T.R.
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE
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RNA SILENCING MEDIATED RESISTANCE AGAINST *PLUM POX VIRUS*

Prepared By
Maryam GHADERI SOHI

Supervisor

Prof. Dr. Mikail AKBULUT
Assoc. Prof . Dr. Kahraman GÜRCAN

(PhD Thesis)

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COMPLIANCE WITH SCIENTIFIC ETHICS

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Signature: 

COMPLIANCE WITH GUIDLINES

The Doctoral Dissertation, entitled as "RNA silencing mediated resistance against *Plum pox virus*" has been prepared according to the postgraduate thesis proposal and thesis writing guidelines of Erciyes University.

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This study, entitled **"RNA silencing mediated resistance against *Plum pox virus*"** that has been prepared by Maryam Ghaderi Sohi under the supervision of, Prof. Dr. Mikail AKBULUT and Assoc. Prof. Dr. Kahraman GÜRCAN has been certified as the Doctoral dissertation in the Department of Biology, Graduate School of Natural and Applied Science at Erciyes University.

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PhD Thesis May2019
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ABSTRACT

Widespread presence of different strains of *Plum pox virus* (PPV), including Didron (D), Marcus (M) and Turkey (T), in different regions of Turkey and the economic importance of stone fruit hosts such as apricots for the country, highlight the necessity of the PPV resistance programs. In this research, to evaluate gene-silencing approach in producing resistance against this virus, five genomic regions including *UTR/P1* (752 pb), *P1/HCPro* (771bp), *HCPro* (649 bp), *HCPro/P3* (594 bp) and *CP* (990bp) derived from PPV-T were amplified by PCR for preparation of hairpin constructs using the Gateway system. The Agrobacterium-mediated transformation system has been used to introduce the constructs to model plant, *Nicotiana benthamiana*. PCR amplification of neomycin phosphotransferase II (*nptII*) gene and the specific genes from regenerated *N. benthamiana* confirmed the successful transformation procedure. In addition, for further confirmation sequencing of insert genes in each arm of hairpin constructs was performed. In order to evaluate the efficiency of virus resistance, the transgenic *N. benthamiana* T1 lines were grown in greenhouse to be inoculated with PPV-D, PPV-M and the PPV-T strains. Based on symptom observation and ELISA test results, several lines of transgenic plants harboring the hairpin constructs *UTR/P1* and *CP*, one line of *HCPro* and two lines of *HCPro/P3* containing hairpin constructs showed the highest level of resistance against PPV infection. The majority of transgenic plants containing the *P1/HCPro* hairpin construct showed PPV symptoms in resistance assay indicating that they were not resistant. Aiming to gain a deeper insight about the RNA-mediated gene silencing defense process in transgenic plants and comparing the potential of hairpin structures in production of transgene-derived siRNAs, the expression profiles of siRNAs and miRNAs in both wild-type (infected and un-infected) and transgenic plants were determined by small RNA high-throughput sequencing. Comparison of miRNA

expression profiles of infected and un-infected wild-type plants revealed that PPV infection led to up/down regulation of some miRNAs. The gene ontology (GO) analysis of predicted miRNA target genes revealed decrease in frequency of genes involved in different biological responses including defense response. In transgenic resistant plants, except for transgenic plants harboring *HCPro* hairpin construct (moderately resistant), the enriched GO terms of predicted miRNA target genes showed a similar pattern to the enriched GO terms of un-infected wild-type plants. This may be interpreted by prevention of PPV establishment in resistant transgenic plants resulting from induced RNAi. In addition, siRNA analysis in transgenic plants revealed various potentials of these hairpin structures in producing siRNAs and their accumulation on target regions. This is the first study of its kind which compares the most common PPV genomic regions used for induction of RNA silencing against PPV by deep sequencing analysis for screening of the siRNA species. Characterization of produced siRNAs in transgenic plants indicated that *UTR/P1* and *CP* hairpin structures with higher capacity of production of siRNAs and dense accumulation of them on target regions are, to date, the best candidates for transformation of stone fruit plants.

Keywords: *Plum pox virus*, RNA silencing, Resistance, siRNA, Deep sequencing

ŞARKA VİRÜSÜNE (PLUM POX) KARŞI RNA SESSİZLEŞTİRİLMESİ ARACILIĞIYLA DİRENÇ GELİŞTİRİLMESİ

Maryam GHADERI SOHI

Erciyes Üniversitesi, Fen Bilimleri Enstitüsü
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ÖZET

Plum Pox Virüsünün Dideron (D), Marcus (M) ve Türkiye (T) ırkını da içeren farklı ırklarının Türkiye'nin değişik bölgelerinde oldukça yaygın oluşu ve kayısı gibi taş çekirdekli meyvelerin ülke için ekonomik önemi, PPV direnç programına ihtiyaç olduğunu vurgulamaktadır. Bu çalışmada, bu virüse karşı direnç geliştirmek amacıyla gen sessizleştirmesi yaklaşımını değerlendirmek için PPV-T genomundan elde edilen *UTR/P*1 (752), P1/HCPro (771), *HCPro* (649) *HCPro/P3* (594) ve *CP* (990 bç) bölgelerini kapsayan 5 bölgesi, saç tokası konsraktı hazırlamak amacıyla PCR ile çoğaltılmıştır. Konstraktın bir model bitki olan *Nicotiana benthamianaya* aktarılması için *Agrobacterium* aracılı transformasyon sistemi kullanılmıştır. Rejenere edilen *Nicotiana benthamiana* bitkisinden *neomycin phosphotransferase* ve aktarılan spesifik gen bölgelerinin PCR ile çoğaltılması transformasyonun başarılı bir şekilde yapıldığını teyit etmiştir. Transformasyonun gerçekleştiği, saç tokası konstraktının her iki koluna klonlanan parçaların dizilenmesi ile daha ileri düzeyde doğrulanmıştır. Transgenic *Nicotiana benthamiana* bitkilerinde virüs direncinin etkinliğini değerlendirmek amacıyla PPV-D, PPV-T ve PPV-M ile inokule etmek için T1 hattı serada yetiştirilmiştir. Semptomların gözlenmesi ve ELISA test sonuçlarına göre *UTR/P1* ve *CP* saç tokası konstraktını içeren çok sayıda transgenik hat, bir *HCPro* ve HCPro/P3 saç tokası konsraktı içeren 2 hat PPV'ye direnç göstermiştir. *P1/HCPro* saç tokası konstraktını taşıyan bitkilerin çoğunluğu direnç deneylerinde PPV semptomu göstermiş olup dirençli bulunmamışlardır. Transgenik bitkilerde RNA aracılı gen susturma yoluyla savunma olgusunun daha iyi anlaşılmasını sağlamak ve saç tokası yapılarının transgenden türeyen suni Si-RNA üretimindeki potansiyellerini karşılaştırmak için, transgenik ve yabani tip bitkilerdeki SiRNA ve miRNA ifade profilleri yüksek verimli küçük RNA dizilemesi ile analiz edilmiştir. Enfekteli ve enfektesiz yabani tip bitkilerin

miRNA ifade profillerinin karşılaştırılması, PPV infeksiyonunun bazı miRNAlar'ın ifadelerinin aşağı ve yukarı yönde düzenlenmesine öncülük ettiğini ortaya çıkarmıştır. Transgenik dirençli bitkilerde tahmin edilen miRNA hedef genlerinin gen ontoloji (GO) analizleri, savunma tepkisini de içeren farklı biyolojik tepkilerle ilişkili genlerin frekansında azalma olduğunu göstermiştir. Transgenik bitkilerin, HCPro (orta derecede dirençli) saç tokası konstraktını taşıyan transgenik bitkiler hariç, tahmin edilen miRNA hedef genlerinin zenginleştitilmiş GO şartları, enfekteli olmayan yabanıl bitkilerin zenginleştirilmiş GO şartları ile benzer desen göstermiştir. Bu durum, dirençli transgenik bitkilerde uyarılmış RNAi den kaynaklanan PPV infeksiyonunun yerleşmesinin önlenmesi şeklinde yorumlanabilir. Ek olarak, transgenik bitkilerde siRNA analizi, siRNAların üretilmesi ve hedef bölgelerde birikmesinde bu saç tokası yapılarının çeşitli potansiyellerini ortaya çıkartmıştır. Bu çalışma, PPV genomunun en yaygın bölgelerini RNA sessizleştirmesinin uyarılması ve oluşturulan siRNA türlerinin taranması için de derin dizilim analizini kullanan ürünün ilk çalışmasıdır. Transgenik bitkilerdeki uyarılmış siRNAlar'ın karakterizasyonu, *UTR/P1* ve *CP* saç tokası yapılarının siRNA üretimi ve hedef bölgelerde birikiminde daha yüksek kapasiteye sahip olduğunu göstermekte ve bu genom bölgelerinin taş çekirdekli meyvelerde transformasyon için en iyi aday olduklarını belirtmektedir.

Anahtar kelimeler: Şarka virüsü, RNA sessizleştirilmesi, Direnç, SiRNA, Derin dizileme

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MATERIALS AND METHODS

LIST OF ABBREVIATIONS

°C	: Centigrade
µl	: Microliter
6K16	: Kilo dalton protein 1
6K2	: 6 kilo Dalton protein 2
ACLSV	: *Apple chlorotic leaf spot virus*
AGO	: Argonaute
Agr	: Agrobacterium
AS	: Acetosyringone
BAP	: 6-Benzylaminopurine
CaMV	: *Cauliflower mosaic virus*
cDNA	: Complementary DNA
Cef	: Cefotaxime
CHS	: Chalcone synthase
CI	: Cylindrical inclusion protein
Cm	: Centimeter
CP	: Coat protein
CSIRO	: Commonwealth Scientific and Industry Research Organization
CTAB	: Cetyl trimethylammonium bromide
CYSDV	:*Cucurbit yellow stunting disorder virus*
d/r	: Direct-repeat
DCL	: Dicer-like
ddH_2O	: Double-distilled H_2O
DIECA	: Sodium diethyl-dithiocarbamate
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
dNTP	: Deoxyribonucleotide triphosphate
Dpi	: Days post-inoculation
DRB	: dsRNA binding
dsRNA	: Double-strand RNA
DTT	: Dithiothreitol
E. coli	*: Escherichia coli*

EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzyme-linked immunosorbent assay
GO	: Gene ontology
GUS	: Beta-glucuronidase
H	: Hour
HC-Pro	: Helper Component proteinase
hc-siRNA	: Heterochromatic small interfering RNA
hpRNA	: Hairpin RNA
HST	: HASTY
Ht-siRNA	: High-throughput small interfering RNA
HYL1	: HYPONASTIC LEAVES1
IgG	: Immunoglobulin G
Ihp RNA	: Intron-spliced hairpin RNA
ihprolC-P*P1*97	: intron hair pin rolC PPV 197
IR	: Inverted-repeat
Kan	: Kanamycin
kCal/mol	: Kilocalorie/ mole
KCl	: Potassium chloride
kDa	: Kilo Dalton
L	: Liter
LB	: Luria-Bertani
LiCl	: Lithium chloride
LIYV	: *Lettuce infectious yellows virus*
Log	: Logarithm
MES	: 2-morpholinoethanesulfonic acid
MFEI	: Minimal folding free energy index
$MgCl_2$	: Magnesium chloride
Min	: Minute
miRNA	: MicroRNA
ml	: Milliliter
mM	: Millimolar
mRNA	: Messenger RNA
MS	: Murashige Skoog

$Na2CO_3$	: Sodium carbonate
$NaHCO_3$	: Sodium Bicarbonate
NaOCl	: Sodium hypochlorite
NAT-siRNA	: Natural antisense short interfering RNA
NBS-LRR	: Nucleotide-binding site-leucine-rich repeat
Ng	: Nano gram
NIaPro	: Nuclear inclusion a protease
NIb	: Nuclear inclusion protein b
Nm	: Nanometer
nptII	: Neomycin phosphotransferase II
nt	: Nucleotide
OD	: Optimum density
ORF	: Open reading frame
PBS	: Phosphate-buffered saline
PCR	: Polymerase chain reaction
PDR	: Pathogen-derived resistance
PDV	: *Prunus dwarf virus*
phasiRNAs	: Phased siRNAs
PIPO	: Pretty interesting potyviral ORF
PIWI	: P-element Induced WImpy
PNRSV	: *Prunus necrotic ring spot virus*
Pol V	: DNA Polymerase V
PPV	: *Plum pox virus*
PPV-An	: *Plum pox virus* Ancestor
PPV-C	: *Plum pox virus* Cherry
PPV-CR	: *Plum pox virus* Cherry Russian
PPV-D	: *Plum pox virus* Dideron
PPV-EA	: *Plum pox virus* El Amar
PPV-M	: *Plum pox virus* Markus
PPV-Rec	: *Plum pox virus* Recombinant
PPV-T	: *Plum pox virus* Turkey
PPV-W	: *Plum pox virus* Winona
PR	: Pathogenesis-related

pre-miRNA : Precursor miRNA
pri-miRNA : Primary miRNA
PTGS : Post-transcriptional gene silencing
PVP : Polyvinylpyrrolidone
PVY : *Potato virus* Y
R : Resistance
RdDM : RNA-directed DNA methylation
RDR : RNA-dependent RNA polymerase
Rif : Rifampicin
RISC : RNA-induced silencing complex
RNA Pol IV : RNA polymerase IV
RNAi : RNA interference
RNase III : Ribonuclease III
Rpm : Revolutions per minute
rRNA : Ribosomal ribonucleic acid
RSS RNA : Silencing suppressor
RSV : *Rice stripe virus*
RT-PCR : Reverse transcription polymerase chain reaction
SDS : Sodium Dodecyl Sulphate.
SE : SERRATE
Sec : Second
siRNA : Small interfering RNA
SMV : *Soybean mosaic virus*
snoRNA :Small nucleolar RNAs
snRNA : Small nuclear RNA
Spec : Spectinomycin
sRNA : Small RNA
tasiRNA : Trans-acting siRNAs
Ta-siRNA : Trans-acting siRNA
TBE : Tris-borate-EDTA
T-DNA : Transfer DNA
TE : Tris-EDTA
TEV : *Tobacco etch virus*

TGH	: G-patch domain protein TOUGH
TG-R	: Transgenic-resistant
TG-S	: Transgenic-susceptible
TMV	: *Tobacco mosaic virus*
Tris-HCl	: Tris hydrochloride
tRNA	: Transfer ribonucleic acid
U/ µL	: Units/µL
UTR	:Untranslated region
V	: Volt
v/v	: Volume/Volume
VPg	: Viral protein genome-linked
vsiRNAs	: Viral small interfering RNAs
VSRs	: Viral suppressors of RNA silencing
w/v	: Weight/Volume
WT	: Wild type
YEP	: Yeast extract peptone
ZYMV	: *Zucchini yellow mosaic potyvirus*
β-ME	: β-Mercaptoethanol

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INTRODUCTION

Plum pox virus (PPV), the causing agent of Sharka was observed and reported for the first time in Bulgaria on *Prunus domestica* cv. Kjustendil (Atanasoff, 1935). PPV is a *Potyvirus* in *Potyviridae* family. The natural host range of PPV comprises the economically important crops like stone fruit trees around the world. Incurable Sharka disease with high potential of crop losses may be considered as a serious restricting agent for mass production of stone fruits around the world. PPV is now widespread almost globally and, except for Australia, it has been detected in all continents (Garcia and Cambra, 2007). Eastern Europe was the first localized PPV epidemic and then, after World War II in the late 1950's, the virus was detected from Western Europe to Germany and Austria. In a few years by the mid-60's, the disease was reported in the Netherlands, Switzerland, Greece, England and Turkey. Then in France, Italy, and Belgium by the early 70's; Spain and Portugal by the early 80's; and by the late 80's it was reported in Egypt, Syria and Cyprus, India and Jordan (Sochor et al., 2012). Later on, PPV was reported in the USA in 1999 (Levy et al., 2000), Canada in 2000 (Thompson et al., 2001), China in 2004 (Navratil et al., 2005), Argentina in 2005 (Dal Zotto et al., 2006) and, finally, it has also been reported in commercial Japanese apricot trees in Tokyo (Maejima et al., 2010).

