PhD THESIS

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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

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

DEVELOPMENT OF POTATO LINES RESISTANT TO COLORADO POTATO BEETLE (CPB) USING RNAi-BASED TRANSFORMATION APPROACH

TAHIRA HUSSAIN

July 2018

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Prof. Dr. Mehmet Emin ÇALIŞKAN

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The study titled "**Development of Potato Lines Resistant to Colorado Potato Beetle Using RNAi based Transformation Approach**" and presented by **Tahira HUSSAIN** with the help of supervisor **Prof.Dr. Mehmet Emin ÇALIŞKAN,** has been accepted as Doctoral thesis by the jury at the **Department of Agricultural Genetic Engineering** of Nigde Ömer Halisdemir University Graduate School of Natural and Applied Sciences.

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This thesis has been found appropriate at the date of 09/07/2018 by the jury mentioned above who have been designated by Board of Directors of Graduate School of Natural and Applied Sciences and has been confirmed with the resolution of Board of Directors dated …./…./20.... and numbered ……)

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THESIS CERTIFICATION

I certify that the thesis has been written by me and that, to the best of my knowledge and belief. All information presented as part of this thesis is scientific and in accordance with the academic rules. Any help I have received in preparing the thesis, and all sources used, have been acknowledged in the thesis.

TAHIRA HUSSAIN

ÖZET

RNAi YAKLAŞIMI KULLANILARAK PATATES BÖCEĞİNE (CPB) DAYANIKLI PATATES HATLARININ GELİŞTIRILMESI

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Ticari olarak kullanılan böceklere karşı dirençli transgenik bitkilerin çoğu, *Bacillus thuringiensis*'ten izole edilen cry gen(ler)ini içerdikleri halde zararlı yönetimindeki sürdürülebilirlik ve dayanıklılık ile ilgili olarak Bt içeren kültür bitkilerinin yoğun olarak yetiştirilmesi yaklaşık yirmi yıldır sorgulanmoktudır. Bu çalışma, RNAi yaklaşımı kullanılarak patates böceğinin (Colorado Potato Beetle - CPB) metamorfoz ile ilişkili Ecdysone reseptörü (EcR) genini yüksek derecede özgünlük ile susturmaya odaklanmıştır. CPB'nin Ecdysone reseptör geninin kısmi cDNA'sı, sens ve anti-sens oryantasyonunda spesifik primerler kullanılarak çoğaltılmış ve bir intronik dizi (pdk) ile çevrili pRNAi-GG vektörüne klonlanmıştır. Agria ve Lady Olympia çeşitlerinin yaprak ve internodal eksplantları, 35S promotörünün kontrolü altındaki EcR fragmanının sens ve antisens oryantasyonunu içeren plazmid pRNAi-CPB'yi barındıran *Agrobacterium* suşu LBA4404 ile enfekte edilmiştir. Neomisin fosfotransferaz (*nptII*) geni, 100 mg L^{-1} konsantrasyonunda bitki seçilim markörü olarak kullanılmıştır. Standart moleküler analizler aracılığıyla elde edilen birincil transformantlarda T-DNA'nın uygun şekilde bitki genomuna entegrasyonunu gösterilmiştir. Her iki çeşitte gelişen transgenik bitkiler, birinci, ikinci ve üçüncü evre CPB larvalarına karşı etkinlikleri açısından değerlendirilmiştir. Yaprak biyotoksisite deneylerinde CPB'nin % 15-80 ölüm oranınına ulaştığı gösterilmiştir. Transgenik bitkiler üzerinde beslenen böceklerde (0.87-4.14X) kontrolle (1.87-6.53X) karşılaştırıldığı zaman larva ağırlığında önemli ölçüde daha az değişiklik gözlemlenmiştir. Ayrıca, transgenik bitkiler üzerinde beslenen CPB larvaları, EcR-dsRNA'nın işlevselliğini gösteren azalmış EcR gen transkriptleri sergilemiştir. Bu çalışma sonucunda, patatesteki böcek zararlılarından kaynaklanan kayıpları azalttığı ve böylece artan ürün verimliliğine yol açtığı gösterilmiştir.

Anahtar Sözcükler: RNAi, Ecdysone reseptörü, Transgenik teknoloji, Böcek direnci, Molting

SUMMARY

DEVELOPMENT OF POTATO LINES RESISTANT TO COLORADO POTATO BEETLE (CPB) USING RNAi-BASED TRANSFORMATION APPROACH

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Most of the commercialized insect resistant transgenic crops express cry gene (s) isolated from *Bacillus thuringiensis*, however, intensive cultivation of *Bt* crops over almost two decades has been questioned regarding its sustainability and durability in pest management. The present study was focused to knock down highly specific molting associated Ecdysone receptor (EcR) gene of Colorado Potato Beetle (CPB) using RNAi approach. The partial cDNA of Ecdysone receptor gene of CPB was amplified using specific primers in sense and anti-sense orientation and cloned in pRNAi-GG vector flanked by an intronic sequence (pdk). Leaf and internodal explants of Agria and Lady Olympia cultivars were infected with *Agrobacterium* strain LBA4404 harboring plasmid pRNAi-CPB with EcR fragment under sense and antisense orientation under the control of 35S promoter. Neomycin phosphotransferase (*nptII*) gene was used as a plant selectable marker at a concentration of 100 mg L^{-1}. The primary transformants obtained showed proper integration of T-DNA in plant genome by standard molecular analysis. The transgenic plants developed out of both cultivars were evaluated for their efficacy against first, second and third instar CPB larvae. The leaf biotoxicity assays revelead 15-80% mortality of CPB. A significantly lower fold change in larval weight was observed in insects fed on transgenic plants (0.87-4.14X) as compare to control (1.87-6.53X). Furthermore, CPB larvae fed on transgenic plants exhibited reduced transcripts of EcR gene indicating the functionality of EcR-dsRNA. The outcome of this research work is believed to reduce loses from insect pests in potato; thus leading to increased crop productivity.

Keywords: RNAi, Ecdysone receptor, Transgenic technology, Insect resistance, Molting

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SYMBOLS AND ABBREVIATIONS

Symbols	Descriptions
FAOSTAT	Food and Agriculture Organization Statistical Databases (United Nations)
DNA	Deoxyribonucleic acid
Ti plasmid	Tumor inducing plasmid
MS medium	Murashige and Skoog medium
2, 4-D	2,4-dichlorophenoxyacetic acid
NAA	1-Naphthaleneacetic acid
BAP	6-Benzylaminopurine
GA3	Gibberellic acid
µM	Micro-molar
mM	Milli-molar
g L^{-1}	Gram per liter
mg L^{-1}	Milligram per liter
ml	Milliliter
µl	Microliter
µmol m^{-2} s^{-1}	Unit of measuring Light (Micromoles per meter square per second)
W	Watt
%	Percent
°C	Degree Centigrade
LB	Luria-Bertani medium
pH	Potential of Hydrogen
NOS	Nopaline Synthase
nptII	Neomycin Phosphotransferase
bp	Base Pair
PCR	Polymerase Chain Reaction
EDTA	Ethylene Diamine Triacetic Acid
ddH_2O	Double-distilled water
RSM	Regeneration Selection Medium

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a significant crop globally among other food crops regarding production and ranks 4th in position after wheat, rice and maize (FAOSTAT, 2018; USDA, 2016). It is one of the most promising crop-plant to overcome the challenges of hunger and poverty round the globe (Bagri et al., 2018). During 2016, global production of potato was 376,826,967 tons (FAOSTAT, 2018). Turkey is an important potato producing country among 156 potato-producing countries worldwide (Göre, 2017). Potato production contributes around 3% to gross national agricultural product (GNP), hence contributing a significant share in Turkish economy (FAOSTAT, 2018; Çalışkan et al., 2010). With the production of 4.8 million tonnes it was cultivated on an area of 172,000 ha in Turkey (Turkstat, 2018).

With the increasing world population, significance of potato is increasing because of nutritional value and other health benefits (Brown, 2005). Apart from its good and high potential of yield, it is very nutritious since tubers are enriched with proteins, carbohydrates, minerals (K, Mg, Mn, Cu, Fe, P) and vitamins (C, B3, B1, K, B6, folate, pantothenic acid) (Caliskan et al., 2010). In the Mediterranean agro-climatic conditions, potato is customarily raised as an early season winter crop and/or spring season crop (Caliskan et al., 2002).

The various biotic and abiotic stresses limit productivity and growth of potato. Estimated quantitative losses due to insect pests are 34% (Oerke et al. 2006). Colorado potato beetle (CPB) *Leptinotarsa decemlineata*, is the most economically important pest in many parts of world including Asia, Europe and North-America. Co-evolution with solanaceous crops which contains diverse secondary metabolites, have made the detoxification mechanism of CPB to survive variety of complex natural and synthetic chemicals. So, there is need of exploring new alternate control tactics (Zhu et al., 2011).

Since ancient times, the classical plant breeding techniques have been a good source of crop improvement. Main concern with the classical plant breeding techniques is that it requires several years of careful greenhouse and field work in developing a plant with

desired traits (DANIDA, 2002). Many breeding attempts have been made to create insect resistance potato plants (Flanders et al., 1992; Raman and Palacios, 1982). However, non-transgenic potato cultivar has not been introduced commercially expressing considerable resistance levels against Colorado potato beetle (Lagnaoui et al., 2001). Biotechnology has assisted classical breeding by providing alternatives for improvement of potato in area of insect resistance. To study the insect resistance, biotechnological approach to utilize genetically modified (GM) crops can play more appropriate role. Due to narrow genetic base of potato, conventional breeding strategies are usually inefficient (Douches et al., 1996). As conventionally breed-potato genotypes have not been released with resistance against CPB (Martínez, 2013)**.**

Genetic engineering technologies have led to discover reproducible and efficient techniques to transfer economically important genes between species and genera which can encode certain traits of interest by breaking barriers across species. These techniques are far better than conventional plant breeding tools by permitting the quick and predictable incorporation of desirable characters in the target species. Insect-pest resistance, herbicide resistance, and biofortification of crop plants are the common applications of genetic engineering (Pimental et al., 1989; Bakhsh et al. 2015).

Number of transformation techniques have been introduced like particle bombardment method (Aragão et al., 2000), microinjection (Crossway et al., 1986), protoplasts transformation using chemicals like calcium phosphate or polyethylene glycol (Datta et al., 1990), electroporation (Fromm et al., 1986), embryogenic suspension culture (Finer and McMullen, 1991) and plant transformation with silicon carbide whiskers (Frame et al., 1994).

Agrobacterium-mediated transformation is among the most promising techniques of plant genetic transformation. It is being used for genetic transformation of many crops. The protocol of *Agrobacterium*-meditated transformation is comparatively simpler, veracious, cheaper, and the foremost; inserts single transgene (Hansen and Wright, 1999). This kind of transformation is the most common in dicotyledonous plants (Fraley et al., 1986). Transformation and regeneration methods have become a primary source of introducing a variety of desirable traits in plants. However, *Agrobacterium*-mediated transformation is not favorable for most of the monocotyledonous plant species. Due to

this reason direct methods to transfer gene have been developed for monocots species. However, these direct gene transfer methods are not favored by most of the scientists owing to high copy number insertion (Christou, 1995; Christou, 1997).

Worldwide farming communities have adopted transgenic crops very rapidly since these were commercialized in 1996. Biotech crops were cultivated at area of about 185.1 million hectares (James, 2014). Out of these 181.5 million hectares 25 million hectares were occupied by the insect resistant crops, whereas around 45 million hectares were cultivated for insect + herbicide resistant crops. Most of these insect resistance transgenic crops harbor insecticidal genes from the bacteria *Bacillus thuriengenesis* (*Bt*) under control of promoter *35S* that induce expression of gene at high level in all types of tissues at different growth stages (Carrière et al., 2016).

The continuous induction of gene and its expression may cause metabolic burden on plant due to continuous synthesis of foreign gene products as compared to temporal expression of toxin and can increase environmental risk of increase resistance in targeted insects. Sustainability of *Bt* crops has been questioned due to increase in resistant of pests against these transgenic crops, as resistance has been reported very frequently (Luttrell et al., 2004; Ali et al., 2006; Tabashnik et al., 2008; Bravo et al. 2008; Zhang et al. 2011; Gassmann et al., 2011; Tabashnik et al., 2013; van den Berg et al., 2013).

In recent years, although the trend of using *Bt* insect resistance crops has been increased widely but now modern genetic technologies have become more imperative to develop crops with insect resistance. To control insects pests RNAi technology is a new prospective used over past decade in this regard. Numbers of studies by Baum et al., (2007), Mao et al., (2011), Zhu et al., (2012), Yao et al., (2013) and Mao et al., (2014) have shown the pathways to use RNAi-based technique to combat insect pests against targeted genes.

RNA interference is emerging as an eco-friendly, efficient and reliable tactic for the control of insects in economic crops. This is a gene knock-down strategy that uses double stranded RNA to hinder the normal gene function directly against a specific gene sequence or promoter region of messenger RNA (mRNA) (Mansoor et al., 2006). RNAi

process consists of three basic steps and this process is known as post transcriptional gene silencing (PTGS). First step involves the slicing of long dsRNA into shorter RNA partial dimers with the help of dicer a ribonuclease III enzyme. For the silencing machinery, double stranded RNA acts as catalyzer whereas the enzyme dicer cuts the dsRNA to shorter 21 to 25 nucleotides. Guide strand of short interfering RNAs (siRNAs) integrates in RNA induced silencing complex (RISC) and precede that complex to interfere with mRNA for further degradation (Tomari, 2005; Hannon, 2002). Research has proven that dsRNA enters the body of CPB via gut lumen followed by induction of an effective systemic RNAi response, depicting that RNAi can be an eco-friendly control tactic for the management of this insect pest (Cappelle et al., 2016; Zhu et al., 2011).

The pest resistance can be enhanced in transgenic plants with the expression of dsRNA of gene isolated from insect. The 20-hydroxyecdysone a ligand receptor complex initiates various physiological and developmental phenomena in insects like molting and metamorphosis. Ecdysone receptor gene (*EcR*) is one of the member of nuclear receptor (NR) superfamily and is ligand-inducible nuclear transcription factor (Hopkins, 2009). Growth stages of insect larvae are marked by series of molts, essential for hardening and expansion of cuticle as the larva grow. These molts are get initiated by the surges of steroid hormones ecdysone, that is converted to an active form 20-hydroxyecdysone (Sehnal, 1989). So Ecdysteroids are critically significant for the growth, development, reproduction, regeneration and molting of crustaceans (LeBlanc, 2007).

The ecdysone factor 20E and its nuclear receptor *EcR*-USP are insect-associated, both are absolute requirement for the insect development, so we have hypothesized that transgenics which express *EcR*-dsRNA, can be promising and efficient way of bringing improvement in terms of pest resistance in plants. The insects which will feed on transgenic plants expressing *EcR*-dsRNA will not be able to complete their life cycle as dsRNA will efficiently inhibit the accumulation and aggregation of *EcR* transcripts and formation of nuclear receptor complex *EcR*-USP in insects. So far constitutive promoters have been widely used in insect resistant transgenes, like *35S CaMV* promotor (Alam et al., 1999), ubiquitin promotor (Tang et al., 2006) and actin promotor (Pan et al., 2006). In particular *35S CaMV* promotor has been widely used in plant

genetic engineering and almost all genetically engineered crops till now utilize *CaMV 35S* promotor. It is now becoming clear that this promotor in not robust and its function can be influenced by many unrecognized environmental and physiological environmental factors (Sunilkumar et al., 2002).

For the plant biotechnology another common group of promotors after the viral promotors are derived from highly expressing plant genes like photosynthetic proteins and seed storage proteins, whose mRNAs have been easily characterized and cloned. For expressing transgenes, actin, tubulin and ubiquitin gene promotors have been used in many plant species. Considerable efforts are under process to discover more environmentally regulated, tissue specific, hormonal or stress responsive genes and promotors (Potenza et al., 2004).

Rubisco activase (*RCA*) is a member of AAA^{+} family of ATPases and is linked to the functionally diverse activities of cells (Portis, 2003). It catalyzes activation of the ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco). It is bifunctional enzyme found in plants' chloroplasts because it not only catalyzes initial CO2 fixation step in Calvin cycle but also function as oxygenase in the photorespiration. Plant leaves predominantly contains the protein termed as Rubisco. *RCA* transcripts' expression can be observed merely in plants' green tissues and regulated by light, which indicates that the RCA promoters are expressed only in the specific organs receiving light and potato is a type of heliophyte species, its growth is dependent on the light. So, these kinds of promotors and their associated sequences are promising candidates for the expression of gene at higher level (Xu et al., 2010).

We have produced two different sets of transgenic potato plants expressing *EcR*-dsRNA under control of constitutive (*35S*) and green tissue specific promoter (*pRCA*), respectively. The use of green tissue specific promoter has been employed to confine expression of dsRNA only in green parts of potato plants. As promoter activity is expected to be only in the green parts, no *RCA* transcripts' accumulation in other plant organs, such as tubers, will be observed. Hence, this genetic engineering technology will not result in transgene expression in the tubers, which are the ultimate organs of potato crop consumed by humans and animals.

1.1 Aims and Objectives

Four main objectives of this study were as follows: Isolation and characterization of the partial cDNA fragment of Ecdysone receptor gene (*EcR*s) encoding Ecdysone receptors in Colorado potato beetle, Isolation of 5'-flanking regions of the potato *RCA* gene promoter to induce green tissue specific expression, Development of RNAi based construct with sense and antisense cDNA of Ecdysone receptors inter spread by an intronic region under control of *35S* and green tissue specific (*pRCA*), Development of transgenic potato plants with constructs *EcR*/dsRNA and further evaluation of putative primary transformants using standard molecular techniques and Leaf bioassay of transgenic cultivars against Colorado potato beetle (*Leptinotarsa decemlineata*).

CHAPTER II

REVIEW OF LITERATURE

2.1 Potato and its Significance

Potato is one of significant and dominant food crops in the world. Its production increased in last fifteen years since it serves as staple food in developing countries to reduce hunger (Thiele et al., 2010). Potato plays a vital role as non-cereal food crop and can be compared with wheat, rice and maize for its role in securing global food system in the developing countries (USDA, 2016; FAOSTAT, 2018). Potato is a complete source of nutrients containing protein (2%), carbohydrates (16%), minerals (1%), dietary fibers (0.6%) and a good amount of vitamin C and antioxidants (Haase 2007; Navarre et al., 2009). There has been a continuous change in pattern of potato consumption in many developing countries. The reason behind is increasing population pressure, fast urbanization, changing consumers and the market preferences (Çalişkan et al., 2010).

2.2 Biotic and Abiotic Stresses in Potato

The numerous biotic (insects, weeds and diseases) and abiotic (temperature, water, salinity and solar radiation) stresses pose significant yield losses in economically important plants worldwide (Shao et al., 2008). Among biotic stress insect pests and the diseases postulate a prolonged threat to the crop plants thus causing about 34% losses of agricultural production all over the world (Oerke, 2006).

Potato is infested by several kinds of insect pests including leaf-feeders like potato leafhopper; insects that attack and damage tubers like wireworms; insect-pests infesting both leaves and tubers like potato tuber moth, black cutworm and insects that carry and transmit viral diseases like green peach aphid. Species of *Lepidoptera* are also known as potato tuberworms as their larvae wades into potato tubers both in field and store. Among potato tuberworms, potato tuber moth and in coleopterans, Colorado Potato Beetles are most common insect pests of potato (Visser, 2005; Rondon, 2010).

2.3 Colorado Potato Beetle

Among the economically important pests, Colorado potato beetle (*Leptinotarsa decemlineata* S) has got more attention of the researchers. In terms of movement, it can not only fly (upto several kilometres) but also can walk (up to hundreds of meters) for feeding, both of its larva and adult forms feed on parts of solanaceous plants including potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersium L*), eggplant (*Solanum melongena* L.) and nightshade (*Solanum nigrum* L) (Jacques, 1988; Boiteau et al., 2003, Alyokin et al., 2008). The leaves of potato plants are consumed heavily by the CBP (adult, beetle as well as black-spotted red larva) that results in 30% to 50% of decrease in total production and occasionally in some lands there is no gain at all (Zhou et al., 2012).

2.4 Control Methods of CPB

Various strategies have been adopted in the integrated pest management for control of agricultural insect-pests like biological control, mechanical control, mass trapping, trench traps, attractants, beits, push-pull strategies, host plant resistance, conventional chemical control and genetic control (Alyokhin et al., 2008; Jaleel et al., 2013; Saeed et al., 2014).

Insecticides have been primarily used for long time to control CPB in the fields (Casagrande, 1987). The first insect on which the insecticides were used on a very large-scale was *L. decemlineatea*, in 1864 (Gauthier et al., 1981). This insect has incredible capability to generate resistance against almost each insecticide that has been used to manage this pest on wide-spectrum (Bishop and Grafius, 1996) and its resistance has shown to increase further with every new insecticide introduced (Forgash 1985; Heim et al., 1990; Ioannidis et al., 1991). So far, it has managed to develop resistance to 52 insecticides from almost all primary insecticide groups since the mid of previous century (Alyokhin et al., 2008)

The recommendation is to discourage resistance development by insecticide rotation, but the situation is becoming worse due to multiple resistances (Sharma, 2013). It is unfortunate for the farmers, who spray their potato fields up to or even more than 10 times in one growth season of 4 to 6 months in some countries, just to fight these pests.

For the farm families and farm workers involved in potato production, various health hazards are associated with the use of the insecticides (Larson et al., 2014).

To cope with the insecticides CPB employs several mechanisms of resistance. Those mechanisms include insensitivity of target site (Malekmohammadi and Galehdari, 2016), decrease in penetration (Argentine, 1994), increase in excretion (Rose and Brindley, 1985) and metabolic detoxification of the insecticides (Ahammad-Sahib, 1994). From these, metabolic resistance is best described mechanism, believed to have ability of neutralizing dietary toxins from ancestor (Dermauw et al., 2012). This success of CPB to overcome insecticides is due to its co-evolution with plants of Solanaceae family as its host (Alyokhin et al., 2015), which produces tremendously toxic compounds recognized as glycoalkaloids (Pelletier, 2013). Metabolic resistance in CPB involved a mechanism to resist against carbamate (Rose and Brindley, 1985), pyrethroid (Argentine, 1989), organophosphate (Ahammad-Sahib, 1994), and abamectin (Gouamene-Lamine, 2003) classes of these insecticides. Additionally, there are few evidence that resistance to neonicotinoid is due to metabolic resistance as well (Mota-Sanchez et al., 2006).

Phase I enzymes comprise of Cytochrome P450s (CYPs) and esterases (ESTs) which play a significantly important role in detoxification and transportation of xenobiotics (Li et al., 2007; Feyeresisen, 2012); reactions of phase II include glutathione S-transferases (GSTs) and the UDP-glycosyltransferases (UGTs) (Enayati et al., 2005; Ahn et al., 2012); and phase III reactions i.e. excretion involves ATP-binding cassette (ABC) transporters (Dermanw and Van, 2014). The diverse metabolic enzymes in insect-pests are the key players in developing resistance to approximately every insecticide being used or being considered for future use (Naqqash et al., 2016).

The concerns have been raised because of possible risks posed to environment and human health due to formulations of more and more frequent yet ineffective pesticides to combat the potato pests which are continuously evolving the mechanisms of resistance in them.

2.5 Early Attempts of Insect Resistant Potatoes

Potato presents unique challenges to the breeders as it is propagated via tuber cutting vegetatively and it does not need to breed for production of homogenous plants with true seeds. As potato belongs to *Solanum tuberosum* so tetraploid nature of potato makes it difficult to breed to transfer desirable and important traits among cultivars. In Central America and Peru many species of Solanum are cultivated as commercial cultivars. These cultivars provide huge range of potential traits for breeding into *Solanum tuberosum*, including qualities of tubers (e.g., color, flesh), resistance to various insect pests and pathogens. Most wild cultivated varieties of Solanum are diploid in nature making it more complicated for breeding. Hence, genetic transformation is a valuable and attractive process to insert candidate gene of desirable trait to develop new cultivars of potato.

To cope with various biotic stresses, plant genetic transformation has developed as a useful tool (Kumar, 2018). Fraley et al., (1983) developed first genetically modified plant of tobacco conferring resistance to antibiotic. Chimeric gene was formed with the fusion of *Agrobacterium tumefaciens* plasmid and the locus having antibiotic resistance. Containing this chimeric region in *Agrobacterium tumefaciens* tobacco plants were infected, and soon after the infection, selection of transformed tobacco cells was carried out using different tissue culture techniques.

In plants, *Agrobacterium* mediated transformation is well established especially in dicots. Various studies revealed that *Agrobacterium* can produce galls in a wide variety of dicots. In monocotyledonous species this gall producing activity of *Agrobacterium* has rarely observed (Fraley et al., 1986). According to Pitzschke et al., (2010) *Agrobacterium* species can infect a wide range of host pants involving most of the dicots. In plants Transformation with *Agrobacterium* involves several steps and plant bacterial interaction (Figure 2.1).

Classical breeding methods have been used to develop various insect/pest resistant cultivars, this method is not only time consuming but also tedious as the complexity increases with addition of other traits. In the last thirty years, resistant genotypes have been established and introduced with the help of transgenic technology, to reduce both production losses and unwanted usage of pesticide (Christou et al., 2006). These

genetically modified plants have become essential part of IPM globally (Kos et al., 2009). Undoubtedly the perfect example is the *Bacillus thuringiensis* (*Bt*) toxins containing plants (*Bt* plants), which has gained considerable success both environmentally as well as economically. *Bt* endotoxins are lethal for main target insects and cause smaller and/or no damage to vertebrates and other non-target organisms. Also, the endotoxin is more detrimental to earlier instars of the insects and as the product is kept within the plant tissues the system is safe environmentally.

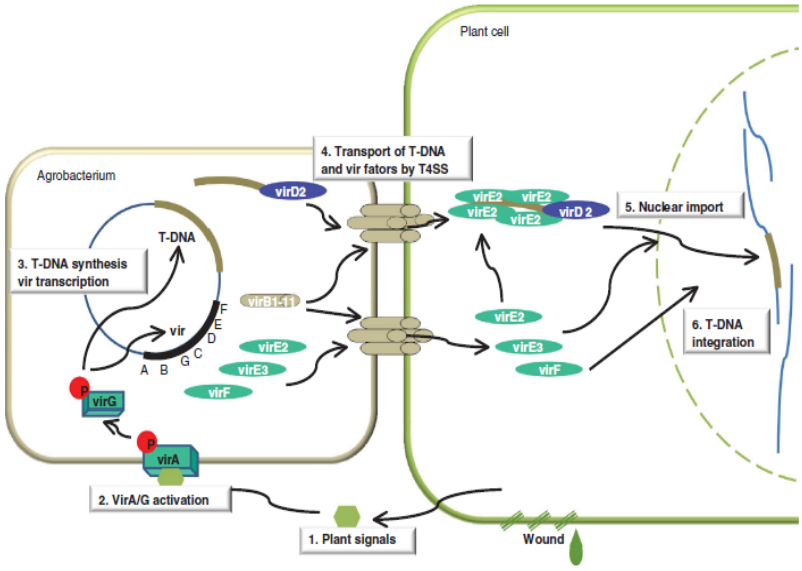


Figure 2.1. Overview of the *Agrobacterium*–plant interaction: 1. Induction of plant signals, 2. Activation of VirA/G, 3. Synthesis of T-DNA followed by vir gene expression in *Agrobacterium*, 4. Through a bacterial type IV secretion system (T4SS) T-DNA and Vir proteins are transferred into the plant cell to assemble a T-DNA/Vir protein complex, 5. The T-DNA complex is imported into the host cell nucleus, 6. The T-DNA becomes integrated into the host chromosomes by illegitimate recombination (Pitzschke et al., 2010).

In 1902, the bacterium *Bacillus thuringiensis* was initially discovered in silkworm rearing unit of Japan. Once again it was isolated in the flour moth population in 1911 and was described by Berliner in Thuringen, Germany. *Bacillus thuringiensis* is a gram-positive bacterium which produces crystalline inclusions of insecticide during the phase of sporulation and these inclusions are structured with protoxin subunits called δ-endotoxins. Almost all *B. thuringiensis* strains form various crystalline proteins (Cry proteins), and each has a specific small host range (Feitelson et al., 1992).

More than 100 different *Bt* formulations are available in diverse strains of *B. thuringiensis* (Sanahuja et al., 2011). Based on host range and protein structural homologies, these genes were first categorized in various classes' *cry*I, *cry*II and *cry*III (Hofte and Whiteley, 1989). Endotoxins viz. *cryl* and *crylll* are active against lepidoptera and coleoptera respectively. In many crops the toxin genes have been expressed from *Bt* and the examples include transgenic cotton (*Gossypium hirsutum*) (James, 2013), maize (Pilcher et al., 1997), chickpea (*Cicer arietinum*) (Acharjee et al., 2010), and sorghum (*Sorghum bicolor*) (Girijashankar et al., 2005).

Development of pest resistance to *Bt* in few insects has threatened effectiveness of the *Bt* toxins. This resistance has been reported in American bollworm (Lepidoptera: Noctuidae) and also in European corn borer (Lepidoptera: Pyralidae) (Ferre and Van Rie, 2002; Baum et al., 2007). Furthermore, there is increased resistance from environmentalists against this technology (Kumar et al., 2008; Benbrook, 2012). This necessitates the development of other alternatives for pest control. There are also many reports showing that intensive cultivation of *Bt* crops are leading to increased pest resistance against the transgenic plants (Bravo et al., 2008; Tabashnik et al., 2008). Recently Zhang et al., (2011) showed an increase of *H. armigera* resistance to the *Bt* cotton. Even cross resistance had been reported in transgenic plants that could express two different *Bt* toxins (Tabashnik et al., 2008).

The genetically engineered crops used against the CPB, comprised of Cry3A endotoxin obtained from the bacterium *Bacillus thuringiensis* var. *tenebrionis* and used to transform potato plants by use of constitutive *35S* cauliflower mosaic virus (*CaMV 35S*) promoter. This approach was quite promising and reliable that the multiple genotypes of these plants like Russet Burbank, Snowden, Atlantic and Superior were first genetically modified potato varieties commercialized by the Monsanto, from 1995 to 2001 in North America (Duncan et al., 2002). Insect pest control has been successful due to the transgenic crops which produce *Bacillus thuringiensis (Bt)* toxins, however their efficacy is decreased in such conditions when the pests develop resistance (Tabashnik et al., 2009).

Transgenic *Bt* potato plants targeting CPB were introduced first time in 1996. Resistance to number of chemical pesticides has been reported in this beetle except *Bt* spray containing toxins of Cry3A. So, transgenic potato expressing *cry3A* gene was

introduced as successful variety to control insects (Perlak et al., 1993). However, many buyers were reluctant to buy genetically modified potatoes due to perceived resistance of public against biotechn crops. The *Bt* potato was then withdrawn from market in year 2001 (Perlak et al., 1993).

The evolved resistance in insect pests, is threatening durability of the transgenic *Bt* crops which containing insecticidal proteins from the *Bacillus thuringensis (Bt)*. Results of about 77 studies gathered from five different continents were analyzed by Tabashnik et al., (2013), which mainly comprised of field analysis for evolved resistance against *Bt* crops and empirical assessment of those factors which affect the resistance or both. Despite of the fact that most of the populations of pest remained susceptible, the resistance evolving in the fields decreased the efficacy of *Bt* crops and this is well documented till now for about populations of 5 insects out of 13 major species of pests evaluated and it depicts out an alarming condition because in 2005 only one pest species was reported to be resistant. Although the researchers are attempting to develop 2nd and 3rd generation insect resistant transgenics however no successful outcome like *Bt* plants has been achieved till now (Christou et al., 2006; Kos et al., 2009).

2.6 RNAi Interference a Breakthrough Technology

RNA interference is type of host induced gene silencing (HIGS) which is conserved in eukaryotes and process was first explained by Fire et al., (1998). It has been emerged as a major tool of plant functional genomics involving production of the double-stranded RNA (dsRNA) having homologous sequence of the gene selected for the post transcriptional gene silencing (PTGS) (Senthil-Kumar et al., 2010). This mechanism has been used extensively as potential tool for gene silencing, determining function of host gene and to improve or create pre-existing traits of plants integrated with stress tolerance and yield aspects also primarily in organisms having no availability of stable transgenics like insects. Thus, phenomenon of the RNAi has gained the attention of biotechnologists for study of functional genomics in a variety of living organisms and has accelerated the usage of functional genomics to the newer levels (Kamthan et al., 2015).

With this discovery, mechanism of the RNAi has been studied widely including insects, yeast, plants and mammals, showing different functional and the evolutionary

conservations (Belles, 2010). For functional genomic studies of insect, exogenous dsRNA should be delivered to the bodies of insects thereby activating siRNA pathway to silence the expression of target genes. dsRNA uptake via micro-injections and feeding either via spraying or plant mediated RNAi triggers the entire body and the sustained repression of mRNA level from targeted gene. This process involves uptaking of the dsRNA from external environment and successive transport of the RNAi signals between the cells and tissues in body (Pecot et al., 2011, Joga et al., 2016).

Due to increase in insect-resistance against *Bt* plants its crucial to develop advance strategies for the integrated pest management. Recently, RNAi technique has been introduced as an attractive method to knock-down the important genes of insect pests for protection of crops (Gordon et al., 2006; Gordon et al., 2007; Price et al., 2008; Huvenne et al., 2010). The knockdown efficiency of many gene in RNAi recalcitrant species of insects is about 60% or less and this silencing is also short termed sometimes (Huvenne and Smagghe, 2010; Li et al., 2013). In some insects, RNAi works quiet efficiently in insects particularly belonging to order Coleoptera (Tomoyasu, 2008)). The broad studies of coleopteran insects like the western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte), red flour beetle (*Tribolium castaneum*) and Colorado potato beetle utilizes RNAi efficiently in both the basic and the applied sciences (Palli, 2012). Although in beetles, the in vivo amplifications of dsRNA or siRNA is not properly explored; in these insects very less quantity of dsRNA appears to be enough for catalyzing response of RNAi. In all the beetles' RNAi does not appear to work consistently. It is shown in many studied that systemic RNAi works well in the most species of beetles, differences in efficiency and effectiveness of oral delivery of dsRNA has been reported, like it works more better in WCR and CPB than in red flour beetle (Baum et al., 2007; Whyard et al., 2009; Zhu et al., 2011).

RNAi is a knockdown method not a knockout: sometimes effect is transient and no total silencing is there as its efficacy also depends on delivery methods and uptake mechanism in the cells of the test organism (Rajagopal et al., 2002, Shakesby et al., 2009). To use this technique as a tool for insect pests control it is suggested that dsRNA should persist till death of insect pests to minimize development of resistance. However, it is not unsolvable problem to utilize RNAi technique as in many of cases, partial silencing of many genes have been reported to cause irreversible lethality thus leading to detrimental effect on insect. This technique has been illustrated after wide application

by studing several targeting genes of seven different orders. Bioassay with transgenic crops showed that insect development can be delays which can lead to reduce damage of plants (Baum et al., 2007; Mao et al., 2007).

