested by avoiding feeding, slow motion of the insect pests, discoloring insect body appearance and growth retardation. Under continuous feeding, insect feeding behavior and physiological status were recorded from close observation within 12 hours intervals. No mortality was recorded from insects feeding on non -transplastomic control plants and caused considerable damage although different lethal effects was recorded from insect pests feeding on transplastomic lines and 100% lethal effect on 1st, 2nd instar larvae and adult CPB population was recorded. Transplastomic line Sa7 (Sante-7) showed more mortality within lowest period whereas transplastomic line SR_4 showed less lethal effect on CPB and took long time period for mortality. For 100% mortality, 1st instar, 2nd instar and adult CPB required time was ranges between 5.33 to 24.67 days. Our findings can be compared with the findings of Salim et al., 2021

Hussein et al., 2017. Study conducted by Salim et al., 2021 and Hussein et al., 2017 confirmed 100% lethal effects on 1st instar to adult CPB where required day lengths were ranges between 6 to 26. Both above mentioned authors developed CPB resistant potato plants with nuclear transformation whereas in our current study target genes were integrated into the plastome owing higher level of protein expression. Besides this, genetic makeup, and promoter types also important factor for these deviation of results. Another factor can be mentioned that is the use of green tissue specific promoter in our construct that confined the expression of gene of interest within the green part of potato and this facility ensured more protein expression in our transplastomic green leaves, as a result, our transplastomic plants ensured 100% lethal effect (LT_{100}) within less time that indicate the more insecticidal protein accumulation in our transplastomic potato leaves.

Increase of insect weight feed on control and transplastomic plants were measured and compared to detect the lethal effects of transplastomic lines on CPB larvae and adult. Larvae and adult CPB feeding on control plants gained more weight compared with transplastomic lines. In our study, larval and adult insects weight gain feed on transplastomic leaves ranges between 1.66- 3.60 mg and 22.33-28.33 mg respectfully. These findings were compared with existing study conducted by Hussain et al., 2019 that was ranges between 3.0 to 6.9 mg and 3.1 to 3.2 mg respectfully where the integrated gene was different than ours. From our current findings we can conclude that insect larvae feeding on transplastomic leaves gained less weight than the findings recorded by Hussain et al., 2019 that indicate more lethal effects of our transplastomic leaves on CPB larvae. Transplastomic lines SR_4, Ch_3, Sa_6 and SR_5 gained more weight whereas K_1, K_3, Ch_4 and Sa_7 lines gained less weight.

Consumption rate of foliage part of control and transplastomic leaves by insect larvae and adult pest was also recorded and compared accordingly. Foliage consumption by larvae on control and transplastomic leaves were ranges between 0.92 to 1.8 cm^2 and 0.78 to 1.56 cm^2 respectively whereas consumption rate by adult insects were ranges between 8.0 to 15.3 cm2. Foliage part of control plants were consumed more compared to transplastomic plants. Our findings were compared with the findings of Cingel et al., 2015 and Salim et al., 2021. Compared to Cingel et al., 2015, our study revealed that insect pests were more suppressed by transplastomic plants and consumption were less.

On the contrary, findings of Salim et al., 2021 correlates with our findings and no significant differences were recorded. Both studies were done by *Agrobacterium* mediated nuclear transformation where our study was chloroplast transformation by biolistic gene gun that ensured more accumulation of insecticidal proteins in the chloroplast genome and finally transplastomic plants incurred more and durable resistant to the CPB.

CHAPTER VI

CONCLUSION

Current thesis work was conducted to develop the insect-resistant transplastomic potato lines to combat the notorious insect pest CPB (*Leptinotarsa decemlineata*). The chloroplast transformation vector was constructed using two insecticidal cry genes *SN-19* and *cry3A*, under the control of green tissue-specific promoter *psbA*. Bi-functional visual and selectable marker *FLARE-S'* (fluorescent antibiotic resistance enzyme, spectinomycin and streptomycin) was used as a selection marker that combines *aadA* (aminoglycoside-3-adenyl-transferase) and *gfp* (green fluorescent protein) and the expression of *FLARE-S'* was controlled by a strong tobacco plastid ribosomal RNA operon promoter *Prrn*. *aadA* Confers resistance to the translation-blocking antibiotic spectinomycin modifies the antibiotic by adenylation and prevents binding to chloroplast ribosomes that allow greening. The transformation vector and the gene of interest were transferred into the chloroplast genome of four potato cultivars by gene gun mediated biolistic bombardment method. Once this vector is inside the chloroplast, there occurs homologous recombination between trnA-trnI (in chloroplast genome) and trnA-GOI-Marker-trnI (in vector). It results in the integration of our GOI and marker gene into the chloroplast genome. Such integration is predetermined and site-specific. Hence, there is no chance of wrong integration.