So far, ten PPV strains have been recognized with genetically distinct properties and they are not essentially distinguishable based on their biological and epidemiological characteristics. These are: PPV-D (Dideron), PPV-M (Marcus), PPV-T (Turkey), PPV-EA (El Amar), PPV-Rec (Recombinant), PPV-W (Winona), PPV-C (Cherry), PPV-CR

(Cherry Russian), PPV-An (Ancestor) and PPV-CV (Cherry Volga) (Chirkov et al., 2018). PPV-M and PPV-D are the most prevalent strains and generally PPV-M is considered as a very severe strain with mostly fast outbreaks. PPV-D is widespread in Europe, while PPV-M is detected mostly in Southern and Central European countries. On the other hand, PPV-D is the strain responsible for most outbreaks outside Europe (Garcia et al., 2014).Genomic sequence analysis of isolates which were collected from Ankara in Turkey showed a unique recombination event in the HC-Pro gene. Therefore, these isolates were classified as a new recombinant strain named as PPV-T (Ulubas et al., 2009).

Since the detection of PPV on plums and apricots in Edirne (the European part of Turkey) (Şahtiyanci, 1969), there have been several surveys indicating the presence of the virus in different regions of Turkey including Central Anatolia, Marmara, Mediterranean, Aegean and Black Sea regions (Şahtiyancı, 1969; Kurçman, 1973; Yürektürk, 1984; Erdiler, 1988; Elibüyük and Erdiller, 1991; Elibüyük, 2003, 2004, 2006; Sertkaya et al., 2003; Koç and Baloğlu, 2006; Candresse et al., 2007; Gümüş et al., 2007; İlbağı et al., 2008; Ulubaş Serçe et al., 2009; Akbaş et al., 2011; Ceylan et al., 2014; İlbağ and Çıtır, 2014, Deligöz et al., 2015; Gürcan 2016 a,b; Gürcan and Ceylan, 2016; Gürcan et al., 2018; Teber et al., 2019). Recent diversity analysis of PPV-M and T isolates from Turkey revealed an old evolutional history of PPV in this country. PPV-T has only been identified in Turkey and not in other countries except for Albania, where only a single PPV-T isolate was detected (Ulubaş Serçe et al., 2009; Palmisano et al., 2015). Phylogenetic analyses of partial sequences of 421 isolates and complete genome sequences of 57 isolates, representing the geographical distribution of PPV-T in Turkey, revealed that within the PPV-T strain several monophyletic and, in some cases, geographically limited groups exist (Ankara-Konya1-Kayseri, Ankara-Balkan, Istanbul, Konya2 and Balkan). In this study it was found that the PPV-T diversity (0.018%) was greater that of PPV strains D and Rec but lower than M strain including the newly described and divergent M-Istanbul isolates and this shows a long evolutionary history of PPV-T. Based on findings of this study, it is assumed that the European part of Turkey in Balkans which is close to Bulgaria, the place that PPV was identified for the first time, is likely to be a center of origin for PPV-T isolates. The most likely scenario

for spread of PPV-T in Turkey is believed to be colonization of different regions by diverse isolates from the Balkans region, followed by secondary local spread (Teber et al., 2018). In another survey, Gürcan et al. (2018) showed a group of divergent PPV-M isolates detected in apricot, peach and plum trees could be evolutionarily successful divergent PPV-M isolates prevalent in Istanbul. This group of divergent Istanbul M isolates formed a monophyletic clade and thereby named as PPV-MIs. European type PPV-M was identified mostly in the newly established peach or apricot orchards in the Western and Mediterranean regions but also in residential gardens of the Thrace region (Gürcan and Ceylan, 2016). PPV-D isolates were mostly identified in residential gardens of Aksaray, Bursa, Eskişehir, Konya, İstanbul, and the Thrace region (Elibuyuk, 2004; Gürcan and Ceylan, 2016). Complete genome analysis of several D isolates in Turkey raised possibility that Turkey could be genetic diversity center for PPV-D isolates (Gürcan and Ceylan, 2017).

Considering diversity and spread of PPV in Turkey, it seems that quarantine and eradication cannot be considered a key solution for controlling PPV infection due to economic consequences of eradication and difficulties in restriction of plant material movement in such condition. Therefore, resistant cultivars and breeding programs play an important role in controlling the virus disease. However, restriction factors such as lack of resistance sources to PPV in peaches (Escalettes et al., 1998; Pascal et al., 2002; Rubio et al., 2012), the absence of resistant cultivars in domestic plumes (Kegler et al., 1998) and high susceptibility rate in European apricots (Kegler et al., 1998; Martínez-Gómez et al., 2000) and in the Irano-Caucasian eco-geographical group apricots (Gürcan et al., 2015; Gürcan et al., 2019) make the breeding program challenging for these important stone fruit plants.

Approaches such as genetic engineering, particularly pathogen-derived resistance (PDR) have been considered as alternative for producing PPV resistant plants around the world. After the first report of expression of viral gene for producing resistance against a virus (Powell et al., 1986), different virus sequences have been employed to produce transgenic plants resistant to viruses. In the case of PPV, resistant plants were obtained both in herbaceous model species and plants in *Prunus* genus using PDR

(Regner et al., 1992; Ravelonandro et al., 1993; Guo and Garcia 1997; Ravelonandro et al., 1997; Jaquet et al., 1998; Tavert-Roudet et al., 1998; Wittner et al., 1998; Barajas et al., 2004). Since identification of post transcriptional gene silencing (PTGS) phenomenon as the main mechanism in inducing resistance in transgenic plants, it has been utilized widely for introduction of resistance against different pathogens including viruses. Among different strategies for induction of PTGS the gene constructs encoding intron-spliced RNAs (ihp RNAs) with a hairpin structure showed to be highly efficient against viruses and also endogenous genes (Smith et al., 2000). So far, as it has been summarized by Ilardi and Tavazza (2015), several PPV-derived sequences have been used as hairpin structure for induction of RNA silencing mechanism in *N. benthamiana* as model plant and in *Prunus* species. In these studies, hairpin structures were derived from isolates of M and D strains. As the results of studies indicate successful PPV resistance by ihpRNA constructs derived from different genomic regions of the virus have been achieved. The *5′ UTR*, P1, HC-Pro, initial part of *P3* and *CP* are the most common genomic regions utilized for hairpin structures for inducing RNA silencing against PPV. However, as the result of studies indicated, the efficiency and potential of these different constructs in induction resistance was not similar. For instance, Di Nicola-Negri et al., (2010) reported that the hairpin *UTR/P1* construct outperformed among other constructs that they had utilized and *N. benthamiana* plants harboring this construct were highly resistant/immune to seven PPV isolates belonging to D, M and Rec strain.

Moreover, different resistant response by different length of hairpin structure has also been reported. In transformation of *N. benthamiana* plants with ihpRNA constructs containing the full-length or the second half of the PPV *CP* gene, the full-length *CP* construct showed a better performance (Hily et al., 2007). Therefore, the choice of target gene and the length of inverted repeat construct seem to be critical for induction of successful and persistent resistance. In order to have a better prospect about underlying mechanism of such RNAi-mediated resistance a deeper investigation at molecular level can be helpful. Deep sequencing technology with the ability of more precise assessment of abundance of produced siRNAs and their accumulation patterns in target regions may provide us more information. The expression profiles of siRNAs

and miRNAs and investigation of probable differences in siRNA accumulation in transgenic plants harboring different hairpin constructs may be helpful in making decision for selection of best hairpin construct which can be used subsequently for stone fruit plants' transformation to obtain resistance against PPV.

In the present study, transgenic *N. benthamiana* plants harboring five different hairpin structures derived from an isolate of PPV-T strain were obtained. After assessment of these transgenic plants by virus inoculation with three different PPV strains, they were subjected to deep sequencing analysis. Profiling of siRNAs in transgenic plants harboring different hairpin constructs revealed that different number of transgene-derived siRNAs were produced in transgenic plants harboring these hairpin constructs. This result indicated the different potential of these hairpin constructs in producing of siRNAs. This is the first study comparing the most common hairpin structures, used for inducing RNAi, in terms of their capacity for producing siRNA species using deep sequencing. Characterization of siRNA population of these transgenic plants indicated that *UTR/P1* and *CP* hairpin structures with higher capacity of production of siRNAs and dense accumulation of these siRNAs on target regions are the best candidates for transformation of stone fruit plants.

1. CHAPTER
LITERATURE REVIEW

1.1 Virion structure

Plum pox virus belongs to the *Potyvirus* genus in *Potyviridae* family. As a typical potyvirus, PPV virions are long, flexuous and rod-shaped of ~660-750 nm in length and 12.5-20 nm in width, shaped by a single coat protein (*CP*) of about 36 kDa. This *CP* encapsicates a single-stranded genomic RNA with positive polarity (Brunt et al., 1996).

1.2 Genome organization of PPV

The genome encodes a single large polyprotein composed of 3125-3143 amino-acids, about 355kDa, which is cleaved by proteinase activity of three virus-encoded proteins including *P1*-Pro, HC-Pro and NIa-Pro to produce 10 proteins. *P1* and *HCPro* are produced by a C-terminal autocatalytic *P1* proteinase and the helper component-proteinase (*HCPro*) activity (García et al., 1993; Ravelonandro et al., 1993). The processing of the rest of PPV polyprotein is done by the C-terminal proteinase domain of the nuclear inclusion a (NIa) protein (NIaPro) (García et al., 1989). Production and maturation of potyviral proteinases from the polyprotein area highly regulated process which plays an important role in controlling of infection (García et al., 1992).

1.2.1 Functions of the gene products

In potyviruses there will be 10 final proteins (Fig. 1.1). P1 is the most variable protein among different potyviruses both in terms of sequence and length (Valli et al., 2007). P1 is involved in host specificity and symptom manifestation (Maliogka et al., 2012; Nagyová et al., 2012). P1 in cooperation with HCPro is active in inhibition of plant post-transcriptional gene silencing (PTGS) defense mechanism (Valli et al., 2006). So far, based on the result of different studies, involvement of HCPro in different virus activities has been reported. Functioning as a suppressor of gene silencing and involvement in long distance movement through the plant have been proposed for this viral protein among all other functions (Roth et al., 2004; Yap et al., 2009). There is limited information about the P3 protein function in *potyviruses*. Like *P1* it is variable among different potyvirus species. In a study, an alternative shorter P3 variant was discovered which was the result of translation frameshift to so-called PIPO (pretty interesting potyviral ORF). It has been shown that P3N-PIPO was essential for virus infectivity because of its involvement in cell-to-cell movement (Wei et al., 2010). Involvement in virus replication has also been proposed for this protein (Klein et al., 1994). The 6K1 peptide seems to be more conserved among potyviruses compared to P3 protein. In a recent study, deletion of 6K1 or a short motif/region of 6K1 in the full-length cDNA clones of PPV abolished viral replication and it revealed that 6K1 is an important viral protein of the potyviral replication complex (Cui and Wang, 2016). The cylindrical inclusion protein (CI) forms pinwheel-shaped cytoplasmic inclusion bodies which is a typical feature of the potyviral infection. This protein has been shown to be required for RNA replication (Fernández et al., 1997). Involvement of CI in cell-to-cell movement has been reported. Moreover, studies have revealed the interaction of CI with some host factors (Jenner *et* al., 2002; Jiménez et al., 2006). Like 6K1, 6K2 contains a central hydrophobic domain. It has been shown that in TEV the 6K2 acts as an endoplasmic reticulum-targeted viral protein for formation of RNA virus replication complexes on membranes (Schaad et al., 1997a). In another virus it has been shown that this protein affects viral long-distance movement and symptom induction independently and in a host specific manner (Spetz and Valkonen, 2004). VPg (viral protein genome-linked) is a protein that covalently linkes to the 5′-terminus of PPV genome through a

tyrosine residue by a phosphodiester bond (Murphy et al., 1991; Puustinen et al., 2002). Result of some studies indicated that the VPg can be used as a primer for initiation of RNA synthesis (Anindya et al., 2005; Puustinen and Mäkinen, 2004). The other proposed function for VPg in potyviruses is cell-to-cell or long-distance movement (Rajamäki and Valkonen, 1999; Schaad et al., 1997b). NIaPro is the enzyme responsible for cleavage of seven out of nine cleavage sites of the potyviral polyproteins. Involvement in virus replication and a DNase activity have also been suggested for the NIaPro (Daròs and Carrington, 1997; Anindya and Savithri, 2004). NIb is the viral RNA-dependent RNA polymerase responsible for potyviruses genome replication (Hong and Hunt, 1996). It is responsible for VPg uridylylation which is used as a primer for initiation RNA synthesis (Merits et al., 1999).

Although the main function of coat protein (CP) of potyviruses is encapsidation of the viral genome, this protein is also involved in several infection-related actions (Salvador et al., 2006; Decroocq et al., 2009). The CP contains three domains. The central domain is called the core CP and interacts with viral RNA in matured virions. This domain in addition to encapsidation is involved in replication and interacts with plasmodesmata in the process of cell-to-cell movement (Varrelmann and Maiss, 2000; Rojas et al., 1997; Dolja et al., 1995). The N- and C- terminal of potyviral CP are exposed on the virion surface (Allison et al., 1985; Baratova et al., 2001; Shukla et al., 1988) and the c-terminal domain have some roles in encapsidation and long-distance movement (Dolja et al., 1995; Kang et al., 2006). The N-terminal is the most variable region of the polyprotein which contains species- and strain-specific epitopes (Shukla et al., 1989). The n-terminal domain may be involved in systemic vascular movement (Dolja et al., 1994; Szathmáry et al., 2009). Moreover, CP of some potyviruses, including PPV, undergoes post-translational modification such as phosphorylation and *O*-GlcNAcylation, which regulate the RNA binding capacity of CP. This may control the amount of genomic RNA that is subjected to translation, replication, propagation and encapsidation (Ivanov et al., 2001; Chen et al., 2005; Fernández-Fernández et al., 2002).

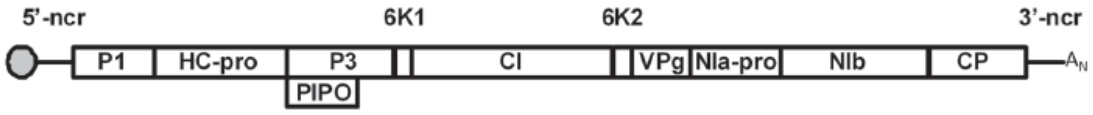


Figure 1.1. Scheme of the PPV genome. The grey circle represents covalently bound VPg (Šubr and Glasa, 2013)

1.3 Pathogenicity, symptoms and transmission

The host range of the virus in the nature is limited to the plants of the genus *prunus* including peaches, plums, apricots, nectarines, almonds and sweet and sour cherry. However, it has a broad experimental host range. In terms of the ability of infection of different hosts, there is different reaction and not all strains and isolates can infect the same host range.

The symptoms that appear on stone fruit trees mostly vary based on various factors such as the strain of the virus, timing of infection, host cultivar, and environmental condition. These symptoms can appear on leaves, fruits, flowers and seeds. On the other hand, the severity of the appeared symptoms depends on the *prunus* species and cultivar, PPV strain, season and location of the host. Symptoms on leaves are chlorotic spots, band or rings, vein clearing or sometimes leaf deformation in peaches. The fruits of apricot and plums may be deformed and show internal browning of the flesh and their stones may show pale rings and spots (Dunez, 1987). Some peach cultivars may show color-breaking symptoms on the flower petals.

PPV is transmitted by different species of aphids in a non-persistent manner which means that the virus can be acquired and transmitted within seconds during aphid feeding. In such cases, it is generally assumed that long distance transmission of the virus is not taking place by the aphids because infectivity by the aphid vector is lost in short time (Wallis et al., 2005); however, some evidences suggest that under specific

environmental conditions some potyviruses may be transported in long distances by migrating aphids (Zeyen and Berger, 1990). But the most common way of long distance spread of the virus is the shipment of uncertified and infected plant materials and root-stocks or grafted seedlings.