2.7 Factors Effecting Efficacy of dsRNA

According to literature five most vital factors have been introduced influencing the silencing effects and the efficiacy of RNAi as a management strategy of insects these factors includes:

2.7.1 Target gene

Target gene (s) selected can significantly affect the efficacy of this technique. If we are targeting a gene related to growth, development or survival of insect like vATPase gene higher mortality percentages can be achieved. While targeting a gene which have secondary effect on survival and/or makes it difficult to get significant mortality as it is a part of complexes of genes like detoxification related genes in which silencing of one gene doesn't affect the insect survival as this process is also being carried out by a variety of genes (Zhu et al., 2011; Kaplanoglu et al., 2017).

2.7.2 Concentration of the dsRNA

Concentration of the dsRNA is very important with respect to targeted gene and the organism. Optimal concentration can induce optimal silencing (Meyering-Vos and Muller, 2007; Shakesby et al., 2009).

2.7.3 Sequence of nucleotides

Targeted sequences not only define off target impacts in the target organism but it may effect other non-target organisms. Knock down of off-target was proven in triatomid bug Rhodnius prolixus: along with the targeted nitroporin 2, two conserved sequences of nitroporin were silenced (Araujo et al., 2006). Vacuolar H^+ ATPase dsRNA of the Colorado potato beetle (*Leptinotarsa decemlineata*), also worked for targeting an ortholog gene in the western corn rootworm (Baum et al., 2007).

2.7.4 Fragment length of dsRNA

Uptake and knock-down efficacy in both unicellular and multicelluar organisms depends on the length of dsRNA fragment (Mao et al., 2007; Saleh et al., 2006). For oral ingestion studies, length of dsRNA can lie between 300–520 bp. Though, a research work has also used only single siRNA (Kumar et al., 2009). Success in clinical research decides whether to use siRNA or not (Castanotto and Rossi, 2009; Kurreck, 2009). According to Saleh et al., (2006), minimum suggested length of dsRNA is 211 bp regarding diligence of the silencing effects, Shakesby et al., (2009) found that the knock-down in aphids' aquaporin persists for about 5 days and was later reduced. According to Turner et al., (2006), impact of dsRNA on pheromone protein binding system in *Epiphyas postvittana* can be related to the under-expression rate of the targeted protein.

2.7.5 Targeted organism's life stage

Despite of the fact that older stages of insects can be easily treated with dsRNA especially with injection, however they are more effective at immature stages. Araujo et al., (2006), conducted a comparative study on 4^{th} and 2^{nd} instar of kissing bug. According to this study, 4^{th} instar had no effect of dsRNA application as compared to the 42% knock-down of 2^{nd} instars. In another study, no silencing was observed in adults of fall armyworm as compared to the strong knock-down of genes in 5^{th} larval instars (Griebler et al., 2008).

Efficacy of RNAi majorly depends on uptake and delivery of dsRNA to cells of target organism. Consequently, its delivery is a key issue in RNAi based host plant resistance techniques. After choosing possible promising target, delivery method of dsRNA to insect is the most important aspect.

2.8 Different Delivery Methods of dsRNA

Delivery methods of dsRNA can be categorized into two: Injection based and Feeding based delivery of RNAi.

2.8.1 Delivery via injection (s)

Microinjections are effective tool of functional genomics, however they can't be used for insect pests' management in the agricultural fields. Additionally, use of microinjections have some bottlenecks like its tricky ways of experimentation and difficult to apply in smaller and aquatic insects (Nunes and Simões, 2009; Walshe et al., 2009). Use of micro-injection to inject dsRNA in larvae and adults of red flour beetle was firstly used to silence genes and study gene function **(**Baum et al., 2007).

Clememts et al., (2017) gave information that around 562 up-regulated mRNA transcripts were found in the imidacloprid resistant population of CPB. The main idea behind this study was conducted to determine role of the up-regulated transcripts in the resistant populations and can be used to re-establish a susceptible strain. Among the up-regulated transcripts, three viz. Comp115309 (cytochrome p450), Comp105889 (cuticular protein) and Comp114026 (glutathione synthetase) were selected for this study using micro-injections for insertion in resistant *L. decemlineata* population. Successful knock-down of the tested transcripts encoding for Comp115309, Comp105889 and Comp114026 in the treated population, resulted in increased susceptibility of the tested populations to the imidacloprid. Additionally, significantly higher mortality was recorded for the three genes as compared to the control.

According to Revuelta et al., (2011), point mutations in the acetylcholinesterase gene (*Ldace2*), an orthologue of Dmace2 gene found in *Drosophila melanogaster*, are responsible for resistance to carbamates and organophosphates in CPB. Sequencing and cloning of *Ldace1* gene, that was previously unknown in CPB, have been reported in this study. All the functional residues of AChE are also a part of the *Ldace1* coding enzyme. Significantly higher expression of *Ldace1* has been observed at larval stages and adult as compared to embryos. The dsRNA targeting AChE was inserted in insect via micro-injection lead to decrease in expression of AChE by 50% while dsRNA targeting *Ldace2* decreased the expression of AChE activity by 85%. *Ldace1* interference resulted in significantly higher mortality (43%) in CPB adults after three days post-injection, which shows its essential role in insects' body. Injection with dsRNA with *Ldace2* was considerably less mortality (29%) than *Ldace2* after seven days of treatment. Treating insects with dsRNA targeting *Ldace1* increased their

susceptibility to organophosphates which suggest that LdAChE1 is the key target of these insecticides.

Yao et al., (2013) targeted two Vacuolar-ATPase namely V-ATPase B and the V-ATPase D of corn plant hopper through oral delivery and the microinjection. Cellular break down of ATP and intercellular protons transport are very important functions performed by an essential enzyme V-ATPase. So, this enzyme can be a promising target for RNAi mediated control of other insect-pests. Both methods used to deliver dsRNA caused significant down-regulation of the transcriptome, where the micro-injections were comparatively more effective. The qRT-PCR analysis showed that there was 27-fold decrease in transcript level of targeted gene.

2.8.2 Oral delivery

It can be divided into 3 main categories viz. sprayable RNAi, Nanoparticles loaded with RNAi and plant mediated transformation with RNAi.

2.8.3 Sprayable RNAi-based

Sprayable products are being considered for commercialization. Some companies have commercialized RNAi based sprays for household pests (Anonymous 2018). These products can be categorized as: (1) resistance repressors, (2) direct control agents, (3), growth enhancers and (4) developmental disruptors (EPA, 2014). The dsRNA spraying can be successful tool to manage only few pest populations in field. Li et al., (2015a) stated that spraying methods do not work affectively in case of piercing-sucking insect pests that feeds on the sap of phloem and the stem borer pests that feeds inside plant stems.

Zhu et al., (2011) showed that feeding of CPB with dsRNA targeting five genes viz. actin, sec 23, vATPase E, vATPase B, and COP β showed significant mortality as compared to control. Significantly higher level of reduction in mRNA level i.e. 93% was reported in case of vATPase B while significantly higher mortality was observed in COP β.

Similarly, Kong et al., (2014) reported that feeding bio-assay using dsRNA to target *LdShd* in the 2nd instar larvae of CPB could successfully down-regulated expression level of *LdShd* to 46.8% after 5 days of feeding and decreased mRNA level upto 59.2% of *ecdysone receptor* (*LdEcR*) gene causing death rate of 80.0% along with delayed development and affected pupation.

Spit et al., 2017 found that two nuclease genes could exclusively expressed in the gut of CPB and it was demonstrated that removal of these nuclease activities in the adults could improve sensitivity towards the dsRNA and resulted in improvement in protection of the potato crop. This study suggested that interfering with the activity of nuclease is a good strategy for improvement of RNAi efficacy in the control of insect pest applications.

Wan et al., (2014) cloned and characterized of full-length gene related to insect ryanodine receptors (RyRs). Introduction of the double-stranded RNA of *LdRyR* via diet significantly downregulated the transcriptome level upto 45% of target gene in immature and the adult stage respectively, and also decreased chlorantraniliprole associated percent mortality.

The dsRNA delivery to the phytophagous insects can be possible by delivering dsRNA by the root uptake or via injections in plant vascular system which can enter the insects scan acquired RNA naturally by sucking and chewing (Andrade and Hunter, 2016).

2.8.4 Nanoparticles loaded with RNAi

Nanoparticles are used to decrease the dsRNA degradation and thus enhance uptake of intact form of dsRNA by a cell. Polymeric nanoparticles are biodegradable so safe for our environment. These can be used for various purposes like for surface modification due to their stability (Vauthier et al., 2003; Herrero-Vanrell et al., 2005).

Chitosan, a polymer is used to encapsulate the dsRNA by Zhang et al., (2010). He worked with loading of this RNAi on nanoparticles against mosquitoes. Loading dsRNA is made possible by self-combination of polycations in order to achieve chitosan nanoparticles. This process is appropriate for long dsRNA and siRNA. Chitosan is combined with food and transferred to larvae through oral ingestion. This method

delivers great assessment of phenotype like behavioral evaluation as this nanoparticle is nontoxic.

Zhang et al., (2015) described that by using Chitosan in *Anopheles gambiae* and *Aedes aegypti*, target genes could successfully knocked-down during the development of larva.

He et al., (2013) also observed clear RNAi gene silencing revealed by the larvae of Asian corn borer fed on the diet containing the combination of FNP and CHT10-dsRNA. As result of this gene silencing, reduction in size of larvae, failure of molting and then death was observed. So, for producing a strong gene knockdown, adding of FNP was necessary.

2.9 Plant Mediated RNAi

The dsRNA expression in transgenic plants against suitable pests' target genes have been reported to protect against insect-pests by highlighting the pathway for development of new generation of insect resistant crops (Price and Gatehouse, 2008; Mutti et al., 2006; Mutti et al., 2008).

Agrobacterium mediated transformation is considered as one of the most efficient method for production of plant mediated RNAi. There is need of further investigation to improve efficiency of RNAi technique as new genetic tool for the better protection of crops. Two key steps of RNAi are: to produce efficient way of the dsRNAs in plants and delivery of these silencing molecules into the gut cells of insect (Mao et al., 2013).

According to Baum et al., (2007), targeting subunit A in ATPase gene using dsRNA expressing transgenic corn plants significantly enhanced the mortality of western corn rootworm. Moreover, targeting the cytochrome P450 monooxygenase gene by dsRNA expressing transgenic Arabidopsis plants significantly decreased the resistance of American bollworm to the sesquiterpene gossypol and enhanced the mortality. Following these successful experiments of RNAi based control of insect-pests, various other research works have laid down the basis for successful implementation of plant-mediated RNAi.

Pitino et al., (2011) selected green peach aphid for gene silencing via plant mediated RNAi technology. For this purpose, *Rack1 and C002* (*MpC002*) genes were selected which are principally expressed in gut and in salivary glands respectively. Double *CaMV 35S* promoter was used to produce dsRNAs. The *Arabidopsis thaliana* and *Nicotiana benthamiana* were transformed via *Agrobacterium tumefaciens*. Aphids were fed on leaves of *both* transgenics producing transiently dsRNAs corresponding to selected genes. The expression levels of *MpC002* and *Rack-1* were reduced up 60% on transgenic plants as compare to control plants.

Xiong et al., (2013) studied effects of a molting related gene in *H. armigera*, via plant-mediated RNAi. Initially four internal fragments of *HaHR3* were silenced by using bacterial expression system. Later on, tobacco was transformed by *agrobacterium*-mediated transformation using the best candidate regarding RNAi efficiency. Decrease in transcriptome level of the targeted gene was observed which resulted in abnormal development and eventually the death of larvae.

According to Mao and Zeng, (2014), the gap gene *hunchback* (*hb*) is very important in tissue differentiation in insects and silencing of this gene can result in deformation and ultimately death of the daughter generation. Considering the importance of green peach aphid, plant-mediated RNAi via transgenic tobacco was developed to target the *hb* gene. Variable patterns of integration were observed in transgenic tobacco. T_2 generation of homozygous transgenics was used for bioassays by releasing neonate aphids on them. It was demonstrated that feeding on transgenic plants significantly down-regulated the transcriptome level of the target gene in test aphids and reduced insect reproduction by plant-mediated RNAi.

Xu et al., (2014) adopted plant-mediated RNAi technology to target carboxylesterase (*CbE E4*) in *Sitobion avenae* which is involved in detoxification of wide range of pesticides. RNAi construct was ligated between the rbcs promoter. Co-transformation of wheat cultivar "Jinghua1" was carried out via biolistic device. Aphids were fed and raised on these transgenic lines. The expression of *CbE E4 in S. avenae* decreased upto 30–60%. Total number of aphids reared on transgenic lines was lower than number reared on the non-transgenics.

According to Yu et al., (2014), the insect ecdysone receptor (*EcR*) plays a significant role in the molting of insects and thus development depends directly on its transcript level. Concluding, this gene can be a promising target for RNAi based management of brown planthopper which is an economic pest of rice plants. A conserved sequence of 360 bp (*NlEcR-c*) common among *NlEcR-A* and *NlEcR-B* was targeted in oral feeding experiments which significantly reduced the transcript levels of *NlEcR*. Additionally, oral ingestion of RNAi also reduced the number of brown planthoppers significantly. Subsequently, *NlEcR* dsRNA expressing transgenic rice plants were developed via *Agrobacterium*-mediated transformation. Effective silencing was obtained in neonate nymphs of brown planthoppers being reared on transgenic plants. Targeted gene expression level was significatly reduced in all of lines tested. Immatures rate of survival was approximately 90%, while the offsprings in the transgenic lines was lesser as compared to control, with a reduction to 44.18-66.27%.

Coleman et al., (2014) used plant-mediated RNA interference (RNAi) to explore the function of gene viz. *MpC002*, or *MpPIntO2* in green peach aphid. The dsRNA was produced under the control of *CaMV 35S* promotor while plants were transformed via *Agrobacterium tumefaciens*. Down-regulation in gene expression was ~70% after 8 days of feeding on transgenic *Arabidopsis thaliana*. After three generation analysis of aphids reared on transgenic plants it was revealed that there was 60% reduction of reproduction levels in case of plants expressing dsMpC002 and 40% reduction of reproduction levels in case of plants expressing dsRack1 and dsMpPIntO2.

Thakur et al., (2014) developed transgenic tobacco lines generating and expressing long dsRNAs precursor to produce siRNA to knockdown *v-ATPaseA* gene in whitefly. The dsRNA was produced using *CaMV 35S* while plants were transformed via *Agrobacterium tumefaciens* strain LBA4404. Host plants successfully derived resistance against whiteflies and decreased expression level of the target in exposed whiteflies was 62%. Mortality rate observed was 15 to 38% after two days of feeding, 26 to 58% on day fourth and 34 to 83% was recorded on sixth day of feeding.

Liu et al., (2015) successfully silenced the *arginine kinase* (*AK*) gene of *Helicoverpa armigera* that encodes phosphotransferase playing vital role in metabolism of cell energy cycle in the invertebrates. *CaMV 35S* promoter was used for production of dsRNA. Transformation of resulted construct was done in *Arabidopsis* plants by the

Agrobacterium-mediated transformation. The transgenics of *Arabidopsis* expressing *HaAK* dsRNA were generated. The maximum recorded mortality rate of first-instar larvae was 55% after 3 days of feeding treatment on transgenic plants as compare to control. The knocked down transcript level of *HaAK was up to 52%.*

Raza et al., (2016) reported that of insect's diet feeding on the phloem mainly depends on uptake of water and sugar that depends on the osmotic pressure in body of insect. Two important genes viz. *aquaporin* (*AQP*) and the sucrase gene *alpha glucosidase* (*AGLU*) were targeted in tobacco plants. *CaMV 35S* promoter and terminators were used for dsRNA production, while ex-plants were transformed and developed by some modifications in the direct method of organogenesis utilizing leaves as explants for transformation via *Agrobacterium*. Both of these genes control maintenance of osmotic pressure in sap sucking insects. Feeding bioassay was performed after six days of feeding transcript level was reduced and mortality rate recorded was more than 70%, on whiteflies fed on the transgenic lines as compared to the control plants.

Poreddy et al., (2017) reported, *Manduca sexta* and *M. quinquemaculata* contains two important genes conferring resistance i.e. cytochrome P450 monooxygenase (CYP6B46-plants) and the lyciumoside-IV-ingestion induced β-glucosidase1 (irBG1-plants). Transgenic *Nicotiana attenuata* plants expressing double-stranded RNA (dsRNA) were produced using *Agrobacterium tumefaciens*. They explored that conserved sequence in transgenic plants can silence the genes in closely related species *M. quinquemaculata* larvae fed on dsRNA expressing transgenics. Targeted transcriptomes were significantly declined viz. 90% in case of CYP6B46 while 80% in case of irBG1-plants. They concluded that the plant mediated RNAi can be specific as well as useful for closely related species sharing same fragment of the target gene. Homologous genes in tomato hornworm were also silenced due to oral ingestion of transgenic plants expressing dsRNA to target irCYP6B46 and irBG1, by using PMRi procedures. Expression of CYP6B46 was reduced to 90%, while expression of BG1 was reduced to 80%. However, this silencing was gene specific, it was not observed in other genes. Concluding, PMRi procedure can be successfully used in RNAi based control of insect pests.

Ibrahim et al., (2017) generated RNAi based plasmid, dsRNAs of novel *v-ATPase* transcripts in the whitefly was designed. Transformation of lettuce was performed by *Agrobacterium* tumefaciens strain EHA105. They found that after five days of exposure to transgenics, insects significantly higher mortality 83.8–98.1% was observed and also reduction in fecundity was calculated as compare to the control lines. qRT-PCR showed decreased in targeted transcriptome level in whiteflies exposed to transgenic lines.

Niu et al., 2017 produced transgenic maize using RNAi technology to target the 2 genes (*dvvgr* and *dvbol*) related to reproduction in western corn rootworm (*Diabrotica virgifera virgifera*) with goal to reduce fecundity of insect. Maize elite-inbred line (PHR03) was transformed using *Agrobacterium tumefaciens*. Results demonstrate that not only adult beetles but also larvae when fed on transgenic plants showed significant reduction in the fecundity. Adults bioassay conducted with different concentrations in artificial diet resulted in reduced mortality ranging between $46.5 \pm 12.5\%$ and $75.4 \pm 11.3\%$.

Maturing and transportation of active lipoprotein lipases need special proteins named as Lipase maturation factor (LMF). Xu et al., (2017) targeted an internal fragment of lmf2-like gene in grain aphid. *Rbcs* promoters was used for ligation of RNAi construct. Co-transformation of wheat cultivar "Jinghua1" was carried out via biolistic device. Transcript level of the targeted gene was significantly down-regulated to 27.6% after 5 days, while it reduced to 57.6% after 10 days of treatment. Aphids' population being reared on transgenics was quite smaller as compared to the population on control, and this difference increased significantly after two weeks. The numbers of ecdysis decreased by 20% in aphids' population reared on the transgenics.

Bhatia and Bhattacharya, (2018) studied that seasonal photoperiodism and shifting of aphid' mode of reproduction from asexual to sexual depends on cuticular protein (CP) in aphids. Arabidopsis plants were transformed using gene fragment of CP in green peach aphid to initiate plant-mediated knockdown. *35S* promoter was used for expression while transformation was carried out using *Agrobacterium* tumefaciens strain GV 3101. Targeted siRNAs were produced by transgenics in the body of aphids being reared on them. Quantitative PCR analysis depicted decreased transcriptome level of *CP* gene in immature aphids fed with the transgenics. Significant decreased

expression level of *CP* gene resulted in noticeable decline (47%) of the aphid population on transgenic plants.

Zhu et al., (2012) established transgenic lines of tobacco plants to express dsRNA of 482 bp fragment of ecdysone receptor (*EcR*). It was used as diet for *Helicoverpa armigera* larvae, it produced molting default resulting larval death. Beet armyworm, *Spodoptera exigua* were also influenced when fed on transgenic lines expressing dsRNA designed for American bollworm. This happened because of larger conserved fragment of *EcR* gene of American bollworm, used to design dsRNA.

Zhang et al., (2015) produced transgenic potato plants expressing dsRNAs targeting actin genes in Colorado potato beetle. Transgenic plants expressing dsRNA silencing actin gene can result in mortality rate of 100% after a short period of feeding.

For the normal development in plants an appropriate RNAi pathway is necessary (Poethig et al., 2006). To increase the resistance against pest plants could be equipped with dsRNAs as the transgenic lines expressing dsRNAs for silencing particular insect genes showed exploitive effects on the gene expression and lead to death in American bollworm (Mao et al., 2007; Mao et al., 2011), the western corn rootworm and pea aphid, respectively (Pitino et al., 2011). As the transferred dsRNA in the transgenic plants is usually from a conserved sequence of target insects which restricts its non-target effects and shows its practical implementation in insect-proof crop development (Gordon et al., 2007; Mao et al., 2011).

2.10 Importance of Promoters in Transgenic Plants

All over the world, the transgenic crops utilize *CaMV 35S* promoter (Odell et al., 1985) to activate transgenes but now it is becoming clearer that this promotor is not appropriate for field since this promotor does not express results strongly as the ones achieved in glasshouse and laboratory studies. In future generations, it has been notified that transgenes activated by constitutive promoter obtained from viruses are silenced by a promoter methylation-driven post-transcriptional regulation (Mette et al., 2000; Elmayan and Vaucheret, 1996). Thus, in insect pests, resistance can be developed even in reduced expression of cry genes when these are activated by *35S* promoter (Stewart et al., 1996; Dutton et al., 2003).

For plant biotechnology, after viral promoters, the highly expressed plant genes have provided the second most common group of promoters (Potenza et al., 2004). Rubisco activase (*RCA*) is from AAA^+ family of the ATPases involved in multiple activities inside the cells (Portis, 2003) and this enzyme is localized in chloroplast and is associated with catalysing the activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). It is primary carboxylating enzyme involved in photosynthesis and it must be activated for the CO_2 fixation. In 1985, the concept of 'activase', which is a protein for the activation of Rubisco, was introduced first mainly based on both biochemical and the genetic studies of a high CO_2-demanding mutant of Arabidopsis (Salvucci et al., 1985). The occurrence and activation of Rubisco was shortly acknowledged after the enzyme was discovered, when stimulation of activity following pre-incubation with HCO_3 and Mg^{2+} was observed by (Pon et al., 1963).

It has been studied that *RCA* shows α- and β-isoforms in most plant species and these two forms share a smaller difference at C-terminus (Salvucci et al., 1987; Portis., 2003; Portis., et al., 2008). These forms are synthesized by alternative splicing but from same gene (Werneke et al., 1988; Pilgrim and McClung, 1993) or are formed by correlated genes (Salvucci et al., 2003). On the other hand, the β-isoform of *RCA* is expressed by only some plants like tobacco, maize, cucumber and bean (Portis., 2003). However, data collectively proved that only in aerial green tissues of plants, expression of *RCA* transcripts is observed and the light regulated (Rundle and Zielinski, 1991; Orozco and Ogren, 1993; Liu et al., 1996; Xu et al., 2010), which shows the organ-specific and light-inducible expression of *RCA* promoters.

Until now *RCA* promoters was found and analysed in spinach (Orozco and Ogren, 1993) and Arabidopsis (Liu et al., 1996). Both promoters are persistant with transcriptional pattern of *RCA* genes and are active only in green tissues of the plants. In this aspect, *RCA* promoter owing to their excellent potential can be utilized in the crop improvement (Qu et al., 2011). A 702 bp green tissue specific promoter of *Rubisco activase gene* (*pRCA*) from potato, has been successfully characterized by the group of Qu et al., (2011); it was planned that this promoter would drive the expression of *EcR*-dsRNA in this research work. Keeping in view the significance of *RCA* promoter regarding plant biotechnology, this research work was conducted to over-express dsRNA via *RCA* promoter targeting *EcR* gene.

2.11 Selected Target Gene

The efficiency of silencing target genes depends directly on the selection of target gene (s). If we are targeting a gene related to growth, development or survival of the insect like vATPase gene, higher mortality percentages can be achieved. While targeting a gene which have secondary effect on survival and/or it is a part of complexes of genes like detoxification related genes in which silencing of one gene doesn't affect the insect survival as this process is being carried out by a variety of genes, are not considerd promising (Zhu et al., 2011; Kaplanoglu et al., 2017).

EcR, a ligand-inducible nuclear transcription factor, is a member of nuclear receptor (NR) superfamily (Hopkins, 2009). Growth of insect larvae is puncuated by series of molts, necessary to allow expentıon of hardened cuticle as the larva become larger. These molts are initiated by surges of the steroid hormones ecdysone, which is converted into an active form 20-hydroxyecdysone (Sehnal, 1989). Growth, development, regeneration, molting, and reproduction in insects depend on ecdysteroids (LeBlanc, 2007). Activity of ecdyosteroids depends on the nuclear heterodimeric complex ecdysone receptor (*EcR*) (Clayton et al., 2001). The step-to-step transcription of genes is induced by the heterodimer complex activates the ecdysteroids, that ultimately direct molting, growth and development (Nakagawa and Henrich, 2009).

Metamorphosis and resulting molting process in insects is controlled by the steroid hormone named 20-hydroxyecdysone (20E) and ecdysone receptor (EcR) and the ultraspiracle (USP). Transcriptome involved in molting and metamorphosis is activated by the 20E-*EcR*-USP, a ligand-receptor complex. This transcriptome results from the transcription of the 20E related genes viz. Br-C, E74, E75, and E93 resulting in desirable changes in insects (Riddiford et al., 2000).

This present study has been designed to target/block highly specific 20-hydroxyecdysone hormone that majorly directs metamorphosis and molting in insects with the help of nuclear receptor complex. Targeting *EcR* would result in larval mortality/suppression when fed in transgenic material; hence a controlled insect-pest activity in potato will result in reducing crop demage incurred on potato due to CPB.

CHAPTER III

MATERIAL AND METHODS

To develop transgenic potato plants that can exhibit resistance against CPB by expressing *EcR*-dsRNA to silence the Ecdysone receptor gene (*EcR*) following material and methods were used.

3.1 Experimental Materials

3.1.1 Plant material

Two potato commercial varieties (Agria and Lady Olympia) were selected for the proposed study. These varieties have been selected on the basis of their agronomic characteristics and good yield potential in the area (Prof. Dr. Mehmet Emin Çaliskan, Personal communication).To propagate experimental explants tubers were taken from green house of Department of Agricultural Genetic Engineering, Faculty of Agricltural Sciences and Technologies, Nigde Ömer Halisdemir University. Sterilization of tubers was done by 4 to 5 times washing with tap water followed by washing with tween 20 (10%) for 5 minutes, 70% ethanol for 5 minutes and hydrogen peroxide (1%) for 1/2 minutes. Established shoot cultures from surface sterile tuber sprouts were multiplied in vitro by subculture of single node stem explants on the basal MS medium (Murashige and Skoog, 1962) broth (Appendix I). Cultures were then grown under controlled environment of growth chamber with conditions set as 25 ± 2°C, light/dark photoperiod of 16/8 hours, 47 µmol $m^{-2}s^{-1}$ irradiance and 58 W fluorescent tubes/lights in growth chambers.

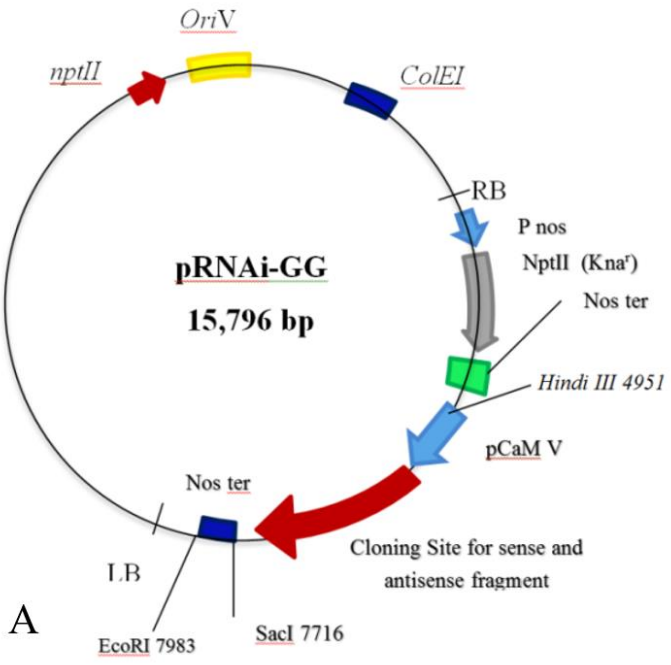
3.1.2 Bacterial material

In this study, plant expression vector (pRNAi-GG) was used to develop ihp RNAi constructs as described by Yan et al (2012) (Figure 3.1A). The propogation of pRNAi-GG was maintained in E.coli (DH5α and DB301) and *Agrobacterium* strain (LBA4404). For the transfer of gene *Agrobacterium tumefaciens* LBA4404 strain was used.

3.2 Description of pRNAi-GG Vector

pRNAi-GG vector was used in present study for developing required constructs in both orientations and were named as pRNAi-CPB and pRNAi-GFP later on. Earlier, pRNAi-GG plasmid was produced using T-DNA vector pBI121 as skeleton (Yan et al. 2012) (Figure 3.1A). Full length sequence and the detailed annotation of pRNAi-GG is available in GenBank (accession number JQ085427).

pRNAi-GG vector consists of a *ccdB* gene, *Pdk* intron, *ccdB* gene cassette between the duplicated *CaMV 35S* promoter and *Nos* terminator (Figure 3.1B). Two BsaI sites flank each *ccdB* gene and are positioned in such way that the recognition sites are eliminated from the vector after proper digestion and ligation of products. The cleavage site sequences of BsaI on the left of the first *ccdB* gene are the same to that on the right of the second *ccdB*, but with different orientation, and the same to the other two BsaI sites. Thus, a single PCR product can replace the two *ccdB* genes simultaneously at both sense and antisense orientations to form the two arms of the hairpin (Figure 3.1B). To make the ihpRNA construct, the target region of the gene of interest can be amplified with BsaI recognition sites flanked at both ends, and the cleavage site sequences (adaptor for pRNAi-GG) are complementary to the appropriate sequences on the vector.



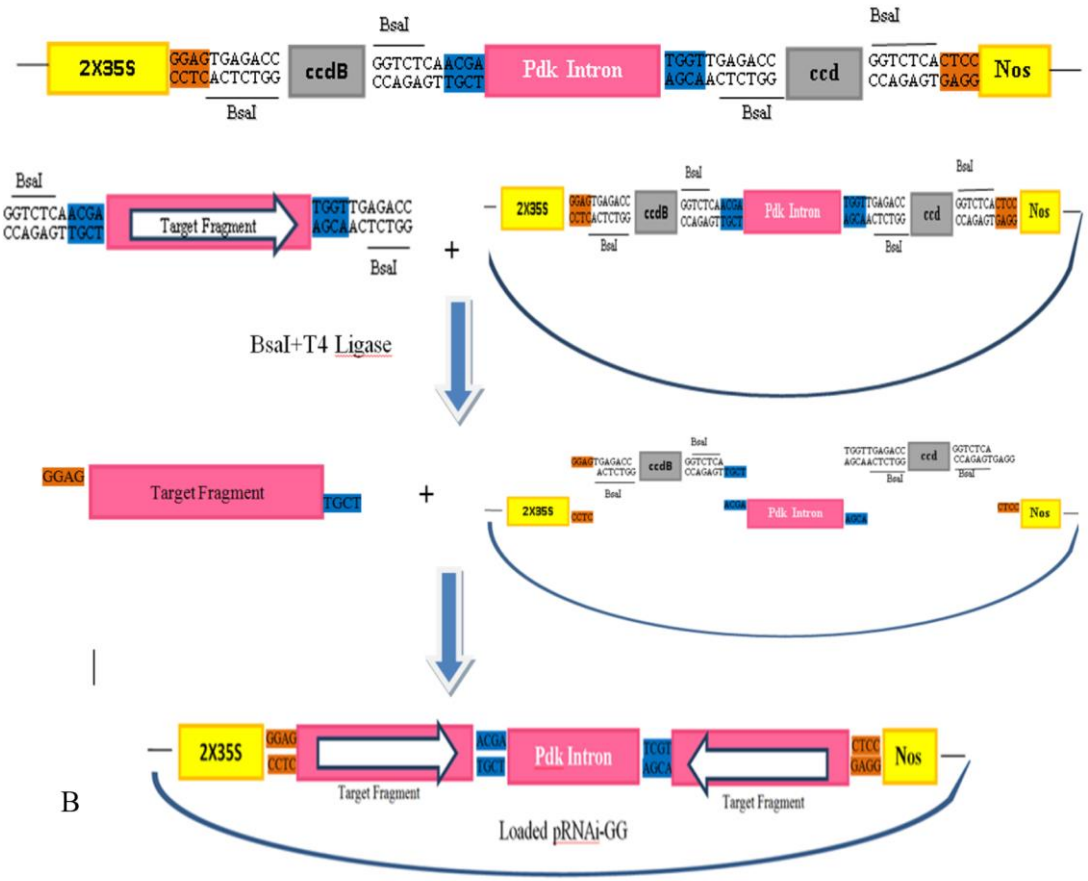


Figure 3.1. pBI121 modified pRNAi-GG constructs used as golden gate cloning. Sense and antisense fragments of a gene cloned in opposite orientations (A) pRNAi-GG contains *35S* promoters, two *ccdB* gene copies, a *pdk* intron, four recognition sites with designed adaptors (BsaI sites) (B). Intron contains chloramphenicol resistant gene. Amplification of gene fragments can be carried out via gene specific primers with BsaI sites and just in one restriction ligation reaction, thus ihpRNA can be constructed.

3.3 Construction of pRNAi-CPB and pRNAi-GFP Vector

For the construction of ihpRNAi vectors containing gene of interest following steps were followed.

3.3.1 Isolation and amplification of Partial cDNA of *EcR* gene of CPB

3.3.1.1 Total RNA extraction

For this purpose, five third instar larvae of Colorado potato beetle (CPB) were taken from entomology laboratory, Department of Plant production and Technologies, Nigde Ömer Halisdemir University and were subjected to RNA isolation using Omega RNA Extraction Kit (Cat. No. R6827-01). The larvae were properly grinded in liquid nitrogen using pre-cooled mortar and pestle and RNA protocol was carried out as per instruction given in manual.

3.3.1.2 Quantification of total RNA

To measure RNA concentrations, spectrophotometer was used (BioSpec-SHIMADZU) available in molecular laboratory.

3.3.1.3 cDNA Synthesis

Further, 2µg of CPB-RNA was used to prepare cDNA via Thermo scientific RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1621) using following reagents.

Table 3.1. Reaction mixture to synthesize cDNA

DNase treated RNA	2µg
Anchored oligo dT primers (20 µM)	1µL
DEPC-treated water	to 12µL

Incubation temperature for the reaction mixture was 70ºC for while the time was five minutes, followed by quick chilling on ice. Then 1µL of ribonuclease inhibitor (20µ/µL), 2µL of 10mM dNTP mix and 4µL of 5X reaction buffer was added and incubated at 37ºC for 5 min. At the end, 1µL of H minus M-MuLV reverse transcriptase (200µ/µL) was added and then mixture was incubated at 42 ºC for 60 min. The heat deactivation of reaction mixture was carried out at 70ºC for 10 min.