Several studies have been conducted with single cry gene integration into the nuclear genomes and even into the chloroplast genome. Still, because of less amount of target protein accumulation into transformed plants and extraordinary and tremendous adaptive malleability of super-pest CPB, it could develop resistance against transgenic /transplastomic crops. Considering this consequence, our current study was designed as the first endeavor to combine two cry proteins (*Cry3A* and *SN-19*) to encode durable resistance against this baleful insect pest CPB. To mitigate these hurdles and drawbacks, we introduced an efficient and prompt *in-vitro* regeneration protocol for transplastomic potato, and we got our 16 PCR positive transplastomic shoots within only 36 to 49 days that is an outstanding achievement ever.

On the other hand, we achieved 85.7% regeneration proficiency with our studied protocol that is also remarkable in terms of existing findings (0.6 % or 1.2%).

These putative transplastomic plants were screened with molecular tools (PCR, southern blot, qRT-PCR, Bradford assay, ELISA, and SDS-PAGE), and stable integrations of target genes were confirmed, and leaf bioassay was conducted with 1st and 2nd instar larvae and adult CPB. Transplastomic lines showed high toxicity to the CPB population. Weight changes of insect pest and foliage consumption rate during feeding on transplastomic and control plants also estimated that was determined by the expression level of foreign protein into the transplastomic leaves. Introduction of green tissue-specific promoter *psbA* into the transformation vector favored transgenes' expression within only green part of potato not into edible tubers. This outcome solves the passive saying of consumes and environmentally concern scholastic groups as well of biosafety council. Besides this, less protein accumulation in the plant genomes because of nuclear transformation incurred less durable insect resistance of transgenic plants to the insect pest and insect pests were capable of developing resistance against transgenic plants over time. Our current study ensured a broad spectrum of foreign protein integration into the chloroplast genome, which is robust and durable resistant to CPB. The successful implementation of this transplastomic technology in potato will help mitigate the CPB mediated potato loss proficiently. However, this transplastomic endeavor will minimize the large-scale dependency on conventional pesticide dependent potato production without adverse effects on flora and fauna of the agricultural ecosystem of Nigde and Turkey.

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APPENDIX

Appendix-I

MS Medium (Murashige and Skoog, 1962) Composition

MS Salts	4.33g L -1
MS Vitamins	1mL L-1
Sucrose	30 g L -1
Plant Agar	8 g L -1
pH	5.6-5.8

Appendix-II

20x SSC

NaCl	350.6g
Na-Citrate	176.4g

Adjust pH to 7.0 with NaOH and make up volume to 2 litter with distilled water.

Pre-Hybridization Buffer

SSC	5X
Blocking reagent	1%
Sarkosyl	0.1%
SDS	0.02%

Dissolve blocking reagent at 65-80℃ with constant shaking and store at -20℃.

Hybridization Buffer

Add 5-20ng of prob per mL of pre-hybridization buffer. Store at -20℃.

5x TBE Buffer

Trimza base	54g
Boric acid	27.5g
0.5M EDTA	20mL

Adjust volume to 1 liter with distilled water.

CURRICULUME VITAE

Md Jakir HOSSAIN was born on ………….., in Rajshahi, ………... He completed his secondary education from ……………. high school, Puthia, Rajshahi in …….. Afterwards, he joined …………… college, Puthia, Rajshahi in ………. for his higher secondary education. Then he was enrolled in the University of Rajshahi, Bangladesh for his undergraduate studies. He completed his B.Sc. (Hons.) from Department of Botany in …………. He completed his M.Sc. and M.Phil. degrees from the same department in …….. and ……. , respectively. He was awarded University merit scholarship for M.Phil. degree. In …….., Mr. Jakir was selected as PhD candidate at Department of Botany , University of Rajshahi and awarded Erasmus credit mobility program under European Union and joined at Meddle East Technical University , Ankara , Turkey for his PhD course work.

In September …….., Mr. Jakir enrolled in Graduate School of Natural and Applied Sciences, Department of Agricultural Genetic Engineering at Niğde Ömer Halisdemir University, Niğde, Turkey to pursue his PhD education under the guidance of Dr. Allah Bakhsh. During his PhD research Jakir worked to develop CPB resistant transplastomic potato lines under a bilateral project between Turkey (TUBITAK-……..) and Pakistan. As a part of this project Mr. Jakir joined Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan for project-based research activities. Jakir is fluent in Bangla and English and knows Turkish and Arabic in intermediate level.

ARTICLES PRODUCED FROM THESIS

Bu tez çalışmasından, 2 (iki) adet uluslararası makale ile 2 (iki) adet uluslararası bildiri üretilmiştir. Bu üretilen çalışmalar aşağıda sunulmuştur.

Hossain, M.J., Aksoy, E., Gökçe, Z.N.Ö., Joyia, F.A., Khan, M.S. and Bakhsh, A., "Rapid and efficient in vitro regeneration of transplastomic potato (Solanum tuberosum L.) plants after particle bombardment", ***Turkish Journal of Agriculture and Forestry***, 45(3), 313-323, 2021.

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