1.4 Management of *Plum pox virus*

Like other plant virus diseases, PPV management is a big challenge considering the fact that such diseases cannot be controlled directly by application of chemicals on infected plants. Sharka disease does not kill the host plants, therefore, once plants are infected they will serve as a permanent reservoir of PPV and considering the easy way of its transmission by aphids in a non-persistent manner, the virus can spread to other stone fruit plants in that area. Thus, PPV control strategies are firstly based on early detection of the infection and prevention of its spread to new areas by periodic surveys of orchards, removal and destruction of infected trees, selection and certification of virus-free plant materials and rootstocks and insecticide treatment to control aphid population though this chemical treatment cannot be effective for a virus like PPV which is transmitted in a non-persistent manner (Perring et al., 1999).

Considering all the difficulties and limitations in controlling of PPV infection, it seems development and application of PPV-resistant stone fruit trees is the only promising approach to control PPV. However, few sources of PPV resistance and problems like incompatibility barriers between species, challenges in selection process such as long generation time of stone fruit trees and the high degree of heterozygosis make the breeding programs inefficient and challenging. Considering all these limitations, genetic engineering technologies for production of PPV-resistant varieties have attracted scientists' attention and there have been different surveys in this respect. In genetic engineering approaches, transgenic plants are produced by introducing genes or genetic materials isolated from other organisms, regardless existence of barriers such as sexual compatibility, into plant genome. Among genetic engineering approaches, introduction of a viral gene to the plant to develop resistant transgenic plants based on the concept of pathogen-derived resistance PDR (Sanford and Johnston, 1985) has been noticed and

employed for many plant viruses including PPV. The RNA-mediated resistance mechanism, known as RNA silencing pathway, is a powerful mechanism which can be used for engineering resistant plants. Introduction of a viral gene into plant genome triggers RNA silencing which is a sequence-specific gene silencing mechanism. This mechanism is a process in which double stranded RNA (dsRNA) triggers the host Dicer-like enzymes to dice the dsRNA into short molecules (small interfering RNAs, siRNAs) of 21-24 nucleotides in length. The produced siRNAs are loaded onto a multi-subunit RNA- Induced Silencing Complex (RISC) which will guide sequence-specific degradation of homologous RNAs (Sijen et al., 2001).

1.5 The history of RNA silencing

RNA silencing is a regulatory and sequence-specific gene inactivation mechanism in eukaryotic organisms. The regulatory effects are induced by small RNA classes which are different in their biogenesis pathways, processes of target regulation and biological pathways they regulate (Ghildiyal and Zamore, 2009). RNA silencing, besides gene regulation, which can take place at different stages including transcription, RNA processing, RNA stability and translation, can also play an important role in host defense mechanism like antiviral defense in plants and invertebrates (Carthew and Sontheimer, 2009; Ding and Voinnet, 2007).

First evidences of RNA silencing were seen in an early study conducted by Matzke et al. (1989) in which *Agrobacterium*-mediated transformation of the tobacco (*Nicotiana tabacum*) was performed by introduction of two T-DNA vectors encoding different selectable markers. In obtained transgenic plants, the selectable marker encoded by the first T-DNA became inactive and this inactivation was shown to be associated with promoter sequence methylation of the selectable marker gene. In this study, it was suggested that the homology in sequence of the nopaline synthase promoter which was present in two T-DNA vectors, was the main reason for methylation of the first vector.

Napoli et al, (1990) suggested the “co-suppression” term for a phenomenon in which the introduced gene in transformed population and the endogenous form of that gene

were silenced or “turned off” to different degrees. The co-suppression phenomenon was dicovered when petunia (*Petunia hybrida*) plants were engineered to overexpress the flower pigmentation gene, chalcone synthase (*CHS*), to intensify the purple coloration of flowers. But surprisingly, the flowers of transgenic plants showed a range of different pigmentations, including deep purple, white and patterns of purple and white. Based on these evidences, it was concluded that the changes in flower color was duo to silencing of introduced and endogenous forms of the *CHS* gene.

For many years the details of the mechanisms which led to co-suppression in plants were not clear. On the other hand, another similar phenomenon was detected in plants which were engineered to express virus-encoded sequences, especially the coat protein (*CP*) gene of the virus genome. The plants which were engineered to express these viral sequences became resistance to the same virus or closely related virus strains. Therefore, this technique, expression of viral genes in plants, was used to induce resistance against corresponding viruses. In such attempt for introduction of resistance against *Tobacco etch virus* (TEV), tobacco plants were transformed with the *CP* sequence of the virus. The transgenic plants initially showed the systemic symptoms of the virus but three to five weeks after the first inoculation, transgenic plants recovered and new leaf tissues were symptom and virus free. The TEV-resistant state did not break after second inoculation with TEV but the transgenic plants were susceptible to a closely related virus, *potato virus Y*. Molecular analysis of recovered tissue revealed that despite the fact that transgene transcription rate in recovered and un-inoculated plants was similar but accumulation of transgene mRNAs in recovered tissue was 12 to 22 fold less than accumulation of transgene mRNA in un-inoculated transgenic tissue. Based on this evidence, it was concluded that the gene silencing or co-suppression was a cytoplasm-localized event and occurred at posttranscriptional level which could target specific RNA sequences for inactivation (Lindbo et al., 1993).

Complementary studies on transgenic petunia plants transformed with *CHS* gene, provided some evidences that transgene-derived RNA might be responsible for the sequence specificity of co-suppression. Molecular analyses of co-suppressed plants indicated that in addition to suppression of CHS mRNA, some truncated transcripts

which also could be found in plants with white flowers, detected in co-suppressed plant lines. It was suggested that by overexpression of the *CHS* transgene some aberrant RNA species were produced as a result of endonucleatic cleavage of secondary structures and these aberrant RNAs were responsible for endogene mRNA degradation by pairing with their complementary regions (Metzlaff et al., 1997). On the other hand, in another study, it was shown that monomeric T-DNA loci were unable to induce CHS silencing in transgenic petunia plants but, multimeric T-DNAs loci in which the T-DNAs were arranged in an inverted-repeat (IR) orientation, were successful in induction of silencing (Stam et al., 1997). These observations brought up the idea of testing the double-stranded RNA (dsRNA) as initiator of gene silencing. Directly introduction of dsRNA for evaluation of its effects as the silencing trigger was carried out in *Caenorhabditis elegans* and the term RNA interference (RNAi) was proposed for the first time for this silencing mechanism (Fire et al., 1998). In an experiment, the transgenic plants containing sense and antisense sequences of *potato virus Y* (PVY) were crossed and these crossed transgenic plants exhibited silencing and protection against the virus which was not seen in parental transgenic plants containing either sense or antisense sequences (Waterhouse et al., 1998). The effect of IR constructs on induction of silencing was more noticed when the result of super-transformation of two transgenic callus lines of rice, expressing a beta-glucuronidase (*GUS*) gene with an inverted-repeat (i/r) *GUS* and direct-repeat (d/r) constructs, revealed that induction of silencing in inverted-repeat configuration was much more than simple sense and antisense constructs. It was also shown that gene silencing was more effective in inverted-repeat configuration compared with direct-repeat configuration (Wang and Waterhouse, 2000).

The next missing pieces of RNAi puzzle were discovering the key elements and their mode of actions in induction of RNA silencing. Detection of small RNA (sRNA) molecules, which direct the sequence specificity in RNA silencing mechanism, was a step in finding missing pieces of this puzzle. Detailed study of three transgene-induced posttranscriptional gene silencing (PTGS) and one virus-induced PTGS led to detection of sRNA molecules with the same length of about 25 nucleotides complementary to the sequences undergoing silencing in each case and it was concluded that these sRNA molecules were most probably synthesized from an RNA template which could explain

the specificity seen in PTGS (Hamilton and Baulcombe, 1999). These sRNA species are known as small-interfering RNAs (siRNAs) right now and later studies revealed the mechanism of their formation. In a survey, Dicer, the evolutionarily conserved enzyme in worms, flies, plants, fungi and mammals with endonuclease activity which specifically cleaves double-stranded RNAs and produces siRNA species was identified. Dicer is a member of the RNase III family with a helicase domain and dual RNase III motifs (Bernstein et al., 2001). Diverse Dicer-like (DCL) enzymes have been identified which indicates their multiple activities. In plants, unlike animals, at least four DCL proteins have been characterized (Margis et al., 2006). It has been shown that different DCL enzymes, (DCL1-DCL4) are involved in processing and formation of diverse siRNAs which are present in plants (Fukudoma and Fukuhara, 2017).

After cleavage of dsRNA and production of sRNAs, the key step in RNAi is assembly of RNA-induced silencing complex (RISC) which is a protein-siRNA complex that can recognize the mRNA target which is complementary to the siRNA sequence. Argonaute, one of the most important proteins in this complex, plays an important role in slicing the targeted mRNA. AGO1 is the most studied member of Argonaute member which has shown to have slicing activity in plants. In a study it was shown that the conserved amino acid residues in PIWI domain of AGO1 is responsible for *in vitro* cleavage of a *mir165* target RNA. It has been demonstrated that AGO1 is associated with miRNAs, endogenous ta-siRNAs and transgene-derived siRNAs but there was no association with virus-specific siRNAs or siRNAs involved in chromatin silencing (Baumberger and Baulcombe, 2005). In Arabidopsis 10 Argonaute proteins have been identified and further studies revealed association of some members of this Argonaute family with different types of RNA silencing. Because of RNA cleavage activity of the first characterized AGO protein, AGO1, these classes of proteins have been characterized as slicer proteins which is not an accurate term for describing these proteins; because further studies showed their involvement in repressing gene expression by other mechanisms as well (Mallory and Vaucheret, 2010).

1.6 RNA silencing in plants

As it was described, RNA silencing was triggered by formation of self-complementary RNA fold-back “hairpin” structures or double-stranded RNA (dsRNA) which gave rise to 20-24 nt small RNAs. Small RNAs derived from single-stranded hairpins are called hairpin RNAs (hpRNAs) and those derived from double-stranded RNA precursors are known as small interfering RNAs (siRNAs). hpRNAs are divided into microRNAs (miRNAs) and everything else (other hpRNAs). siRNAs in plants can be categorized to heterochromatic siRNAs (hc-siRNAs), secondary siRNAs and natural antisense transcript siRNAs (NAT-siRNAs) (Axtell, 2013).

1.6.1 SiRNAs and their biogenesis

siRNAs are produced by processing of long perfectly double-stranded RNAs. Plants like RNA viruses, fungi, protists and some lower animals like *Caenorhabditis elegans*, have a conserved RNA-dependent RNA polymerase (RDR) which is able to convert single stranded RNAs into dsRNAs. *A. thaliana* contains six RDRs. RDR1, RDR2, and RDR6 are the ones involved in formation of dsRNA molecules which are subsequently processed into different types of siRNAs (Willmann et al., 2011).

1.6.1.1 Heterochromatic siRNAs

Heterochromatic siRNAs are mostly 23-24 nt molecules in length which are derived from intergenic or repetitive genomic regions and transposable elements, and at homologous loci they are acting by imposing some repressive chromatin modifications such as DNA methylation and histone modification (Law and Jacobsen, 2010; Matzke et al., 2009).

For biogenesis of ht-siRNAs specific members of RDR, DCL and AGO gene families are required. Transcription of ht-siRNAs from methylated DNA templates takes place by RNA Pol IV to generate long single-stranded RNA transcripts (Mosher et al., 2008). These RNA transcripts are converted into dsRNA by involvement of RDR2 and

subsequently processed into 24nt siRNA duplexes with the help of DCL3 and loaded into AGO4 (Haag et al., 2012). AGO4 by interaction with DNA-dependent RNA polymerase, Pol V, makes a complex consisted of many other associated factors which functions as effector for RNA-directed DNA methylation (RdDM) (El-Shami et al., 2007).

siRNA-mediated epigenetic modification is significantly important for genome stability over generations. hc-siRNAs play an important role in genome stability by silencing the transposable elements (Xu et al., 2013). In addition, results of some studies indicate that hc-siRNAs are also participating in controlling of expression of a large number of endogenous genes in plants (Liu et al., 2004; Chan et al., 2006).

1.6.1.2 Secondary siRNA

Secondary siRNAs, like other siRNAs, derive from a dsRNA precursor which its synthesis is triggered by one or more upstream small RNAs. For most known secondary siRNAs, this upstream sRNAs are either miRNAs or other secondary siRNAs. The biogenesis of secondary siRNAs requires components of both siRNA and miRNA pathways (Peragine et al., 2004; Allen et al., 2005). Trans-acting siRNAs (tasiRNAs) are 21-nt class of secondary siRNAs which are generated by transcription activity of RNA polymerase II from genetic loci known as *TAS* genes (Vazquez et al., 2004; Allen et al., 2005; Peragine et al., 2004). The produced *TAS* precursor RNA is targeted by specific miRNAs and then the cleaved transcripts function as template for dsRNA synthesis by RDR6. Subsequently, the 21-nt siRNAs are processed by DCL4 (Peragine et al., 2004; Xie et al., 2005; Yoshikawa et al., 2005; Adenot et al., 2006; Hiraguri et al., 2005). Many secondary siRNAs are phased (phasiRNAs) meaning their first nucleotide occur every 21 or 22 nucleotides from miRNA-cleavage site (Chen et al., 2007)

The secondary siRNAs are well conserved in flowering plants and also other diverged lineages. *A. thaliana*, the plant with well-studied tasiRNAs, contains four families of *tasiRNA PRECURSOR* (*TAS*) genes. *TAS3* family of secondary siRNA loci, triggered by miR390, produces two trans-acting siRNAs that are involved in the regulation of

organ polarity, meristem identity, and developmental timing (Axtell et al., 2006). *TAS4* secondary siRNA locus is targeted by miR828 and produces a trans-acting siRNA that targets MYB transcription factor and affects anthocyanin production in Arabidopsis (Luo et al., 2012). On the other hand, genomic data obtained from a larger number of plant species have indicated that Arabidopsis is exploiting a small number of secondary sRNAs and other genomes contain hundreds or thousands of them. In dicots, these secondary sRNAs target several large and conserved families of genes encoding disease resistance proteins and transcription factors (Fei et al., 2013). miRNAs and secondary siRNAs can target leucine-rich repeat (NB-LRR) disease resistance genes (Zhai et al., 2011).

1.6.1.3 NAT-siRNA

Natural antisense transcript siRNAs (NAT-siRNAs) are a subset of siRNAs which the dsRNA precursors are produced by hybridization of two separately transcribed complementary RNAs. In *cis*-NAT-siRNAs separate RNAs which are derived from transcription of pposite strands of the same locus hybridize but, in *trans*-NAT-siRNAs the hybridization occurs between genes that possess no overlap. It has been demonstrated that abiotic and biotic stresses trigger the nat-siRNAs and their accumulation in specific developmental stages has been reported (Katiyar-Agarwal et al., 2006; Borsani et al., 2005; Jin et al., 2008; Ron et al., 2010; Zubko et al., 2007). It was assumed that in contrast with other types of siRNAs, formation of the dsRNA precursors are not RDR dependent. However, the results of several studies, revealed RDR of many cis-NAT-siRNAs (Axtell, 2013). Amplification of siRNAs which is triggered by initial hybridization of cis-NAT mRNAs was suggested to be the reason of RDR dependency (Zhang et al., 2012). However, there are no supporting evidences for this hypothesis and further investigation about cis-NAT-siRNAs biogenesis process is needed.