3.3.1.4 Polymerase chain reaction (PCR)

cDNA was diluted to 1:20 to be used as template for PCR reaction. Primers (Table 3.2) were designed to amplify 445 bp internal fragment of *EcR* gene (Accession No. AB211192; from 551-995 bp). Recognition sites of BsaI restriction enzyme were introduced in forward and reverse primers to follow golden gate way cloning strategy (Yan et al., 2012). Firstly, a gradient PCR was carried out to optimize annealing temperature of primers ranging from 55-70ºC. PCR was performed in the reaction volume of 20 µL containing cDNA template of 20ng, primers (forward and reverse) 50 pM each, dNTPs 100 µM, 1X PCR Buffer (1.5mM MgCl2, 50 mM KCl and 10mM Tris-HCl) and 1 unit Pfu Polymerase. The PCR conditions were set as 94ºC for 4 minutes as first step, 94ºC for 15 sec as 2nd step, following 55-70ºC for 15 sec and 72ºC for 30 sec (for 35 cycles). The amplified PCR fragments were determined by resolving on 1% agarose gel and observed under the UV light.

Table 3.2. Primer list used in the study. Primers sequence, annealing temp. and product size has been provided

Primer	**Sequence (5-3)**	**Ann. temp (^{o}C)**	**Product (bp)**
EcR-F	5'-ACA GGTCTCAGGAGGCATCACGCGAGGAATCTGA-3'	60	~445
EcR-R	5'-ACA GGTCTCATCGTTCCGTTTGACAGCGCATTG-3'	-----	-----
pRCA-F	5'-AAATCTAACCTCATATTAAAGTG-3'	44	~702
pRCA-R	5'-CATTCTTGATGACTAAAATTGATG-3'	-----	-----
P21	5'-ACCATTTACGAACGATAGCC-3'	55	
P22	5'-GTAAAACGACGGCCAGTG-3'	-----	
P24	5'-CATTTTAGCTTCCTTAGCTCC-3'	-----	
P25	5'-CATTTGGATTGATTACAGTTGG-3'	-----	
nptII-F	5'-TTGCTCCTGCCGAGAAAG-3'	55	~ 450
nptII-R	5'-GAAGGCGATAGAAGGCGA-3'	-------	
ChvA-F	5'-CGAAACGCTGTTCGGCCTGTGG-3'	65	~890
ChvA-R	5'-GTTCAGCAGGCCGGCATCCTGG-3'		
GFP-F	5'-ACAGGTCTCAGGAGCAGATCATATGAAGCGGCACG-3'	54	~390
GFP-R	5'-ACA GGTCTCATCGTAAGGGCAGATTGTGTGGAGA-3'	-----	

3.3.1.5 DNA fragment purification from agarose gel

The amplified partial fragment of *EcR* gene was excised from agarose gel and subjected to gel purification. Gel purification was carried out using ThermoScientific Gene JET Gel Extraction Kit (Cat. No. K0692) and concentration of eluted DNA was measured with nanodrop.

3.3.2 Amplification of Fragment of Green Fluorescent Protein (*GFP*) Gene

RNAi construct containing dsRNA-GFP was used as a control in experiments. Gene specific primers as shown in (Table 3.2) containing overhangs of Bsa1 enzyme were used to amplify 390 bp fragment of *GFP* using pBIN61-GFP plasmid as template. pBIN61-GFP plasmid was already available in Plant Transformation Laboratory, Department of Agricultural Genetic Engineering, Nigde Ömer Halisdemir University. PCR was conducted using conditions as described earlier in 3.3.1.4 with annealing temperature of 54 ^{o}C. No non-specific amplification was also observed. Pfu polymerase with proof reading ability was used in all PCR reactions.

3.4 Construct Development

To make ihpRNA constructs using pRNAi-GG vector a single PCR product is required for cloning the gene of interest by golden gate cloning in both orietnations as described earlier. PCR products (*EcR*-CBP and *GFP*) amplified by gene specific primers with overhangs of Bsa1 were used to perform single step restriction ligation reaction. The reaction to make ihpRNA constructs was set as mentioned in (Table 3.3)

Table 3.3. Reaction mixture to construct ihpRNA

Purified PCR product	50 ng
pRNAi-GG vector	200 ng
BsaI enzyme (NEB)	5 units
T4 DNA ligase (Promega high concentration ligase 20 u/µl)	10 units
Ligation buffer (Promega)	1X

The restriction ligation was incubated at 37^{0}C for three hours, followed by the incubation for 5 minutes at 50^{0}C (final digestion) and 5 minutes incubation at 80^{0}C

(heat inactivation). The ihpRNA constructs for silencing were produced by this restriction ligation reaction and were named as pRNAi-CPB and pRNAi-GFP.

3.4.1 Ligation confirmation of *GFP* and CPB

The orientation of sense and antisense ligated fragments were confirmed by PCR, using vector specific primers P21 and P24 to confirm insertion on one side and P25 and P22 for confirmation of gene insertion on other side of intron. The ligation showed bands by resolving on agarose gel as shown in (Figure 3.2).

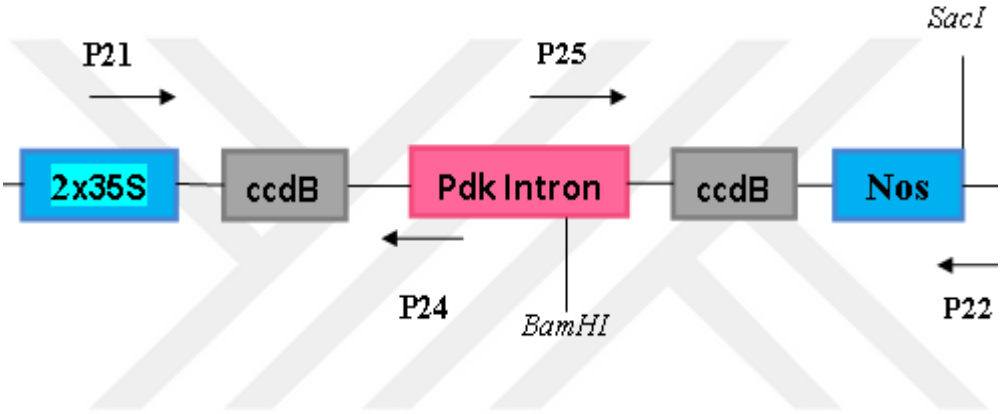


Figure 3.2. The position of restriction sites and the primers on pRNAi-GG utilized for identification of the recombinant pRNAi-GG vector. Insert reverse primers P21 and P22 were designed for identification of the recombinant pRNAi-GG.

3.4.2 Transformation of constructs to *E.coli*

Transformation of PCR confirmed ligated mixture into *E.coli* (DH5α or DB3.1) cells was performed using standard protocol of transformation. Competent cells stored at -80°C were thawed on the ice for approximately 20-30 mins. Meanwhile plates of LB medium with 25 mg L^{-1} kanamycin and 5 mg L^{-1} of chloramphenicol were removed from the storage at 4ºC and were allowed to 25-28ºC. 3 µl of ligation product (normally 10 pg - 100 ng) was mixed into eppendorf containing 50 µL of competent cells and tapped gently. Then the competent cells or DNA mixture was incubated on ice for 30 mins. Afterwards, heat shock was performed by placing tube into 42ºC water bath for about 30-60 secs and tubes were placed back on the ice for about 2 min. 500 µl of LB or SOC media (with no antibiotics) was added to the bacteria and grew at 37ºC in shaking incubator for 45-60 min. Transformation was spread on plate containing LB agar with

25 mg L^{-1} kanamycin and 5 mg L^{-1} chloramphenicol and were Incubated at 37ºC overnight.

3.4.3 *E.coli* Colony confirmation of pRNAi-GFP and pRNAi-CPB

Next day, several colonies were observed on LB agar plate with appropriate antibiotics. Some colonies were selected randomly and subjected to colony PCR using plasmid back bone specific primer i.e P21-P24. PCR reaction was carried out in the volume of 20 µL containing a E.coli colony, primers (forward and reverse) 1 uM each, dNTPs of 0.2 µM, 1X PCR Buffer, $MgCl_2$ 1mM, and 1 unit Pfu Polymerase. The PCR was done at 94ºC for 4 minutes, 94ºC for 15 sec, 55-70^{o}C for 15 sec and 72ºC for 30 sec followed by 34 times. The positive colonies were inoculated in LB broth medium containing the 25 mg L^{-1} kanamycin and 5 mg L^{-1} of chloramphenicol and incubated at 37^{0}C overnight using gently shaking. Next day, plasmid extractions were done using ThermoScientific GeneJET plasmid Miniprep Kit (Cat. No. K0503).

3.4.4 Restriction Digestion conformation of the pRNAi-GG, pRNAi-CPB and pRNAi-GFP

The positive clones from PCR were further confirmed by the restriction digestion analysis with *BamHI* and SacI. Reaction of restriction digestion was set as shown in (Table 3.4).

Table 3.4. Restriction digestion reaction

DNA to be digested	1ug
10X Fast digest green buffer	2ul
BamHI (1u/ul)	1ul
SwaI (10u/ul)	0.1ul
H2O	To make volume up to 20 ul

Reaction samples were kept at 37°C for about 2 hours. The results were then analyzed by resolving digested samples on the 1% agarose gel.

3.5 Electroporation of pRNAi-CPB and pRNAi-GFP in *Agrobacterium* Strain

Electroporation of newly developed constructs was performed in *Agrobacterium* competent cells of LBA4404. Competent cells of LBA4404 were mixed with 2 μL of plasmid (10-20 ng) and were placed on ice. The electroporation device Bio-Rad (# 165-2660) was set at capacitance of 25 μF, voltage 2.2 kV with 200 ohms resistance capacity. After electroporation, 500ul to 1mL of SOC solution was added and then incubated at 28ºC for 2-3 hours at 200 rpm. Cultures were spread on LB plates containing medium with 25 mg L^{-1} kanamycin and 5 mg L^{-1} of chloramphenicol for selection of the transformed cells. These plates were then kept for 24-48 hours at 28ºC. The positive clones were confirmed performing colony PCR using P21-P24 primers.

Glycerol stock preparation

Cultures were prepared from the positive colonies in LB broth containing appropriate antibiotics until reached to optimal optical density. Glycerol stocks of *Agrobacterium* LBA4404 harboring pRNAi-CPB and control pRNAi-GFP were prepared adding 500 ul of overnight cultured bacteria to 500 ul of 50% glycerol and stored at -80^{0}C for future use.

3.6 Construction of pRNAi-GG Vector with Tissue Specific Promoter (*pRCA*)

One of the objectives of study was to express RNAi construct under control of Rubisco activase green tissue specific promoter (*pRCA*). For this purpose, *35S CaMV* promoters was excised from pRNAi-GG using *HindIII* and *SwaI* and replaced with *pRCA*. Following steps were involved to fulfill this task.

- Isolation of *pRCA* promoter from Potato Cv. Lady Olympia
- Digestion of pRNAi-GG with *HindIII* and *SwaI*
- Ligation of digested pRNAi-GG and isolated *pRCA* promoter

3.6.1 Isolation and amplification of *pRCA* promoter from Potato

The leaves of the Lady Olympia cultivated *in vitro* were excised and grinded in liquid nitrogen. DNA isolation was performed from the samples using the Thermo scientific Genomic DNA Extraction Kit (Cat No. K 0512). For PCR amplification of the *pRCA*

promoter region (702 bp), region-specific primers having *HindIII* and *SwaI* cleavage regions were used at their ends. PCR conditions used were similar as mention in 3.3.1.4

3.6.2 Digestion of pRNAi-GG with *HindIII* and *SwaI*

pRNAi-GG plasmid was excised with *HindIII* and *SwaI* restriction enzymes to remove *35S* promoter from plasmid. *SwaI* digests pRNAi-GG downstream to 2X*35S* promoter.

3.6.3 Ligation of digested pRNAi-GG and isolated *pRCA* promoter

HindIII-SwaI digested pRNAi-GG plasmid was excised from gel and was subjected to DNA purification kit (Cat. K0692). The predigested insert (*RCA*) and vector (pRNAi-GG) were subjected to ligation reaction. Ligation reaction was as follow:

- Insert (Promoter *RCA*) 2 µl (15 ng)
- Plasmid (pRNAi-GG 1 µl (100 ng)
- 10X Ligase Buffer 1.0 ul
- T4 DNA Ligase (3U/µl)
- H_2O until volume to make 10ul reaction

Ligation reaction was incubated at 4°C for overnight for further procedure.

3.6.4 Transformation to *E.coli*

The ligated product was transformed in DB3.1 strain of E.coli following the protocol of transformation as mention in 3.4.2

3.6.5 *E.coli* Colony conformation

Following day, colonies were screened using colony PCR. Standard colony PCR reaction was followed to screen colonies as mentioned in 3.4.3

3.7 Construction of pRCA-pRNAi-CBP Expression Vector

As described earlier in 3.7 the *35S* promoter was successfully excised from pRNAi-GG construct using *HindIII* and *SwaI* restriction enzymes and replaced it with green tissue specific promoter *pRCA* (Figure 3.3).

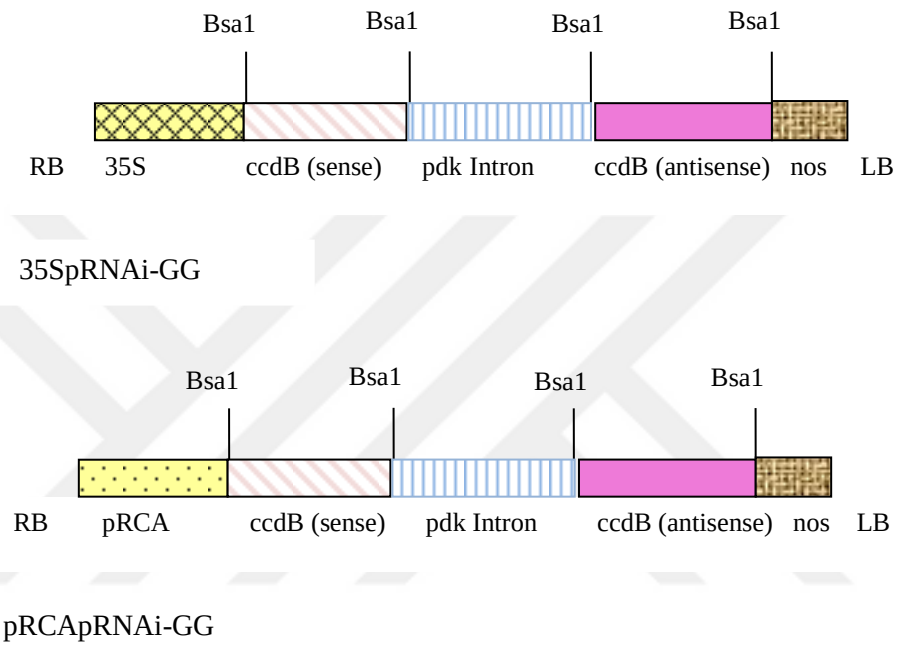


Figure 3.3. Schematic representation of pRNAi-GG under the control of *35S* and *pRCA* promoters

In order to construct, pRCA-pRNAiCPB vector, following steps were followed:

3.7.1 Cleavage of 35spRNAi-CPB with *HindIII* and *SwaI*

The *35S* promoter was removed using *HindIII* and *SwaI* restriction enzymes from pRNAi-CPB (already developed) plasmid. *SwaI* digests pRNAi-GG downstream to 2X*35S* promoter 3.7.2.

3.7.2 Purification from Agarose gel

The pRNAi-CPB plasmid digested by *HindIII-SwaI* was resolved on 1% agarose gel at 80V for 90 min and was analyzed under the UV-light. The gel slices were excised using

sterile blade and DNA was isolated and purified from the gel using DNA extraction kit (Cat. K0692). Gel purified pre-digested *pRCA* promoter fragment and pRNAi-CPB were run on 1% agarose gel to check quality and quantity of fragment prior to ligation reaction.

3.7.3 Ligation reaction

The semi-truncated *RCA* promoter and digested pRNAi-CPB vector were subjected to ligation reaction. Ligation reaction mixture was set as mention in table given. The ligation reaction was incubated overnight at 4 °C.

Table 3.5. Ligation reaction mixture

Contents	Quantity
Amplified (Promotor *RCA*)	2 µl (20 ng)
Plasmid (pRNAi-GG)	1 µl (100 ng)
10X (Ligase buffer)	1.0 µl
T4 DNA Ligase (3U/µl)	1.0 µl
H2O	Until the volume to complete 10 µl

3.7.4 Transformation to *E.coli*

The ligation product was transformed into JM109 E. coli strain.

3.7.5 *E.coli* colony confirmation of pRCApRNAi-CPB

Using promoter specific primers, colonies were screened by colony PCR. In this context, standard colony PCR method was applied.

3.7.6 Preparation and confirmation of clones

Couple of colonies were found positive (Figure 4.14). Two colonies with sharp bands were inoculated with LB broth supplemented with appropriate antibiotic (kanamycin 25 mg L^{-1}). Plasmid extraction was done 3.4.4

3.7.7 Restriction digestion conformation

Extracted Plasmid from positive clones was further subject to restriction digestion with *HindIII* and *SwaI*. Reaction mixture used is given in (Table 3.6).

Table 3. 6. Restriction digestion mixture

DNA to be digested	1ug
10X Fast digest green buffer	2ul
BamHI (1u/ul)	1ul
SwaI (10u/ul)	0.1ul
H2O	To make volume upto 20 ul

Reaction samples were kept at 37°C for about 2 hours. The results were then observed by resolving digested samples on the 1% agarose gel.

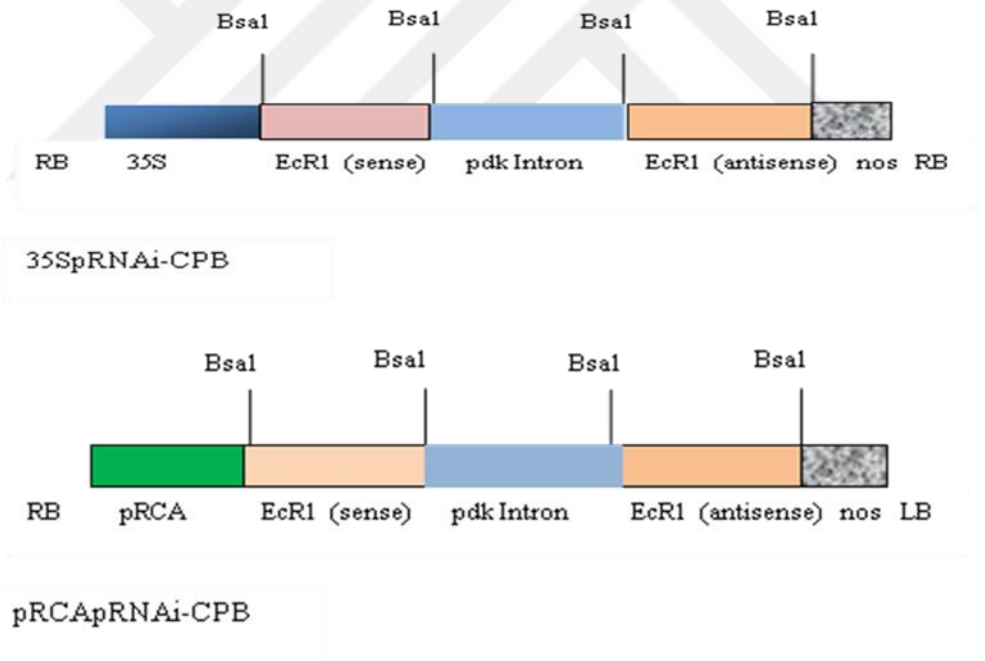


Figure 3.4. Schematic representation of pRNAi-CPB under the control of *35S* and *pRCA* promoter

3.7.8 Electroporation

Electroporation of pRCApRNAi-CPB was done with same protocol as described for the electroporation of 35SpRNAi-CPB and 35SpRNAi-GFP

3.8 Transformation of Potato Cultivars with 35SpRNAi-CPB, 35SpRNAi-GFP and pRCA-pRNAi-CPB and pRNAi (as mock) Constructs

3.8.1 Preparation of plant material

Prior to transformation selected varieties were established as mention in 3.1.1.

3.8.2 Explant propagation

Explants were propagated as mentioned in 3.1.1. Leaf discs and internode were used for the transformation.

3.8.3 *Agrobacterium* culture preparation

The glycerol stocks of *Agrobacterium* strain LBA4404 harboring RNAi constructs were streaked on Luria-Bertani Agar (LB Agar) (Appendix-II) plates having kanamycin 50 mg L^{-1} , chloramphenicol 5 mg L^{-1} and Rifampicin 100 mg L^{-1}. one colony of each construct from plate was inoculated in 10 ml of Luria-Bertani broth (LB broth) (Appendix-II) along with mentioned appropriate antibiotics and prepared cultures were incubated overnight in thermos-shaker at 28C.

3.8.4 Co-cultivation Medium

Potato cultivars were subjected to *Agrobacterium* mediated genetic transformation using Leaf discs as well as internodal (4-6 mm) parts as explants. *Agrobacterium* strain containing targeted constructs (35SpRNAi-CPB, 35SpRNAi-GFP and pRCApRNAi-CPB) were inoculated with explants for 30-40 minutes with mid shaking in liquid medium having *Agrobacterium* suspension (O.D 0.6) without antibiotic followed by incubation on cocultivation medium for three days approximately. Co-cultivation medium contained MS salts including 3% sucrose, 8 g/l agar and 100 uM acetosyringone. pH of the medium was set to 5.5 – 5.8. Co-cultivation was carried out in growth chamber using standard cultural conditions at 16/8-hour day / night, 47 μmol / m2 / s light intensity and 25 ± 2 ° C temperature.

3.8.5 Regeneration selection medium (RSM)

After cocultivation, the explants were washed with antibiotic (Ducid or Sulcid) for 5-10 min and blotted dry on sterile filter paper or tissue paper and cultured on Callus Inducing Medium (MS supplemented with BAP 2 mg L^{-1}, NAA 0.2 mg L^{-1}, kinetin 1mg L^{-1}, Trans-Zeatin 2 mg L^{-1}, Sulcid 500 mg L^{-1} (to suppress the growth of *Agrobacterium*) and Kanamycin 100 mg L^{-1} (for plant selection).

3.8.6 Calculation of callus induction and shoots

Average number of callus induction and shoot regeneration was calculated as per given formula:

$$\frac{\text{Number of callus/ total shoots obtained}}{\text{Total number of Ex-plants/No of calli}} \times 100$$

3.8.7 Transfer to shoot/Root medium

With well-developed calli, explants were sub-cultured to shoot induction medium (MS supplemented with BAP 1 mg L^{-1}, GA3 0.1 mg L^{-1}, kanamycin 100 mg L^{-1}, Sulcid 500 mg L^{-1}. Green shoots (1-2 cm) were cut out and shifted to larger glass jars having root inducing medium (MS supplemented with NAA 0.1 mg L^{-1}, Sulcid 500 mg L^{-1} and Kanamycin 100 mg L^{-1}).

3.8.8 Acclimatization

The putative transgenic plantlets with well-developed roots and size were shifted into pots having soil mixture of perlite, peat and vermiculite (1:1:1 v/v/v) and were first shifted to growth chamber for acclimitizatio (25 ± 2°C, light/dark photoperiod of 16/8 hours, 47 µmol $m^{-2}s^{-1}$ irradiance and 58 W fluorescent tubes/lights) and then were transferred in the green house [25 ± 1°C (day) and 20 ± 1°C (night) and 50% humidity] to set seeds.

3.9 Molecular Analysis of Primary Transformants

3.9.1 DNA extraction and PCR Assays

The putative transgenic plants developed out of Agria and Lady Olymia were first acclimatized in growth chamber. A total of 300 plants (175 Lady Olympia and 125 Agria) were acclimatized. The leaf samples were collected and subjected to various molecular analyses for ectopic gene integration and expression in host genome. DNA extractions were carried out using Thermoscientific plant genomic DNA extraction kit (Cat.No.0722).

PCR analysis of transgenic plants was conducted with difference primers to confirm presence of *EcR* gene fragment in sense orientation (P21-P24 primer), antisense orientation (P22-P25), gene specific primers (*EcR* gene and *GFP*), and plant selectable marker gene (*nptII*) in host genome. PCR using *ChvA* gene-specific primers for *Agrobacterium* contamination was also done. (Figure 3.5) represents the primers location within T-DNA region.

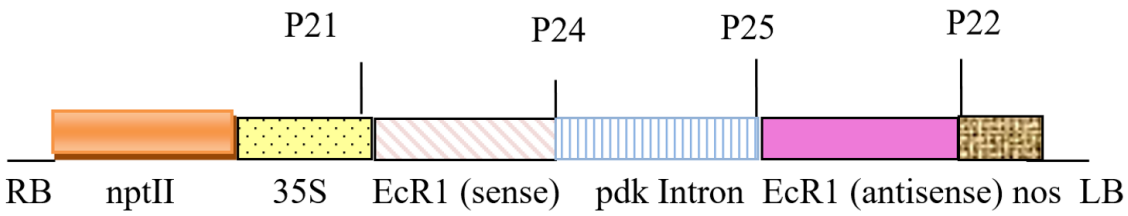


Figure 3.5. Location of primers in T-DNA region

3.9.2 Gel electrophoresis

Gel electrophoresis was done using preparing 1% agarose gel and 0.5x TBE buffer (Appendix-II). Ethidium bromide with concentration of 0.5-1ug/mL was added. Gel was detremined at 80-100V for 30-45 min.

3.9.3 Calculation of Transformation Efficiency

Transformation efficiency was calculated, after compilation of all data. For this purpose, Mean Transformation Efficiency (MTE) was calculates by counting number of the transgenic plants regenerated (nTPR) in total number of all explants used (tnEU). Calculations were done by using these calculated values in formula:

$$MTE\ (\%) = (nTPR / tnEU) \times 100$$

3.10 Southern Blot Analysis

Southern blot was carried out according to modified method of Southern (1975) with genomic DNA samples isolated from positive transgenic plant leaves and non-transgenic control plants. DNA extraction were carried out using Thermoscientific plant genomic DNA extraction kit (Cat. No. 0722). Approximately 10µg DNA of transgenic plant samples were digested with *HindIII* and *BamH1* to release fragment of *EcR* gene from 35SpRNAi-CPB plasmid. The digested DNA samples were resolved in 1% agarose gel using 1X TBE buffer at 20V for 10h.

After running, gel was denatured with mixture of 1.5M NaCl and 0.5M NaOH for about 30 minutes and depurination was done using 1.5M HCl for 10 minutes. Then neutralization of gel was performed with the 0.5M Tris-HCl and 1.5M NaCl with pH 7.5. Digested samples of DNA were then transferred to the Hybond-N+ Nylon Membrane (Amersham) with sol. 0f 20X SSC (Appendix-III) by the capillary action. DNA was fixed on the membrane by exposing membrane to UV light for about 5 minutes.

Membrane was placed in hybridization tube along with pre-hybridization solution and was treated for an hour at 65ºC. Hybridization with the probe was done for 18 hours. User guide of Fermentas Biotin Chromogenic Detection Kit (Cat# K0661) was used for the detection. 1X blocking/washing buffer (30ml) was used to wash membrane with moderate shaking. Washing was followed by dipping the membrane in blocking solution with moderate shaking, for 30 minutes. After removing the blocking solution properly, membrane was saturated with 20 µL of diluted streptavidin AP conjugate followed by washing with 1X blocking/washing buffer. Afterwards, washing buffer was

removed and detection buffer (30ml) was added for 30 minutes. Finally, 1X NBT/BCIP was prepared and dissolved in detection buffer to carry out enzymatic reaction which needs incubation of membrane in 20 ml of 1X NBT/BCIP.

3.11 Probe Labeling

Labeling of probe for detection of *EcR* gene was done using Thermo scientific Biotin DecaLabel DNA labeling kit (Cat #K0651). According to instructions given in manual 20ul of template total100ng, 10ul of decanucleotides in the 5X reaction buffer along with nuclease free water up to 40ul in the PCR tube. Tube was then vortexed to spun down. PCR tube was set and incubated in the PCR machine at 100°C for 5-10 min and chilled down quickly on the ice. After spinning the mixture was transferred to eppendorf tube. To the eppendorf 5ul Biotin Labeling mix and 1ul Klenew fragment 5U was added. Tube was spun down with all added contents, and was incubated for 2 to 5 hours at 37°C. 1ul of 0.5M EDTA with pH 8.0 was used to stop reaction. The labeled probe estimation was done by spotting that on the nitrocellulose membrane with the DNA labelled as control (from kit) in sequential dilutions then detection was performed using Thermos Scientific Biotin Chromogenic Detection Kit (Cat# K0661).

3.12 Real-Time Quantitative PCR (qRT-PCR)

Total RNA used was extracted from PCR positive transgenic plants using AMRESCO RiboZol™ RNA Extraction Reagent. Furthermore, 1µg of RNA was used to convert into cDNA following protocol as described in Fermentas cDNA synthesis kit (Cat. No. 1621). qRT-PCR contents 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 were 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 was determined

according to the 2-ΔΔCt* proportional calculation method (Livak and Schmittgen, 2001).

3.13 Lab Bio-toxicity Assays

Leaf feeding bioassay method was used to evaluate whether resistance to targeted insect pest has been developed in transgenic potato plants expressing *EcR*-dsRNA under control of both promoters, larvae of Colorado potato beetle were released randomly on plucked mature leaves to feed on. Also, fresh leaves from primary transformants were taken in petri plates with moist filter paper and pre-starved 2^{nd} or 3^{rd} instar larvae of targeted insect pests were fed on them. After three to four days of feeding, data was collected whether the plants expressing *EcR* dsRNA exhibited resistance against the insects in comparison with control (Transformed plants expressing *GFP*/dsRNA and non-transformed plants). Mortality rate of CPB larvae were noted according to the formula as follows:

$$\%\text{Mortality} = (\text{No. of dead larvae} / \text{Total no. of larvae}) \times 100$$

3.13.1 Analysis of fold change in weight

Effect of the transgenic plants on CPB larvae (1^{st}, 2^{nd}, 3^{rd}) was analyzed. Five larvae were placed on the plates having leaves of different transgenic plants along with control after taking initial weight of them. Petri plates were then placed in the growth chamber with temperature 28±1, humidity 60±5 and 16/8 h day/night period. Plates were parafilmed to avoid the escape of larvae. After three days of feeding larvae were recollected and weighed again. Increase in weight was recorded by using formula:
Fold change = (Final weight-initial weight)/Initial weight

3.13.2 Evaluation of *EcR* transcripts of CPB fed on transgenic plants

It was hypothesized that molting defects in targeted pest larvae will be resulted from the suppression of its *EcR* mRNA level after feeding on leaves of the transgenic potato plants expressing *EcR*-dsRNA. To counter check the hypothesis, *EcR* mRNA level in insects larvae was measured by quantitative real-time PCR (qRT-PCR). For this purpose, collection of larvae was done to perform extraction of total RNA after feeding

on leaves of transgenic potato plants, expressing the dsRNA for designated time period for qRT-PCR analysis. Total RNA was extracted by an efficient RNA isolation kit according to instructions of manufacturer. First stranded cDNA, was made from the RNA primed by oligo dT using MMLV reverse transcriptase. Furthermore, qRT-PCR was performed in 20 ul volume by using SYBR Green Master Mix and then analysis of data was done using Real-Time PCR Detection System. Quantitative real time data was also be collected to investigate activity of both promoters.

CHAPTER IV

RESULTS

4.1 Cloning of *EcR* and *GFP* gene Fragment in pRNAi-GG

For the cloning purpose amplification of *EcR* gene of CPB and *GFP* was done using cDNA and Plasmid as template.

4.1.1 Amplification of *EcR* gene of CPB

Total RNA from CPB larvae was isolated, converted to cDNA and was used as template to amplify partial fragment of *EcR* of CPB. Gene specific primer containing overhangs of BsaI site were used to amplify desired *EcR* fragment of gene. Required band of 445 bp was amplified and obtained in all the samples.

Amplification of gene was done at 60 °C as Initial gradient PCR results showed that a temperature of 60^{o}C worked better for amplification of *EcR* fragment. So, another PCR was done to amplify target fragment (Figure 4.1). Amplified product was eluted from gel by Thermo Scientific Gene JET Gel Extraction Kit (Cat. No. K0692). Size and quality of estimation of eluted DNA was performed by running 2ul of eluted product on 1% of gel at 90V for about 30 min. Eluted fragment was further used for cloning purpose.

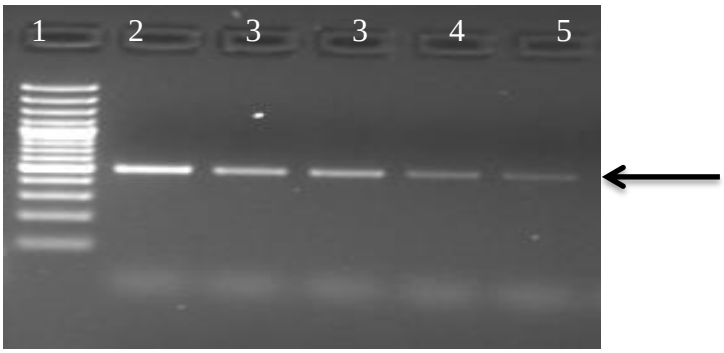


Figure 4.1. Amplification of partial cDNA fragment of *EcR* gene of CPB. Lane 1: 100 bp plus ladder thermo scientific, Lane 2-6: 445 bp amplified fragment from cDNA samples of CPB

4.1.2 Amplification of fragment of *GFP*

RNAi construct containing dsRNA/*GFP* was planned to be used as a control in experiments. Gene specific primers containing overhangs of Bsa1 enzyme were used to amplify 390 bp fragment of *GFP* using pBIN61-GFP plasmid as template. PCR was performed with annealing temperature of 54 ^{o}C (Figure 4.2). No specific amplification was also observed. Pfu polymerase with proof reading ability was used in all PCR reactions. The amplified band was cut from the gel and elution of DNA was done using Thermo Scientific Gene JET Gel Extraction Kit (Cat. No. K0692). Size and quality of Eluted DNA was assessed by resolving 2ul of eluted product on 1% of gel at 90V for about 30 min. Eluted fragment was further used for cloning purpose.

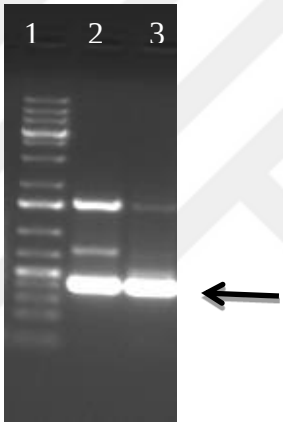


Figure 4.2. Amplification of *GFP* gene fragment using pBIN61-GFP plasmid as template. Lane 1: 1 Kb plus ladder thermo scientific, Lane 2-3: 390 bp amplified fragment of *GFP* gene.

4.1.3 Construction of pRNAi-CPB and pRNAi-GFP

Golden Gate (GG) cloning approach was used to make ihpRNA constructs using pRNAi-GG vector. For silencing the gene of interest a single product of PCR is required for this purpose. PCR products (*EcR*-CBP and *GFP*) amplified by gene specific primers with overhangs of Bsa1 were used to perform single step restriction ligation reaction. Gel eluted amplified fragments were used and ligation was preceded. Prior to ligation concentration of both the fragments and vector were determined. The vector to insert ratio was considered as 1:1.

4.1.4 Ligation confirmation of *GFP* and CPB

The sense and antisense orientation of ligated fragments were confirmed with the ligation mixture using vector specific primes P21 and P24 to confirm insertion on one side and P25 and P22 for confirmation of gene insertion on other side of intron (Figure 4.3). The ligation showed estimated bands with agarose gel electrophoresis as shown in (Figure 4.4).

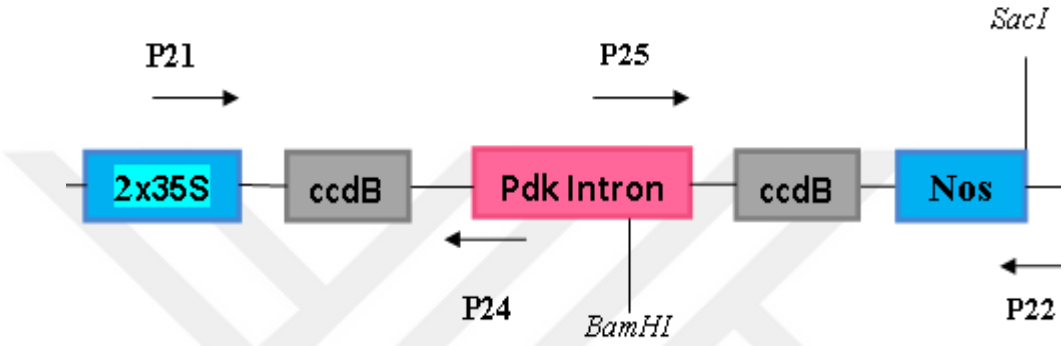


Figure 4.3. Position of restriction sites and primers on pRNAi-GG utilized to identify recombinant pRNAi-GG vector.

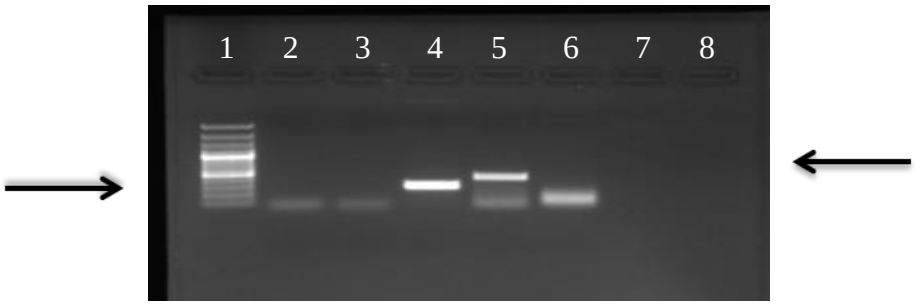


Figure 4.4. PCR confirmation of *EcR* fragment using ligated product as template with P21 and P24 primers. Lane 1: 100 bp plus ladder (Thermo scientific), Lane 2-4: amplified *GFP*, Lane 5-7: amplified CPB. Lane 4: showing ~390 bp amplified *GFP* sense fragment and lane 5 showing ~445 bp amplified *EcR* sense fragment of CPB were marked as positives.

4.1.5 Transformation and colony confirmation of *E.coli*

The ligated product was then transformed to E. coli (DH5α or DB3.1) competent cells with the standard protocol of transformation. The positive clones of pRNAi-GFP and

pRNAi-CBP were confirmed by colony PCR using P21-24 primer along with positive control (pRNAi-GG).

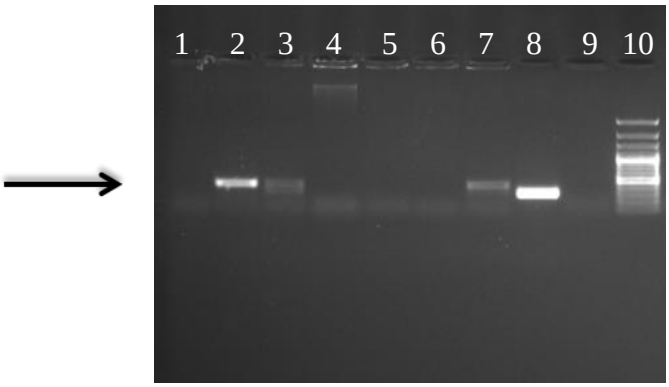


Figure 4.5. Colony PCR showing amplification of *GFP* fragment. Lane 1-7: ~390 bp amplified *GFP* sense fragment, clones 2, 3 and 7 were marked as positive, Lane 8: pRNAi-GG vector control showing amplification of ccdB gene (~300 bp), Lane 9: Negative control and Lane 10: 100 bp plus ladder (Thermo scientific).

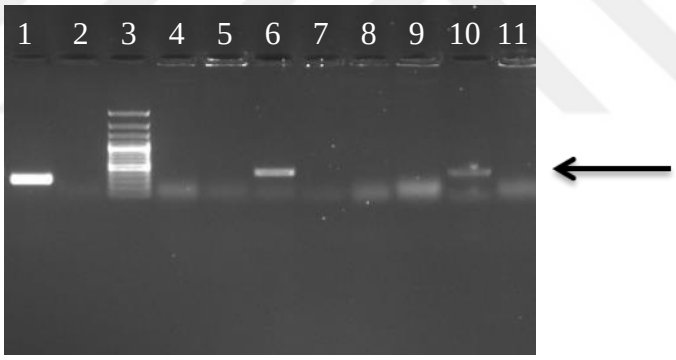


Figure 4.6. Colony PCR showing amplification of fragment of *EcR* gene of CPB. Lane 1: pRNAi-GG vector control showing amplification of ccdB gene (~300 bp), Lane 2: Negative control, Lane 3: 100 bp plus ladder (Thermo scientific), Lane 4-11: ~445 bp amplified *EcR* sense fragment of CPB, clone 6 and 10 were marked as positive.

4.1.6 Further confirmation of clones (pRNAi-CPB and pRNAi-GFP) in pRNAi-GG

Clones were further confirmed using gene specific primers of CPB and *GFP*. (Figure 4.7) shows the amplification of *EcR* fragments of CPB (A), and *GFP* (B) from pRNAi-CPB and pRNAi-GFP plasmids using gene specific primers.

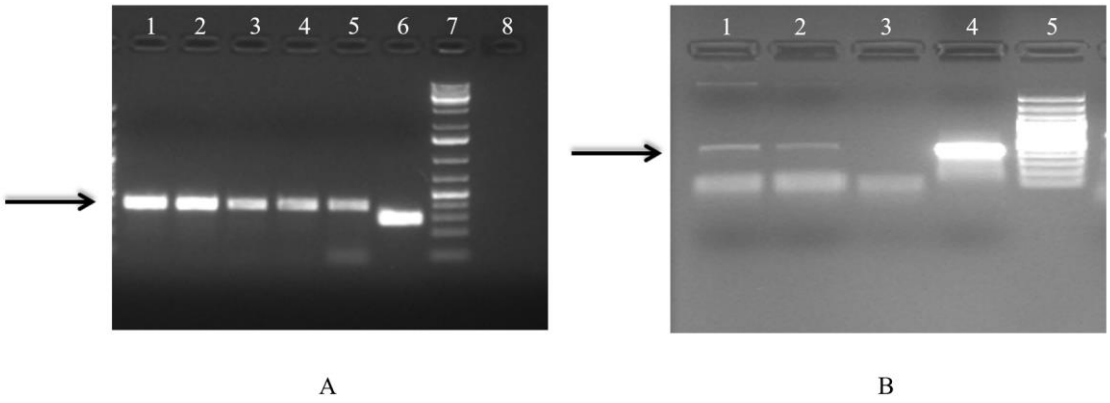


Figure 4.7. Amplification of *EcR* (445) and *GFP* (390) fragments from pRNAi-CPB and pRNAi-GFP with gene specific primers, (A) Lane 1-5: positive clones of pRNAi-CPB, Lane 6: Positive control, Gel eluted fragment of CPB-*EcR* gene, Lane 7: 100 bp plus ladder (Thermo scientific), (B) Lane 1-2: positive clones of pRNAi-GFP, Lane 3: Negative control, Lane 4: Positive control, gel eluted fragment of *GFP*.

4.1.7 Antisense clone confirmation

The clone was subjected to PCR with P25 and P22 primers as well to confirm antisense fragments. (Figure 4.8) shows the amplification of *EcR* fragment in reverse orientation, additional base pairs show DNA bases amplified from NOS terminator (~264 bp), also some spacer sequences (~313) from plasmids before ccdB gene. The control pRNAi-GG showed a band of ~960 bp as expected using online NCBI information (JQ085427.1) while our cloned showed relatively lesser band of ~760 bp (445 bp of *EcR* fragment+41 bp spacer+264 nos fragment).

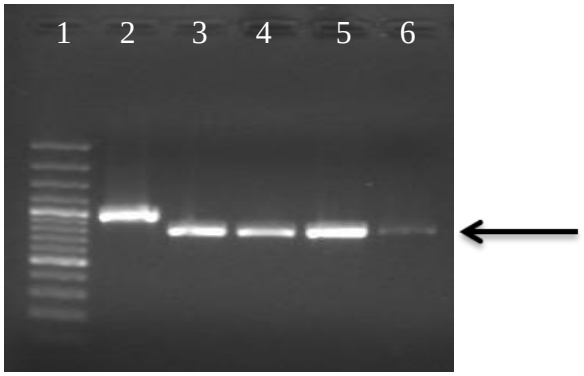


Figure 4.8. Amplification of antisense *EcR* fragment from pRNAi-CPB using P25 and P22 primers, Lane 1: 100 bp plus ladder (Thermo scientific), Lane 2: control pRNAi-GG, Lane 3-6: pRNAi-CPB.

4.1.8 Restriction Digestion conformation of the pRNAi-GG, pRNAi-CPB and pRNAi-GFP

The clones were further confirmed by restriction digestion analysis with *BamHI* and SacI. *BamHI* digests pRNAi-GG plasmid at 2554 base whereas SacI cuts at 3804 bases of plasmid. As expected ~1.250 kb was excised from control while pRNAi-CPB and pRNAi-GFP yielded less fragment (~1,050 bp) (Figure 4.9).

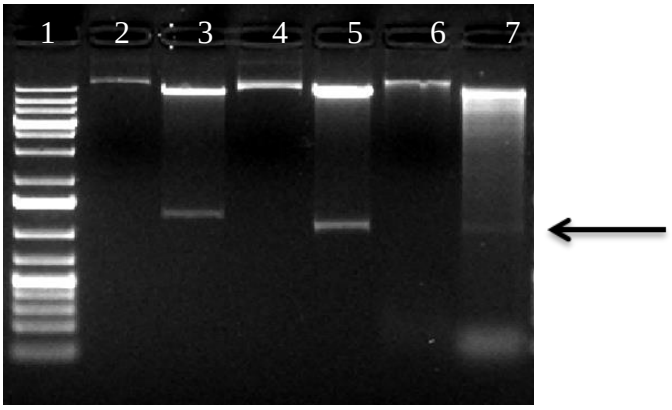


Figure 4.9. Restriction analysis of pRNAi-GG, pRNAi-CPB and pRNAi-GFP. Lane 1: 1 kb plus ladder, Lane 2: undigested pRNAi-GG, Lane 3: BamH1-SacI digested pRNAi-GG, Lane 4: undigested pRNAi-CBP, Lane 5: digested pRNAi-CPB, Lane 6: undigested pRNAi-GFP, Lane 7: digested pRNAi-GFP

4.1.9 Electroporation and colony confirmation of *Agrobacterium*

Electroporation of newly developed plasmids in LBA4404 competent cells of *Agrobacterium* was performed. After electroporation and incubation culture was spread on LB plates containing appropriate antibiotics. Transformed cells were incubated for 24-48 hours at 28ºC. Cultures were then prepared from the positive colonies in LB broth containing appropriate antibiotics until reached to optimal optical density. The positive clones were confirmed doing colony PCR using P21-P24 primers.

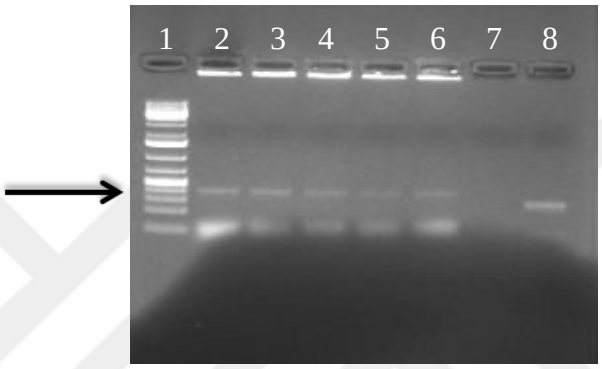


Figure 4.10. Colony PCR showing amplification of fragment of *EcR* gene of CPB. Lane 1: 1 kb plus ladder (Thermo scientific), Lane 2-6: ~445 bp amplified *EcR* sense fragment of CPB, Lane 7: Negative control, Lane 8: pRNAi-GG vector control showing amplification of ccdB gene (~300 bp), all clones were marked as positive.

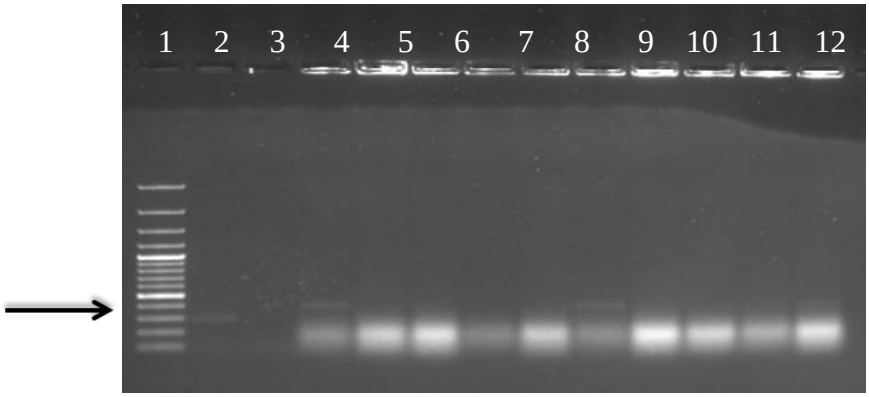


Figure 4.11. Colony PCR showing amplification of *GFP* fragment with p21 and p24 primers. Lane 1: 100 bp plus ladder (Thermo scientific), Lane 2: pRNAi-GG vector control showing amplification of ccdB gene (~300 bp), Lane 3: Negative control, Lane 4-13: ~390 bp amplified *GFP* sense fragment, clones 4, 6 and 13 were marked as positive**.**

Glycerol stocks of *Agrobacterium* LBA4404 harboring pRNAi-CPB, and control pRNAi-GFP were prepared and stored at -80^{0}C for future use.

4.2 Construction of pRNAi-GG Vector with Tissue Specific Promoter (*pRCA*)

To express RNAi construct under control of Rubisco activase green tissue specific promoter (*pRCA*), amplification of *pRCA* promoter was done from Potato Cv. Lady Olympia and *35S CaMV* promoters was excised from pRNAi-GG using *HindIII* and *SwaI* and replaced with amplified *pRCA*. Digestion of pRNAi-GG with *HindIII* and *SwaI* was done and ligation of digested pRNAi-GG with isolated *pRCA* promoter was performed.

4.2.1 Amplification and gel elution of *pRCA* promoter

For the amplification of *pRCA* promoter a gradient PCR at higher temperature (55-70^{o}C) with specific primers (Table 3.2) did not yield required band. A PCR reaction at lower temperature 44^{o}C resulted in required band of (702 bp) with no any nonspecific amplification with Pfu polymerase (Figure 4.12). The amplified product was eluted from gel by Thermo Scientific Gene JET Gel Extraction Kit (Cat. No. K0692). Size and quality of Eluted DNA was estimated by running the 2ul of eluted product on 1% of gel at 90V for about 30 min.

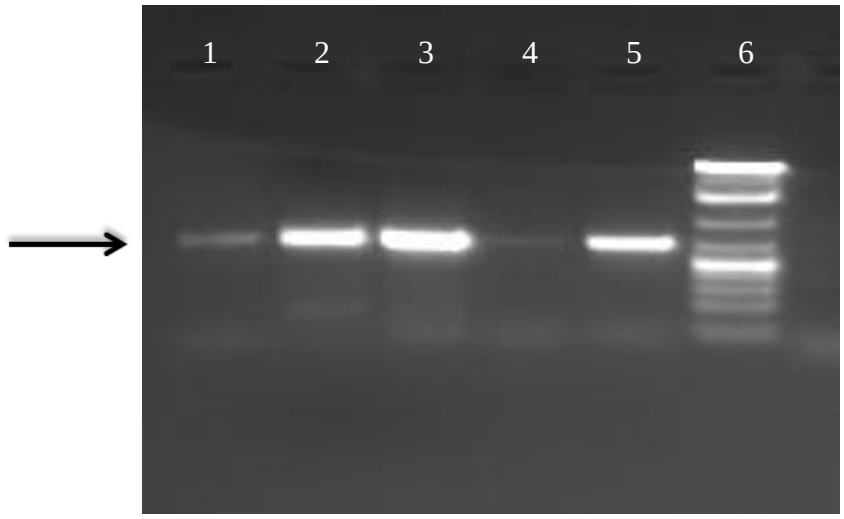


Figure 4.12. The amplification of *pRCA* fragment from Lady Olympia cultivar. Lane 1-5: 702 bp amplified *pRCA* fragment from DNA samples of Lady Olympia. Lane 6: 1 Kb plus ladder thermo scientific

4.2.2 Digestion of pRNAi-GG with *HindIII* and *SwaI*

pRNAi-GG plasmid was digested with *HindIII* and *SwaI* restriction enzymes to remove *35S* promoter from plasmid. *SwaI* digests pRNAi-GG downstream to 2X35S promoter**.** An expected band of ~832 bp (662 bp of *35S* promoter+ remaining spacer sequences from plasmid upstream to ccdB gene) was released from plasmid.

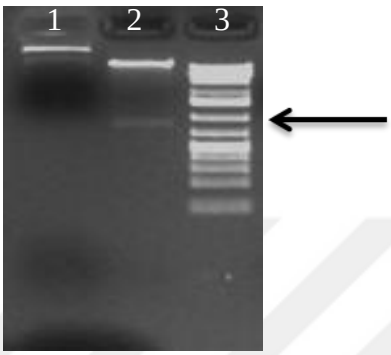


Figure 4.13. Restriction analysis of pRNAi-GG with *HindIII* and *SwaI*. Lane 1: undigested pRNAi-GG, Lane 2: digested pRNAi-GG, Lane 3: 1 Kb plus ladder thermo scientific**.**

4.2.3 Ligation of digested pRNAi-GG and isolated *pRCA* promoter

HindIII-SwaI digested pRNAi-GG plasmid was excised from gel and was subjected to DNA purification kit (Cat. K0692). The predigested insert (*RCA*) and vector (pRNAi-GG) were subjected to ligation reaction. The ligated product was transformed to DB3.1 strain of E.coli. Following day, colonies were screened using colony PCR. Standard colony PCR reaction was followed to screen colonies.

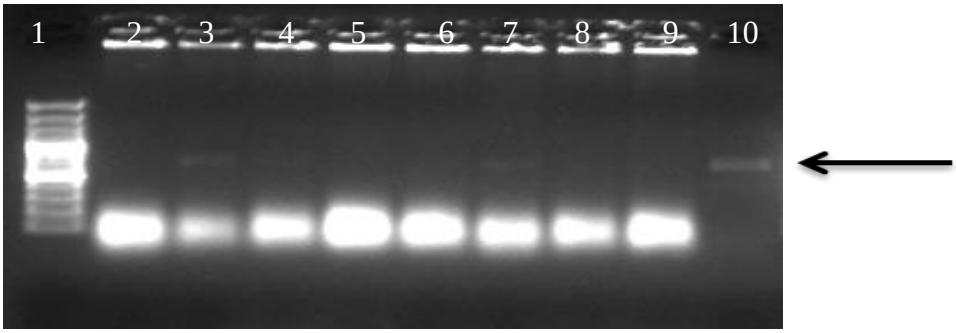
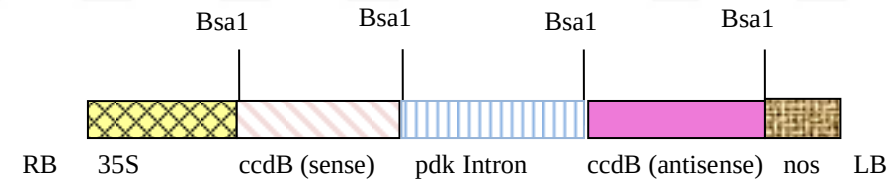


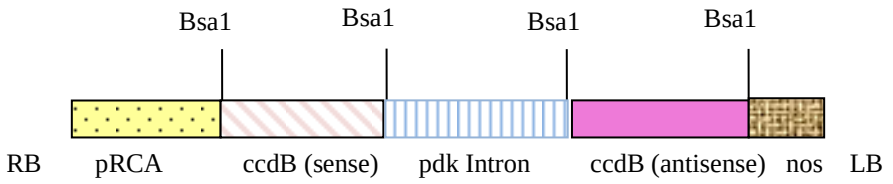
Figure 4.14. Colony PCR to screen correct clone of pRNAi-GG with *pRCA* promoter using gene specific primer of promoter. Lane 1: 100 bp plus ladder (Thermo), Lane 2-9: tested colonies, Lane 10: Positive control, gel eluted *pRCA* fragment.

4.3 Construction of pRCA-pRNAi-CBP Expression Vector

As described in 3.7.2, *35S* promoter was successfully excised from pRNAi-GG construct using *HindIII* and *SwaI* restriction enzymes and replaced it with green tissue specific promoter *pRCA* (Figure 4.15)



35SpRNAi-GG



pRCApRNAi-GG

Figure 4.15. Schematic representation of pRNAi-GG under the control of *35S* and *pRCA* promoters

4.3.1 Cleavage of 35spRNAi-CPB with *HindIII* and *SwaI*

The *35S* promoter was removed using *HindIII* and *SwaI* restriction enzymes from pRNAi-CPB (already developed) plasmid. *SwaI* cuts the pRNAi-GG plasmid from the bottom of the 2X*35S* promoter region (Figure 4.13). As shown in the (Figure 4.16), the expected band at ~ 832 bp (662 bp fragment of the *35S* promoter + spacer sequences at the top of the ccdB gene) was removed from the plasmid.

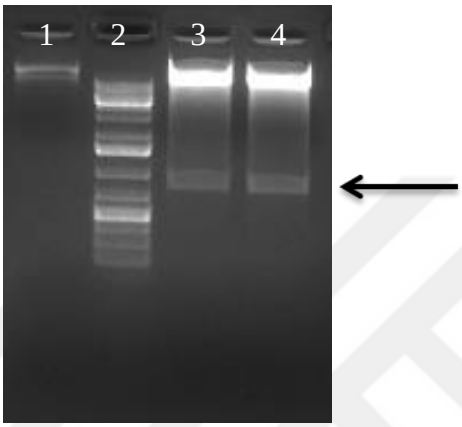


Figure 4.16. Analysis of pRNAi-CPB plasmid by *HindIII* and *SwaI* enzymes. Lane No. 1: undigested pRNAi-CPB, Lane No. 2: 1 Kb + marker (thermo scientific), Lane No. 3-4: truncated pRNAi-CPB plasmid.

4.3.2 Conformation of amplified *pRCA* and digested 35spRNAi-CPB

The pRNAi-CPB plasmid digested by *HindIII-SwaI* was isolated from gel and purified by DNA purification kit (Cat. K0692). Gel purified pre-digested *pRCA* promoter fragment and pRNAi-CPB were run on 1% agarose gel to check quality and quantity of fragment prior to ligation reaction.

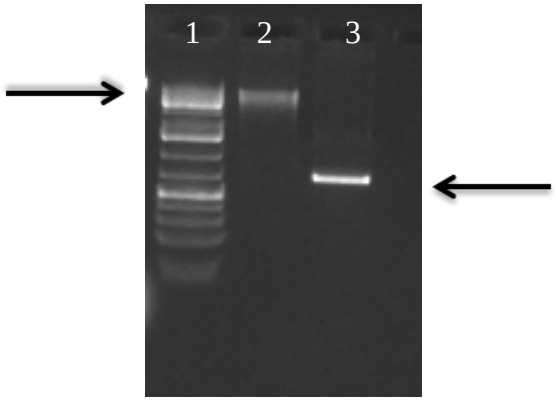


Figure 4.17. *HindIII* and *SwaI* digested plasmid and promoter fragment prior to ligation. Lane No. 1: 1 Kb + marker (thermo scientific), Lane no.2: *HindIII* and *SwaI* digested gel purified 35SpRNAi-CPB, Lane NO. 3: *HindIII* and *SwaI* digested gel purified *pRCA* fragment.

4.3.3 Ligation of digested pRNAi-CPB and isolated *pRCA* promoter

The semi-truncated *RCA* promoter and pRNAi-CPB vector were subjected to ligation reaction. The ligation reaction was kept overnight at 4°C. The ligation product was transformed into JM109 E. coli strain. Using promoter specific primers, colonies were screened by colony PCR. Standard colony PCR method was followed. Few number of colonies were found positive (Figure 4.18). Two colonies with sharp bands were inoculated with the LB broth supplemented with appropriate antibiotic (kanamycin 25 mg L^{-1}). Plasmid extracted from two clones was further subject to restriction digestion with *HindIII* and *SwaI* and required bands were digested (Figure 4.19)

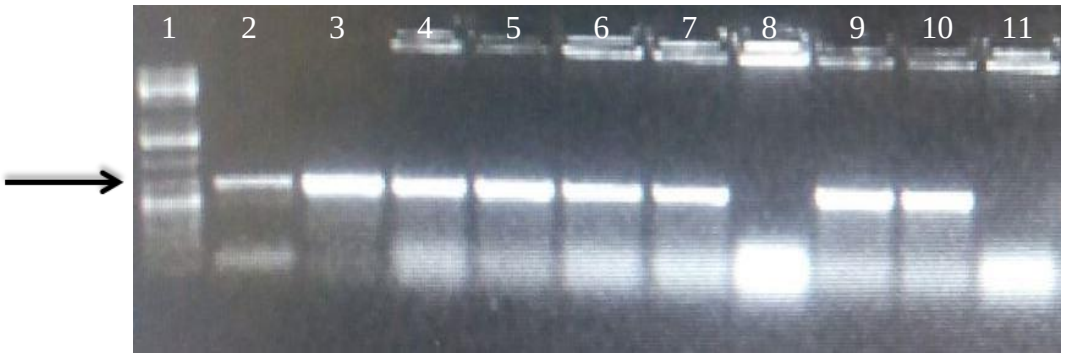


Figure 4.18. Colony PCR results showing *pRCA* promoter fragment. Lane no. 1: 1 kb + DNA marker (Thermo scientific), Lane No. 2-3: pRCA-pRNAi-GG vector control, Lane no. 4-10: test columns.

4.3.4 Digestion analysis of pRCApRNAi-CPB plasmid

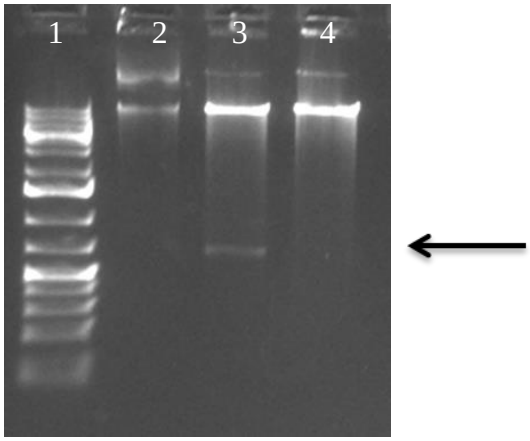


Figure 4.19. Digestion analysis of pRCApRNAi-CPB plasmid with *HindIII* and *SwaI* enzymes. Lane No. 1: 1 Kb + marker (thermo scientific), Lane No. 2: unreacted pRCApRNAi-CPB, Lane No. 3-4: truncated pRCApRNAi-CPB plasmid

4.4 Electroporation and Colony Confirmation of *Agrobacterium*

The positive clone was electroporated in *Agrobacterium* strain LBA4404 cells. Colonies were further screened using *pRCA* primers in colony PCR. The glycerol stocks were then prepared and stored at -80°C for further use.

The schematic representation of both constructs is shown below:

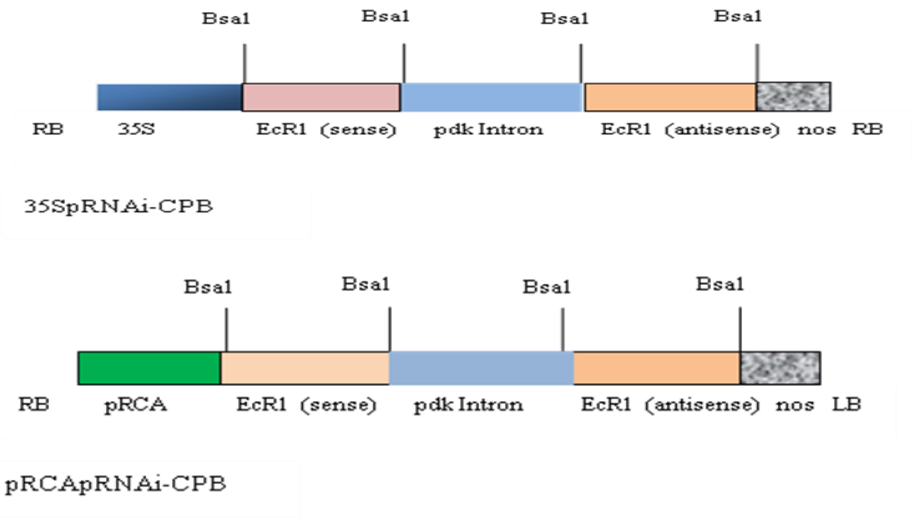


Figure 4.20. Schematic representation of pRNAi-CPB under the control of *35S* and *pRCA* promoter.

4.5 Transformation of Potato Cultivars with 35SpRNAi-CPB, 35SpRNAi-GFP and pRCApRNAi-CPB and pRNAi (as mock) Constructs

4.5.1 Explant propagation

Surface sterilization of tubers was done for the further propagation of explants. Leaf discs and internode were used for the transformation, internode being used as efficient explants with more regeneration efficiency.

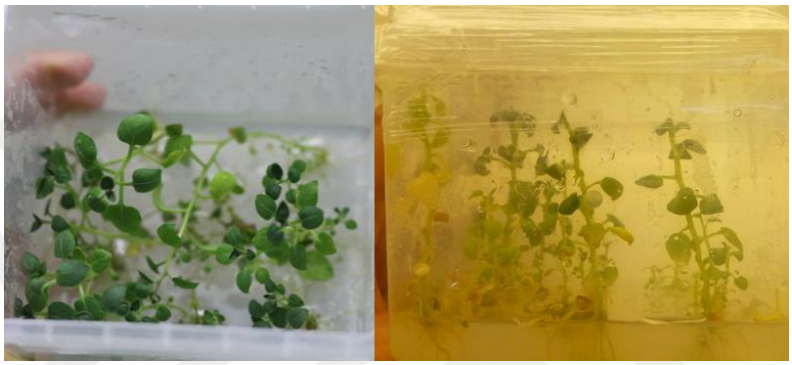


Figure 4.21. *In-vitro* grown plants for transformation experiments

4.5.2 Inoculation and Co-Cultivation with *Agrobacterium* Suspension

Leaf discs and internode explants were subjected to inoculation with the overnight grown *Agrobacterium* culture suspension.

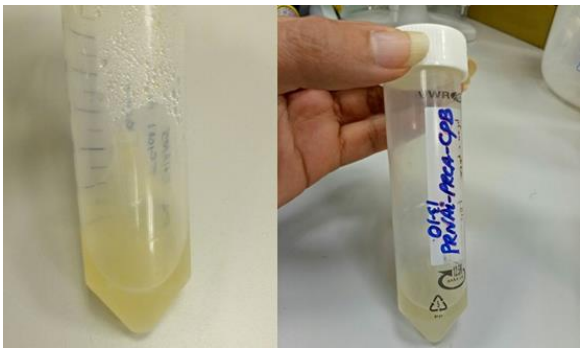


Figure 4.22. Overnight grown *Agrobacterium* (LBA4404) culture

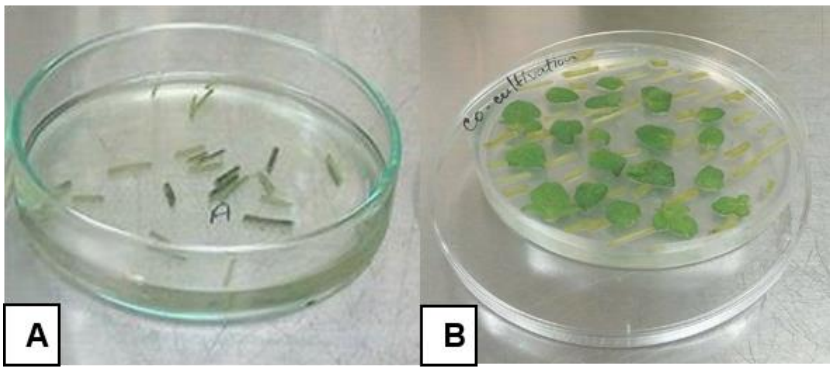


Figure 4.23. Inoculation of explants with *Agrobacterium* (LBA4404) suspension (A), Explants on co-cultivation medium after inoculation (B)

4.5.3 Cultivars Response on the Regeneration Selection Media

After the transformation, plants were kept in the growth chamber on regeneration selection medium with selectable markers and appropriate antibiotics to get callus from the transferred plant cells. For each transformation three replications were made having 20 explants in each replication, out of 20 explants 10 leaves and 10 intenodes were used. Total 60 explants were used for each experiment. Simililar procedure was followed for each construct. Total 1500 explats of 35SpRNAi-CPB, 1200 of pRCApRNAi-CPB, 1000 0f pRNAi-GG and 1000 of 35SpRNAi-GFP were used.

GFP plants of lady Olympia were used. Similar number of explants were used for each construct in case of Agria plants. Callus induction was observed after 3-4 weeks and average callus induction was calculated as 69.73% for 35SpRNAi-CPB, 65.2% of pRCApRNAi-CPB, 59.73 of pRNAi-GG and 75.1% of 35SpRNAi-GFP for Lady Olympia and 65.40 of 35SpRNAi-CPB, 60% of pRCApRNAi-CPB, 55.4% 0f pRNAi-GG and 69.23 of 35SpRNAi-GFP for Agria. Average number of shoots per explant were also calculated and 2-4 number of shoots were obtained from each explant for both varieties as shown in (Table 4.1 and Table 4 .2).

Transformed cells started to show callus induction after 3-4 weeks of inoculation. After 2-3 weeks of callus induction embryogenic growth was shown by explants. From those embryonic cells there was shoot formation and transgenic candidate shoots were counted. In the plant cells that did not produce callus, darkening was observed after 1-2 weeks of inoculation and it was determined that callus could not be formed.