1.6.2 miRNAs and their biogenesis pathways

Studies about the position of miRNAs in plants indicated that, in contrast with animal miRNAs, they are mostly located in intergenic regions with some exceptions like their

location in intronic regions of known genes. Therefore, it is assumed that most of them have unique transcriptional unit (Graffiths-Jones et al., 2008). It is revealed that some of plants' miRNAs genes are in a close conjunction, suggesting that they are expressed polycistronically. However, polycistronic management of mirRNAs in plants is not very frequent compared to the number of polysictronic management of miRNAs in animals (Merchan et al., 2009). In plants, miRNAs are expressed by transcription of a primary miRNA transcript (pri-miRNA) by involvement of RNA polymerase II (Kim et al., 2011). pri-miRNAs are endogenous hairpin-shaped transcripts. They typically have imperfect fold-back structures, which are processed into step-loop precursors. In nucleus, the pri-miRNAs transcript are recognized and cleaved by DCL1 to produce ~70-nucleotide precursor miRNA (pre-miRNA) molecules. The next stage is cleavage and liberation of mature miRNAs from pre-miRNA stem loop structures by involvement of four types of DCL enzyme (Vazquez et al., 2008). Studies on different stages of miRNA biogenesis indicated that some RNA binding proteins are also required for pri-miRNAs' processing and miRNAs' accumulation. The G-patch domain protein TOUGH (TGH) (Ren et al., 2012b), the zinc finger protein SERRATE (SE) (Lobbes et al., 2006), and the dsRNA binding domain (DRB) protein HYPONASTIC LEAVES1 (HYL1/DRB1) (Vazquez et al., 2004) are the detected RNA binding proteins which their interactions with DCL1 have been demonstrated. HYL1 and SE promote the accuracy of pri-miRNAs' processing by DCL1 (Dong et al., 2008; Manavella et al., 2012a) and TGH enhances DCL1 activity in pri-miRNAs' processing (Ren et al., 2012b). The miRNA duplexes are then transferred to cytoplasm. For some miRNAs in plants HASTY (HST), the exportin 5 (the nuclear exporter of pre-microRNAs in animals) homolog, can be responsible for nuclear export (Park et al., 2005). In cytoplasm after separation of mature miRNA guide strands from passenger strands, they are incorporated into AGO protein to form the RISC. Most miRNAs are loaded into AGO1; however, different studies have shown that other AGOs also can bind with miRNAs (Ji et al., 2011; Maunoury and Vaucheret, 2011).

Plant miRNAs are mostly 21 nt molecules in length which some evidences like their high complementarity to the targets throughout their length (Fahlgren and Carrington, 2010) and sequencing analysis of the degraded target mRNAs (Addo-Quaye et al.,

2008), indicated that large number of miRNAs' targets undergo slicing. However, evidences such as detection of some cases of target regulation at protein level with no considerable changes in mRNA level (Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008; Dugas and Bartel, 2008; Beauclair et al., 2010), and identification of *Arabidopsis* mutants which were impaired in gene expression at protein level but not at mRNA level and involvement of miRNAs in this process (Brodersen et al., 2008; Yang et al., 2012; Li et al., 2013), suggested that plant miRNAs can also block translation. Plant miRNAs are involved in various biological processes including controlling of expression of genes encoding transcription factors, stress response proteins and other proteins that play roles in development, growth and physiology of plants (Roger and Chen, 2013).

1.7 RNA silencing induced by exogenic nucleic acids

As it was mentioned, the discovery of RNA silencing phenomenon accured at sense transgenes' studies, where the transgenes with overexpressed pigmentation enzyme gene not only self-silenced but also caused induction of silencing of the endogenous counterparts (Napoli et al., 1990). Since discovery of RNA silencing triggered by dsRNA and identification of various sRNA pathways in plants, artificially induction of RNA silencing for different purposes have been widely used. Triggering of RNAi in plants have been done by their engineering with sense transgenes to over-express the target transcripts or integration of antisense transgenes into plant genome to produce transcripts that are complementary to their target transcripts (Waterhouse et al., 2001). However, after discovery of dsRNA-induced gene silencing, long self-complementary hairpin RNAs (hpRNAs) which are comprised of sense and antisense sequences of a portion of a target gene have been widely used for different purposes including viral gene silencing in plants. Sense and antisense sequences which are separated by a non-complementary spacer region, in the transcribed RNAs in plant, are complementary and will produce typical hpRNA constructs (Smith et al., 2000).

Construction of virus-derived hpRNAs and transformation of plants with these constructs for expression of dsRNAs induces RNA silencing mechanism and the

transgenic plants can silence the gene of that virus. This will result in resistance against virus infection.

1.8 PPV resistance by RNA silencing mechanism

1.8.1 Application of CP and other PPV genes for induction of resistance

After discovery of pathogen-derived resistance or PDR, this strategy became popular and applied for different viruses. Considering all difficulties of transformation and regeneration of recalcitrant fruit trees, the primary studies were done on *Nicotiana* species and in particular on *N. benthamiana*. In the first attempts of PPV-derived resistance, like the first successful PDR by introduction of *tobacco mosaic virus* (TMV) coat protein *CP* gene to tobacco plants (Powell et al., 1986), PPV coat protein was the first genome fragment used to obtain resistance against the virus in plants. The expression of *CP* in transgenic plants resulted in exhibition of different degrees of resistance to PPV such as reduction of accumulation of the virus and inhibition of systemic spread, delay in symptom appearance, inhibition of virus accumulation and no symptom appearance (Regner et al., 1992; Ravelonandroet al., 1993).

However, considering possible biological risk of engineered viral *CP* like changing epidemiological characteristics of related viruses through heteroencapsidation[1] (Lecoqet al., 1993), an alternative method like transformation of *N. benthamaina* plants with modified PPV *CP* gene was performed to reduce the biological risks. These modifications were deletions in DAG amino acid motif which is involved in aphid transmission and *CP* regions which are required for capsid assembly. The results showed that two lines were highly resistant and the detected immunity was correlated with accumulation of transgene copies and low or undetectable transgene RNA levels. This evidence indicated that the described resistance was RNA-mediated which could be classified as a "sense suppression" or homology-dependent resistance (Jacquet et al., 1998b). The successful introduction of resistance in some cases in *nicotiana* plants led

[1] . Heteroencapsidation is the encapsidation of the genome of the superinfecting virus by the CP of the other virus.

to attempts for engineering of *Prunus* plants with *CP* gene for production of resistance (Laimer da Camara Machado et al., 1992; Scorza et al., 1994; Malinowski et al., 1998). Among the transgenic *Prunus* plants, the transgenic plum trees containing the PPV-*CP* gene showed to accumulate various levels of PPV-*CP* RNA and *CP*. Transgenic clones C-2, C-3 and C-4 accumulated high levels of PPV-*CP* RNA and PPV-*CP* and in particular, C-5 accumulated low levels of RNA and had undetectable *CP* (Scorza et al., 1994; Malinowski et al., 1998). Since then, the C-5 clone has been extensively evaluated under greenhouse and field conditions and the results indicated that the C-5 clone was highly resistant to PPV infection both in natural viruliferous and graft-inoculated infection conditions (Ravelonandroet al., 1997; Malinowski et al., 2006; Polak et al., 2008). In addition, the C-5 clone resistance in natural was also stable and efficient when it was challenged with different combination of viruses such as *Prunus necrotic ring spot virus* (PNRSV), *Apple chlorotic leaf spot virus* (ACLSV), *Prunus dwarf virus* (PDV), and PPV-D (Zagrai et al., 2008) and PPV-Rec (Polak et al., 2008).

CP gene was not the only region of genome used as source for introduction of resistance and other genomic region were also used to evaluate their resistance potentials. These genomic regions include the *P1, HC, CI, NIb* (Tavert-Roudet et al., 1998; Barajas et al., 2004; Wittner et al., 1998; Guo and García, 1997; Guo et al., 1999) and after challenging with PPV, responses varied from susceptibility to recovery or immunity. In transgene plants transformed with PPV full-length helper-component proteinase (*HCPro*) gene, the gene silencing suppressor (Tenllado et al., 2003), correlation between induced resistance and lower accumulation of transgene mRNA in *N. benthamiana* was observed. In addition, accumulation of short-interfering RNAs (siRNAs) was also seen and it was assumed that PPV resistance was caused by a post-transcriptional gene silencing (PTGS) process which can induce silencing under certain conditions (Barajas et al., 2004). Based on these results, it was clear that the induced resistance was not protein-mediated but it was the result of a RNA silencing pathway which was assumed to be triggered by strong elicitors of PTGS, such as formation of long segments of double-stranded RNAs (dsRNAs). After showing the ability of gene constructs encoding intron-spliced RNAs with the hairpin structure (ihpRNAs) in

induction of PTGS with almost 100% efficiency (Smith et al., 2000), several constructs expressing PPV-derived ihpRNAs were developed to obtain resistance against the virus.

1.8.2 Application of intron-spliced hairpin RNAs (ihpRNAs)

Self-complementary hairpin RNAs (hpRNAs) contain two complementary regions that form double-stranded structures separated by a short loop. Therefore, hairpin constructs are produced by insertion of a spliceosomal intron in the loop region located between two complementary sequences and forms intron-hairpin RNA (ihpRNA) structure. Using viral sequences from isolates belonging to PPV-D or PPV-M strains as the source, several ihpRNA constructs have been designed to evaluate the efficiency of introduced resistance by RNA silencing mechanism. The first PPV ihpRNA construct was produced by application of a 197 bp sequence (from base 134 to 330) of a PPV-D isolate. The two inverted repeats separated by DNA sequence of rolA intron and this hairpin construct was under the control of the rolC promoter to confer systemic resistance in transgenic *N. benthamiana* plants. The transgenic progeny of *ihprolC-PP197* plants were resistant to PPV systemic infection and SiRNAs of 23–25 nt homologous to the PPV sequence used in the *ihprolC-PP197* construct were detected in transgenic plants before and after inoculation (Pandolfini et al., 2003). The successful introduced resistance against PPV in transgenic plants containing ihpRNA construct, supported the fact that the resistance in transgenic plants was not protein-mediated and further studies were carried out to evaluate the efficiency of the PTGS mechanism. Selection of genomic regions as the source for ihpRNA construct may play an important role in inducing resistance. Di Nicola-Negri et al. (2005) chose the *P1* and the *HCPro* genes as potential RNA silencing targets considering that these parts of the genome are the most conserved regions between PPV-D and PPV-M (Glasa et al., 2004), they are involved in the RNA silencing suppression mechanism (Pruss et al., 1997; Kasschau and Carrington, 1998), *P1* and *HCPro* are the first two ORF products of the PPV genome and they are responsible for processing themselves at their respective C-termini (Lopez-Moya et al., 2000). In this study, four regions of PPV genome covering the *P1* and *HCPro* genes; UTR/*P1*, *P1*/*HCPro*, *HCPro* and *HCPro/P3*, were amplified from ISPaVe, an Italian PPV-M isolate. The results indicated that transgenic plants conferred

efficient resistance to homologous PPV-M isolate (Di Nicola-Negri et al., 2005). However, in order to choose the best hairpin construct candidate for introduction resistance in *Prunus spp*., the transgenic *N. benthamiana* h-UTR/*P1*, h-*P1*/*HCPro*, h-*HCPro* and h-*HCPro/P3* plants were challenged with molecularly typed nine different PPV isolates which were originally collected from five stone fruit species and the results indicated that all transgenic plants were resistant to seven isolates of D, M and Rec strains and in particular, the h-*UTR/P1* plants were also fully resistant to PPV-C and -EA isolates which are phylogenetically distantly related strains to PPV-M (Di Nicola-Negri et al., 2010).

Despite the promising approach of ihp-mediated virus resistance, there have been some evidences of instability of the PTGS under some abiotic and biotic stress conditions (Szittya et al., 2003; Wu et al., 2008; Zhong et al., 2013). In order to evaluate this reverse effect on PTGS in transgenic PPV resistant plants, the h-UTR/*P1*-plants were grown at low (15°C) or high (30°C) temperatures and the results of resistant assays showed that they were fully resistant to multiple PPV challenges like different PPV inoculum concentrations and inoculation with distantly related PPV-C isolate. Moreover pre-infection of h-*UTR/P1* plants with a virus belonging to *Cucumovirus*, *Potyvirus*, or *Tombusvirus*, which can encode viral suppressors of RNA silencing (VSRs), did not affect PPV resistance (Di Nicola et al., 2014). Based on these results, transgenic plums containing the hairpin h-*UTR/P1* construct were successfully developed which were shown to be resistant after assessment by grafting the transgenic plants on PPV infected plants (Monticelli et al., 2012; García-Almodóvar et al., 2015).

Except than the *P1* and the *HCPro* genes as the source for hairpin construct, the *CP* gene was also considered as the source for ihpRNA construction. In order to test the effects of different length of PPV-*CP* dsRNAs, in a study, two types of hairpin constructs produced using the full length and the second half of the PPV *CP* gene. The results indicated that there was a correlation between the length of the *PPV* sequence in the ihpRNA constructs and the frequency of transgenic-resistant plants. The transformed plants with the full-length *CP* produced a higher percentage of resistant

lines. Therefore, the full-length *CP* construct was applied for plum transformation (Hily et al., 2007).

Later, the transgenic plums with the full-length *CP* hairpin construct were inoculated with three widespread PPV strains (D, M, and Rec) and the atypical EA strain. For evaluation of resistance of full-length *CP* hairpin construct by PPV-C isolate which is unable to infect *P. domestica*, the PPV ihpRNA-*CP N. benthamiana* was used. The stable resistance in transgenic plants confirmed the efficiency of the PPV-*CP* ihpRNA construct for targeting the PPV genome and introduction of resistance (Ravelonandro et al., 2014).

In another study, comparison of two ihpRNA constructs; the 5′ portion of the *P1* gene and the 3′ portion of *CP*, derived from a Canadian PPV-D isolate revealed a better resistance performance in ihpRNA*P1* construct in model plant. Therefore, this construct was chosen to transform plum. Challenge of the transgenic plum lines via chip-budding of a Canadian PPV-D isolate showed that PPV was undetectable in five out of 10 transgenic lines (Wang et al., 2009).

A hairpin structure targeting two parts of a viral genome simultaneously or a chimeric hairpin structure consisting of genes of more than one virus are strategies used for induction of resistance against one virus or several viruses that were used for hairpin construction. In a study, a triple-intron double-hairpin construct targeting the *P1* and *CP* sequences of PPV simultaneously was produced and used for transformation of *N. benthamiana* and plum (*Prunus domestica* L.). The produced transgenic plants in resistance assays showed an efficient resistance to PPV (Wang et al., 2013). In another study, a chimeric ihpRNA construct which was made by fusion of sequences from the most important six stone fruit-infecting viruses, including the *CP* gene of PPV-D, was used for transformation of *N. benthamiana*. The selected T3 homozygous plants from obtained transgenic plants were assayed in multi-virus resistance tests and in challenge with a PPV-D isolate, they did not show any symptoms (Liu et al., 2007).

1.8.3 Deep sequencing analysis for characterization of transgenic siRNA population

After viral infection, the RNA silencing machinery in plants is triggered to initiate the defense mechanism which is directed by viral small interfering RNAs (vsiRNAs). The new technology of deep sequencing provides the advantage of screening of siRNA species in plants under different conditions. Profiling and characterization of siRNA species during viral infection provides a precise assessment of the siRNA species produced as a consequence of viral infection and also in transgenic plants containing the ihpRNA constructs.

In a survey, characterization of produced transgene-derived siRNAs in transgenic *N. benthamiana* plants transformed with hairpin structures harboring two silencing motif-rich regions of the PPV coat protein (*CP*) gene and virus-derived siRNAs in wild-type plants inoculated with a PPV, was done by large-scale small RNA sequencing. siRNA data processing of infected transgenic-susceptible (TG-S) lines and untransformed (WT) plants showed high level of 21- and 22-nt siRNAs that targeted the whole PPV genome but, 24-nt siRNAs were absent in these individuals. On the other hand, infected transgenic-resistant (TG-R) plants accumulated 21- to 24-nt siRNAs that targeted the selected motif-rich regions in virus genome which led to prevention of viral multiplication. The results indicated that although large set of siRNAs were present in both types of susceptible plants (TG-S and WT), they failed to prevent viral multiplication and it was concluded that viral suppressors of RNA silencing (VSRs) won the battle and they were successful in disturbing host RNAi mechanism. In contrast, the RNAi performed successfully by transgenic-derived siRNAs in transgenic-resistant (TG-R) plants. The produced siRNAs targeted viral genome and prevented PPV multiplication. In addition, the decrease of 24-nt siRNA species in susceptible plants raised the questions about the possible role of DNA methylation processes and their relevance in plant defense against RNA viruses because this species of siRNAs is involved in DNA methylation mechanism (Montes et al., 2014).