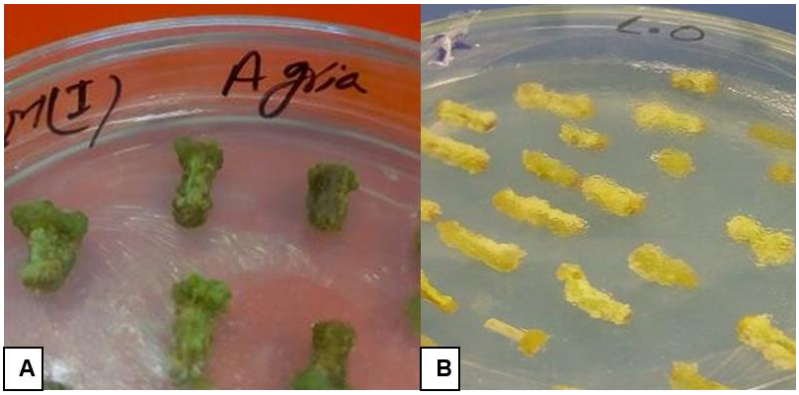


Figure 4.24. Callus induction after 3-4 weeks on RSM, Agria (A), Lady Olympia (B)

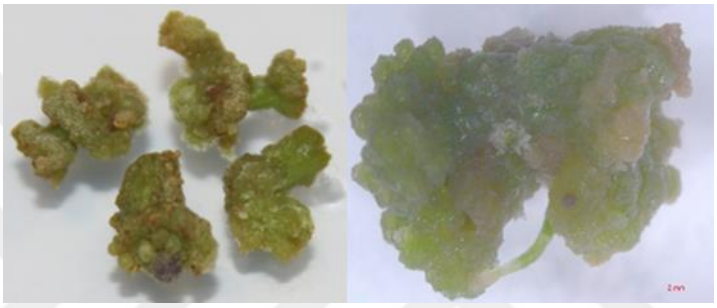


Figure 4.25. Embryogenic induction in callus

4.5.4 Transfer to shoot/Root medium

Shoots from transformed callus were sub-cultured to shoot induction medium then after 3 weeks well-developed shoots were transferred to the root induction medium.

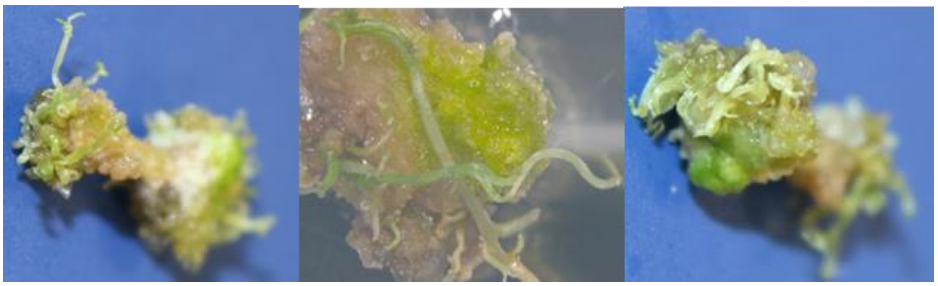


Figure 4.26. Shoot regeneration of Agria and Lady Olympia from Embryos on shoot medium

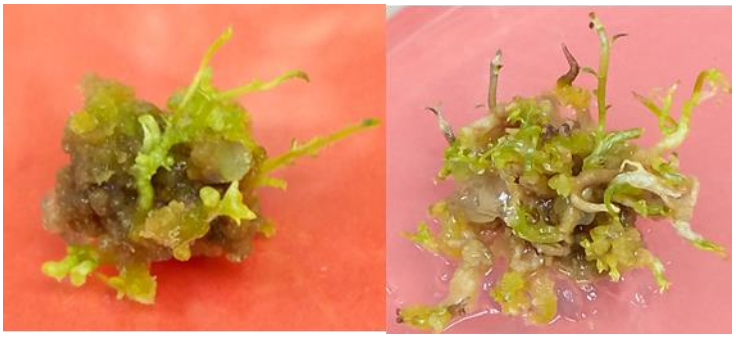


Figure 4.27. Ready regenerated shoots of Agria and Lady Olympia from callus to transfer to root medium

Figure 4.28. Regeneration of in-vitro putative transgenic plants of Agria and Lady Olympia on root medium

4.6 Acclimatization

A total of 300 plants were acclimatized out of which 175 plants of Lady Olympia (60, 50, 45and 20 of 35SpRNAi-CPB, pRCApRNAi-CPB, pRNAi-GG and 35SpRNAi-GFP respectively) and 125 plants of Agria (50, 30, 25 and 20 of 35SpRNAi-CPB, pRCApRNAi-CPB, pRNAi-GG and 35SpRNAi-GFP respectively) were shifted to green house as mentioned in (Table 4. 4). The putative transgenic plants developed out of Agria and Lady Olymia were first acclimatized by transferring in soil in growth chamber. Then plantlets with well-developed roots and size were transferred and grown in the greenhouse environment gradually. The transgenic candidate plants that were transferred to the pot were frequently followed and observed to grow well by meeting the need for irrigation.

Figure 4.29. Overview of the putative transgenic plants of Agria and Lady Olympia plants in growth chamber

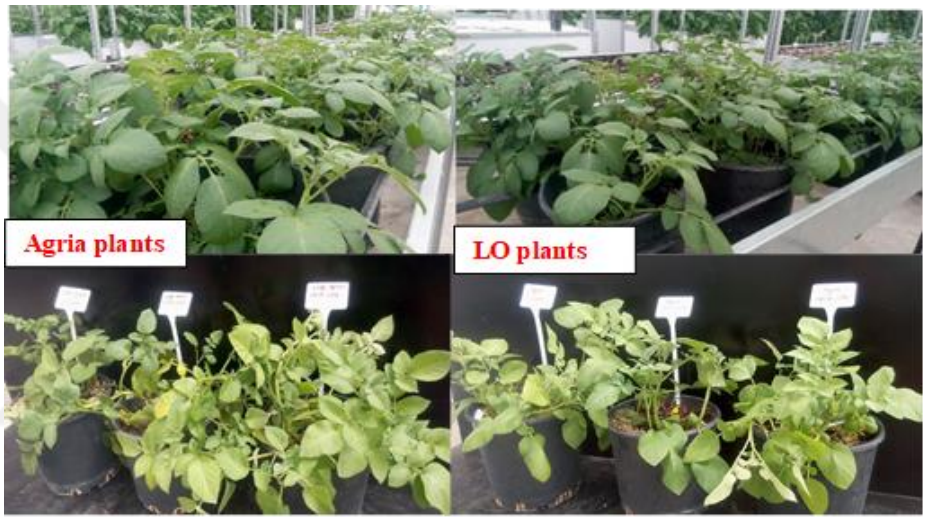


Figure 4.30. Well acclimatized primary transformants of Agria and Lady Olymia in green house

4.7 Transformation Data

The data of transformed plantlets for all constructs of both varieties Lady Olympia and Agria is shown in (Table 4.1 and Table 4.2) below.

Table 4.1. Transformation data 35SpRNAi-CPB and pRCApRNAi-CPB

Cultivars	35SpRNAi-CPB			pRCApRNAi-CPB		
	No. of Explants	Callus formation %	Average number of shoots / explants	No. of Explants	Callus Formation (%)	Average number of shoots / explants
Lady Olympia	1500	69.73	2.20	1200	65.2	3.5
Agria	1500	65.40	2.77	1200	60.0	2.90

Table 4.2. Transformation data pRNAi-GG and 35SpRNAi-GFP

Cultivars	pRNAi-GG			35SpRNAi-GFP		
	No. of Explants	Callus formation %	Average number of shoots / explants	No. of Explants	Callus Formation (%)	Average number of shoots / explants
Lady Olympia	1000	59.73	3.20	1000	75.1	1.95
Agria	1000	55.40	3.77	1000	69.23	1.80

4.8 Molecular Analysis of Primary Transformants

4.8.1 DNA extraction and PCR Assays

A total of 300 plants (175 Lady Olympia and 125 Agria) were acclimatized. The leaf samples were collected and subjected to various molecular analysis for ectopic gene integration and expression in host genome. DNA extractions were carried out using Thermo scientific plant genomic DNA extraction kit (Cat. No. 0722). Extracted DNA concentration was checked on 1% gel.

PCR analysis of transgene plants was conducted with different primers to confirm presence of *EcR* gene in sense orientation (P21-P24 primer), antisense orientation (P22-P25), gene specific primers (*EcR* gene and *GFP*), plant selectable marker gene (*nptII*) in host genome. **(**Figure 4.31) represents the primers location within T-DNA region. The information of primer sequence, annealing temperature and product size has been provided in (Table 3.2).

P21-24 and P25-22 showed required bands in sense and antisense orientation (Figure 4.32 and 4.33) While amplification with gene specific primers (*EcR* and *GFP*) and *nptII* further confirmed results (Figure 4.34-4.36). Only the plants positive for both orientations along with marker genes were selected and subjected to further assays. Candidate transgenic plants were analyzed by PCR using *ChvA* gene specific primers for *Agrobacterium* contamination (Figure 4.37). The plants that showed positive amplification with *ChvA* gene were discarded and not considered for further studies.

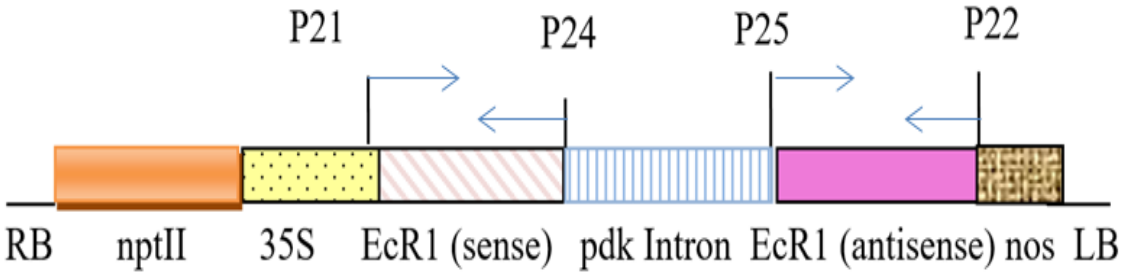


Figure 4.31. Schematic representation of T-DNA region of 35SpRNAi-CPB plasmid and location of different primers to confirm orientation of insert.

4.8.2 PCR confirmation of pRNAi-CPB

The positive transgenics were confirmed by PCR using P21-P24 primers along with positive control (pRNAi-GG).

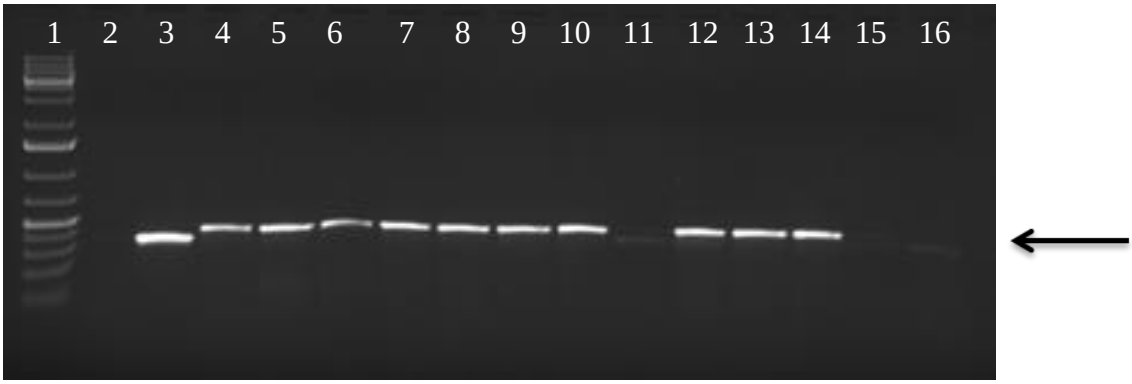


Figure 4.32. Amplification of sense orientation of *EcR* fragment in putative transgenic plants using P21 and P24 primers. Lane no1: 1 kb plus DNA marker (Thermo scientific), Lane no 2: Negative control, Lane no 3: pRNAi-GG control, Lane no. 4-9: Lady olympia Transformants, Lane no 10-16: Agria transformants.

DNA from transgenics were subjected to PCR with P25 and P22 primers as well to confirm antisense fragments. The (Figure 4.33) shows the amplification of both *EcR* fragments in reverse orientation, additional base pairs show DNA bases amplified from NOS terminator (~264 bp), also some spacer sequences (~313) from plasmids before ccdB gene. The control pRNAi-GG showed a band of ~960 bp as expected using online NCBI information (JQ085427.1) while our cloned showed relatively lesser band of ~760 bp (445 bp of *EcR* fragment+41 bp spacer+264 nos fragment).

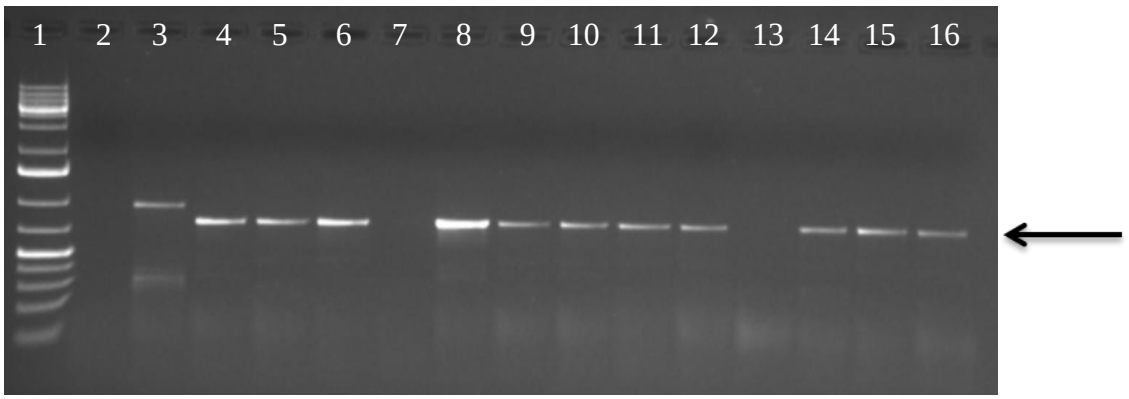


Figure 4.33. Amplification of antisense orientation of *EcR* fragment in putative transgenic plants using P25 and P22 primers. Lane no 1: 1 kb plus DNA marker (Thermo scientific), Lane no 2: Negative control, Lane no 3: pRNAi-GG control, Lane no. 4-9: Lady Olympia Transformants, Lane no 10-16: Agria transformants.

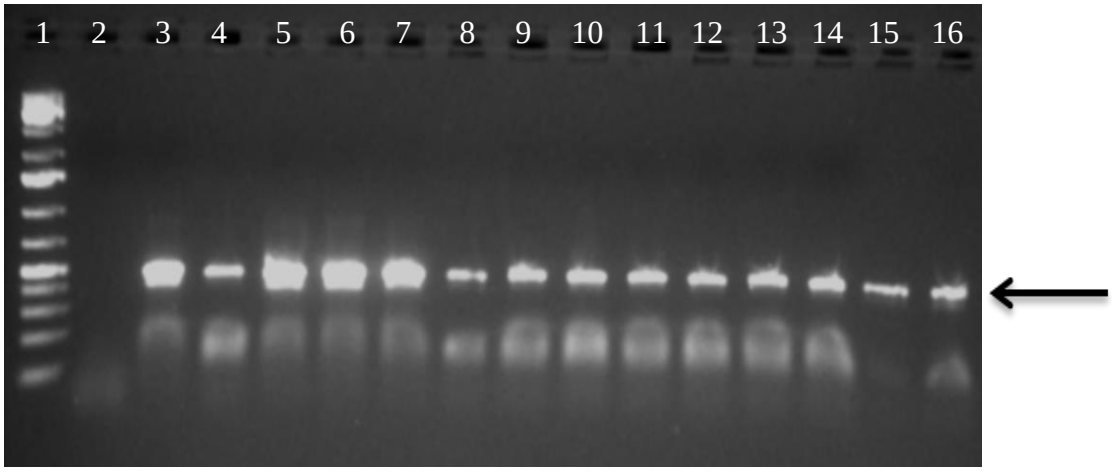


Figure 4.34. Amplification of *EcR* fragments in putative transgenic plants using gene specific (sense) primers. Lane no: 1 kb plus DNA marker (Thermo scientific), Lane no 2: Negative control, Lane no 3: pRNAi-GG control, Lane no. 4-9: Lady Olympia Transformants, Lane no 10-16: Agria transformants.

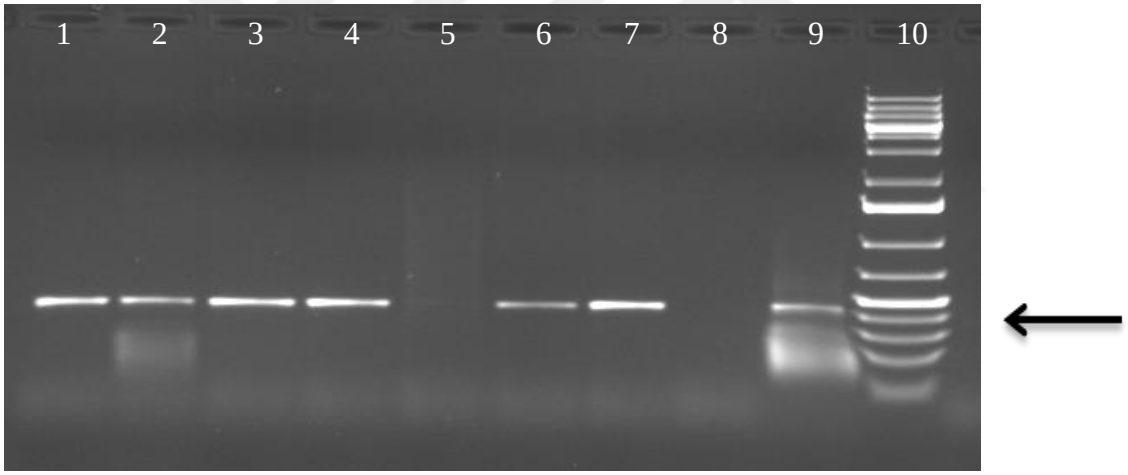


Figure 4.35. Amplification of *GFP* fragment in putative transgenic plants using gene specific (sense) primers. Lane no 1-8: Primary Transformants, Lane no 8: Negative control, Lane no 9: 35SpRNAi-GFP plasmid, Lane no 10: 1 kb plus DNA marker (Thermo scientific).

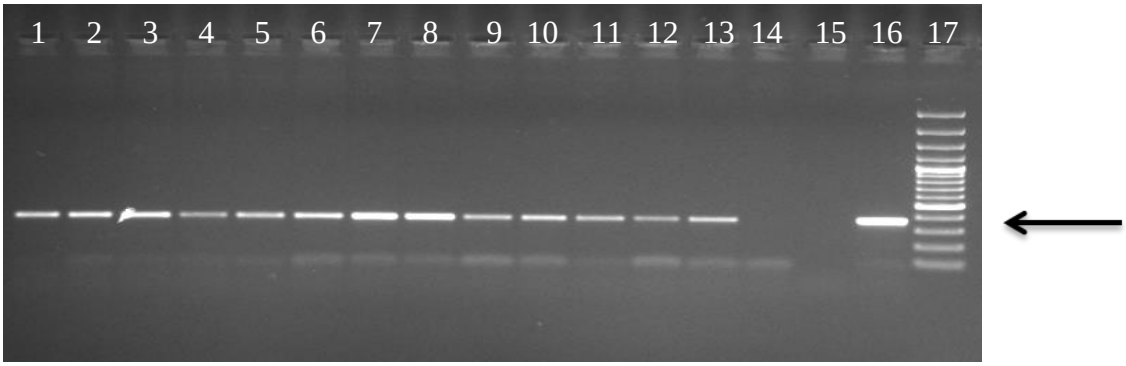


Figure 4.36. Amplification of *nptII* gene fragment in putative transgenic plants using gene specific primers. Lane no 1-14: Primary Transformants of Agria and Lady Olypmia, Lane no 15: Negative control, Lane no 16: 35SpRNAi-GG plasmid positive control, Lane no 17: DNA ladder mix (Thermo scientific)

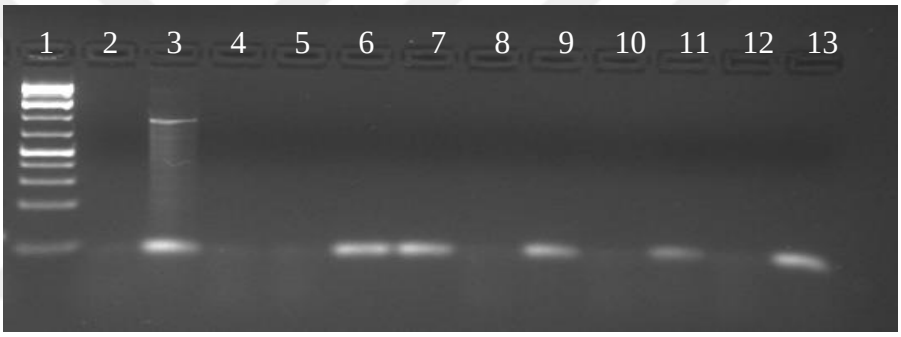


Figure 4.37. PCR assay to analyze *ChvA* gene presence in primary transformants putative transgenic plants. Lane no 1: 1 kb plus DNA ladder (Thermo scientific), Lane no 12: Negative control, Lane no 3: *Agrobacterium* chromosal DNA as positive control, Lane no 4-7: Primary Transformants of Lady olympia, Lane no 8-13: Agria transformants.

4.8.3 Confirmation of pRCApRNAi-CPB with gene specific primers

Putative transgenic plants were further subjected to PCR for confirmation with gene specific primers of *pRCA* and same constructs were also checked with *EcR* primers to confirm the presence of *EcR*-CPB gene in transgenic plants. (Figure 4.38) shows the amplification of *pRCA* fragment and (Figure 4.39) shows the amplification of *EcR* gene from constructs of pRCApRNAi-CPB.

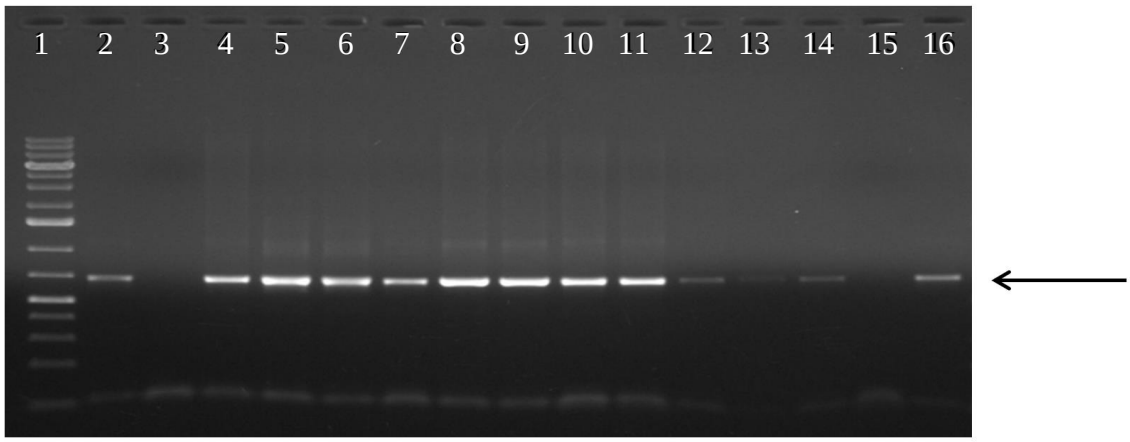


Figure 4.38. PCR showing amplification of fragment of *pRCA* promotor. Lane 1: 1kb plus ladder (Thermo scientific) Lane 2: Positive control showing amplification of ~702 bp *pRCA* gene, Lane 3: Negative control, Lane 4-9: Lady Olympia primary Trnsformants, Lane 10-16: Agria primary Transformants showing ~702 bp amplified fragment from pRCApRNAI-CPB.

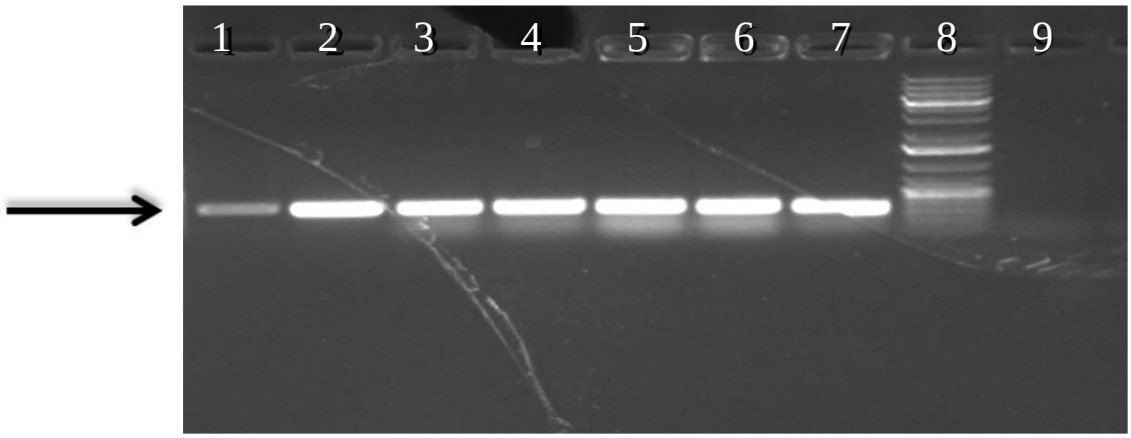


Figure 4.39. PCR showing amplification of fragment of *EcR* gene of CPB with gene specific primers in pRCApRNAi-CPB. Lane 1-6: ~445 bp amplified fragment of *EcR*-CPB from pRCApRNAi-CPB, Lane 7: Positive control showing amplification of *EcR* gene, Lane 8: 100 bp plus ladder (Thermo scientific) Lane 9: Negative control.

The summary of the results of different Primary transformants developed out of both potato cultivars based on PCR analysis has been presented in (Table 4.3). Results showed that some of the transformants either showed gene of interest in sense or antisense orientation as well as escapes were also recorded.

Table 4.3. Summary of the results of different Primary transformants developed out of potato cultivars based on PCR analysis

S. No.	Plant Name	Constructs	P21-P24	P25 - P22	Gene Specific	npt II	*ChvA*
1	A-1	35SpRNAi-GFP	+	+	+	+	-
2	A-2	35SpRNAi-GFP	+	+	+	+	-
3	A-3	35SpRNAi-GFP	+	+	-	+	-
4	A-4	35SpRNAi-GFP	+	-	+	-	-
5	A-5	35SpRNAi-GFP	+	+	+	+	-
6	A-1 (5)	35SpRNAi-CPB	+	+	+	+	-
7	A-2	35SpRNAi-CPB	+	+	+	+	-
8	A-3	35SpRNAi-CPB	+	-	-	-	+
9	A-4	35SpRNAi-CPB	+	+	+	+	-
10	A-5	35SpRNAi-CPB	+	+	+	+	-
11	A-6	35SpRNAi-CPB	-	-	+	ND	+
12	A-7	35SpRNAi-CPB	-	-	+	ND	+
13	A-8	35SpRNAi-CPB	+	+	+	+	-
14	A-9	35SpRNAi-CPB	+	+	+	+	-
15	A-10	35SpRNAi-CPB	+	+	+	+	-
16	LO-1	35SpRNAi-GFP	+	+	+	+	-
17	LO-2	35SpRNAi-GFP	+	+	+	+	-
18	LO-3	35SpRNAi-GFP	+	-	-	-	-
19	LO-4	35SpRNAi-GFP	-	-	-	-	-
20	LO-5	35SpRNAi-GFP	+	+	+	+	-
21	LO-1	35SpRNAi-CPB	+	+	+	+	-
22	LO-2	35SpRNAi-CPB	+	+	+	+	-
23	LO-3	35SpRNAi-CPB	-	+	+	+	-
24	LO-4	35SpRNAi-CPB	+	-	+	+	-
25	LO-5	35SpRNAi-CPB	+	+	+	+	-
26	LO-6	35SpRNAi-CPB	+	+	+	+	-
27	LO-7	35SpRNAi-CPB	+	+	+	+	-
28	LO-8	35SpRNAi-CPB	+	-	-	-	+

Table 4.3. (Continue) Summary of the results of different Primary transformants developed out of potato cultivars based on PCR analysis

29	LO-9	35SpRNAi-CPB	-	-	+	ND	-
30	LO-10	35SpRNAi-GFP	+	+	+	+	-
31	Ag-*pRCA*1	pRCApRNAi-CPB	+	+	+	+	-
32	Ag-*pRCA*2	pRCApRNAi-CPB	+	+	+	+	-
33	Ag-*pRCA*3	pRCApRNAi-CPB	+	-	+	+	-
34	Ag-*pRCA*4	pRCApRNAi-CPB	+	+	+	+	-
35	Ag-*pRCA*5	pRCApRNAi-CPB	+	+	+	+	-
36	Lo-*pRCA*1	pRCA-pRNAi-CPB	+	+	+	+	-
37	Lo-*pRCA*2	pRCApRNAi-CPB	+	+	+	+	-
38	Lo-*pRCA*3	pRCApRNAi-CPB	+	+	+	+	-
49	Lo-*pRCA*4	pRCApRNAi-CPB	-	+	+	+	-
40	Lo-*pRCA*5	pRCApRNAi-CPB	+	+	+	+	-

*A (Agria), LO (Lady Olympia), ND (not detected)

4.8.4 Transformation efficiency

Transformation efficiency of transgenic Lady Olympia plants from green house was calculated as 34.2%, 28.5%, 25.71% and 11.42% for 35SpRNAi-CPB, pRCApRNAi-CPB, pRNAi-GG and 35SpRNAi-GFP respectively. Similarly transformation efficiency for Agria was calculated as 40%, 24%, 20% and 16% for 35SpRNAi-CPB, pRCApRNAi-CPB, pRNAi-GG and 35SpRNAi-GFP constructs respectively as mention in (Table 4.4)

Table 4.4. Transformation efficiency of L.O and Agria with all constructs based on PCR assays

Cultivar	Total # of Transgenic plants	35SpRNAi-CPB		pRCApRNAi-CPB		pRNAi-GG		pRNAi-GFP	
		T.P	T.E %	T.P	T.E %	T.P	T.E %	T.P	T.E %
L.O.	175	60	34.2 (4)	50	28.5 (4.16)	45	25.71 (4.5)	20	11.4 (2)
Agria	125	50	40 (3.3)	30	24 (2)	25	20 (2)	20	16 (2)

*T.P (Transgenic Plants), T.E (Transformation Efficiency %), Transformation efficiency in brackets shows calculations based on total number of explant used in all experiments.

4.9 Southern Blot Analysis

Few PCR positive plants of Agria (Ag1-Ag3) and Lady Olympia (LO1-LO3) were selected to proceed further for confirmation and integration of transgene in plants genome by Southern blot. Southern integration analysis was performed according to the method of Southern (1975). The genomic DNA from plant samples was isolated from PCR positive transgenic plant leaves. DNA extraction was carried out using Thermo scientific plant genomic DNA extraction kit (Cat. No.0722). Approximately 10μg DNA of transgenic plant samples were digested with *HindIII* and BamH1 to release fragment of *EcR* gene from 35SpRNAi-CPB plasmid. Standard procedures were followed further to analyze copy number of introduced *EcR* fragment in primary transformants. Earlier, probe (PCR amplified *EcR* fragment) was labelled by Fermentas Biotin DecaLabel™ DNA Labeling Kit (Cat #K0651) following instructions provided in manual. Hybridization with the probe was done for 16-18 hours. Detection procedure was followed after hybridization, according to Fermentas Biotin Chromogenic Detection Kit (Cat# K0661). All PCR positive plants also showed signal on membrane indicating integration of introduced cassette in host genome (Figure 4.40).

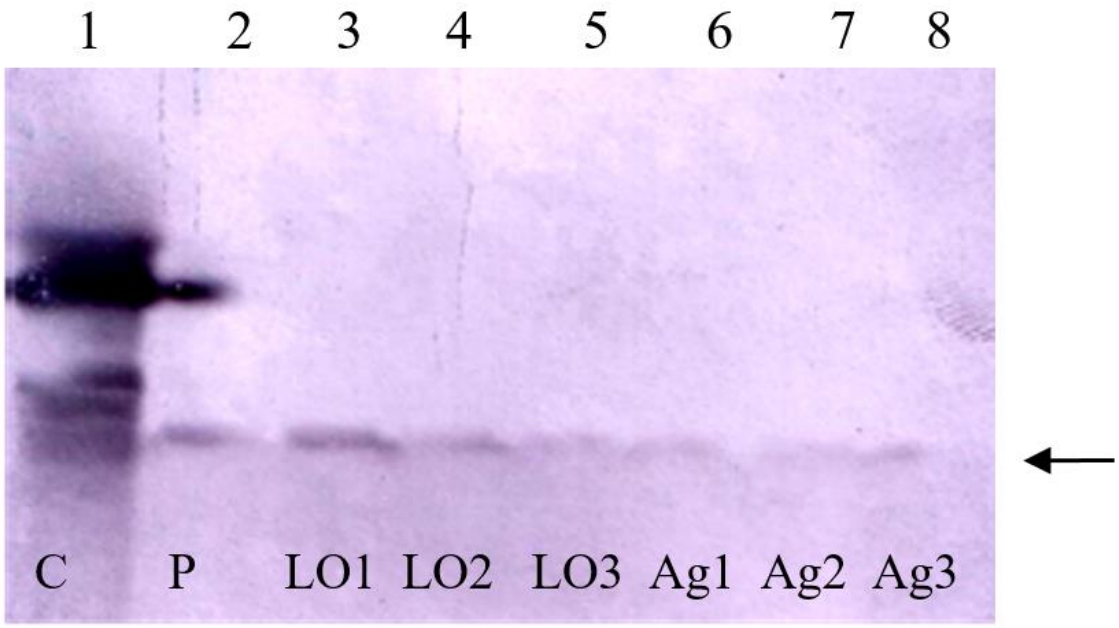
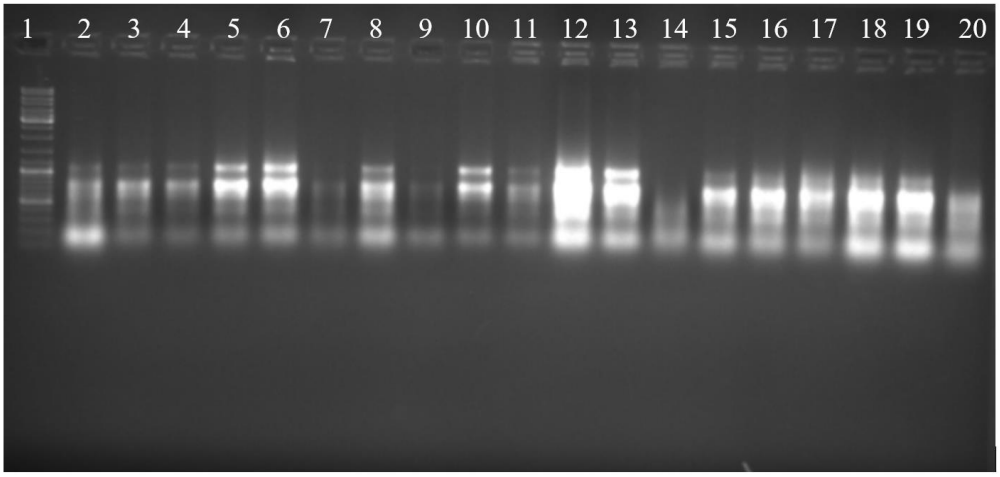


Figure 4.40. Southern blot analysis of PCR positive plants. Transformants of Lady Olympia and Agria were detected Lane 1: Lambda *HindIII* marker, Lane 2: plasmid 35SpRNAi-CPB, Lane 3-5: Lady Olympia and Lane 3-8: Agria putative transgenic plants.