In the study of Di Nicola-Negri et al. (2010) where the h-*UTR/P1* construct among other constructs (h-*P1*/*HCPro*, h-*HCPro* and h-*HCPro*/*P3*) was able to confer resistance to a broad range of PPV strains including PPV-C and EA, in order to find out whether the homologous stretches between PPV-C or EA and the hairpin constructs were also sites for production of potentially effective siRNAs, two siRNAs prediction tools, siRNA design and Block-iTTM RNA designer were used. The results of prediction tools indicated the presence of effective h-*UTR/P1* derived siRNAs partially homologous (1–2 mismatch) to PPV-C and EA sequences. Based on the results of in silico prediction of potentially effective ISPaVe44 siRNAs, it was concluded that the h-*UTR/P1* construct is able to confer complete resistance to a broad range of PPV strains.

The above mentioned studies were the only ones using the deep sequencing technique for investigation of diversity and complexity of siRNAs in PPV infected and PPV-derived transgenic plants.

2. CHAPTER
MATERIALS AND METHODS

2.1 Isolation and amplification of *plum pox virus* (PPV) gene fragments

2.1.1 Virus source

An aged apricot tree heavily infected with PPV and located in Erciyes University Campus was used as virus source. PPV infectivity in the tree was previously tested (Ceylan et al., 2014). The PPV-T isolate from the tree was used for the amplification of the five different gene fragments for construction of intron hairpin RNA vectors.

2.1.2 Primers

For amplification of desired PPV fragments, primers were designed from PPV-T genome sequence, isolate AbTk with accession EU734794 in GeneBank, using the Primer Premier 5.0 software (Dieffenbach, 1995). As potential silencing targets, five primer pairs; four of which covering the *P1* and HC-Pro genes including *UTR/P1* (752 pb), *P1/HCPro* (771bp), *HCPro* (649 bp), *HCPro/P3* (594 bp) (Di Nicola-Negri et al., 2005) and one pair covering *CP* (990 bp) were designated. The oligonucleotide primers were synthesized by Integrated DNA Technology Inc. The attB1 and attB2 sites which serve as the binding sites for recombination proteins were added to the 5' end of each forward and reverse primer, respectively. The recombination sites are required for Gateway Technology which is based on the bacteriophage lambda site-specific recombination system (Ptashne, 1992).

Table 2.1. Primer sequences for amplification of desired fragments

Fragment size	Name	Sequences
752 pb	Forward (UTR/*P1*)	5´GGGG ACA AGT TTG TAC AAA AAA GCAGGC **AAATATAAAAACTCAACACAACATTCA** 3´
	Reverse (UTR/*P1*)	5´GGGG AC CAC TTT GTA CAA GAA AGC TGG GT **CGCTTACGCCCTATGATCTC** 3´
771bp	Forward (*P1*/*HCPro*)	5´GGGG ACA AGT TTG TAC AAA AAA GCAGGC **TAGTCGGTCACTACACGACGA** 3´
	Reverse (*P1*/*HCPro*)	5´GGGG AC CAC TTT GTA CAA GAA AGC TGGGT **TCATTGATCTTGTTGAGATGG** 3´
649 bp	Forward (*HCPro*)	5´GGGG ACA AGT TTG TAC AAA AAA GCAGGC **TGATGAGCGCACAAGACTACA** 3´
	Reverse (*HCPro*)	5´GGGG AC CAC TTT GTA CAA GAA AGC TGGGT **AACCTGCCTTTGCTATGAACA** 3´
594 bp	Forward (*HCPro*/*P3*)	5´GGGG ACA AGT TTG TAC AAA AAA GCAGGC **TTCACAAAGACAGTGCGTGA** 3´
	Reverse (*HCPro*/*P3*)	5´GGGG AC CAC TTT GTA CAA GAA AGC TGG GT **AACTGCTGCATGTTCGTCAA** 3´
990 bp	Forward (*CP*)	5´GGGG ACA AGT TTG TAC AAA AAA GCAGGC **GCTGATGAAAAGGAGGACGA** 3´
	Reverse (*CP*)	5´GGGG AC CAC TTT GTA CAA GAA AGC TGG GT **CTCCCCTCACACCGAGGA** 3´

Note: The underlined suquences are attB1 and attB2 sites

2.1.3 RNA isolation

Total RNA was isolated from the leaf samples of tree infected with the PPV-T isolate using Qiagen RNA isolation kit. 100 mg of leaf tissue was weighed and ground thoroughly in liquid nitrogen with a mortar and pestle. The tissue powder and liquid nitrogen were decanted into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube. After evaporation of liquid nitrogen, 450 µl RTL buffer containing β-Mercaptoethanol (β-ME) (10 µL β-ME per 1 ml buffer RLT) was added to tissue powder and vortexed vigorously. For better disruption of tissue it was incubated

for 1-3 min at 56°C. Then, the lysate was transferred into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. The supernatant of the flow-through was transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. 0.5 volume of ethanol (96-100%) was added to the cleared lysate and mixed immediately by pipetting. The mixture was transferred to an RNeasy spin column placed in 2 ml collection tube and centrifuged for 15 s at ≥10,000 rpm. Then, the flow-through was discarded. 700 µl of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at ≥10,000 rpm. The flow-through was discarded. Then, 500 µl of RPE buffer containing ethanol (4 volumes of ethanol 96-100% added previously) was added to the RNeasy spin column and centrifuged for 15 s at ≥10,000 rpm. The flow-through was discarded again. Washing the RNeasy spin column with RPE buffer with the same amount was repeated for the second time and after centrifugation, the RNeasy spin column was carefully removed from the collection tube so that the column does not contact the flow-through and placed in a new 2 ml collection tube and centrifuged at full speed for 1min to eliminate any possible carryover of buffer RPE. Then, the RNeasy spin column was placed in a new 1.5 ml collection tube and 30µl RNase free water was added directly to the spin column membrane and centrifuged for 1 min at ≥10,000 rpm to elute the RNA. The isolated RNA was kept at -20°C until use.

2.1.4 RT-PCR amplification of PPV gene fragments

Synthesis of cDNA from the extracted total RNA of PPV-T was performed using SuperScript^{TM} II reverse transcriptase (Invitrogen). 12 µl RNA, 200 ng random primer and 1 µl 10mM dNTP were added to a nuclease free PCR tube and the mixture was incubated for 5 min at 65°C. After incubation, the tube was immediately cooled down on ice. Keeping the tube on the ice, 4 µl 5x first-strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 2 µL 0.1 M DTT and 1 µl RNaseOut water were added and mixed properly by pipetting. The tube was incubated for 3 min at 25°C. Eventually, after addition of 1 µl of SuperScript^{TM} II reverse transcriptase and mixing well by pipetting, the cDNA synthesis was performed in thermocycler by incubation for 12 min at 25°C, 50 min at 42°C and 15 min at 70°C.

The produced cDNA was applied as template for PCR reaction using the designed five primer pairs for amplification of five different PPV-T gene fragments to be used for hairpin construction. In a sterile 0.2 ml PCR tube the following amount of reagents were added: 1.5µl 10X Taq Buffer, 0.9 µl 25 mM $MgCl_2$, 1.5 µl 2 mM dNTP, 0.2 µl 10 µM forward primer, 0.2 µl 10 µM reverse primer, 0.3 µl 5 U/µL Taq DNA Polymerase, 1 µl cDNA and nuclease free water to a final volume of 15 µL. The PCR reaction was performed in a Biorad Thermocycler at 94°C for 90 sec as the initial denaturation step, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 90 sec and extension at 72°C for 90 sec and ending with final extension at 72°C for 5 min. The PCR products were mixed with loading buffer and loaded onto a 1.2% (w/v) agarose gel to be checked by electrophoresis. Electrophoresis was performed in Tris-borate-EDTA (TBE) buffer at 90 V for 1 hour and the gel was visualized by Gel Doc™ XR+ Gel documentation system. After confirmation of presence of expected single band for each fragment, the PCR products were purified using MinElute PCR Purification Kit – QIAGEN.

2.2 Vector construction

2.2.1 Materials

2.2.1.1 Template

The five PPV-T PCR-amplified and purified gene fragments in previous section were used as templates for making hairpin RNA (hpRNA) constructs, using Gateway cloning system for triggering RNA interference (RNAi).

2.2.1.2 Bacteria and plasmid

For hpRNA construction, pDONR™ 221 vector was purchased from Thermo Fisher Scientific corporation. This vector contains attP site that in a recombination reaction (BP reaction) with an attB substrate (attB-PCR product) produces an attL-containing entry clone. The destination vector Phellsgate12, was provided by Waterhouse lab (Commonwealth Scientific and Industry Research Organization (CSIRO), Australia).

The Phellsgate12 (destination vector) contains two attR sites in reverse orientation and in a recombination reaction (LR reaction) with an att-L substrate (entry clone), which contains the gene of insert, creates intron-containing hairpin RNA.

One Shot® TO*P1*0 Electrocomp™ *Escherichia coli* was purchased from Thermo Fisher Scientific corporation for cloning and plasmid propagation.

2.2.2 BP recombination reaction

The BP recombination reaction facilitates transfer of a gene of interest in the format of attB-PCR product to an attP-containing donor vector to create an entry clone. BP Clonase™ II Enzyme Mix (Invitrogen) was used for BP recombination reaction. The BP reaction for each of five fragments consisted of 5 µL purified attB- PCR product, 1 µL of 150 ng/µL pDONR221 and adequate TE buffer, PH 8.0 to bring the volume to 8 µL. Then, 2 µL of BP Clonase™ II enzyme mix was added to each sample and reactions were incubated at 25°C for 1 hour. To stop the reaction, 2 µl of proteinase K solution was added to each of them and incubated at 37°C for 10 min.

2.2.3 *E. coli* transformation

Once the BP recombination reaction was done for each of five attB-PCR products, they were used for transformation of competent *E. coli* and selection of entry clones using the appropriate antibiotic. To do so, 1 µL of the BP recombination reaction of each sample produced in previous section, was added into a 0.1cm–gap sterile electroporation cuvette containing 50 µl of electrocompetent *E. coli* and mixed gently. Electroporation was done using MicroPulser™ Electroporation Apparatus (Biorad). Subsequently, 450 µl of room temperature S.O.C medium was added to each cuvette immediately and the solutions were transferred to a 15ml Falcon tube and shook at 37°C for 1 hour to allow expression of the antibiotic resistance marker. Then, 50, 100 and 150 µl of each solution was spread on pre-warmed Luria-Bertani (LB) agar plates containing 50 µg/ml kanamycin (Kan) and incubated at 37°C overnight.

2.2.4 Colony PCR for ligation confirmation of entry clones

After overnight incubation and observation of single colonies on LB agar plates, some individual colonies were selected randomly and sub-cultured to be used for colony PCR using the gene specific primers of each fragment. The PCR reactions were carried on in a sterile 0.2 ml PCR tube containing the following amount of reagents: 1.5µl 10X Taq Buffer, 0.9 µl 25 mM $MgCl_2$, 1.5 µl 2 mM dNTP, 0.2 µl 10 µM forward primer, 0.2 µl 10 µM reverse primer, 0.3 µl 5 U/µL Taq DNA Polymerase, an individual colony picked up with a sterile toothpick and nuclease free water to a final volume of 15 µL. Colony PCR was carried out by an initial heating step at 96°C for 10 min and subsequently, the normal PCR reaction was performed in a Biorad Thermocycler at 94°C for 1:30 min as the initial denaturation step, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 90 sec and extension at 72°C for 90 sec and ending with final extension at 72°C for 5 min. Electrophoresis of PCR products were performed as described previously.

2.2.5 Plasmid isolation from entry clones

After confirmation of the positive colonies for each fragment with colony PCR, the sub-cultured bacteria from relative single colony were used for the plasmid isolation procedure using Sigma GenElute™ Plasmid Miniprep Kit. To do so, using the positive sub-cultured bacteria, fresh single colonies were grown again on a LB agar plate containing 50µg/ml kanamycin. Subsequently, a single colony was picked up from each plate and suspended in a 15 ml falcon tube containing LB liquid medium with selective antibiotic and incubated at 37°C by shaking at 225 rpm overnight. Then, 2 ml of overnight cultures containing entry clones were pelleted by centrifugation for 1 min at 10,000 rpm and the supernatants were discarded. The bacterial pellets were completely re-suspended with 200 µl of Resuspension Solution by vortex to make them homogeneous. Then, the re-suspended cells in each tube were lysed in 200 µl Lysis Solution and the contents were mixed gently by inversion (6-8 times) until the mixtures become clear and viscous. The cell decries were precipitated by addition of 350 µl of Neutralization/Binding Solution and inversion of tubes for 4-6 times. The cell debris

was then pelleted by centrifugation for 10 min at ≥ 10,000 rpm. In this step, to prepare the GenElute Miniprep Binding Columns which were provided by the kit, 500 µl of the Column Preparation Solution was added to each miniprep column and centrifuged at ≥10,000 rpm for 30 seconds to 1 minute and the flow-through liquid was discarded. Subsequently, the cleared lysates were transferred to the prepared column and centrifuged at ≥ 10,000 rpm for 1 min and the flow-through liquid was discarded from each tube. The columns were washed by addition 750 µl of diluted Washing Solution and centrifugation at ≥10,000 rpm for 30 seconds. The flow-through liquid was discarded and the tubes were centrifuged again at maximum speed for 1 to 2 minutes without any additional Washing Solution to remove excess ethanol. Eventually, the columns were transferred to a fresh collection tube and 50 µl distilled water was added to the columns and centrifuged at ≥ 10,000 rpm for 1 min. The isolated plasmids were stored at -20 to be used for LR recombination reaction for making destination vectors.

2.2.6 LR recombination reaction and making destination vectors

The LR recombination reaction is between the entry clone and a destination vector. The following components were added to 5 1.5 ml microcentrifuge tubes: 5 µl of purified plasmid DNA of each 5 entry clone (50-150 ng/µL), 1 µL of Phellsgate12 destination vector (300 ng/reaction) and adequate TE buffer, PH 8.0 to bring the volume to 8 µL. Eventually, 2 µL of LR Clonase™ enzyme mix was added to each tube and reactions were incubated at 25°C overnight. To stop the reaction, 2 µl of proteinase K solution was added to each reaction and incubated at 37°C for 10 min. One microliter of the reaction was then used to transform One Shot® TO*P10* Electrocomp™ *E. coli*. Subsequently, positive clones were grown on an LB agar plate containing 100 µg/ml Spectinomycin (Spec).

2.2.7 Colony PCR for ligation confirmation of destination vectors

After observation of single colonies on LB agar plates containing selective antibiotic (Spec), some individual colonies were selected randomly, sub-cultured and subjected to colony PCR as it was explained in section 2.2.4 using the gene specific primers. Electrophoresis of PCR products were performed as described previously.

2.2.8 Plasmid isolation from destination vectors

Like the plasmid isolation procedure from entry clones, after confirmation of positive colonies for each fragment with colony PCR of destination vectors, the sub-cultured bacteria from relative single colonies were used for plasmid isolation procedure using Sigma GenElute™ Plasmid Miniprep Kit. The isolated plasmids were stored at -20 to be transferred into *Agrobacterium* for *Agrobacterium*-mediated gene transfer.

2.3 *Agrobacterium*-mediated gene transfer

2.3.1 Materials

Agrobacterium tumefaciens strain *EHA*105 was used for preparation of *Agrobacterium* competent cells.

2.3.2 Preparation of *Agrobacterium* competent cells for electroporation

A. tumefaciens strain EHA105 was streaked on a YEP agar plate and incubated at 28°C for 48 hour. A single colony was picked and inoculated in 20 ml YEP medium containing 50 mg/l rifampicin (Rif), with constant shaking at 28°C, 220 rpm overnight to become saturated. Subsequently, 1ml of the culture was inoculated in 200 ml YEP medium containing 50 mg/L Rif and shaking at 28°C, 220 rpm overnight until OD600 = 0.5-1. The final culture was spun at 4°C, 5000 rpm for 10 min to collect the cells. The cell pellets were rinsed with 250 ml cold sterile ddH_2O three times and finally, the pellets were rinsed by 10% glycerol and re-suspended in 800 µL cold sterile 10% glycerol. After that, the cell suspension was divided to 50 µL aliquots that were quickly frozen in liquid nitrogen and stored at -80^oC to be used for transformation procedure.

2.3.3 Transformation of *Agrobacterium* by electroporation

Five microcentrifuge tubes containing 50 µl *A. tumefaciens* electrocompetent cells were thawed on ice and 5 µl of each of purified destination vectors prepared in section 2.2.6 were added to the tubes and mixed gently. The mixtures were then transferred to the

electroporation cuvettes and tapped the suspensions to the bottom of the tubes. The MicroPulser™ Electroporation Apparatus (Biorad) was set to "Agr" and the electroporation was performed. 100 µl of cold YEP was added to the DNA/*Agrobacterium* mixture and mixed gently by pipetting. After that, the mixtures were added to a 10 ml tube containing 900 µL YEP and then shaken at 28°C, 220 rpm for 3 hours. Subsequently, 50, 100 µl of each culture was spread on YEP agar plates containing 50 µg/ml Rifampicin (Rif) and 100 µg/ml (Spec) and incubated inverted at 28°C for 2 days.

2.3.4 Colony PCR for confirmation of presence of destination vectors in *A. tumefaciens*

Similar to previous colony PCR procedure, after observation of single colonies on YEP agar plates containing selective antibiotics (Rif and Spec), some individual colonies were selected randomly, sub-cultured and subjected to colony PCR using the gene specific primers. Electrophoresis of PCR products were performed with conditions described previously in section 2.1.4.

2.4 *N. benthamiana* transformation

2.4.1 Materials

2.4.1.1 Media

Pre-culture medium: Murashige Skoog (MS) medium supplemented with 5µM growth regulator solution 6-Benzylaminopurine (BAP).

Co-culture medium: MS medium supplemented with 5µM (BAP), 3.7 g/L 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.4) and 38 mg/L of acetosyringone (AS)

Selection medium: MS medium supplemented with 5µM (BAP), 100 mg/L Kan and 500 mg/L Cefotaxime (Cef).

Rooting medium: MS medium supplemented with 100 mg/L Kan and 500 mg/L Cefotaxime (Cef).

2.4.1.2 Bacterium

A. tumefaciens strain EHA105 harboring the five different constructed hairpin structures (h-UTR/*P1*, h-*P1*/*HCPro*, h-*HCPro*, h-*HCPro/P3*and h-*CP*) were utilized for *N. benthamiana* transformation.

2.4.1.3 Plant material and growth conditions

For sterilization, *N. benthamiana* seeds were treated with 75% (v/v) absolute ethanol for 1 min followed by another treatment with 0.75% NaOCl for 20 min and eventually washed three times with sterile water. Subsequently, the sterilized seeds were sowed on MS medium (pH 5.8) containing 4.4 g/L powdered MS medium (salts and vitamins), 30 g/L sucrose and 8 g/L agar. The aseptic seeds germinated and the seedlings were cultured in a growth chamber at 26 ± 1 °C under a photoperiod of 16 h-light /8 h-dark.

2.4.2 Leaf Explant Preparation

After germination of *N. benthamaiana* seeds and their growth in aseptic condition, the half mature leaves were removed and cut into pieces of 0.5×0.5 cm^2. The explants were placed on the pre-culture medium with adaxial side up. The explants on pre-culture medium were cultured at 25°C under 12 hour light regime for 2 days.

2.4.3 Preparation of *Agrobacterium* Culture

The transformed *A. tumefaciens* strain EHA105 with five different constructed hairpin structures, were streaked separately on YEP agar plates supplemented with 50 µg/ml (Rif) and 100 µg/ml (Spec) and incubated at 28°C for 2 days until the colonies were available. A single colony from each plate was picked and inoculated into 2 ml YEP medium containing 50 µg/ml (Rif) and 100 µg/ml (Spec) and shaken at 28°C, 220 rpm overnight. Then, 50 µl of each culture was inoculated into 50 ml new YEP medium

containing antibiotics and incubated at 28°C. Subsequently, the cells in the cultures were harvested by centrifugation at 9000 rpm and the bacterial pellets were suspended to final OD_{600} of 0.5 to 1 in liquid cocultivation medium and kept on ice for 2 hours.

2.4.4 Plant Transformation

The prepared *Agrobacterium* inoculum was transferred into a sterile petri plate and the precultered explants in pre-culture medium previously, were transferred to the petri plate and soaked for 30 min. Subsequently, the explants were blotted on a sterile filter paper and then placed onto cocultivation medium containing (AS) which was overlaid with a single piece of sterile Whatman filter paper. The explants were co-cultured at 28ºC for 2 days in the dark.

2.5 Regeneration of transformed explants

After two days, the explants were transferred to the selection medium containing antibiotics. Up to 10 explants were transferred to each plate containing selection medium and they were incubated at 28°C under an 18-h light regime. The explants were transferred to a fresh selection medium after two weeks. After about a month, the appeared shoots from each explant were excised individually once they reached the length greater than 3 mm and transferred to rooting medium supplemented with the appropriate selection agents. Rooting medium was poured in a deep vessel to give room for sufficient plantlet development. The rooting stage was carried on under the same environmental conditions applied for regeneration stage. Following about 2 weeks, the plantlets with well-established root system were removed from rooting medium and after washing off the excess agar from the roots, the plantlets were transferred to the pots containing soil. The pots were watered and transferred to a chambers covered with the lid to keep the humidity inside. The chambers were kept under an 18-h light regime and temperature of 28°C. Once the plantlets were fully acclimated, the lids were removed. When the plantlets became mature enough, leaf samples were collected to be used for DNA isolation for subsequent analysis.

2.6 DNA isolation from transgenic plants

A modified CTAB isolation method (Lefort and Douglas, 1999) was used for DNA extraction of collected samples from transgenic plants. 100 mg of fresh leaf sample of each of transgenic plant was ground in liquid nitrogen. 1 mL of DNA extraction buffer [50mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 0.7 M NaCl, 0.4 M LiCl, 1 % w/v CTAB (hexadecyltrimethylammonium bromide), 1 % w/v PVP 40, 2 % w/v SDS] and 10 µL of β-mercaptoethanol (1 % final concentration) was added to each tube containing the ground plant powder and the mixture was vortexed for 5 s, mixed by 2-3 inversion and then incubated for 15 min at 65°C in a water-bath. Then, 0.5 mL of chloroform/isoamylalcohol (24:1) was added to the tube. The mixture was inverted thoroughly until making an emulsion and then rested on ice for 30 min. After ice incubation, the mixture was centrifuged for 5 min at 14000 rpm. Subsequently, the supernatant was transferred to new 1.5 ml Eppendorf tube and an equivalent volume of isopropanol was added to tube. The tube was kept at -20ºC for 2 hours and then centrifuged for 1 min at 14000 rpm. The DNA pellet was washed with 70% ethanol and centrifuged for 1 min at 14000 rpm. Eventually, the ethanol was withdrawn and the pellet allowed to be dried. The DNA pellet was re-suspended in 50 µl distilled water and kept in -20 until use.

2.7 Characterization of transgenic NB plants

In order to confirm the presence of the transgenes in transformed *N. benthamiana* PCR amplification of the insert genes and the neomycin phosphotransferase II (*nptII*) gene by gene specific and *nptII* primers were performed.

Table 2.2. Primer sequences of the neomycin phosphotransferase II (*nptII*) gene

Name	Primer sequences	Fragment size
nptII (Forward)	5’GAGGCTATTCGGCTATGACTG3’	650bp
nptII (Reverse)	5’ATCGGGAGCGGCGATACCGTA3’	

2.8 Sequencing

In addition to PCR amplification of the insert genes, sequencing of the insert genes were performed in both arms of the hairpin structure to confirm the presence and their correct orientation.

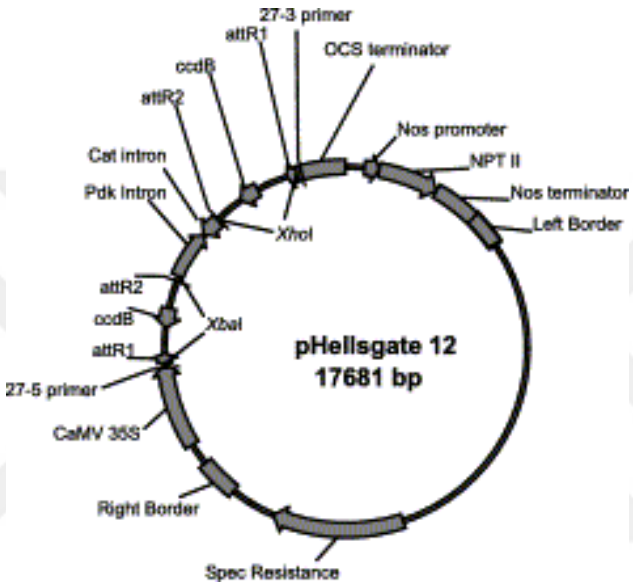


Figure 2.1. Map of Phellsgate 12 vector

Sequencing was performed using the gene specific forward and reverse primers. In addition, the gene specific reverse primer and primers which cover the terminal part of the CaMV35S promoter (P27-5) and initial part of ocs terminator (P27-3) in the PHELLSGATE 12 vector were used for amplification and sequencing of the insert gene flanked by the vector sequence regions (Helliwell and Waterhouse, 2003)

Table 2.3. Primer sequences used for sequencing

Name	Primer sequences
P27-5	5'GGGATGACGCAATCC3'
P27-3	5'GAGCTACACATGCTCAGG3'

2.9 Seed collection

After confirmation of presence of transgenes in regenerated *N. benthamiana* (T_0), the plants were allowed to grow and after self-pollination the seeds were collected from each transgenic plant separately to be used for next stage analysis.

2.10 Resistance assay

2.10.1 Preparation of T_1 plants

The obtained *N. benthamiana* T_1 progenies in each of five different transgenic plants were germinated to obtain mature T_1 lines to be inoculated in resistance assay. Germination of T1 progenies was performed three times for conducting the resistance assay with each of three PPV strains; PPV-T, PPV-M and PPV-D. In each round of germination, nine replicates (nine individual plants) for each line of transgenic plants were considered for resistance assay. The wild-type healthy seeds were also grown as control plants.

2.10.2 Evaluation of T_1 lines by PCR

Total DNA of grown T_1 progenies was isolated in each round of germination using the DNA extraction method explained in section 2.6 PCR was carried out using the gene specific primers for amplification of insert genes in transgenic *N. benthamiana*. Plants carrying the insert genes considered as transgenic and those with no insert gene amplification considered as non-transgenic plants.

2.10.3 Inoculation of T_1 with three PPV strains

In order to evaluate the efficiency of introduced resistance in transgenic plants by triggering the RNAi mechanism, the T_1 progenies in each round of germination were inoculated with three PPV strains including PPV-T, PPV-M and PPV-D after

determination of transgenic and non-transgenic plants by PCR. The inoculation test by each virus strain was performed mechanically. The PPV infected leaves of *N. benthamiana* of each virus strain, which was kept in lab as the source, were ground in PBS (phosphate-buffered saline) PH 7.2 supplemented with 2% (w/v) polyvinylpyrrolidone (PVP), and 0.2% (w/v) sodium diethyl-dithiocarbamate (DIECA). The prepared inoculum was rubbed on *N. benthamiana* leaves which had been lightly dusted with 400-grit carborundum. 30 min after inoculation, the leaves were washed by sprinkling of water and the inoculated plants were kept at environmental condition of 15 hour photoperiod and 24ºC temperature.

2.10.4 Evaluation of resistance by ELISA test

Except visual observation of symptoms on inoculated test plants, virus infection was also evaluated by DAS-ELISA at 14 days post-inoculation (dpi). From each line of transgenic and inoculated plants, 5 individuals were selected for ELISA test. Leaf samples were collected from each plant and they were ground in extraction buffer (PBS pH: 7.2-7.4, % 2 PVP, % 0.2 bovine serum albumin) at 1:10 ratio (tissue weight in g: buffer volume in ml). The polyclonal Anti-PPV-IgG was diluted 1:200 in coating buffer (1.59 g/L Na2CO_3, 2.93 g/L $NaHCO_3$, pH 7.4) and 100 µl was added into each well of a microtitre plate. The plate was then incubated at 37 °C for 4 hours with parafilm sealing. After incubation, the plate was washed three times with PBS-Tween 20 (0.05%). 100 µl of prepared plant extracts were added to each well and the plate was incubated at 4ºC overnight. Then, the plate was washed with washing buffer for three times and each well was filled with 100 µl of diluted Anti-PPV-MAB 5B-Conjucate (1:200) in conjugate buffer (PBS pH:7.2-7.4, % 2 PVP, % 0.2 bovine serum albumin). The plate was incubated for 4 hours at 37ºC and it was rinsed again with washing buffer after incubation. Finally, for enzymatic reaction the substrate pNPP (para-nitrophenylphosphate) was dissolved in substrate buffer (97 ml/L diethanolamine, 0.2 g/L $MgCl_2$×6 H_2O, pH 9.8) to give a final concentration of 1mg/ml. 100 µl of the prepared substrate solution was added to each well and the plate was incubated in the dark at room temperature. The color development was measured photometrically at 405 nm by ELISA reader after 30-120 min. Sample OD405 value higher than twice that of

NCx (average OD405 value of the negative control), designated as positive (Clark and Adams, 1977).

2.10.5 RT-PCR analysis of resistant transgenic plants

After getting ELISA results at 60 dpi, 5 plants of the most resistant lines of transgenic plants with 5 different hairpin constructs were selected and after their RNA isolation, they were subjected to RT-PCR using *P1*/P2 primers for amplification of 243bp fragment of *CP* of the virus. In transgenic plants harboring the *CP* hairpin construct, primer pairs amplifying virus fragment not located in *CP* region was used. This analysis was done to evaluate accumulation of virus at molecular level in the transgenic resistant lines.

2.11 Library preparation and small RNA sequencing

2.11.1 Sampling

Once the last resistance assay was performed with the PPV-M strain of the virus, at 21 dpi, leaf pools of selected lines of transgenic plants containing different hairpin constructs (UTR/*P1*, *P1*/*HCPro*, *HCPro*, *HCPro/P3* and *CP*), infected (WT+) and uninfected wild-type (WT-) control plants were made by collection of three different leaves from three plants of above mentioned groups (i.e., three leaves from each plant, and nine leaves per line). The sampling was done for RNA isolation and preparation of them for small RNA sequencing.

2.11.2 RNA extraction and quality control

Total RNA of collected samples was extracted using mirVana™ miRNA isolation kit. 100mg of each leaf pool was ground in liquid nitrogen. 10 volumes of Lysis/Binding Buffer per tissue mass was added to the tube and mixed rapidly. Then, 1/10 volume of miRNA Homogenate Additive was added to tissue lysate, and mixed well by vortexing or inverting the tube several times and then the mixture was left on ice for 10 min. After that, a volume of Acid Phenol: Chloroform that was equal to the lysate volume before

addition of the miRNA Homogenate Additive was added and vortexed for 30-60 sec. The mixture was centrifuged for 5 min at 10,000 rpm to separate the aqueous and organic phases. The aqueous phase was transferred to a fresh tube and 1.25 volumes of room temperature 100% ethanol was added to the aqueous phase. For each sample, a Filter Cartridge was placed into one of the Collection Tube and the lysate/ethanol mixture was added onto the Filter Cartridge and centrifuged for 15 sec at 10,000 rpm. The flow-through was discarded. 700 µL miRNA Washing Solution 1 was added to the Filter Cartridge and centrifuged for ~5–10 sec. The flow-through was discarded from the Collection Tube. Then, the filter was washed twice with 500 µl washing solution 2/3 and after discarding the flow-through from the last washing step, the Filter Cartridge was replaced in the same Collection Tube and spun for 1 min to remove residual fluid from the filter. Subsequently, the Filter Cartridge was transferred into a fresh Collection Tube and 30 µL of pre-heated (95°C) nuclease-free water was added to the center of the filter. The tube was spun for ~20–30 sec at maximum speed to recover the RNA. The purity of isolated RNA samples was evaluated by measuring absorbance ratio at 260/280 nm, using the BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan), with a threshold of 1.2-2.