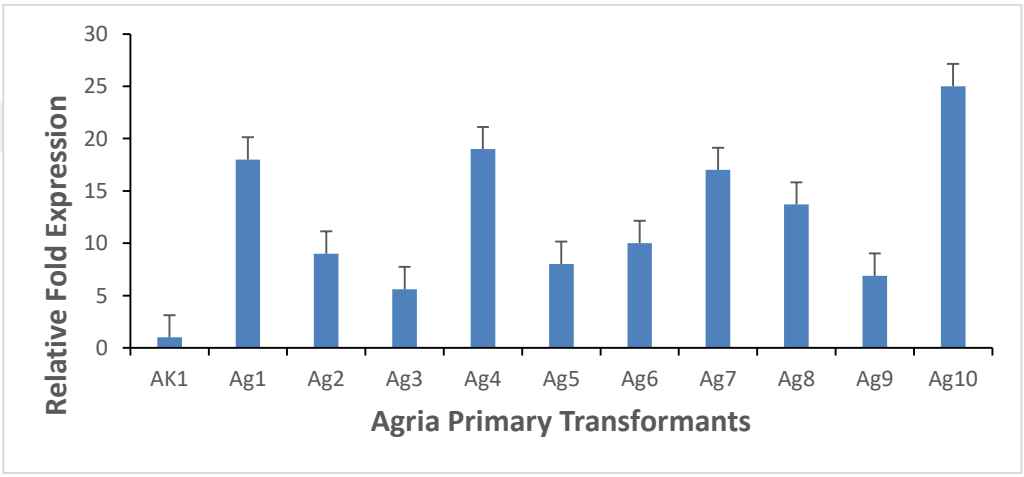
4.10 Real-Time Quantitative PCR (qRT-PCR)

Total RNA used was extracted from PCR positive transgenic plants using AMRESCO RiboZol™ RNA Extraction Reagent (Figure 4.41A). Furthermore, 1µg of RNA was used to convert into cDNA following protocol as described in Fermentas cDNA synthesis kit (Cat. No. 1621). 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 was determined according to the 2-ΔΔCt proportional calculation method (Livak and Schmittgen, 2001). Quantitative Realtime PCR (qRT-PCR) results were significantly higher when the *EcR* gene expressions of the transgenic plants were compared to the control plants. In lady Olympia transformants, 34 folds expression was recorded whereas in case of Agria 23 folds increased expression was recorded.

A



B



C

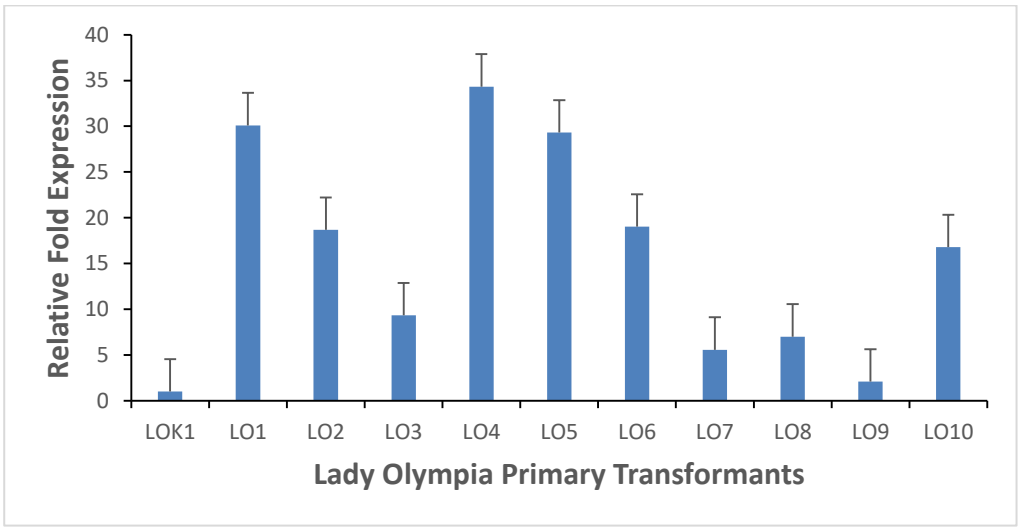


Figure 4.41. qPCR analysis of Agria and Lady Olympia transgenic plants. Total RNA isolated from plants (A). Coefficients of *EcR* transcript levels determined by qRT-PCR in candidate transgenic plants compared to control plants (B-C). Transgenic plants of Agria and Lady Olympia showed enhanced expression though it varied among plants.

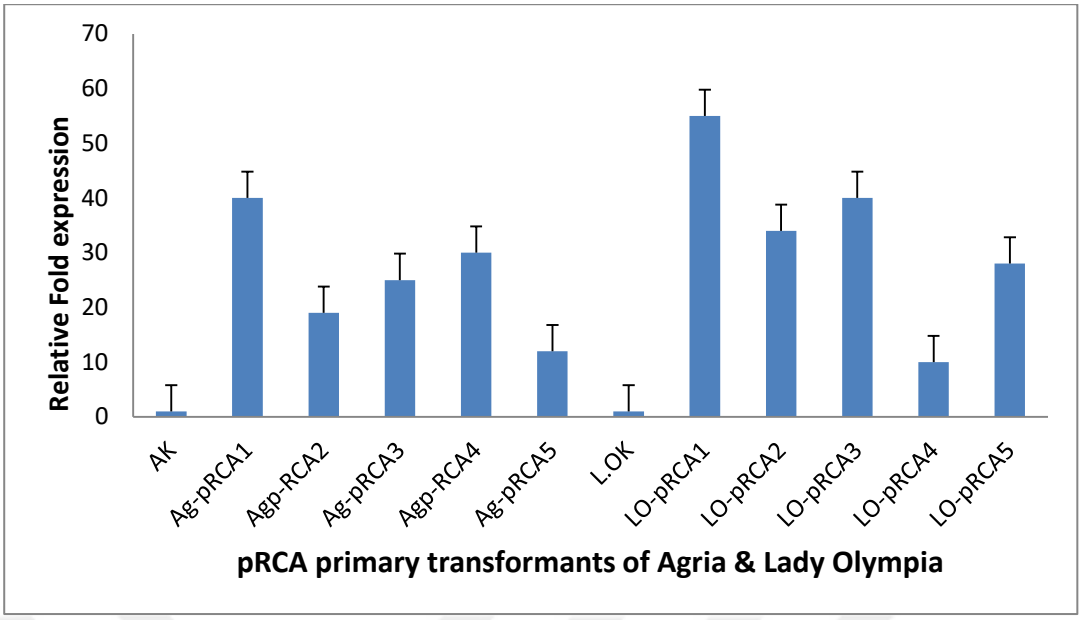


Figure 4. 42. qPCR analysis of *pRCA* transgenic plants. Coefficients of *EcR* transcripts under control of *pRCA* promotor by qRT-PCR in candidate transgenic plants compared to control plants. Transgenic plants of Agria and Lady Olympia showed enhanced expression as compared to control.

4.11 Leaf Bioassays

To evaluate the efficacy of plant mediated *EcR* gene silencing in CPB. Feeding leaf bioassays was performed. For this purpose, rearing of CPB insects was done in controlled conditions (Figure 4.43) and first, second and third instar larvae were starved for 3 to 4 hours before starting feeding assays. Bioassays were performed in two groups one as transgenic and other as control group. For the target gene five instar larvae of CPB with similar size for each trial were chosen and put in the plastic petri plates along with the leaves of transgenic and control plants (Figure 4.44-4.46). Three replicates were performed for each transgenic plants with total of total 15 larvae for each replica. After putting larvae and leaves petri plates were placed in the incubator (Temperature= 28±1 °C; RH: 60%). All the assays were carried out for 3 days consecutively and data was collected after every 24 hours. Each day, the condition of beetles was noticed in both tested and control groups. The mortality rate was recorded and calculated as shown in (Table 4.5-4.7).

Figure 4.43. Rearing of CPB insects on non-transgenic potato plants

4.11.1 First instar leaf bioassay analysis

There was significant difference regarding percent mortality among different transgenic plants expressing dsRNA. After 24 hours of feeding, significantly higher mortality percentage (20.00%) was recorded in Ag1, LO2, LO4 and Ag-*pRCA*. While, significantly lower mortality was observed in Ag2, Ag3, Ag4, LO1, LO3 and LO-*pRCA* ranging between 5% and 15%. However, no mortality was observed in control. After 48 hours of exposure, significantly higher mortality (25%-30%) was observed in Ag1, LO2, LO3, LO4 and Ag-*pRCA*. Significantly lower mortality was observed in rest of the observed lines ranging between 15%-20%. However, there was no mortality in control. After 72 hours of consecutive feeding, higher mortality was observed in LO2 and LO4 viz. 70%-80% while significantly lower mortality (20.00%) was observed in Ag2 and Ag3 as shown in (Table 4.5 and Figure 4.44).

Table 4.5. Summary of the results of mortality percentage of first instar larvae of CPB

Mortality percentage (%) 1st instar			
Variety	24 hours	48 hours	72 hours
Ag1	20.00a	30.00a	40.00b
Ag2	10.00ab	20.00ab	20.00cd
Ag3	5.00ab	15.00ab	20.00cd
Ag4	10.00ab	20.00ab	30.00bc
LO1	10.00ab	20.00ab	40.00b
LO2	20.00a	30.00a	80.00a
LO3	15.00ab	25.00a	45.00bc
LO4	20.00a	30.00a	70.00a
Ag-*pRCA*	20.00a	25.00a	50.00b
LO-*pRCA*	15.00ab	20.00ab	40.00b
Control	0.00b	0.00b	0.00c

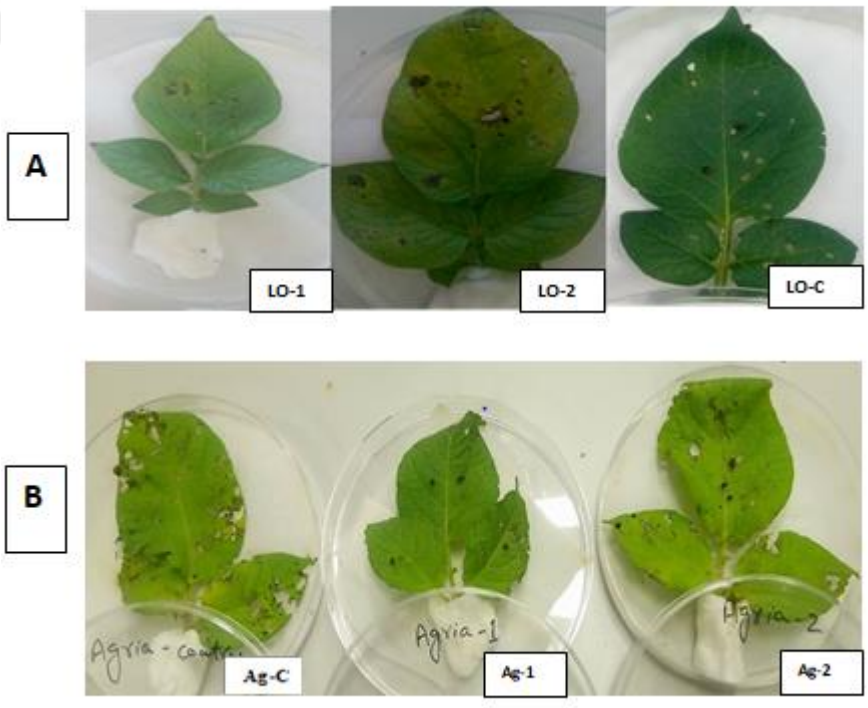


Figure 4. 44. Leaf biotoxicity assay of first instar CPB feeding on Lady olympia (A) and Agria (B) transformants

4.11.2 Second instar leaf bioassay analysis

In case of second instar analysis after 24 hours of feeding no mortality was found but after 48 hours of feeding significant mortality of 20% was found in Ag1, LO1, LO4 and LO-*pRCA*. In rest of transgenic lines (Ag3, Ag4, LO2, LO3 and Ag-*pRCA*) significantly lower mortality of 10% to 15% was observed. While no mortality was observed in Ag2 and control plants. Similarly mortality data was observed after 72 hours of continues feeding and significant mortality of about 30% to 40% was observed in Ag1, LO1 and LO4. Whereas, significantly lower values of 15% to 25% was observed in rest of plants with no mortality in control as shown in (Table 4.6 and Figure 4. 45).

Table 4.6. Summary of the results of mortality percentage of 2nd instar larvae of CPB

Mortality percentage (%) 2nd instar			
Variety	24 hours	48 hours	72 hours
Ag1	0.00a	20.00a	30.00a
Ag2	0.00a	0.00b	20.00ab
Ag3	0.00a	15.00ab	15.00ab
Ag4	0.00a	15.00ab	25.00ab
LO1	0.00a	20.00a	30.00a
LO2	0.00a	15.00ab	25.00ab
LO3	0.00a	10.00ab	20.00ab
LO4	0.00a	20.00a	40.00a
Ag-*pRCA*	0.00a	15.00ab	30.00a
LO-*pRCA*	0.00a	20.00a	25.00ab
Control	0.00a	0.00b	0.00b

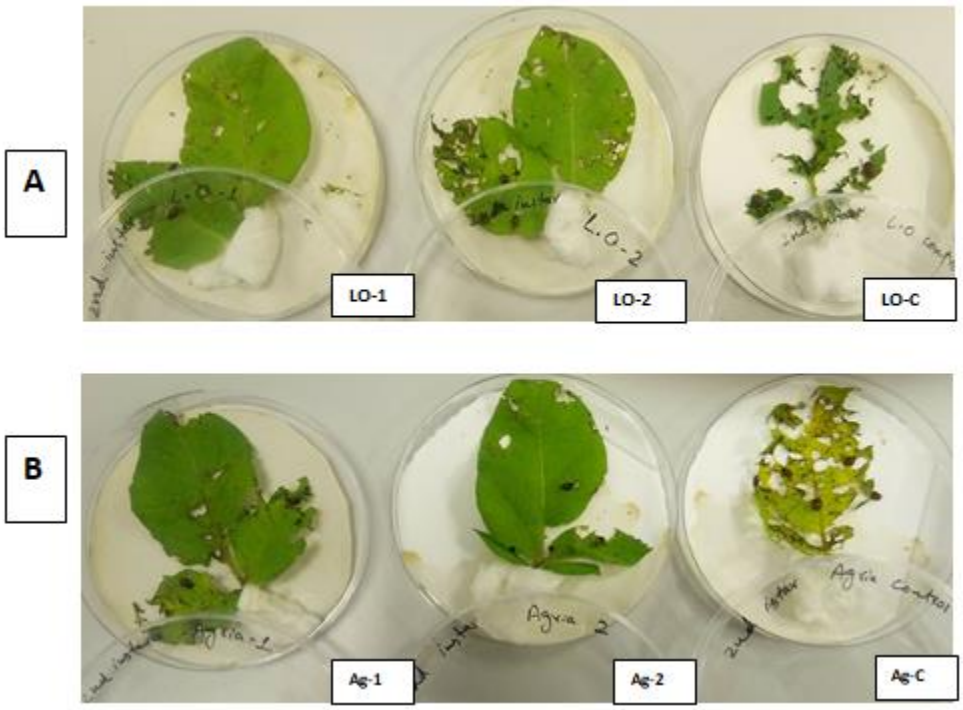


Figure 4.45. Leaf biotoxicity assay of second instar CPB feeding on Lady olympia (A) and Agria (B) transformants

4.11.3 Third instar leaf bioassay analysis

Third instar analysis showed no mortality after 24 hours but significant mortality of 20 % in two plants LO2 and LO-*pRCA* was observed after 48 hours of feeding and remaining varieties showed non-significant mortality of 10% while Ag2 and control didn't show any mortality. After 72 hours of feeding mortality was calculated and 35-60% highly significant values were found in Ag1, Ag4, LO1, LO2, LO4, Ag-*pRCA* and LO-*pRCA* varieties. However, significantly lower values of 25% to 30% mortality were found in case of Ag2, Ag3, LO3 and no mortality was noticed in control as shown in (Table 4.7 and Figure 4.46).

Table 4.7. Summary of the results of mortality percentage of first 3rd instar larvae of CPB

Mortality percentage (%) 3rd instar			
Variety	24 hours	48 hours	72 hours
Ag1	0.00a	15.00ab	40.00b
Ag2	0.00a	0.00b	30.00b
Ag3	0.00a	15.00ab	25.00bc
Ag4	0.00a	10.00ab	40.00b
LO1	0.00a	20.00a	60.00a
LO2	0.00a	10.00ab	40.00ab
LO3	0.00a	15.00ab	30.00b
LO4	0.00a	10.00ab	50.00ab
Ag-*pRCA*	0.00a	10.00ab	35.00ab
LO-*pRCA*	0.00a	20.00b	40.00ab
Control	0.00a	0.00b	0.00c

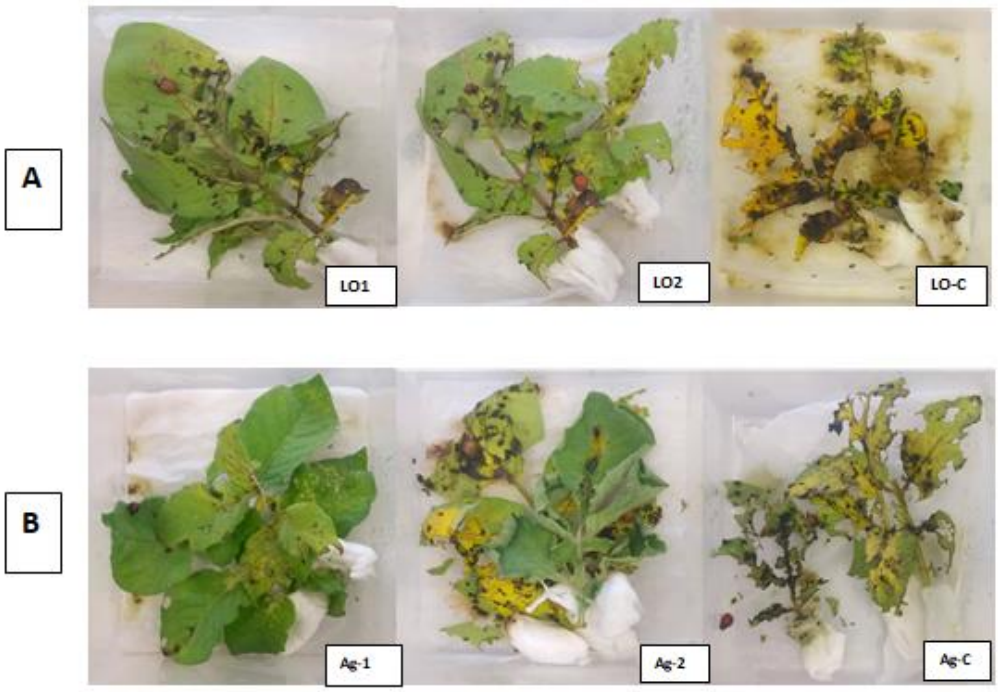


Figure 4.46. Leaf biotoxicity assay of third instar CPB feeding on Lady olympia (A) and Agria (B) transformants

4.12 Fold change in weight of different larval instars

There was significance difference in fold increase of weight in different larval instars when fed on different transgenic lines. In first instar, significant increase in weight viz. 6.53X in Agria control while 6.45X in L.O control was observed when fed on control plants as compared to the transgenics expressing dsRNA. Transgenic plants with higher expression supported lesser increase in weight as compared to the low expressing transgenics. In first instar analysis of Ag4 and LO4 there was significantly lesser increase in weight viz.1.83X and 2.44X.

In case of second instar larval observation significantly more increase in weight was observed when fed on control plants i.e. 3.03X in Agria control and 7.94X in LO control as compared to the transgenics. Similarly, in highly expressing transgenic plants like Ag1 and LO1 relatively lesser increase in weight (1.35X in Ag1 and 2.65X in LO1) was observed. Likewise, significantly higher increase in weight was observed in 3rd instar larvae when exposed to control plants (1.87X in Ag control and 2.23X in LO control). In highly expressing transgenics viz Ag4 and LO4 fold increase in weight was significantly lesser i.e. 0.87X and 1.13X respectively.

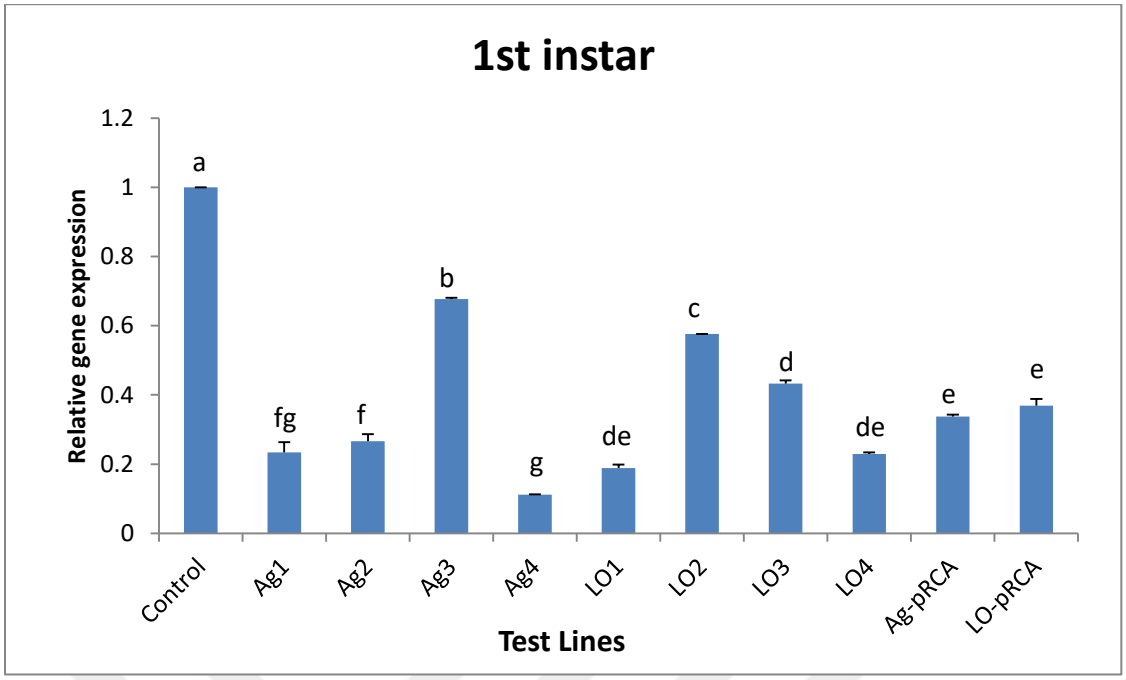
Table 4. 8. Fold change in weight of different instars on Lady olympia (A) and Agria (B) transgenic plants.

Genotype	1st instar	2nd instar	3rd instar
Ag-C	6.53a	3.03e	1.87c
Ag1	1.91g	1.35i	1.10h
Ag2	1.35i	1.91j	1.22f
Ag3	3.03e	3.00f	1.41e
Ag4	1.83h	1.83k	0.87i
Ag-*pRCA*	2.44f	2.44h	1.63d
LOC	6.45b	7.94a	2.23a
LO1	1.24j	2.65g	1.15g
LO2	4.14c	3.43c	1.96b
LO3	3.00e	3.84b	1.86c
LO4	2.44f	3.34d	1.13gh
LO-*pRCA*	3.34d	2.00i	1.83c

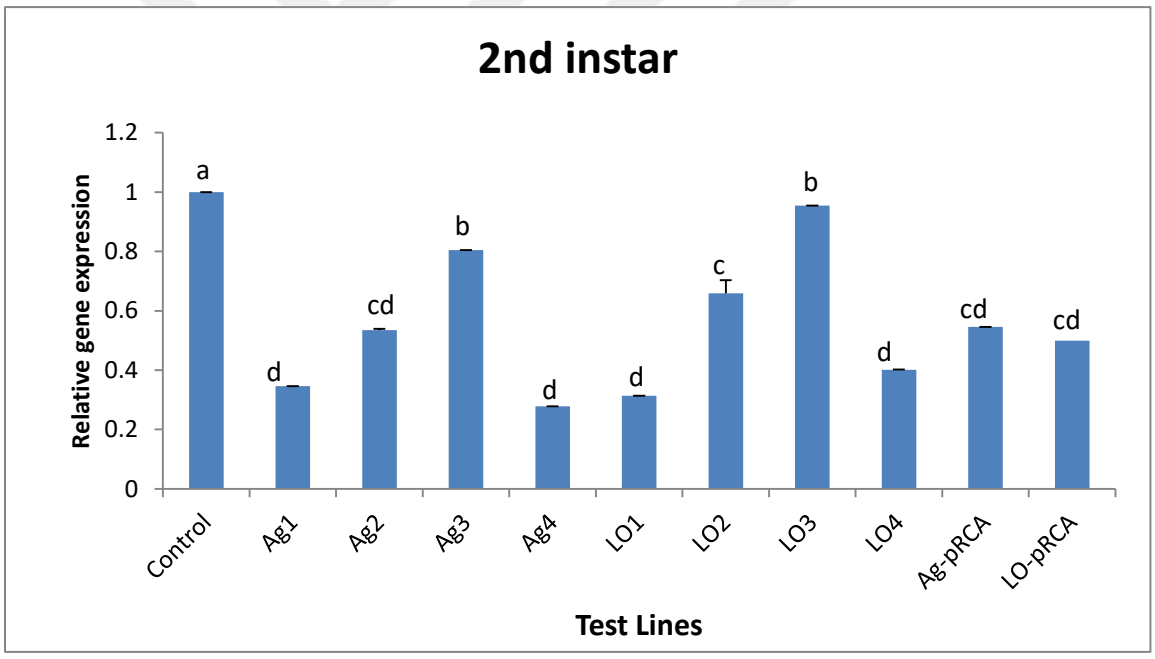
4.13 Feeding RNAi Effects on Relative Expression of *EcR*

After three days of consective feeding on transgenic lines there was significant decrease in expression level of target gene as compare to the control in all of three instars. In the first instar significantly lower expression level (0.11) was observed in Ag4, while significantly higher level of expression (0.67) was observed in Ag3. Similarly in the second instar transcript levels were significantly decreased to minimum in the transgenic plants Ag1, Ag4, LO1 and LO4. The higher expression was observed in Ag3 and LO3. In the third instar, there was less change in overall transcript levels after three days of consective feeding on transgenic lines. Relatively lower expression (0.28) was observed in Ag4, while significantly higher expression (0.88) was recorded in LO3. The relative expression of *EcR* in insect after three days of feeding is shown in (Figure 4.47).

A


1st instar
Relative gene expression
1.2
1
0.8
0.6
0.4
0.2
0
a
fg
f
b
g
de
c
d
de
e
e
Control
Ag1
Ag2
Ag3
Ag4
LO1
LO2
LO3
LO4
Ag-pRCA
LO-pRCA
Test Lines

B


2nd instar
Relative gene expression
1.2
1
0.8
0.6
0.4
0.2
0
a
d
cd
b
d
d
c
b
d
cd
cd
Control
Ag1
Ag2
Ag3
Ag4
LO1
LO2
LO3
LO4
Ag-pRCA
LO-pRCA
Test Lines

C

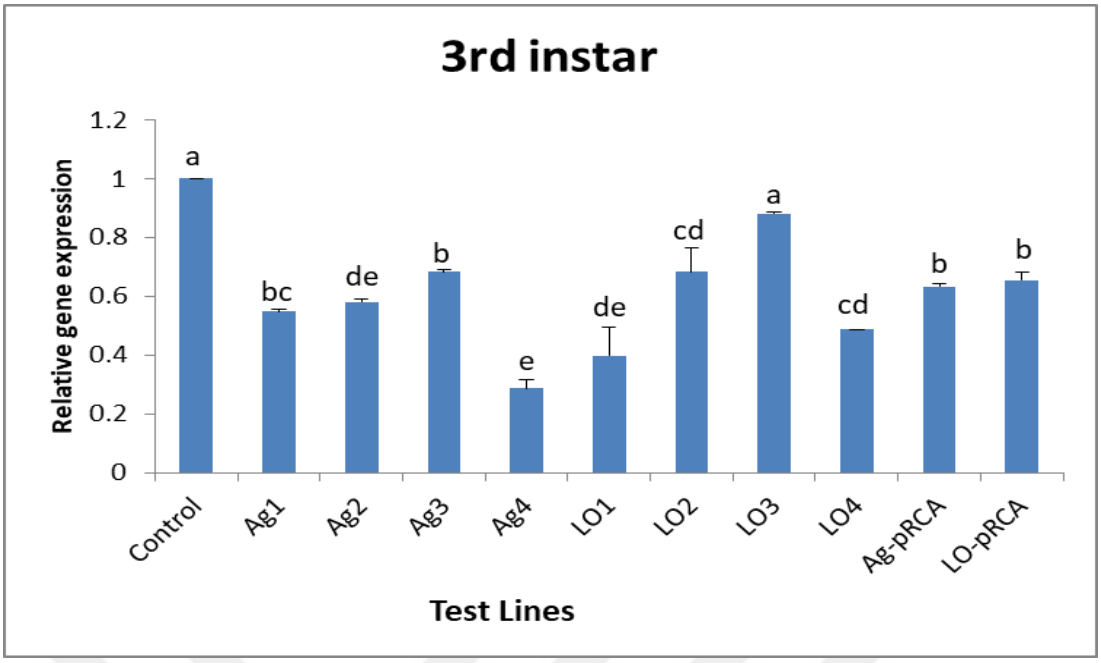


Figure 4.47. qPCR analysis of *EcR* gene of CPB fed on transgenic leaves. (A-C) Coefficients of *EcR* transcript levels of CPB determined by qRT-PCR in first, second and third instar CPB larvae fed on transgenic plants along with control.

CHAPTER V

DISCUSSION

RNA interference is a sequence specific silencing mechanism that is catalyzed by introduction of transgenes in the form of inverted repeats to result in dsRNA transcripts (Lindbo and Dougherty, 2005). Transgenic plants expressing non-endogenous dsRNA can be created via *Agrobacterium tumefaciens* mediated transformation which either can make a single sequence consisting of a long hairpin (hpRNA) (Horsch et al., 1984; Guo et al., 2015; Mamta et al., 2015), or two complementary sequences in the cytoplasm (Kumar et al., 2012). RNAi expressing transgenic plants gave satisfactory protection against western corn rootworm and American bollwoorm in the initial experiments conducted by Baum et al. and Mao et al., respectively (Baum et al., 2007; Mao et al., 2007). Following their work various research works have been conducted by different researchers to target various insect-pests like *Myzus persicae* (Mao and Zeng, 2014), *N. lugens* (Yu et al., 2014), *S. avenae* (Xu et al., 2014), *H. armigera* (Liu et al., 2015) and aphids (Coleman et al., 2015).

The undertaken study exhibited functionality of *EcR*-dsRNA using plant mediated transgenic technology as possible way to inhibit molting process of CPB. As a matter of fact, expressing dsRNA to control insect-pests via gene silencing can prove a ecofriendly promising tool for management of insect-pests. Additionally, it offers high specificity, no production of foreign proteins and there is no count of target genes. The length and the amount of dsRNA also its stability in planta and in gut of targeted insects are vital factors determining success of plant mediated RNAi strategies as compared to conventional transgenic crops (Zhang et al., 2017). Large scale production of transgenic plants using plant mediated technology could become commercial in near future. Although the mechanism of RNAi in insect is still not known, plant mediated RNAi has been already considered as a potential tool for application in insect pest control (Kourti et al., 2017)

Using this technique, insect resistance potato lines were produced to express a transgene in transgenic plants that lowered the transcript levels of target *EcR* gene when digested by the insect pests. In this study, plant-mediated RNAi successfully triggered silencing

of the targeted genes and hence cause significant mortality in the treated beetles as compared to control ones, showing an effective RNAi response in CPB.

For RNAi, proper selection of target gene is challenging in order to obtain encouraging results. Rong et al., 2014 reported that ecdysone receptor (*EcR*) is a potential target for RNAi based pests control. The ligand receptor complex 20E-*EcR*-USP initiates various physiological and developmental phenomena in insects like molting and metamorphosis. Thus, *EcR* is a main component of molting process in growth stages of insect larvae making it a promising target. As CPB is devasting pest of potato worldwide and likewise in Turkey, therefore, we selected *EcR* gene of CPB because compounds acting on the *EcR* will be only affective to insects and other arthropods making *EcR* a noticeable target for safer and specific insecticides (Dhadialla et al., 1998).

Golden gate cloning is a molecular approach to make RNAi constructs. This cloning strategy depends on the type IIs restriction enzymes and can be used for effective guided sequences of gene fragments in a single step of restriction-ligation (Engler et al., 2008; Engler et al., 2009). Moreover, by designing proper sites of cleavage, two digested gene fragments can be used to ligate a product without having the restriction sites. Therefore, both restriction and ligation can be carried out in a single restriction-ligation step. Depending on these features of Golden Gate cloning, we chosed this system to generate ihpRNA constructs in single reaction using pRNAi-GG vector. A single PCR amplified product of gene of interest was used in single tube restriction ligation reaction and a single step transformation (Yan et al., 2012). Following strategy of Yan et al., (2012) we developed our RNAi constructs (Figure 4.15) under the control of *35S* and *pRCA* promoter.

An efficient plant transformation system is a powerful tool that can be used to knock out or introduce the gene in plants by providing opportunities to manipulate trait of interest from species. Genetic engineering of potato has been achieved by direct DNA uptake into the protoplasts, delivery of DNA by biolistics (particle bombardment), and by *Agrobacterium*-mediated transformation (Van Eck, 2018). In the present study, *Agrobacterium*-mediated transformation of potato was performed as described by De Block et al., (1988) and Beaujean et al., (1998) with some modifications adopted in our laboratory. *Agrobacterium tumefaciens* strain LB4404 was used for this purpose as our

previous studies revealed that LBA4404 is more efficient among other strains (Bakhsh et al., 2014). Transformation of experimental plants Lady Olympia and Agria was performed using MS medium supplemented with growth hormones and optimized kanamycin concentrations. 1500 and 1200 explants (leaves and internodes) were transformed from each variety with *35S* and *pRCA* respectively. Besides that 1000 explants from each cultivar were also transformed with control constructs. Different researchers have reported different transformation efficiencies in potato using different explants (Veale et al., 2012; Soto et al., 2007; Beaujean et al., 1998; Wenzler et al., 1989; Hameed et al. 2017). Overall transformation efficiency in our experiments was calculated as 3.12%. According to some previous reports there are different ways to calculate genetic transformation activities of different plants (Bakhsh et al., 2012; Wang et al., 2011). The various factors like type of vector and *Agrobacterium* strain used, explant type, explant size, varietal genetic background and gelling agent etc. can affect the *Agrobacterium* mediated efficiency of potato transformation (Frary and Earle, 1996, Rao et al., 2009).

A total of 300 PCR positive plants belonging to different constructs were transferred to the greenhouse. The transgenic plants grew well while no any abnormal phenotypes were observed. Some transformants surviving on kanamycine selection in in-vitro culture conditions did not show amplification of *EcR* fragment. Such plants were considered as negative and scored as escape. Although, it has been recognized that kanamycin use can establish good regeneration selection system for transgenic plants (Sohail et al., 2012; Bakhsh et al., 2014), but such escapes were noticed in our study. Similarly, escapes have also been reported in some earlier studies (Horsch et al., 1985; McCormick et al., 1986; Bakhsh et al 2018). To get efficient and improved selection system for obtaining primary transformants, development of satisfactory in vitro transformation system is a critical method. Experimentally, sensitivities of different explants to kanamycin have been recorded different (Zhang et al., 2001). The T-DNA instability, non-rigorous selections, genomic rearrangements and chimeras might be possible reasons for escapes (McHugen and Jordan, 1989). In our study, only positive plants with introduced gene(s) were marked and subjected to the further studies.

The primary transformants developed out of both cultivars were analyzed performing PCR, Southern blot and leaf bioassay for the integration and expression of gene(s) within T-DNA region. *Agrobacterium* ability depends on activation of vir genes along

with *ChvA* genes to infect host plant. *ChvA* act as a promotor for attachment of host cell to bacteria Douglas et al., (1985). Findings of PCR should show amplicon of about 890 bp using *ChvA* gene specific primers in case of contamination (Nain et al., 2005). Therefore, PCR analysis was performed using *ChvA* to confirm *Agrobacterium* contamination in transgenic plants (Figure 4.37). Our results showed no contamination due to *Agrobacterium* and hence it was determined that *EcR* gene fragment has been introduced in transgenic plants. Total 300 numbers of plants were transferred to green house on basis of initial pcr analysis. Out of 300, 175 plants of Lady Olympia and 125 plants of Agria were recorded as positive by further molecular analysis.

Southern blot analysis of PCR positive plants revealed the integration of transgene in plant genome. A detection of~445 bp fragment on the membrane conformed integration of *EcR* gene in transgenic plants. The presence of expected hybridization signal in transformed plants showed that *EcR* gene was successfully integrated in the potato genome (Zha et al., 2011).

Real-time quantitative PCR (qRT-PCR) has now emerged as standard molecular technique to determine relative expression of target gene (Maqbool et al., 2010; Rao et al., 2011). qRT-PCR analysis was performed to diagnose expression levels of *EcR*-CPB gene. PCR positive plants were subjected to the qRT-PCR analysis and as result, a remarkably high expression of *EcR*-CPB gene was noticed in transgenic plants as compared to control plants. The qRT-PCR results of transgenic plants expressing ds*EcR* under the control of *pRCA* also showed enhanced expression in transgenic plants. Expression level of gene was different in various transgenic plants (Figure 4.41 and 4.42). Our results are in agreement with previous studies of Li et al., (2017). According to the authers, expression level of any gene can vary in different transgenic lines.

As gene expression may varies with the nucleotide sequences of gene, the promotor, and the insertion point of gene in DNA of transgenic variety, copy numbers of transgenes, internal environment of cell as well as other environmental factors (Hobbs et al., 1993; Agaisse et al., 1995; Guo et al., 2015; Rao, 2005). Therefore, wherever transferred gene is in plant genome, the alteration in nucleotide sequence of transferred gene might alter levels of genes expression depending on number of transgene (Rao et al., 2011).

To drive the transgene almost all transgenic crops all over the world utilize *CaMV 35S* promotor (Odell et al., 1985). It is becoming clear that function of this *35S CaMV* promotor may be influenced by undefined environmental and physiological factors (Sunilkumar et al., 2002). This can also be the reason of different expression level of gene in transgenics.

Rubisco activase (*RCA*) is a member of AAA^+ family of ATPases and is linked to the functionally diverse activities of cells (Portis, 2003). *RCA* catalyzes activation of the ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) that is bifunctional enzyme found in plant chloroplast because it not only catalyzes initial CO2 fixation step in Calvin cycle but also function as oxygenase in the photorespiration. The promoter of *RCA* has been reported to express in specific organs (Xu et al., 2010). In this research work *35S* promotor was replaced with *pRCA* promoter to induce expression of dsRNA in green parts of the plants.

Once transgenic plants were confirmed for transgene integration and expression, leaf biotoxicity assays were performed by allowing feeding of 1^{st}, 2^{nd} and 3^{rd} instar CPB larvae on these transgenic plants. A variation in mortality of CPB was recorded with different instars (Figure 4.44-4.46 and Table 4.5-4.7). Mortality of CPB larvae established the functionality of *EcR*-dsRNA in CPB though the efficiency of dsRNA remained variable among different instars and different transgenic plants. Earlier reports are also in agreement with our results. Zhu et al., (2012) reported mortality of *Helicoverpa armigera* upto 40% when transgnic tobacco lines expressing ds*EcR* were fed to the pest.

The incubated CPB larvae on transgenic plants were analysed for the weight gain before and after the experiment. There was significantly higher fold increase in weight ranging between 1.87X–6.53X in different larval instars on control plants as compared to the larvae fed on different transgenic lines, where weight gain ranged between 0.87–4.14X. These results are comparable to the findings of Zhu et al. (2011), Xiong et al. (2013) and Jin et al. (2015). Higher expression of dsRNA resulted in more weight loss due to retarted growth owing to the fact that *EcR* gene is crucial for insect growth (Zhu et al. 2011; Zhu et al. 2012).

The transcript level of *EcR* gene were analyzed in CPB larvae fed on transgenic as well as control plants to evaluate the effect of ds*EcR*. Results revealed significant reduction of *EcR* transcripts in larvae (Figure 4.47). Our results established that the expression of dsRNA in transgenic plants was directly propostional to the percent mortality of insects (Upadhyay et al., 2011; Wuriyanghan et al., 2011; Zha et al., 2011). These findings are in accordance with the previous research works (Zha et al., 2011; Xiong et al., 2013; Xu et al., 2014). Higher expression also affected the weight gain in different larval instars of CPB (Mao et al., 2011). These results are in agreement with the previous findings of Jin et al., (2015), Mao et al., (2011) and Asokan et al., (2014).

CHAPTER VI

CONCLUSION

This PhD dissertation work was conducted to inhibit the transcripts level of highly expressive Ecdysone receptor (*EcR*) gene involved in insect molting process using plant mediated RNAi approach. This is very first study of using RNAi technology to block metamorphogenesis related gene of CPB that is notorious insect pests of potato in Turkey as well as in the world. RNAi constructs were developed using golden gate cloning approach and transferred to two potato cultivars by *Agrobacterium* mediated transformation. This thesis also reported the use of tissue specific promoter (*pRCA*) for the first time to induce the expression of dsRNA in potato. A total of 300 PCR positive plants were recovered that belong to different constructs (35SpRNAi-CPB, pRCApRNAi-CPB, pRNAi-GG and pRNAi-GFP). The overall transformation efficiency was calculated as 3.1%.

The molecular and leaf bioassays of transgenic plants were performed with different instars of CPB in order to evaluate the efficacy of *EcR*-dsRNA of CPB. Total 20-80% of mortality was recorded in first instar larvae while 15-40% mortality was observed in case of second instar larvae. Similarly 25-60% was observed from third instar larvae. Total change in weight was also calculated after three days of feeding and significantly lesser gain in weight was observed upto 0.87X–4.14X.

Our results show that CPB larvae feeding on transgenic potato plants expressing *EcR*-dsRNA halted metamorphosis, affected their body weight and larvae were not able to shift to their next instar. Moreover, the utilization of a confined green tissue specific transgene in part of the study is an ideal strategy to develop transgenic potato plants against insect pests since the transgene will be expressed and active only in green parts of the plants, not the tubers that are the edible part of the crop.

These transgenic potato plants developed showed encouraging resistance against CPB. A 34% of potato crop losses are estimated due to these insect pests. Hence, the outcomes of this research work will eventually help reducing potato losses, and will lead to the remarkable boom in crop productivity.

REFERENCES

Acharjee, S., Sarmah, B. K., Kumar, P. A., Olsen, K. and Mahon, R., "Expression of a sequence-modified cry2Aa gene for resistance to Helicoverpa armigera in chickpea (*Cicer arietinum* L.)." ***Plant Science*** 178: 333–339, 2010.

Agaisse, H. and Lereclus, D., "How does Bacillus thuringiensis produce so much insecticidal crystal protein?." ***Journal of Bacteriology*** 177.21: 6027, 1995.

Ahammad-Sahib, K. I., Hollingworth, R. M., Whalon, M. E., Ioannidis, P. M. and Grafius, E. J., "Polysubstrate monooxygenases and other xenobiotic-metabolizing enzymes in susceptible and resistant Colorado potato beetle." ***Pesticide Biochemistry and Physiology*** 49, 1–12, doi:10.1006/pest.1994.1029 1994.

Ahn, S.-J., Vogel, H. and Heckel, D. G., "Comparative analysis of the UDP-glycosyltransferase multigene family in insects." ***Insect Biochemistry and Molecular Biology***. 42, 133–147, doi:10.1016/j.ibmb.2011.11.006 2012.

Alam, M. F., Abrigo, E., Datta, K., Datta, S.K., Oliva, N., Tu, J. and Virmani, S.S., "Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice." ***Plant Cell Reports*** 18.7-8: 572-575, 1999.

Ali, M.I., Luttrell, R.G. and Young, S.Y., "Susceptibilities of Helicoverpa zea and Heliothis virescens (Lepidoptera: Noctuidae) populations to Cry1Ac insecticidal protein." ***Journal of Economic Entomology*** 99: 164e175, 2006.

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P.J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T.T., Nugent, G., Raemakers, K., Romano, A., Somers, D.A., Stoger, E., Taylor, N. and Visser, R., "Particle bombardment and

the genetic enhancement of crops: myths and realities", ***Molecular Breeding,*** V.15, P.305-327, 2005.

Alyokhin, A., "The Red Queen in a potato field: integrated pest management versus chemical dependency in Colorado potato beetle control." ***Pest Management Science*** 71, 343–356, doi:10.1002/ps.3826, 2015.

Alyokhin, A., Baker, M., Mota-Sanchez, D., Dively, G. and Grafius, E., "Colorado potato beetle resistance to insecticides." ***American Journal of Potato Research*** *85*(6), pp.395-413, 2008.

Andrade, E. C. and Hunter, W. B., "RNA Interference – Natural Gene-Based Technology for Highly Specific Pest Control (HiSPeC)," http://dx.doi.org/10.5772/61612**,** 2016.

Anonymous,"*USDAERSPotatoes*"https://www.ers.usda.gov/topics/crops/vegetables-pulses/potatoes.aspx, January 14, 2018.

Anonymous, https://www.rnagri.com/, 2018

Aragão, F.J.L., Sarokin, L., Vianna, G.R. and Rech, E.L., "Selection of transgenic meristematic cells utilizing herbicidal molecule results in the recovery of fertile transgenic soybean (Glycine max L.) plants at a high frequency", ***Theoretical and applied genetics*** V.101, P.1-6, 2000.

Araujo, R.N., Santos, A., Pinto, F.S., Gontijo, N.F., Lehane, M.J. and Pereira, M.H., 2006. "RNA interference of the salivary gland nitrophorin 2 in the triatomine bug Rhodnius prolixus (Hemiptera: Reduviidae) by dsRNA ingestion or injection." ***Insect Biochemistry and Molecular Biology*** 36, 683–693, 2006.

Argentine, J. A., Clark, J. M. and Ferro, D. N., "Genetics and synergism of resistance to azinphosmethyl and permethrin in the Colorado potato beetle (Coleoptera: Chrysomelidae)." ***Journal of Economic Entomology*** 82, 698–705, doi:10.1093/jee/82.3.698 1989.

Argentine, J. A., Zhu, K. Y., Lee, S. H. and Clark, J. M., “Biochemical mechanisms of azinphosmethyl resistance in isogenic strains of Colorado potato beetle.” ***Pesticide Biochemistry and Physiology*** 48, 63–78, doi:10.1006/pest.1994.1008 1994.

Asokan, R., Chandra, G.S., Manamohan, M., Kumar, N.K. and Sita, T., “Response of various target genes to diet-delivered dsRNA mediated RNA interference in the cotton bollworm, Helicoverpa armigera.” ***Journal of pest science*** 87(1), pp.163-172, 2014.

Bagri, D.S., Upadhyay, D.C., Jain, S.K. and Upadhyay, C.P., “Biotechnological improvement of nutritional and therapeutic value of cultivated potato.” ***Frontiers in bioscience*** *(Scholar edition), 10*, pp.217-228, 2018.

Bakhsh, A., Anayol, E. and Ozcan, S. F., "Comparison of transformation efficiency of five *Agrobacterium tumefaciens* strains in Nicotiana tabacum L", ***Emirates Journal of Food and Agriculture*** 26(3), 259-264, 2014.

Bakhsh, A., Anayol, E., Özcan, S.F., Hussain, T., Aasim, M., Khawar, K.M. and Özcan, S., “An insight into cotton genetic engineering (Gossypium hirsutum L.): current endeavors and prospects.” ***Acta physiologiae plantarum***, *37*(8), p.171, 2015.

Bakhsh, A., Dinc, T., Hussain, T., Demirel, U., Aasim, M. and çalışkan, M. E., “Development of transgenic tobacco lines with pyramided insect resistant genes. ***Turkish Journal of Biology,*** 42(2), 2018.

Bakhsh, A., Siddique, S. and Husnain, T., "A molecular approach to combat spatiotemporal variation in insecticidal gene (Cry1Ac) expression in cotton", ***Euphytica*** 183(1), 65-74, 2012.

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. and Roberts, J., “Control of coleopteran insect pests through RNA interference.” ***Nature Biotechnology*** 25:1322–6, 2007.

Beaujean, A., Sangwan, R. S., Lecardonnel, A. and Sangwan-Norreel, B. S., "*Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation." ***Journal of experimental Botany***, 49(326), 1589-1595, 1998.

Belles, X., "Beyond *Drosophila:* RNAi, In Vivo and Functional Genomics in Insects." ***Annual Review of Entomology*** 55:111–28, 2010.

Benbrook, C. M., "Impacts of genetically engineered crops on pesticide use in the US--the first sixteen years." ***Environmental Sciences Europe*** 24.1: 24, 2012.

Bhatia, V. and Bhattacharya, R., "Host mediated RNAi of cuticular protein gene impaired fecundity in green peach aphid Myzus persicae. ***Pest management science*** 2018.

Bishop, B. A. and Grafius, E. J., "Insecticide resistance in the Colorado potato beetle." ***Chrysomelidae biology*** 1 (1996): 355-377, 1996.

Boiteau, G., Alyokhin, A. and Ferro, D. N., "The Colorado potato beetle in movement." CP Alexander review, *The* ***Canadian Entomologist*** 135: 1 – 22, 2003.

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., "Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (Diabrotica virgifera virgifera LeConte)." ***PLoS ONE*** 7:e47534. doi: 10.1371/journal.pone.00 47534, 2012.

Borges, F., and Martienssen, R. A., "The expanding world of small RNAs in plants." ***Nature Reviews Molecular Cell Biology*** 16, 727–741. doi: 10.1038/nrm4085, 2015.

Bravo, A. and Soberon, M., 2008. "How to cope with insect resistance to *Bt* toxins?" ***Trends Biotechnol*** 26: 573–579, 2008.

Brown, C.R., “Antioxidants in potato.” ***American Journal of Potato Research*** 82: 163–172, 2005.

Bucher, G., Scholten, J. and Klingler, M., “Parental RNAi in Tribolium (Coleoptera).“ ***Current Biology*** 12, R85–R86 2002.

Caliskan, M. E., Caliskan, S. and Gunel, E., "Effects of different mulching treatments on crop growth and tuber yield in early potato production." ***Proceedings of Third National Potato Congress*** *(23-27 September 2002, Izmir-Turkey)*. 2002.

Çalışkan, M. E., Onaran, H. and Arıoğlu, H., "Overview of the Turkish potato sector: Challenges, achievements and expectations." ***Potato research*** 53.4: 255-266, 2010.

Cappelle, K., de Oliveira, C.F.R., Van Eynde, B., Christiaens, O. and Smagghe, G., “The involvement of clathrin mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut.” ***Insect Molecular Biology*** 0, n/a-n/a. doi:10.1111/imb.12222, 2016.

Carrière, Y., Fabrick, J.A. and Tabashnik, B.E., “Can pyramids and seed mixtures delay resistance to *Bt* crops?” ***Trends in biotechnology,*** *34*(4), pp.291-302, 2016.

Casagrande, R. A., “The Colorado Potato Beetle: 125 Years of Mismanagement” 0013 8754/87/0142-0150$02.00/0*,* ***Entomological Society of America****,* 142-150*,* 1987.

Castanotto, D. and Rossi, J.J., “The promises and pitfalls of RNA-interference-based therapeutics.” ***Nature*** 457, 426–433, 2009.

Christou, P., “Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment”, ***Euphytica****,* V.85, P. 13–27, 1995.

Christou, P., Capell, T., Kohli, A., Gatehouse, J.A. and Gatehouse, A.M., "Recent developments and future prospects in insect pest control in transgenic crops." ***Trends in Plant Science*** 11: 302–308, 2006.

Christou, P.,"Rice transformation – bombardment", ***Plant Molecular Biology*** V.35, P. 197–203, 1997.

Clayton, G. M., Peak-Chew, S. Y., Evans, R. M. and Schwabe, J. W., "The structure of the ultraspiracle ligand-binding domain reveals a nuclear receptor locked in an inactive conformation." ***Proceedings of the National Academy of Sciences of the United States of America*** *USA* 98, 1549–1554, https://doi.org/10.1073/pnas.041611298 2001.

Clements, J., Schoville, S., Peterson, N., Huseth, A.S., Lan, Q. and Groves, R.L., "RNA interference of three up-regulated transcripts associated with insecticide resistance in an imidacloprid resistant population of Leptinotarsa decemlineata." ***Pesticide biochemistry and physiology***, 135, pp.35-40, 2017.

Coleman, A. D., Wouters, R. H., Mugford, S. T. and Hogenhout, S. A., "Persistence and transgenerational effect of plant-mediated RNAi in aphids." ***Journal of Experimental Botany*** 66(2):541–8, 2015.

Coleman, A.D., Wouters, R.H.M., Mugford, S.T. and Hogenhout, S.A., "Persistence and transgenerational effect of plant-mediated RNAi in aphids. ***Journal of Experimental Botany*** 66: 541-548, 2014.

Crossway, A., Oakes, J. V., Irvine, J. M., Ward, B., Knauf, V. C. and Shewmaker, C. K., "integration of foreign DNA following microinjection of tobacco mesophyll protoplasts", ***Molecular Genetics and Genomics*** V.202, P. 179-185, 1986.

Dai, S., Zheng, P., Marmey, P., Zhang, S., Tian, W., Chen, S., Beachy, R.N. and Fauquet, C., "Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment", ***Molecular Breeding*** V.7, P.25-33, 2001.

DANIDA, “Assessment of Potentials and Constraints for Development and Use of Plant Biotechnology in Relation to Plant Breeding and Crop Production in Developing Countries”, ***Royal Danish Ministry of Foreign Affairs, Copenhagen***, Denmark, 2002. www.icsu.org/1_icsuscience/GMO/html/DANIDA%20Biblio%20Entry.htm

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M. F., Oliva, N. and Datta, S. K., “Constitutive and tissue-specific differential expression of the cryIA (b) gene in transgenic rice plants conferring resistance to rice insect pest.” ***Theoretical and Applied Genetics,*** 97(1-2), 20-30, 1998.

Datta, S., Peterhans, A., Datta, K. and Potrykus, I., “Genetically Engineered Fertile Indica-Rice Recovered From Protoplasts”, ***Bio Technology*** V.8, P.736-740, 1990.

De Block, M., “Genotype-independent leaf disc transformation of potato (Solanum tuberosum) using *Agrobacterium tumefaciens*.” ***Theoretical and Applied Genetics*** 76: 767–774, 1988.

Dermauw, W. Wybouw, N., Rombauts, S., Menten, B., Vontas, J., Grbić, M., Clark, R. M., Feyereisen, R. and Leeuwen, T. V., “A link between host plant adaptation and pesticide resistance in the polyphagous spider mite Tetranychus urticae.” ***Proceedings of the National Academy of Sciences*** USA 110, E113–E122, doi:10.1073/pnas.1213214110 2012.

Dermauw, W. and Van Leeuwen, T., “The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance.” ***Insect Biochemistry and Molecular Biology*** 45, 89–110, doi:10.1016/j.ibmb.2013.11.001 2014.

Dhadialla, T. S., Carlson, G. R. and Le, D. P., “New insecticides with ecdysteroidal and juvenile hormone activity.” ***Annual Review of Entomology*** 43: 545–569, 1998.

Douches, D.S., Maas, D., Jastrzebski, K. and Chase, R.W., “Assessment of potato breeding progress in the USA over the last century.” ***Crop Science*** 36: 1544-1552, 1996.

Douglas, C. J., Staneloni, R. J., Rubin, R. A. and Nester, E. W., "Identification and genetic analysis of an *Agrobacterium* tumefaciens chromosomal virulence region", ***Journal of Bacteriology*** 161(3), 850-860, 1985.

Duncan, David R., "Field performance of transgenic potato, with resistance to Colorado potato beetle and viruses." ***Hort Science*** 37.2: 275-276, 2002.

Dutton, A., Romeis, J. and Bigler, F., “Assessing the risks of insect resistant transgenic plants on entomophagous arthropods *Bt*-maize expressing Cry1Ab as a case study.” ***Bio Control***, *48*(6), pp.611-636, 2003.

E. P. A., “RNAi Technology as a Pesticid” : Problem Formulation for Human Health and Ecological Risk Assessment. Submitted to the ***FIFRA Scientific Advisory Panel,*** 2014.

Elmayan, T. and Vaucheret, H., “Expression of single copies of a strongly expressed *35S* transgene can be silenced post-transcriptionally.” ***The Plant Journal*** 9: 787-797, 1996.

Enayati, A. A., Ranson, H. and Hemingway, J., “Insect glutathione transferases and insecticide resistance.” ***Insect Molecular Biology*** 14, 3–8, doi:10.1111/j.1365-2583.2004.00529.x 2005.

Engler, C., Gruetzner, R., Kandzia, R. and Marillonnet, S., "Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. ***PLoS One*** 4: e5553. 15, 2009.

Engler, C., Kandzia, R. and Marillonnet, S., “A one pot, one step, precision cloning method with high throughput capability. ***PLoS One*** 3: e3647, 2008.

FAOSTAT., “Statistical data.” Food and Agriculture Organization of the United Nations, Rome, 2017.

Feitelson, J. S., Payne, J. and Kim, L., “Bacillus thuringiensis: Insects and beyond.” ***Bio Technology*** 10, pp. 271-275, 1992.

Ferré, J., and Van Rie, J., "Biochemistry and Genetics of Insect Resistance to B acillus thuringiensis." ***Annual review of entomology*** 47.1: 501-533, 2002.

Feyereisen, R., “Insect CYP genes and P450 enzymes in Insect.” ***Molecular Biology and Biochemistry*** (ed. Gilbert, L.) 236–316 Elsevier, 2012.

Finer, J. J., and McMullen, M. D., “Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. ***In Vitro Cellular & Developmental Biology-Plant***, 27(4), 175-182, 1991.

Fire, A., Albertson, D., Harrison, S. W. and Moerman, D. G., “Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle.” ***Development*** 113, 503–514, 1991.

Fire, A., Si, Q. X., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C., “Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.” ***Nature*** 391:806–811, 1998.

Flanders, K. L., Hawkes, J. G., Radcliffe, E. B., and Lauer, F. I., “Insect resistance in potatoes: sources, evolutionary relationships, morphological and chemical defenses, and eco-geographical associations.” ***Euphytica*** 61(2), 83-111, 1992.

Forgash A. J., "Insecticide resistance in the Colorado potato beetle." ***Insecticide resistance in the Colorado potato beetle*** 704: 33-52, 1985.

Fraley, P.T., Rogers, S.G. and Horsch, R.B., “Genetic transformation in higher plants.” ***Critical Reviews in Plant Sciences*** V.4, P.1-46, 1986.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C., "Expression of bacterial genes in plant cells", ***Proceedings of the National Academy of Sciences of the United States of America,*** V.80 (15), P.4803–4807, 1983.

Frame, B. R., Drayton, P. R., Bagnall, S. V., Lewnau, C. J., Bullock, W. P., Wilson, H. M. and Wang, K., "Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation." ***The Plant Journal*** 6(6), 941-948, 1994.

Frary, A. and Earle, E.D., "An examination of factors affecting the efficiency of*Agrobacterium*-mediated transformation of tomato." ***Plant Cell Reports*** *16*(3-4), pp.235-240, 1996.

Fromm, M.E., Taylor, L.P. and Walbot, V., "Stable transformation of maize after gene-transfer by electroporation." ***Nature,*** V.319, P.791-793, 1986.

Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S. and Dunbar, M.W., "Field evolved resistance to *Bt* maize by western corn rootworm." ***PLoS One*** 6, e22629, 2011.

Gatehouse, A.M.R., Hilder, V.A. and Boulter, D., "Potential of plant-derived genes in the genetic manipulation of the crops for insect resistance, in: Biotechnology in Agriculture N° 7: Plant genetic manipulation for crop protection", ***CAB International,*** pp.155-181, 1992.

Gauthier, N. L., Hofmaster, R. N. and Semel, M., "History of Colorado potato beetle control." ***Advances in potato pest management. Hutchinson Ross, Stroudsburg,*** *PA*: 13-33, 1981.

Girijashankar, V., Sharma, H. C., Sharma, K. K., Sivarama, P. L. and Royer, M., "Development of transgenic sorghum for insect resistance against the spotted stem borer (Chilo partellus)." ***Plant Cell Reports*** 24: 513–522, 2005.

Gordon, K.H. and Waterhouse, P.M., “RNAi for insect-proof plants.” ***Nature Biotechnology*** 25: 1231–1232, 2007.

Gordon, K.H. and Waterhouse, P.M., “Small RNA viruses of insects: expression in plants and RNA silencing.” ***Adv Virus Res*** 68: 459–502, 2006.

Göre, M.E., “Fungal seedborne pathogens infecting potato seed tubers from Turkey, 2011–2014.” ***Journal of Plant Diseases and Protection***, *124*(6), pp.539-551, 2017.

Gouamene-Lamine, C. N., Yoon, K. S. and Clark, J. M., “Differential susceptibility to abamectin and two bioactive avermectin analogs in abamectin-resistant and -susceptible strains of Colorado potato beetle, Leptinotarsa decemlineata (Say) (Coleoptera: Chrysomelidae).” ***Pesticide Biochemistry and Physiology*** 76, 15–23, doi:10.1016/S0048-3575(03)00053-1 2003.

Graves, A.F. and Goldman S.L., “The transformation of Zea mays seedlings with *Agrobacterium* tumefaciens”, ***Plant Molecular Biology*** V.7, P.43–50, 1986.

Griebler, M., Westerlund, S.A., Hoffmann, K.H. and Meyering-Vos, M., “RNA interference with the allatoregulating neuropeptide genes from the fall armyworm Spodoptera frugiperda and its effects on the JH titer in the hemolymph.” ***Journal of Insect Physiology*** 54, 997–1007, 2008.

Grishok, A., Tabara, H. and Mello, C. C., “Genetic requirements for inheritance of RNAi in C. elegans.” ***Science*** 287:2494–2497, 2000.

Guo, W.Z., Sun, J., Guo, Y.F. and Zhang, T.Z., “Investigation of different dosages of inserted *Bt* genes and their insect-resistance in transgenic *Bt* cotton. *Yi chuan xue bao= **Acta genetica Sinica** 28*(7), pp.668-676, 2001.

Guo, Z., Kang, S., Zhu, X., Xia, J. and Wu, Q., “The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management.” ***Scientific Reports*** 5: 13728, 2015.

Haase, N.U., “The canon of potato science: 50. The nutritional value of potatoes.” ***Potato Research*** *50*(3-4), p.415, 2007.

Hameed, A., Tahir, M.N., Asad, S., Bilal, R., Van Eck, J., Jander, G. and Mansoor, S., “RNAi-mediated simultaneous resistance against three RNA viruses in potato.” ***Molecular biotechnology*** *59*(2-3), pp.73-83, 2017.

Hannon, G. J., “RNA interference.” *Nature* 418:244–51. pmid:12110901, 2002.

Hansen, G. and Wright, M., "Recent Advances in the transformation of plants”, ***Trends In Plant Science*** V.4, P.226-231, 1999.

He, Y., Fang, J., Taatjes, D. J. and Nogales, E., “Structural visualization of key steps in human transcription initiation.” ***Nature***. 28;495(7442):481-6. doi: 10.1038/nature11991. Epub 2013 Feb 27, 2013.

Heim, D. C., Kennedy, G. G. and Vanduyn, J. W., "Survey of insecticide resistance among North Carolina Colorado potato beetle (Coleoptera: Chrysomelidae) populations." ***Journal of economic entomology*** 83.4: 1229-1235, 1990.

Herrero-Vanrell, R., Rincón, A. C., Alonso, M., Reboto, V., Molina-Martinez, I. T. and Rodríguez-Cabello, J. C., “Self-assembled particles of an elastin-like polymer as vehicles for controlled drug release.” ***Journal of Controlled Release*** 20;102(1):113-22, 2005.

Hess, A.M., Prasad, A.N., Ptitsyn, A., Ebel, G.D., Olson, K.E., Barbacioru, C., Monighetti, C. and Campbell, C.L., “Small RNA profiling of Dengue virus-mosquito interactions implicates the PIWI RNA pathway in anti-viral defense.” ***BMC microbiology***, *11*(1), p.45, 2011.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T., “Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA”, ***Plant Journal,*** V.6, P. 271-282, 1994.

Hobbs, S., Thomas, L. A., Warkentin, D. and Catherine, M. O., "Transgene copy number can be positively or negatively associated with transgene expression." ***Plant molecular biology*** 21.1: 17-26, 1993.

Höfte, H.R. and Whiteley., "Insecticidal proteins of Bacillus thuringiensis." ***Microbiology Reviews*** 53, pp. 242-255, 1989.

Hopkins, P., "In Ecdysone: Structures and Functions (ed Guy Smagghe)." Ch. 3, 73–97 ***Springer Netherlands***, 2009.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. R. and Fraley, T., "A simple and general method for transferring genes into plants." ***Science*** 227: 1229-1231, 1985.

Horsch, R. B., Fraley, R. T., Rogers, S. G., Sanders, P. R., Lloyd, A. and Hoffmann, N., "Inheritance of functional foreign genes in plants. ***Science*** 223: 496–498, 1984.

Huvenne, H. and Smagghe, G., "Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review." ***Journal of Insect Physiology*** 56: 227–235, 2010.

Ibrahim, A.B., Monteiro, T.R., Cabral, G.B. and Aragão, F.J., "RNAi-mediated resistance to whitefly (*Bemisia tabaci*) in genetically engineered lettuce (Lactuca sativa)." ***Transgenic research***, *26*(5), pp.613-624, 2017.

Ioannidis, P. M., Grafius, E. and Whalon, M. E., "Patterns of insecticide resistance to azinphosmethyl, carbofuran, and permethrin in the Colorado potato beetle (Coleoptera: Chrysomelidae)." ***Journal of Economic Entomology*** 84.5: 1417-1423, 1991.

Jacques, Jr. and Richard, L., ***The potato beetles***. No. 3. Taylor and Francis, 1988.

Jaleel, W., Saeed, S. and Naqqash, M. N., “Biology and bionomics of Dysdercus koenigii F. (Hemiptera: Pyrrhocoridae) under laboratory conditions.” ***Pakistan Journal of Agricultural Sciences*** 50: 373-378, 2013.

James, C., “Global Status of Commercialized Biotech/GM Crops.” 2015 ***ISAAA Brief*****.** ISAAA, 2015.

James, C., “Global status of commercialized biotech/GM crops: 2011**.” ISAAA Brief No. 47** ***ISAAA, Ithaca,*** **NY**, 2014.

James, C., “Global Status of Commercialized Biotech/GM Crops: 2011. ***ISAAA Brief No.*** 46. ISAAA, Ithaca, NY, USA, 2013.

Jancova, P., Anzenbacher, P. and Anzenbacherova, E., „Phase II drug metabolizing enzymes.“ ***Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech. Repub.*** 154, 103–16, doi:10.5507/bp.2010.017 2010.

Jin, S., Singh, N.D., Li, L., Zhang, X. and Daniell, H., “Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts Helicoverpa armigera larval development and pupation.” ***Plant biotechnology journal***, 13(3), pp.435-446, 2015.

Jindra, M., "Gene regulation by steroid hormones: vertebrates and insects." ***European Journal of Entomology*** 91: 163-163, 1994.

Joga, Mallikarjuna R., Zotti, M. J., Smagghe, G. and Christiaens O., "RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far." ***Frontiers in physiology*** 7: 553, 2016.

Kamthan, A., Chaudhuri, A., Kamthan, M. and Datta, A., “Small RNAs in plants: recent development and application for crop improvement.” ***Front. Plant Sci.*** 6:208. doi: 10.3389/fpls.2015.00208, 2015.

Kaplanoglu, E., Chapman, P., Scott, I.M. and Donly, C., “Overexpression of a cytochrome P450 and a UDP-glycosyltransferase is associated with imidacloprid resistance in the Colorado potato beetle, Leptinotarsa decemlineata.” ***Scientific reports****, 7*(1), p.1762, 2017.

Katoch, R. and Thakur, N., “Insect gut nucleases: a challenge for RNA interference mediated insect control strategies.” ***International Journal of Biochemistry and Biotechnology*** 1, 198–203, 2012.

Klein, T.M., Wolf, E., Wu, R. and Sanford, J., *Nature,* V. 327, P. 70–73, 1987.

Koelle, M. R., "The Drosophila *EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily." ***Cell*** 67.1: 59-77, 1991.

Kong, Y., Liu, X. P., Wan, P. J., Shi, X. Q., Guo, W. C. and Li, G. Q., “The P450 enzyme Shade mediates the hydroxylation of ecdysone to 20-hydroxyecdysone in the Colorado potato beetle, Leptinotarsa decemlineata.” ***Insect molecular biology***, *23*(5), pp.632-643, 2014.

Kos, M., van Loon, J.J., Dicke, M. and Vet, L.E., “Transgenic plants as vital components of integrated pest management. ***Trends Biotechnol*** 27: 621–627, 2009.

Kourti, A., Swevers, L. and Kontogiannatos, D., “In Search of New Methodologies for Efficient Insect Pest Control:“The RNAi “Movement”. ***In Biological Control of Pest and Vector Insects*** *InTech,* 2017.

Kumar, A. and Pandey, S. K., “Potato production: Harbinger of agricultural sustainability.” ***Indian Farming*** 58(9): 3–7, 2008.

Kumar, M., “Plant Regeneration and Genetic Transformation in Buckwheat (Fagopyrum spp.), A Multipurpose Gluten Free Crop of High Nutraceutical Importance: A Critical Review.” ***Annals of Plant Sciences,*** *7*(1), pp.1954-1963, 2018.

Kumar, M., Gupta, G.P. and Rajam, M.V., “Silencing of acetylcholinesterase gene of Helicoverpa armigera by siRNA affects larval growth and its life cycle.” ***Journal of Insect Physiology*** 55, 273–278, 2009.

Kumar, P., Pandit, S. S., and Baldwin, I. T., “Tobacco rattle virus vector: a rapid and transient means of silencing Manduca sexta genes by plant mediated RNA interference.” ***PLoS One***, 7(2), e31347, 2012.