2.11.3 Small RNA sequencing

The isolated RNA samples were submitted to LC Science Company for library construction and small RNA sequencing. Small RNA libraries were prepared using TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, USA). Briefly, as it was described in protocol of TruSeq Small RNA Sample Prep Kits, adapters were ligated to the 3' and 5' ends of 1 µg of isolated RNA and then cDNA constructs were created by reverse transcription followed by amplification using 2 primers that anneal to the adapter ends to enrich RNA fragments with adapter molecules on both ends. Gel purification of amplified cDNA constructs were done by running them on 6 % Novex TBE PAGE Gels (Life Technologies). Small RNA libraries were selected, cut and purified by ethanol precipitation. Quality control and validation of libraries were performed via fragment analysis using a Bioanalyzer (Agilent Technologies, Santa

Clara, CA, USA). The validated libraries were then sequenced by single-end sequencing 50bp on an Illumina Hiseq 4000 at LC Sciences.

2.12 Data processing

Raw reads were subjected to ACGT101-miR[1] (LC Sciences, Houston, Texas, USA) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Clean reads ranging from 18-25 nt in length were used for downstream analyses.

2.12.1 Identification of conserved and novel miRNAs

The valid sequences were analyzed by aligning against miRNA database[2] (miRBase 21.0) (Griffiths-Jones et al., 2008). Length variation at both 3' and 5' ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to specific species mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known specific species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p-derived miRNA candidates. The remaining sequences were mapped to other selected species precursors (with the exclusion of specific species) in miRBase 21.0 by BLAST search, and the mapped pre-miRNAs were further blasted against the specific species genomes to determine their genomic locations.

The unmapped sequences were blasted against the specific genomes, and the hairpin RNA structures containing sequences were predicated from the flank 120 nt sequences using RNAfold software[3]. The criteria for secondary structure prediction were: (1) number of nucleotides in one bulge in stem (≤12) (2) number of base pairs in the stem

[1] . An in-house program ACGT101-miR to delete low-quality reads, 3' adapter sequences, and contaminations.
[2] . miRBase is the primary public repository and online resource for microRNA sequences and annotation (http://mirbase.org/)
[3] . The RNAfold web server predicts secondary structures of single stranded RNA or DNA sequences (http://rna.tbi.univie.ac.at/cgi- bin/RNAWebSuite/RNAfold.cgi)

region of the predicted hairpin (≥16) (3) cutoff of free energy (kCal/mol ≤-15) (4) length of hairpin (up and down stems + terminal loop ≥50) (5) length of hairpin loop (≤200). (6) number of nucleotides in one bulge in mature region (≤4) (7) number of biased errors in one bulge in mature region (≤2) (8) number of biased bulges in mature region (≤2) (9) number of errors in mature region (≤4) (10) number of base pairs in the mature region of the predicted hairpin (≥12) (11) percent of mature in stem (≥80). Minimal folding free energy index (MFEI) is an important factor for miRNA identification (Bonnet et al., 2004). Novel miRNAs were only considered when the MEFIs of their pre-miRNAs were ≥0.80 and their normalized copy number was required to be ≥10.

2.12.2 miRNA expression profiling analysis

To compare the expression levels of the miRNAs among libraries a chi-square[1] test and Fisher's exact[2] test were performed after the raw data were normalized from high-throughput sequencing. The log2 ratio was used as the threshold to detect differences in miRNA expression levels. The miRNAs were considered to be significantly up-regulated or down-regulated when the p values of both the chi-square test and Fisher's exact test were ≤0.05, the |log2 ratio| was ≥1 and more than 100 reads were present in at least one library after normalization (Cao et al., 2014).

2.12.3 Target prediction and enrichment analysis

To predict the genes targeted by most aboundant miRNAs, computational target prediction algorithms TargetFinder[3] (https://github.com/carringtonlab/TargetFinder/) were used to identify miRNA binding sites. Then, GO enrichment analysis[4] of target

[1] . The Chi Square Test is a test that involves the use of parameters to test the statistical significance of the observations under study

[2] . Fisher's exact test is a statistical test used to determine if there are nonrandom associations between two categorical variables

[3] . Plant small RNA target prediction tool

[4] . One of the main uses of the GO is to perform enrichment analysis on gene sets. For example, given a set of genes that are up-regulated under certain conditions, an enrichment analysis will find which GO terms are over-represented (or under-represented) using annotations for that gene set.

genes was performed using the Gene ontology knowledgebase (http://www.geneontology.org).

2.12.4 Small interfering RNA (siRNA) detection

In order to detect the siRNAs in transgenic plants containing PPV derived hairpin constructs, the obtained clean reads of each library were aligned to PPV-M isolate isMrPl220 genome (Acc. Number MF370984) using Bowtie v1.2.1.1 and Geneious Prime softwares (Langmead et al., 2009; Kearse et al., 2012). This isolate was the one used for inoculation in PPV-M strain resistance assay.

3. CHAPTER

RESULTS

3.1 Amplification and cloning of PPV fragments

Amplification of selected genomic regions from PPV-T isolate by designated primers covering the *CP* gene and four other fragments located in the *P1* and HC-Pro genes, UTR/*P1*, *P1*/*HCPro*, *HCPro* and *HCPro/P3* (Di Nicola-Negri et al., 2005) was carried out by RT-PCR. Gel electrophoresis of the PCR products confirmed amplification of gene fragments with expected size *UTR/P1* 752pb, *P1/HCPro* 771bp, *HCPro* 649bp, *HCPro/P3* 594bp and *CP* 990bp (Figure 3.1).

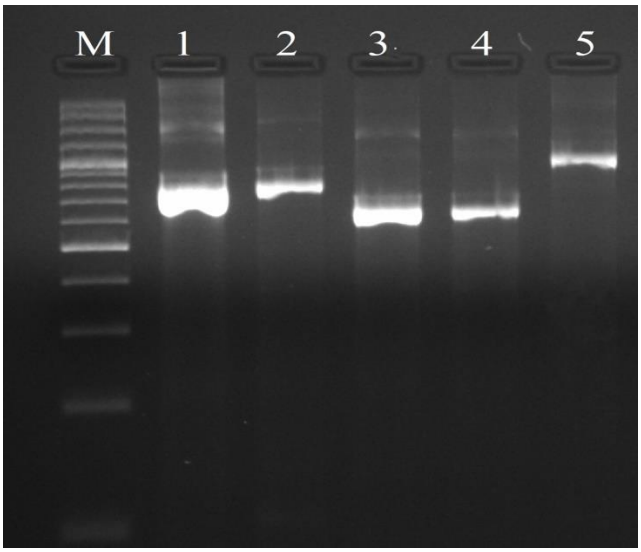


Figure 3.1. Amplification of genes of interest for hiarpin construction. Lanes 1: *UTR/P1* 752pb, 2: *P1/HCPro* 771bp, 3: *HCPro* 649bp, 4: *HCPro/P3* 594bp and 5: *CP* 990bp. M: 100bp DNA ladder

3.2 Construction of hairpin vectors

After amplification of expected gene fragments by RT-PCR, they were applied for hairpin construction using the gateway technology. The PCR products were purified with MinElute PCR Purification Kit (QIAGEN) and used for BP reaction.

3.2.1 Construction of entry clones in BP recombination reaction

Purified PCR products were used for BP recombination reaction with pDONR221 to generate entry clones. The entry clones were transformed into One Shot® TO*P10* Electrocomp™ *E. coli* and the colonies were checked by colony PCR using the specific primers for each fragment (Figure 3.2).

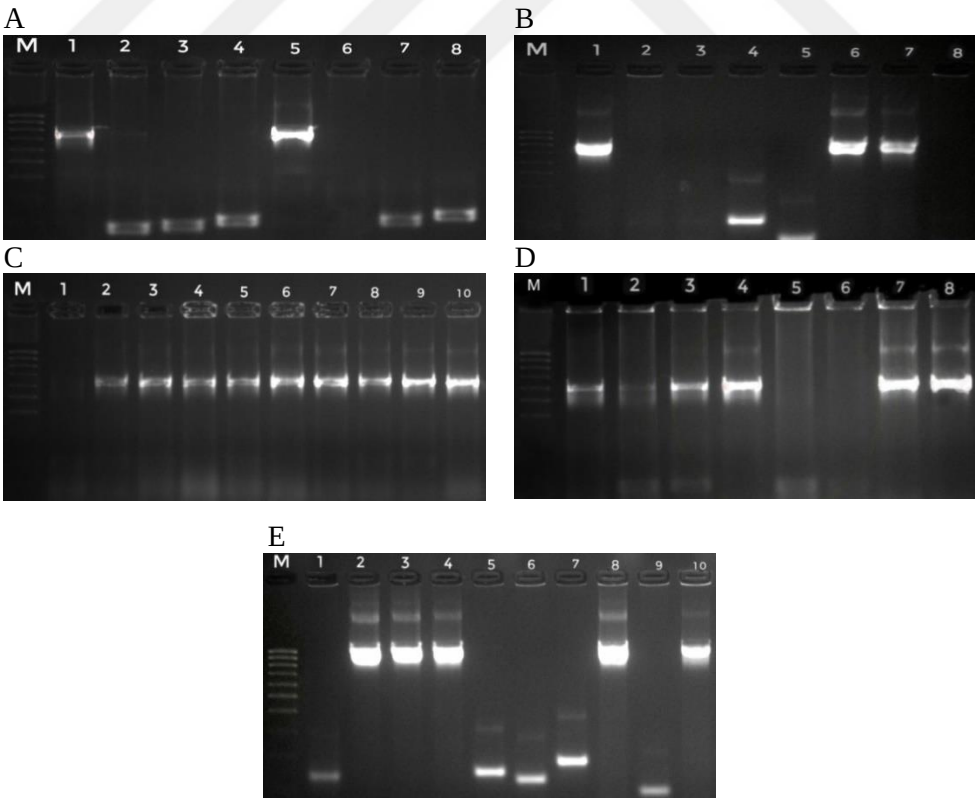


Figure 3.2. Agarose gel electrophoresis of colony PCR product for entry clones. Positive clones of A) *UTR/P1* construct. B) *P1/HCPro* construct. C) *HCPro* construct. D) *HCPro/P3* construct. E) *CP* construct. M: 100 bp DNA ladder

The entry clone vectors were extracted with GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The purified plasmids were used for LR reaction and preparation of destination clones.

3.2.2 Construction of destination vectors in LR recombination reaction

The LR recombination reactions were performed with the purified plasmid of entry clones and pHellsgate12 to generate the destination clones. The destination clones were transformed into One Shot® TOP*1*0 Electrocomp™ *E. coli* and the colonies were checked again by colony PCR using the specific primers for each of fragments (Figure 3.3). After identification of positive colonies, the destination vectors were extracted using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich).

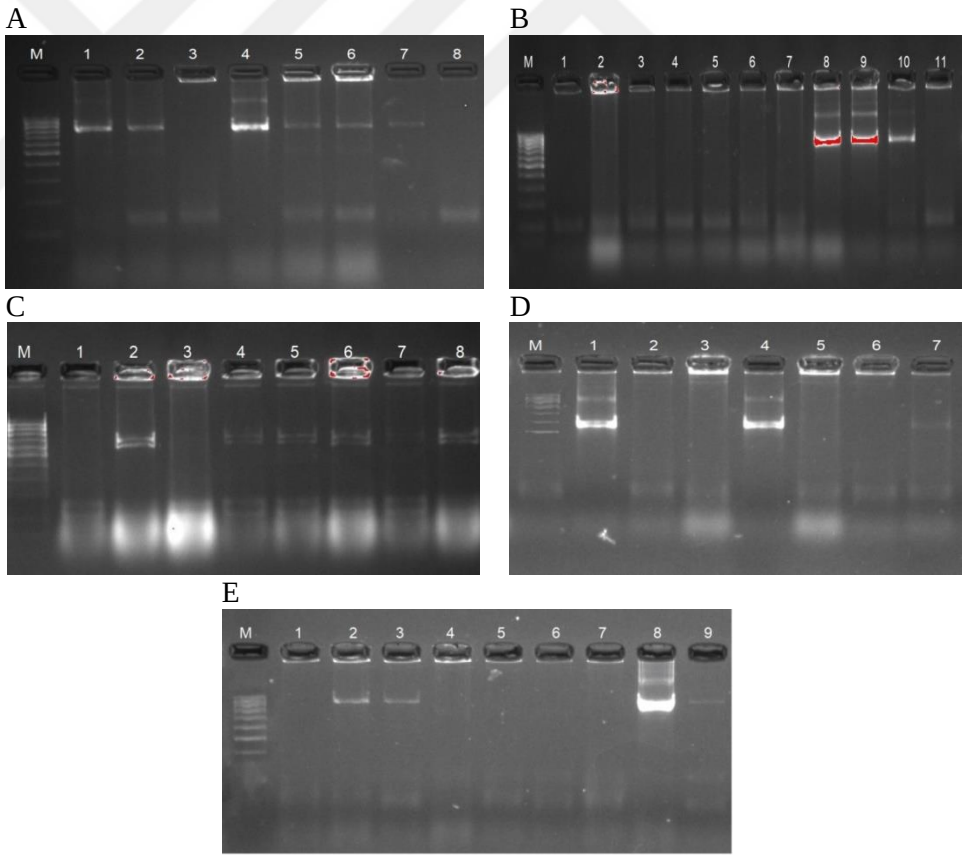


Figure 3.3. Agarose gel electrophoresis of colony PCR product for destination vectors. Positive clones of A) UTR/*P1*(752 pb) construct B) *P1*/HCPro (771 pb) construct C) HCPro (591 pb) construct D) HCPro/*P3*(594pb) construct E) CP (990pb) construct. M: 100pb DNA ladder.

3.3 Transfer of constructs to *Agrobacterium*

After preparation of competent cells of *A. tumefaciens* strain EHA105, transfer of purified destination vectors to competent cells were carried out by electroporation. The positive colonies were determined by PCR colony using the gene specific primers (Figure 3.4).