Kumar, S., Chandra, A. and Pandey, K. C., "Bacillus thuringiensis (*Bt*) transgenic crop: an environment friendly insect-pest management strategy." ***Journal of Environmental Biology*** 29.5: 641-653, 2008.

Kurreck, J., “RNA interference: from basic research to therapeutic applications.” ***Angewandte Chemie-International Edition*** 48, 1378–1398, 2009.

Lagnaoui, A., Canedo, V. and Douches, D.S., “Evaluation of *Bt*-cry1la1 (cryV) transgenic potatoes on two species of potato tuber moth, Phthorimaea operculella and Symmetrischema tangolias (Lepidoptera: Gelechiidae) in Peru.” ***CIP Program Report*** 1999-2000, CIP, Lima, 2001.

Larson, P.S., Minakawa, N., Dida, G.O., Njenga, S.M., Ionides, E.L. and Wilson, M.L., “Insecticide-treated net use before and after mass distribution in a fishing community along Lake Victoria, Kenya: successes and unavoidable pitfalls.” ***Malaria journal*** 13(1), p.466, 2014.

LeBlanc, G. A., “Crustacean endocrine toxicology: a review.” ***Ecotoxicology*** 16, 61–81, https://doi.org/10.1007/s10646-006-0115-z 2007.

Li, H., Guan, R., Guo, H. and Miao, X., “New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests. ***Plant Cell Environ***. 38, 2277–2285. doi: 10.1111/pce.12546, 2015a.

Li, J., Wang, X. P., Wang, M. Q., Ma, W. H. and Hua, H. X., “Advances in the use of the RNA interference technique in Hemiptera.” ***Insect Sci*ence** 20, 31–39. doi: 10.1111/j.1744-7917.2012.01550.x, 2013.

Li, X., Schuler, M. A. and Berenbaum, M. R., "Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics." ***Annual Review of Entomology*** 52, 231–253, doi:10.1146/annurev.ento.51.110104.151104 2007.

Li, Y., Wang, K., Lu, Q., Du, J., Wang, Z., Wang, D. and Li, H., "Transgenic Nicotiana benthamiana plants expressing a hairpin RNAi construct of a nematode Rs-cps gene exhibit enhanced resistance to Radopholus similis." ***Scientific reports***, 7(1), 13126, 2017.

Lindbo, J. A. and Dougherty, W. G., "Plant pathology and RNAi: a brief history." ***Annual Review of Phytopathology*** 43:191–204, 2005.

Liu, F., Wang, X. D., Zhao, Y. Y., Li, Y. J., Liu, Y. C and Sun, J., "Silencing the HaAK gene by transgenic plant-mediated RNAi impairs larval growth of Helicoverpa armigera." ***International journal of biological sciences*** 2015; 11(1):67–74, 2015.

Liu, Z., Taub, C.C. and McClung, C.R., "Identification of an Arabidopsis thaliana ribulose-1,5-bisphosphate carboxylase/ oxygenase activase (*RCA*) minimal promoter regulated by light and the circadian clock. ***Plant Physiology*** 112: 43-51, 1996.

Luo, Y., Wang, X., Wang, X., Yu, D. and Chen, B., "Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding." ***Insect Molecular Biology***, 22:574-583, 2013.

Luttrell, R.G., Ali, I. and Allen, K.C., " Resistance to *Bt* in Arkansas populations of cotton bollworm." In: Richter, D.A. (Ed.), Proceedings of the 2004 Beltwide Cotton Conferences, San Antonio, TX, January 5e9, 2004. ***National Cotton Council of America, Memphis*** TN, pp. 1373e1383, 2004.

Malekmohammadi, M. and Galehdari, H., "Target site insensitivity mutations in the AChE enzyme confer resistance to organophosphorous insecticides in Leptinotarsa decemlineata (Say)." ***Pesticide Biochemistry and Physiology*** 126, 85–91, doi:10.1016/j. pestbp.2015.08.002 2016.

Mamta, B., Reddy, K. R. K. and Rajam, M. V., “Targeting chitinase gene of Helicoverpa armigera by host-induced RNA interference confers insect resistance in tobacco and tomato.” ***Plant Molecular Biology*** 90: 281–292, 2015.

Mansoor, S., Amin, I., Hussain, M., Zafar, Y. and Briddon, R. W., “Engineering novel traits in plants through RNA interference.” ***Trends in Plant Science*** 11:559–65. pmid:17030144, 2006.

Mao, J. and Zeng, F., “Plant-mediated RNAi of a gap gene-enhanced tobacco tolerance against the Myzus persicae.” ***Transgenic Research*** 23: 389–396, 2014.

Mao, Y. B., Cai, W. J., Wang, J. W., Hong, G. J. and Tao, X. Y., “Silencing a cotton bollworm P450 monooxygenase gene by plantmediated RNAi impairs larval tolerance of gossypol.” ***Nature Biotechnology,*** 25:1307-1313, 2007.

Mao, Y. B., Tao, X. Y., Xue, X. Y., Wang, L. J. and Chen, X. Y., “Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms.” ***Transgenic Research*** 20:665-673, 2011.

Mao, Y.B., Xue, X.Y., Tao, X.Y., Yang, C.Q., Wang, L.J. and Chen, X.Y., “Cysteine protease enhances plant-mediated bollworm RNA interference.” ***Plant molecular biology***, *83*(1-2), pp.119-129, 2013.

Maqbool, A., Abbas, W., Rao, A. Q., Irfan, M., Zahur, M., Bakhsh, A., Riazuddin, S. and Husnain, T., "Gossypium arboreum GHSP26 enhances drought tolerance in Gossypium hirsutum", ***Biotechnology Progress*** 26(1), 21-25, 2010.

Martínez, I.R., “Glycoalkaloid profiling of potato genotypes from the north dakota state university potato breeding program (Doctoral dissertation, North Dakota State University). Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann., P. *et al*. 2007. Control of coleopteran insect pests through RNA interference.” ***Nature Biotechnology*** 25: 1322–1326, 2013.

Mazier, M., Pannetier, C., Tourneur, J., Jouanin, L., Giband, M., "The expression of Bacillus thuringiensistoxin genes in plant cells." ***Biotechnology Annual Review*** 3, pp. 313-347, 1997

McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., and Fraley, R., "Leaf disc transformation of cultivated tomato (L. esculentum) using *Agrobacterium tumefaciens*." ***Plant Cell Reports***, 5(2), 81-84, 1986.

McHughen, A., and Jordan, M. C., "Recovery of transgenic plants from "escape" shoots. ***Plant cell reports***, 7(8), 611-614, 1989.

Murashige, T., and Skoog, F., "A revised medium for rapid growth and bio assays with tobacco tissue cultures." ***Physiologia plantarum***, 15(3), 473-497, 1962.

McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R. and Fraley, R., "Leaf disc transformation of cultivated tomato (L. sculentum) using *Agrobacterium tumefaciens*. ***Plant Cell Reports*** 5: 81-84, 1986.

Mette, M. F., Aufsatz, W., Van der Winden, J., Matzke, M. A. and Matzke, A. J. M., "Transcriptional silencing and promoter methylation triggered by double-stranded RNA." ***The EMBO J,*** 19: 5194-5201, 2000.

Meyering-Vos, M. and Muller, A., "RNA interference suggests sulfakinins as satiety effectors in the cricket Gryllus bimaculatus." ***Journal of Insect Physiology*** 53, 840– 848, 2007.

Molla, M.M.H., Nasiruddin, K.M., Al-Amin, M. and Haque, M.S., "*Agrobacterium*-mediated transformation in potato. ***Thai Journal of Agricultural Science***, 44(2), pp.93-102, 2011.

Mota-Sanchez, D., Hollingworth, R. M., Grafius, E. J. and Moyer, D. D., "Resistance and cross-resistance to neonicotinoid insecticides and spinosad in the Colorado potato beetle, Leptinotarsa decemlineata (Say) (Coleoptera:

Chrysomelidae).” ***Pest Management Science*** 62, 30–37, doi:10.1002/ps.1120, 2006.

Mutti, N. S., Louis, J., Pappan, L. K., Pappan, K., Begum, K., Chen, M. S., Park, Y., Dittmer, N., Marshall, J., Reese, J. C. and Reeck, G. R., “A protein from the salivary glands of the pea aphid, Acyrthosiphon pisum, is essential in feeding on a host plant.” ***Proceedings of the National Academy of Sciences*** USA 105:9965–9969, 2008.

Mutti, N. S., Park, Y., Reese, J. C. and Reeck, G. R., “RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, Acyrthosiphon pisum.” ***Journal of Insect Science*** 6:1–7, 2006.

Nain, V., Jaiswal, R., Dalal, M., Ramesh, B. and Kumar, P. A., "Polymerase chain reaction analysis of transgenic plants contaminated by*Agrobacterium*", ***Plant Molecular Biology Reporter*** 23(1), 59-65, 2005.

Nakagawa, Y. and Henrich, V. C., “Arthropod nuclear receptors and their role in molting.” ***FEBS.J.*** 276,61286157,https://doi.org/10.1111/j.17424658.2009.07347.x 2009.

Naqqash, M. N., Gokce, A., Bakhsh, A. And Salim M., "Insecticide resistance and its molecular basis in urban insect pests." ***Parasitology research*** 115.4 (2016): 1363-1373, 2016.

Navarre, D.A., Goyer, A. and Shakya, R., “Nutritional value of potatoes: vitamin, phytonutrient, and mineral content. In ***Advances in potato chemistry and technology*** (pp. 395-424), 2009.

Niu, X., Kassa, A., Hu, X., Robeson, J., McMahon, M., Richtman, N.M., Steimel, J.P., Kernodle, B.M., Crane, V.C., Sandahl, G. and Ritland, J.L., “Control of Western Corn Rootworm (Diabrotica virgifera virgifera) Reproduction through Plant-Mediated RNA Interference.” ***Scientific reports*** *7*(1), p.12591, 2017.

Nicot, N., Hausman, J. F., Hoffmann, L., and Evers, D., "Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress." ***Journal of experimental botany*** 56(421), 2907-2914, 2005.

Nunes, F. M., and Simões, Z. L., "A non-invasive method for silencing gene transcription in honeybees maintained under natural conditions." ***Insect Biochemistry and Molecular Biology*** 39, 157–160. doi: 10.1016/j.ibmb.2008. 10.011, 2009.

Odell, J.T., Nagy, F. and Chua, N.H., "Identification of DNA sequences required for activity of the cauliflower mosaic virus *35S* promoter." ***Nature*** 313: 810–812, 1985.

Oerke, E. C., "Crop losses to pests", ***Journal of Agricultural Science***, 144, 31-43, 2006.

Orozco, B.M. and Ogren, W.L., "Localization of light-inducible and tissue-specific regions of the spinach ribulose bisphosphate carboxylase/oxygenase (rubisco) activase promoter in transgenic tobacco plants." ***Plant Moleculae Biology*** 23: 1129-1138, 1993.

Palli, S. R., "RNAi methods for management of insects and their pathogens." ***CAB Rev***, 7:1-10, 2012.

Pan, G., Zhang, X., Liu, K., Zhang, J., Wu, X., Zhu, J. and Tu, J., "Map-based cloning of a novel rice cytochrome P450 gene CYP81A6 that confers resistance to two different classes of herbicides. ***Plant molecular biology*** 61(6), pp.933-943, 2006.

Pecot, C. V., Calin, G. A., Coleman, R. L., Lopez-Berestein, G. and Sood, A. K., "RNA interference in the clinic: challenges and future directions." ***Nature Reviews Cancer*** 11, 59–67. doi: 10.1038/nrc2966, 2011.

Pelletier, Y., Horgan, F. G. and Pompon, J., "Potato resistance against insect herbivores: resources and opportunities in Insect Pests of Potato." ***Global***

Perspectives on Biology and Management **(**eds. Giordanengo, P., Vincent, C. and Alyokhin, A.) 439–462 Elsevier, 2013.

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever, D. and Fischhoff, D.A., "Genetically improved potatoes: protection from damage by Colorado potato beetles." ***Plant molecular biology***, *22*(2), pp.313-321, 1993.

Pilcher, C. D., Rice, M. E., Obrycki, J. J. and Lewis, L. C., "Field and laboratory evaluations of transgenic Bacillus thuringiensis corn on secondary Lepidopteran pests (Lepidoptera: Noctuidae)." ***Journal of Economic Entomology*** 90: 669–678, 1997.

Pilgrim, M. L. and McClung, C. R., "Differential involvement of the circadian clock in the expression of genes required for ribulose-1,5-bisphosphate carboxylase/oxygenase synthesis, assembly, and activation in Arabidopsis." ***Plant Physiology*** 103:553–564, 1993.

Pimental, D., Hunter, M.S., LaGro, J.A., Efroymsoun, R.A., Landers, J.C., Mervis, F.T., McCarthy, C.A. and Boyal, A.E., "Benefits and risks of genetic engineering in agriculture", ***Bio Science***, V.39, P. 606-614, 1989.

Pitino, M., Coleman, A. D., Maffei, M. E., Ridout, C. J. and Hogenhout SA., "Silencing of aphid genes by dsRNA feeding from plants." ***PLoS ONE***, 6:e25709, 2011.

Pitzschke, A. and Hirt, H., "New Insights into an Old Story: *Agrobacterium*-Induced tumour formation in plants by plant transformation", *The* ***EMBO Journal,*** V. 29(6), P. 1021–1032, 2010.

Poethig, R.S., Peragine, A., Yoshikawa, M., Hunter, C., Willmann, M. and Wu, G., "The function of RNAi in plant development, Cold Spring Harbour Symposia." ***Quantitative Biology*** 71, 165-170, 2006.

Pon, N. G., Rabin, B. R. and Calvin, M., “Mechanism of the carboxydismutase reaction. I. The effect of preliminary incubation of substrates, m*et al* ions, and enzyme on activity.” ***Biochemical Journal*** 338: 7–19, 1963.

Poreddy, S., Li, J. and Baldwin, I.T., “Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature.” ***BMC plant biology*** *17*(1), p.199, 2017.

Portis, A. R. Jr., “Rubisco activase - rubisco’s catalytic chaperone.” ***Photosynthesis Research*** 75: 11-27, 2003.

Portis, A.R. Jr., Li, C., Wang, D. and Salvucci, M. E., “Regulation of rubisco activase and its interaction with rubisco.” ***Journal of Experimental Botany*** 59: 1597-1604, 2008.

Potenza, C., Aleman, L. and Sengupta-Gopalan, C., “Invited review: targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation.” ***In Vitro Cellular and Developmental Biology-Plant*** *40*(1), pp.1-22, 2004.

Price, D. R. and Gatehouse, J. A., “RNAi-mediated crop protection against insects.” ***Trends Biotechnol*** 26:393–400, 2008.

Qu, D., Song, Y., Li, W.M., Pei, X.W., Wang, Z.X., Jia, S.R. and Zhanget, Y.Q., “Isolation and characterization of the organ-specific and lightinducible promoter of the gene encoding rubisco activase in potato (*Solanum tuberosum*).” ***Genetics and molecular research*** 10: 621–631, 2011.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P. and Bhatnagar, R.K., “Silencing of midgut aminopeptidase N of Spodoptera litura by double-stranded RNA establishes its role as Bacillus thuringiensis toxin receptor.” ***Journal of Biological Chemistry*** 277, 46849–46851, 2002.

Raman, K. V., and Palacios, M., “Screening potato for resistance to potato tuberworm.”***Journal of Economic Entomology***, *75*(1), 47-49, 1982.

Rangasamy, M. and Siegfried, B. D., “Validation of RNA interference in western corn rootworm Diabrotica virgifera virgifera LeConte (Coleoptera, Chrysomelidae) adults. ***Pest Management Science*** 68, 587–591. doi: 10.1002/ ps.2301, 2012.

Rao, A. Q., Bakhsh, A., Kiani, S., Shahzad, K., Shahid, A. A., Husnain, T. and Riazuddin, S., “The myth of plant transformation”. ***Biotechnology advances*** 27(6), 753-763, 2009.

Rao, A. Q., Bakhsh, A., Nasir, I. A., Riazuddin, S. and Husnain, T., "Phytochrome B mRNA expression enhances biomass yield and physiology of cotton plants", ***African Journal of Biotechnology*** 10(10), 1818-1826, 2011.

Rao, C. K. "Transgenic *Bt* technology: 3. Expression of transgenes." Foundation for Biotechnology Awareness and Education. Available: http://www. fbae. org/2009/FBAE/website/special-topics_views_***transgenic_bt_technology3***. html 2005.

Raza, W.,Ling, N., Liu, D., Wei, Z., Huang, Q. and Shen, Q., “Volatile organic compounds produced by *Pseudomonas fluorescens* WR-1 restrict the growth and virulence traits of *Ralstonia solanacearum”* ***Microbiological Research*** 192, 103–113, 2016.

Revuelta, L., Ortego, F., Díaz-Ruíz, J.R., Castañera, P., Tenllado, F. and Hernández-Crespo, P., "Contribution of Ldace1 gene to acetylcholinesterase activity in Colorado potato beetle.” ***Insect biochemistry and molecular biology***, 41(10), pp.795-803, 2011.

Riddiford, L. M., Cherbas, P. and Truman, J. W., “Ecdysone receptors and their biological actions.” ***Vitamins and Hormones*** 60, 1-73, 2000.

Rondon, S.I., “The potato tuberworm: A literature review of its biology, ecology, and control.” ***American Journal of Potato Research*** 87: 149–166, 2010.

Rose, R. L. and Brindley, W. A., “An evaluation of the role of oxidative enzymes in Colorado potato beetle resistance to carbamate insecticides.” ***Pesticide Biochemistry and Physiology*** 23, 74–84, doi:10.1016/0048-3575(85)90080-X 1985.

Rowe, R. C. and Powelson, M. L., “Potato early dying: management challenges in a changing production environment”, ***Plant Disease*** *86*(11), 1184-1193, 2002.

Rrchards, G. P., “Insect hormones in development. ***Biological Reviews*** 56. 501-549, 1981.

Rundle, S. J. and Zielinski, R. E., “Organization and expression of two tandemly oriented genes encoding ribulosebisphosphate carboxylase/oxygenase activase in barley.” ***Journal of Biological Chemistry*** *266*(8), 4677-4685, 1991.

Saeed, S., Saeed, Q., Saeed, R., Shafiq, M., Jaleel, W., Ishfaq, M., Naqqash, M. N. and Iqbal, M.., “Impact of various diets on biological parameters of Chrysoperla carnea Stephen (Neuroptera: Chrysopidae) adults under controlled conditions.” ***Applied Sciences and Business Economics*** 1: 1-9, 2014.

Sambrook, J., Russell, D. W., and Maniatis, T., “Molecular cloning” vol. 1-3. Cold Spring Habour ***Laboratory Press*** New York, 2001.

Saleh, A. A., Watanabe, S., Annaluru, N., Kodaki, T. and Makino, K., “Construction of various mutants of xylose metabolizing enzymes for efficient conversion of biomass to ethanol.” ***Nucleic Acids Symposium Series*** *(Oxf)* 279-80, 2006.

Salvucci, M. E., Portis, A. R. Jr. and Ogren, W. L., “A soluble chloroplast protein catalyzes ribulosebisphosphate carboxylase/oxygenase activation in vivo.” ***Photosynthesis Research*** 7: 193–201, 1985.

Salvucci, M. E., van de Loo, F. J. and Stecher, D. S., “Two isoforms of Rubisco activase in cotton, the products of separate genes not alternative splicing. ***Planta*** 216: 736–744, 2003.

Salvucci, M. E., Werneke, J. M., Ogren, W. and Portis, Jr. A. R., “Purification and species distribution of rubisco activase”, ***plant physiology*** 84, 930-936, 1987.

Sanahuja, G., Banakar, R., Twyman, R. M., Capell, T. and Christou, P.,"Bacillus thuringiensis: a century of research, development and commercial applications." ***Plant biotechnology journal*** 9.3: 283-300, 2011.

Sehnal, F., "Hormonal role of ecdysteroids in insect larvae and during metamorphosis. İn Koolman J. (ed); Ecdysone. From Chemistry to Mode of Action. ***Georg thieme Verlag*** Stuttgart.pp.271-278, 1989.

Senthil-Kumar, M. and Kirankumar S. M., "RNAi in plants: recent developments and applications in agriculture." ***Gene silencing: theory, techniques and applications***: 183-199, 2010.

Shakesby, A.J., Wallace, I.S., Isaacs, H.V., Pritchard, J., Roberts, D.M. and Douglas, A.E., “A water-specific aquaporin involved in aphid osmoregulation.” ***Insect Biochemistry and Molecular Biology*** 39, 1–10, 2009.

Shao, H.B. Chu, L.Y. Jaleel, C.A. and Zhao, C.X., “Water-deficit stress-induced anatomical changes in higher plants”, ***Journal of Plant Pathology and Microbiology*** 331, 215-225, 2008.

Sharma, S.K, “Effect of cutworm population and shoot damage in potato on the tuber Yield.” ***Potato Journal*** 40: 114-121, 2013.

Sohail, M. N., Karimi, S. M., Asad, S., Mansoor, S., Zafar, Y. and Mukhtar, Z., “Development of broad-spectrum insect-resistant tobacco by expression of synthetic cry1Ac and cry2Ab genes.” ***Biotechnology Letters*** 34: 1553-1560, 2012.

Soto, N., Enríquez, G. A., Ferreira, A., Corrada, M., Fuentes, A., Tiel, K. and Pujol, M., “Efficient transformation of potato stems segments from cultivar

Désirée using phosphinothricin as selection marker.” ***Biotecnología Aplicada*** 24(02), 139-144, 2007.

Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G. and Vanden, B. J., “Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*.” ***Insect Biochemistry and Molecular Biology*** *Biol.* 81 103–116. 10.1016/j.ibmb.2017.01.004, 2017.

Stalinski, R., Frederic L., Guillaume, T. and Laurence D., "Receptors are affected by selection with each Bacillus thuringiensis israelensis Cry toxin but not with the full *Bt*i mixture in Aedes aegypti." ***Infection, Genetics and Evolution*** 44: 218-227, 2016.

Stewart Jr, C.N., Adang, M.J., All, J.N., Raymer, P.L., Ramachandran, S. and Parrott, W.A., “Insect control and dosage effects in transgenic canola containing a synthetic Bacillus thuringiensis cryIAc gene.” ***Plant physiology***, *112*(1), pp.115-120, 1996.

Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C. and Rathore, K.S., “Developmental and tissue-specific expression of *CaMV 35S* promoter in cotton as revealed by GFP.” ***Plant molecular biology*** *50*(3), pp.463-479, 2002.

Tabashnik, B.E., Brévault T. and Carrière, Y., “Insect resistance to *Bt* crops: lessons from the first billion acres.” ***Nature Biotechnology*** 31, 510e521, 2013.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W. and Carriere, Y., “Insect resistance to *Bt* crops: evidence versus theory.” ***Nature Biotechnology*** 26: 199–202, 2008.

Tabashnik, B.E., Van Rensburg, J.B.J. and Carrière, Y., “Field-evolved insect resistance to *Bt* crops: definition, theory, and data. ***Journal of economic entomology*** 102(6), pp.2011-2025, 2009.

Tang, W., "Development of insect-resistant transgenic indica rice with a synthetic cry1C* gene." ***Molecular Breeding*** 18.1: 1, 2006.

Terenius, O., Papanicolaou, A., Garbutt, J. S., Eleftherianos, I. and Huvenne, H., "RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design." ***Journal of Insect Physiology*** 57:231-245, 2011.

Tabashnik, B. E., and Johnson, M. W., "Laboratory selection for pesticide resistance in natural enemies." **In *Applications of genetics to arthropods of biological control significance*** (pp. 91-106). CRC Press, 2018.

Thakur, N., Upadhyay, S. K., Verma, P. C., Chandrashekar, K., and Tuli R., "Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of v-ATPase A gene." ***PLoS ONE***, 9:e87235, 2014.

Thiele, G. Theisen, K. Bonierbale, M. and Walker, T. "Targeting the poor and hungry with potato science", ***Potato Journal*** 37 75-86, 2010.

Timmons, L., Court, D. L. and Fire, A., "Ingestion of bacteria expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans." ***Gene*** 263:103–112, 2001.

Tomari, Y. and Zamore, P. D., "Perspective: machines for RNAi." ***Genes and Development*** 19:517–29, 2005.

Tomoyasu, Y. and Denell, R.E., "Larval RNAi in Tribolium (Coleoptera) for analyzing adult development." ***Development Genes and Evolution*** 214, 575–578 2004.

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M. and Grossmann, D., "Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium." ***Genome Biology*** 2008:9. Identfied genes involved in RNAi response in Tribolium, 2008.

TÜİK, “Hayvansal Üretim İstatistikleri.” "***Türkiye İstatistik Kurumu Haber Bülteni***." *Sayı* 18851: 13, 2014.

Turkstat, http://www.turkstat.gov.tr/PreHaberBultenleri.do?id=24581, 2017.

Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P. and Newcomb, R.D., “RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. ***Insect Molecular Biology*** 15, 383–391, 2006.

Upadhyay, S.K., Chandrashekar, K., Thakur, N., Verma, P.C., Borgio, J.F., Singh, P.K. and Tuli, R., “RNA interference for the control of whiteflies (Bemisia tabaci) by oral route.” ***Journal of biosciences*** 36(1), pp.153-161, 2011.

USDA., *https://www.nass.usda.gov/Publications/Ag_Statistics/2016/index.php*, 2016.

Van den Berg, J., Hilbeck, A. and Bøhn, T., “Pest resistance to Cry 1Ab *Bt* maize: field resistance, contributing factors and lessons from South Africa.” ***Crop Protection*** 54, 154e160, 2013.

Van Eck, J., “Genome editing and plant transformation of solanaceous food crops.” ***Current opinion in biotechnology*** *49*, pp.35-41, 2018.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C., Vernhettes, S., “Transgene-induced gene silencing in plants”, ***Plant Journal*** V.16, P.651-659, 1998.

Vauthier, C., Dubernet, C., Chauvierre, C., Brigger, I. and Couvreur, P., “Drug delivery to resistant tumors: the potential of poly(alkyl cyanoacrylate) nanoparticles.” ***Journal of Controlled Release***. 5;93(2):151-60, 2003.

Veale, M. A., Slabbert, M. M. and Van Emmenes, L., “Agrobacterium-mediated transformation of potato cv. Mnandi for resistance to the potato tuber moth (Phthorimaea operculella).” ***South African journal of botany*** 80, 67-74, 2012.

Visser, D., “Guide to Potato Pests and Their Natural Enemies in South Africa.” ***Arc- Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria***, South Africa, 105 p, 2005.

Visweshwar, R., Sharma, H. C., Akbar, S. M. and Sreeramulu, K., "Elimination of gut microbes with antibiotics confers resistance to Bacillus thuringiensis toxin proteins in Helicoverpa armigera (Hubner)." ***Applied biochemistry and biotechnology*** 177.8: 1621-1637, 2015.

Voinnet, O., “Non-cell autonomous RNA silencing.” ***FEBS Letters*** 579:5858–5871, 2005.

Walshe, D.P., Lehane, S.M., Lehane, M.J. and Haines, L.R., “Prolonged gene knockdown in the tsetse fly Glossina by feeding double stranded RNA.” ***Insect Molecular Biology*** 18, 11–19, 2009.

Wan, P. J., Guo, W. Y., Yang, Y., Lü, F. G., Lu, W.P. and Li, G. Q., “RNAi suppression of the ryanodine receptor gene results in decreased susceptibility to chlorantraniliprole in Colorado potato beetle Leptinotarsa decemlineata.” ***Journal of insect physiology***, *63*, pp.48-55, 2014.

Wang, B., Ma, Y., Zhang, Z., Wu, Z., Wu, Y., Wang, Q. and Li, M., "Potato viruses in China", ***Crop Protection*** 30(9), 1117-1123, 2011.

Wattilon, B., Kettmann, R., Boxus, P. and Burny, A., “Developmental and circadian pattern of rubisco activase mRNA accumulation in apple plants.” ***Plant Molecular Bioogy.*** 23, 501-509, 1993.

Wenzler, H., Mignery, G., May, G. and Park, W., “A rapid and efficient transformation method for the production of large numbers of transgenic potato plants.” ***Plant Science,*** *63*(1), pp.79-85, 1989.

Werneke, J. M., Zielinski, R. E. and Ogren, W. L., “Structure and expression of spinach leaf cDNA encoding ribulosebisphosphate carboxylase/oxygenase activase. ***Proc Natl Acad Sci USA*** 85: 787-791, 1988.

Whangbo, J. S. and Hunter, C. P., "Environmental RNA interference." ***Trends in Genetics*** 24, 297–305. doi: 10.1016/j.tig.2008.03.007, 2008.

Whyard, S., Singh, A. D. and Wong, S., "Ingested double-stranded RNAs can act as species-specific insecticides." ***Insect Biochem Molecular Biology*** 39:824-832, 2009.

Wilson, R. C. and Tegg, S. R., 2012. "In vitro Cell Selection Techniques for Enhancing Disease Resistance – ***Case Study: Common Scab Resistance in Russet Burbank*.**" pp. 327-345, 2012.

Wuriyanghan, H., Rosa, C. and Falk, B.W., "Oral delivery of double-stranded RNAs and siRNAs induces RNAi effects in the potato/tomato psyllid, Bactericerca cockerelli." ***PloS ONE*** 6(11), p.e27736, 2011.

Xiong, Y. H., Zeng, H. M., Zhang, Y. L., Xu, D. W. and Qiu, D. W., "Silencing the HaHR3 gene by transgenic plant-mediated RNAi to disrupt Helicoverpa armigera development. ***International journal of biological sciences*** 9:370-381, 2013.

Xu, K., He, B., Zhou, S. and Li, Y., "Cloning and characterization of the rubisco activase gene from Ipomoea batatas (L.) Lam." ***Molecular Biology Reports*** 37: 661-668, 2010.

Xu, L., Duan, X., Lv, Y., Zhang, X., Nie, Z., Xie, C., Ni, Z. and Liang, R., "Silencing of an aphid carboxylesterase gene by use of plant-mediated RNAi impairs Sitobion avenae tolerance of Phoxim insecticides**.** ***Transgenic Research*** 23(2):389–96, 2014.

Xu, L., Hou, Q., Zhao, Y., Lu, L., Li, B., Ni, Z. and Liang, R., "Silencing of a lipase maturation factor 2-like gene by wheat-mediated RNAi reduces the survivability and reproductive capacity of the grain aphid, Sitobion avenae. ***Archives of insect biochemistry and physiology***, *95*(3), 2017.

Yan, P., Shen, W., Gao, X., Li, X., Zhou, P. and Duan, J., "High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants." ***PLoS One***, *7*(5), p.e38186, 2012.

Yao, J., Rotenberg, D., Afsharifar, A., Barandoc-Alviar, K., Whitfield, A.E., "Development of RNAi Methods for Peregrinus maidis, the Corn Planthopper. ***PLoS ONE*** 8(8): e70243. doi:10.1371/journal.pone.0070243, 2013.

Yu, R., Xu, X., Liang, Y., Tian, H., Pan, Z., Jin, S., Wang, N. and Zhang, W., "The insect ecdysone receptor is a good potential target for RNAi-based pest control. ***International journal of biological sciences***, *10*(10), p.1171, 2014.

Zha, W., Peng, X., Chen, R., Du, B. and Zhu, L., "Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect Nilaparvata lugens." ***PLoS ONE***,6:e20504, 2011.

Zhang, B. H., Liu, F., Liu, Z. H., Wang, H. M. and Yao, C. B., "Effects of kanamycin on tissue culture and somatic embryogenesis in cotton. ***Plant Growth Regulation*** 33:137–149, 2011.

Zhang, H., Yin, W., Zhao, J., Jin, L. and Yang, Y., "Early warning of cotton bollworm resistance associated with intensive planting of *Bt* cotton in China." ***PLoS ONE*** 6: e22874, 2011.

Zhang, J., Khan, S.A., Hasse, C., Ruf, S., Heckel, D.G. and Bock, R., "Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids." ***Science*** *347*(6225), pp.991-994, 2015.

Zhang, J., Khan, S.A., Heckel, D.G. and Bock, R., "Next-generation insect-resistant plants: RNAi-mediated crop protection." ***Trends in biotechnology*** 35(9), pp.871-882, 2017.

Zhang, M., Hanna, M., Li, J., Butcher, S., Dai, H. and Xiao, W., "Creation of a hyperpermeable yeast strain to genotoxic agents through combined inactivation of PDR and CWP genes." ***Toxicol Sci*** 113(2):401-11, 2010.

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y.T., He, J., Gao, M., Xu, F., Li, Y., Zhu, Z. and Li, X., “Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors.” ***Proceedings of the National Academy of Sciences***, *107*(42), pp.18220-18225, 2010.

Zhang, Z., Coyne, D.P. and Mitra, A., “Factors affecting *Agrobacterium*-mediated transformation of common bean. ***Journal of the American Society for Horticultural Science*** *122*(3), pp.300-305, 1997.

Zhou, Z., Pang, J., Guo, W., Zhong, N., Tian, Y., Xia, G. and Jiahe, W., "Evaluation of the resistance of transgenic potato plants expressing various levels of Cry3A against the Colorado potato beetle (Leptinotarsa decemlineata Say) in the laboratory and field." ***Pest management science*** 68.12: 1595-1604, 2012.

Zhu, F., Xu, J. J., Palli, R., Ferguson, J. and Palli, S. R., “Ingested RNA: interference for managing the populations of the Colorado potato beetle, Leptinotarsa decemlineata.” ***Pest management science*** 67:175-182, 2011.

Zhu, J.Q., Liu, S., Ma, J., Zhang, J.Q., Qi, H.S., Wei, Z.J., Yao, Q., Zhang, W.Q., Li, S., “Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene *EcR*”. ***PLoS ONE****:* 7. p. e38572 http://dx.doi.org/10.1371/journal.pone.0038572, 2012.

APPENDIX

Appendix-I

MS Medium (Murashige and Skoog, 1962) Composition

MS Salts	4.33g L^{-1}
MS Vitamins	1mL L^{-1}
Sucrose	30g L^{-1}
Gelrite	3g L^{-1}
pH	5.6-5.8

Appendix-II

LB Broth (1L)

Yeast extract	5g
Bactotrypton	10g
NaCl	10g

Dissolved in 1 liter of distilled water, adjusted pH 7.5 and autoclaved.

LB AGAR (1L)

LB containing 15g L^{-1} of Bacto Agar

Appendix-III

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

Tahira HUSSAIN was born on June 20, 1987 in Lahore, Pakistan. She completed her higher secondary education from Crescent Public School, Lahore in 2004. Afterwards she joined Himayat-e-Islam Khawateen College, Lahore Pakistan in 2006 for her undergraduate studies. She completed her B.Sc (Hons) in Botany from Department of Botany, Kinnaird College for Women, Lahore Pakistan in 2010. She completed her Masters degree from Department of Environment Sciences, Kinnaird College for Women, Lahore Pakistan in 2012. She enrolled in Graduate School of Natural and Applied Sciences, Department of Agricultural Genetic Engineering at Niğde Ömer Halisdemir University, Niğde, Turkey to pursue her PhD education under the guidance of Prof. Dr. Mehmet Emin ÇALIŞKAN. During her PhD thesis research she worked development of potato lines resistant to Colorado potato beetle (CPB) using RNAi-based transformation approach. She knows English, Urdu and Punjabi languages.