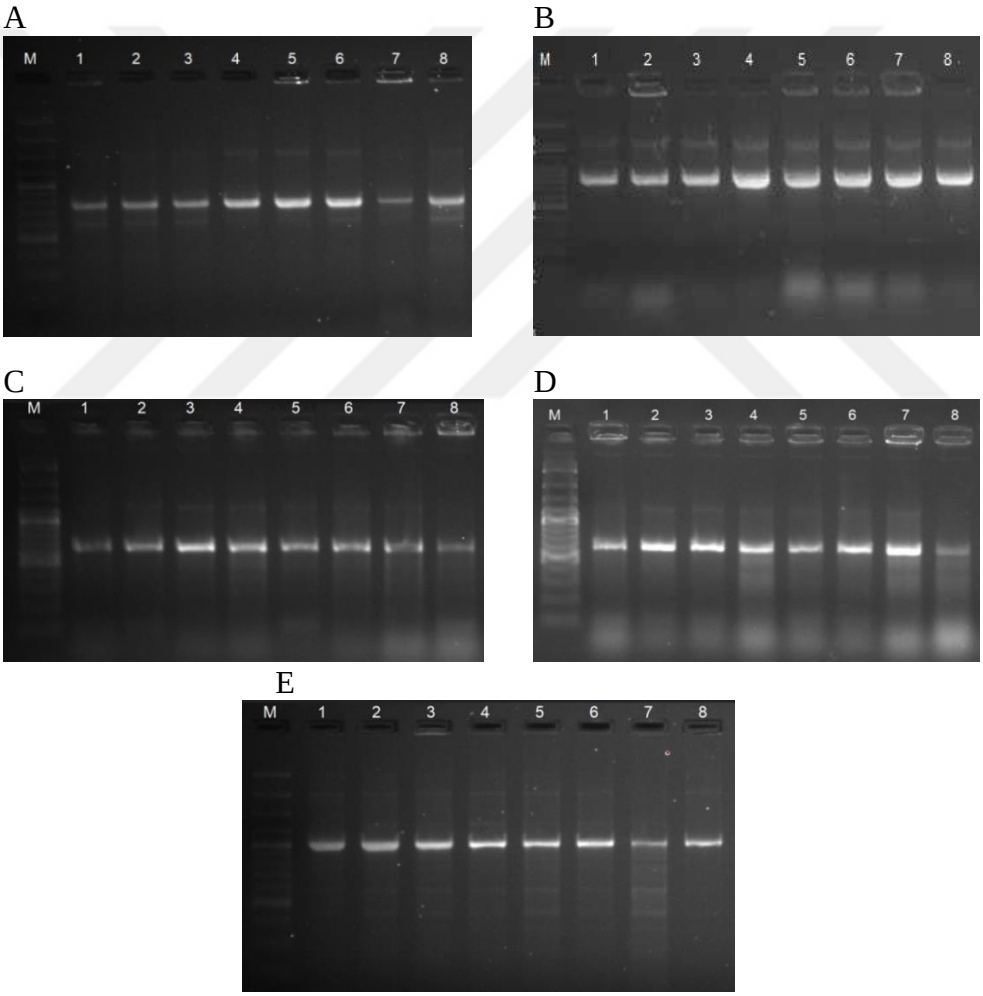
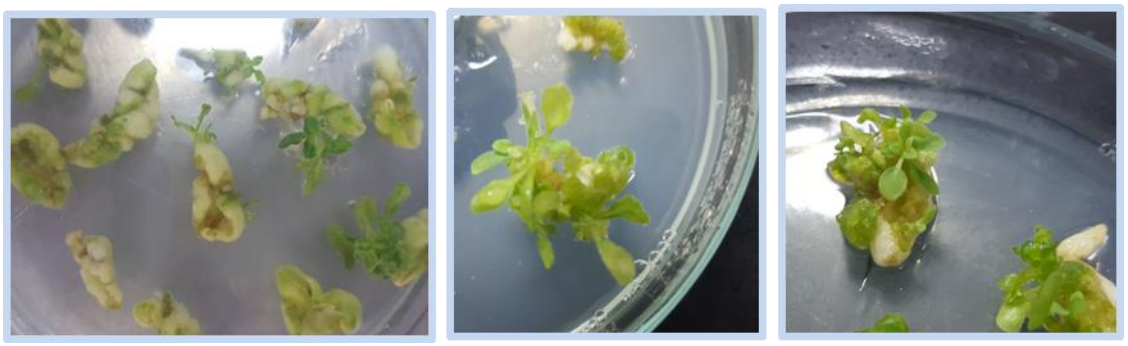


Figure 3.4. Agarose gel electrophoresis of colony PCR products of *Agrobacterium*. Positive clones of A) *UTR/P1*(752 pb) construct B) *P1/HCPro* (771 pb) construct C) *HCPro* (591 pb) construct D) *HCPro/P3* (594pb) construct E) *CP* (990pb) construct. M: 100 bp DNA ladder.

3.4 *Agrobacterium*-mediated *N. benthamiana* transformation

The transformed *Agrobacterium* with five different hairpin constructs were used for *N. benthamiana* transformation using the *Agrobacterium* transformation procedure described by Clemente (2006). After about a month, the shoots were produced on the leaf explants of *N. benthamiana* in the selective medium containing 100 mg/L Kan and 500 mg/L Cef (Figure 3.5A) . Shoots with the length greater than 3 mm were excised and transferred to a new MS medium containing 100 mg/L Kan and 500 mg/L Cef for rooting induction (Figure 3.5B). Plantlets with well-developed root systems were transferred to soil in a chamber with 18-h light regime and temperature of 28°C (Figure 3.5C). Once the plantlets were fully acclimated, they were transplanted to pots and grown under standard condition of greenhouse. The seeds of the self-polinated transgenic plants were collected to be used for evaluation of putative induced resistance in resistance assay.

A



B

C

Figure 3.5. Different stages of regeneration of transgenic plants. A) Generation of shoots from explants. B) excised shoots in rooting medium. C) Generation of roots and transfer of mature plants to soil.

3.5 PCR screening for confirmation of transgenes

At this stage, the putative T_0 transgenic plants were tested for the presence of the introduced genes with gene specific primers and the neomycin phosphotransferase II (*nptII*) gene by PCR. Amplification of desired fragments and the (*nptII*) gene confirmed the presence of genes of interest in each of transgenic plants (Figure 3.6).

As the results of PCR showed, 10 lines of transgenic plants harboring *UTR/P1*, seven of *P1/HCPro*, five of *HCPro*, 6 of *HCPro/P3* and 11lines transgenic plants harboring CP were obtained.

In addition, plants with positive PCR results and containing the insert genes were subjected to sequencing analysis for further confirmation of the presence of inserts and their correct orientation. The results of sequencing confirmed the presence and correct orientation of each construct. Based on these results, it was concluded that the transformation procedure was successfully performed and transgenic plants harboring desired genes were obtained. Subsequently, they were used in resistance assay for evaluation of induced resistance.

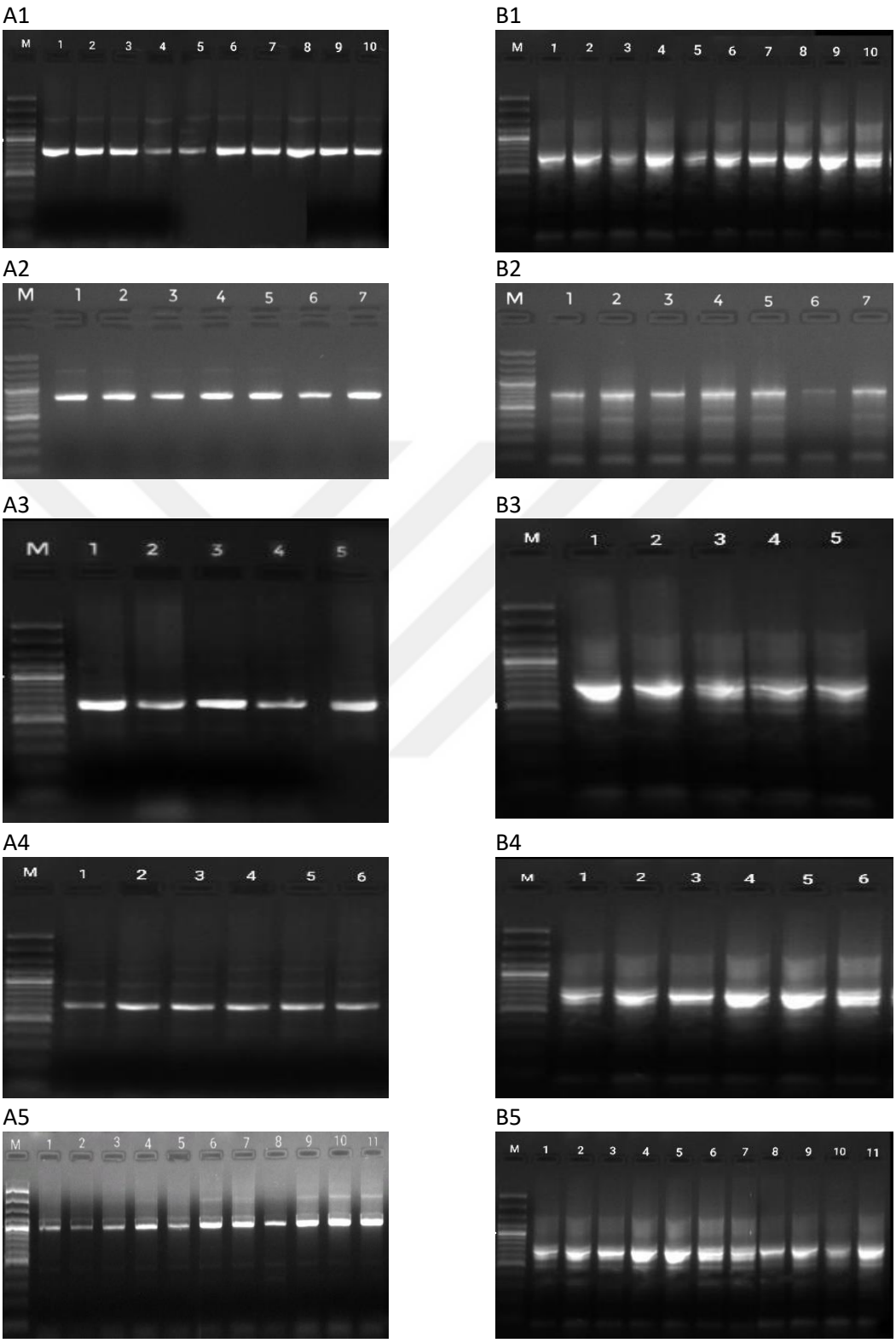


Figure 3.6. Confirmation of transgenic plants by gene specific and *nptII* primers. A1 to A5: *UTR/P1* (752bp), *P1/HCPro* (771bp), *HCPro* (649bp), *HCPro/P3* (594bp) and *CP* (990bp). B1 to B5: 650bp (*nptII*) gene. M: 100bp DNA ladder.

3.6 Resistance assay of transgenic plants

The T1 progeny of confirmed transgenic lines in each construct were planted three times for inoculation with three strains of the virus. A total of nine wild-type control plants and nine replicates per transgenic lines for each construct were planted and after PCR with gene specific primers, the transgenic ones were determined. The transgenic and control plants were inoculated separately with PPV-T, PPV-D and PPV-M strains of the virus.

After inoculation, plants were monitored for detection of virus symptoms and 15 dpi the symptoms were observed on wild-type control plants. The control groups, composing 9 plants allocated for each of the virus strain, developed chlorotic mosaic with dark green islands and leaf puckering (Figure 3.7).

In transgenic plants transformed with different hairpin constructs, various level of reaction to the virus was observed. Generally, the virus symptoms were almost absent in different lines of transgenic plants containing *UTR/P1* and *CP* hairpin constructs. This observation was consistent in thier inoculation with all three strains of the virus. On the other hand, the seven different lines of transgenic plants containing *P1/HCPro* hairpin construct, showed the highest level of virus symptoms in each of inoculation trials with three strains of the virus. In transgenic plants containing *HCPro* hairpin construct, out of five different inoculated lines, line (8-1) with one infected plant in all three inoculation tests showed a better performance in resistance assays . In *HCPro/P3* containing transgenic plants, among six inoculated lines, no visible virus symptom was seen in replicates of two lines (5-4 and 5-6) in each round of inoculation with three strains of the virus.

Figure 3.7. PPV symptoms on control plants.

3.7 Evaluation of resistance by DAS-ELISA test

Evaluation of resistance/susceptibility was performed by DAS-ELISA at 14 dpi in each of three virus inoculation trials. Samples were collected from five replicates of each line of transgenic plants for DAS-ELISA assay. As it was expected based on symptom observations, the replicates of plant lines derived from transgenic plants containing *UTR/P1* and *CP* hairpin constructs, were mostly virus free. Except two individual plants of line 18-2 and 18-6 in inoculation with T strain, all five selected plants in 10 obtained lines containing *UTR/P1* construct, were virus free in ELISA test after three virus inoculation trials

The ELISA results for transgenic plants containing *CP* hairpin construct also indicated of high percentage of resistant plants. Among 11 obtained lines in transgenic plants containing *CP* hairpin construct, all five selected plants of 4-3, 11-3 and 21-5 lines, were virus free in ELISA test after inoculation with three strains of the virus. Two infected plants in each of 3-1 and 4-1 lines and one infected plant in line 12-2 were screened in M inoculation trial. In line 11-1 and 21-3 one infected plant in D and T

inoculated plants were detected, respectively. Line 20-3 with one PPV-D infected plant and one PPV-M infected plant was the only line with infected plants in two virus inoculation trials and therefore, considered as the most susceptible line among others.

In transgenic plants containing *HCPro* and *HCPro/P3* hairpin constructs, the obtained lines showed different level of resistance. In *HCPro* transgenic plants among five lines inoculated with three strains of the virus (D,T and M), plants of one lines showed higher degree of resistance compared to others. Among five plants evaluated by ELISA, Line 8-1 with one infected plant in D strain inoculation and no infected plants in inoculation with T and M strain considered as the most resistant line.

Among six lines obtained containing *HCPro/P3* hairpin construct and inoculated with three strains of the virus, two lines showed a higher level of resistance. Based on ELISA test results, lines 5-4 and 5-6 with no virus detection in inoculation with three strains of the virus, considered as the most resistant lines. In transgenic plants containing *P1/HCPro* hairpin construct, the situation was different and no significant resistance was seen in any of seven obtained lines (Table 3.1).

Table 3.1. Resistance analysis of transgenic *N. benthamiana* plants harboring hairpin constructs challenged with three PPV virus strains

Hairpin constructs		PPV-D		PPV-T		PPV-M	
		Infected /transgenic plants	ELISA positive plants	Infected /transgenic plants	ELISA positive plants	Infected /transgenic plants	ELISA positive plants
UTR/P1 transgenic lines	10-4	0/6	0/5	0/5	0/5	0/8	0/5
	11-1	0/6	0/5	0/7	0/5	0/7	0/5
	12-3	0/8	0/5	0/9	0/5	0/9	0/5
	12-4	0/7	0/5	0/9	0/5	0/9	0/5
	12-5	0/9	0/5	0/9	0/5	0/8	0/5
	15	0/8	0/5	0/8	0/5	0/9	0/5
	18-2	0/6	0/5	1/7	1/5	0/5	0/5
	18-3	0/6	0/5	0/5	0/5	0/6	0/5
	18-5	0/5	0/5	0/8	0/8	0/6	0/5
	18-6	0/7	0/5	1/6	1/5	-	-
CP transgenic lines	3-1	0/6	0/5	0/6	0/5	2/9	2/5
	4-1	0/9	0/5	0/6	0/5	2/9	2/5
	4-3	0/9	0/5	0/9	0/5	0/6	0/5
	11-1	1/8	1/5	0/9	0/5	0/9	0/5
	11-3	0/9	0/5	0/8	0/5	0/7	0/5
	12-2	0/9	0/5	0/5	0/5	1/6	1/5
	20-1	0/8	0/5	1/8	1/5	0/8	0/5
	20-3	1/8	1/5	0/7	0/5	1/8	1/5
	21-3	0/9	0/5	1/8	1/5	0/6	0/5
	21-4	1/9	1/5	0/8	0/5	-	-
	21-5	0/5	0/5	0/9	0/5	0/9	0/5
HCPro transgenic lines	2	2/5	2/5	-	-	3/9	2/5
	6	-	-	0/5	0/5	1/5	1/5
	7-1	1/5	1/5	0/5	0/5	1/7	1/5
	7-2	1/7	1/5	1/7	1/5	2/7	2/5
	8-1	1/9	1/5	0/8	0/5	0/8	0/5
HCPro/P3 transgenic lines	1-2	5/7	5/5	2/6	2/5	5/5	5/5
	5-3	5/8	5/5	2/7	2/5	5/5	5/5
	5-4	0/8	0/5	0/9	0/5	0/7	0/5
	5-6	0/5	0/5	0/8	0/5	0/8	0/5
	5-8	3/8	3/5	0/5	0/5	7/7	5/5
	5-9	-	-	2/7	2/5	6/6	5/5
P1/HCPro transgenic lines	1	8/9	5/5	5/9	3/5	9/9	5/5
	2	8/8	5/5	9/9	5/5	8/8	5/5
	4	4/9	4/5	6/8	3/5	9/9	5/5
	6	1/9	1/5	8/8	5/5	7/9	5/5
	7	5/9	5/5	8/9	4/5	8/8	5/5
	8	4/9	4/5	9/9	5/5	8/8	5/5
	13	3/9	3/5	8/8	5/5	-	-
Wild type		9/9	5/5	9/9	5/5	9/9	5/5

3.8 RT-PCR of selected transgenic plants

In order to evaluate accumulation of virus at molecular level, reverse transcriptase PCR using *P1*/P2 primers for five selected plants in most resistant lines of transgenic plants harboring UTR/*P1*, *HCPro*, *HCPro/P3* constructs was performed 60 dpi. Due to location of *P1*/P2 primers in *CP* region another set of primers used for transgenic plants harboring *CP* hairpin construct. The results showed no amplification of virus fragments in plants harboring UTR/*P1*, *HCPro/P3* and *CP* hairpin constructs. But, in transgenic plant harboring *HCPro* hairpin construct the virus fragment amplification was observed (Figure 3.8). Therefore, *HCPro* containing transgenic plants considered as moderate resistant plants.

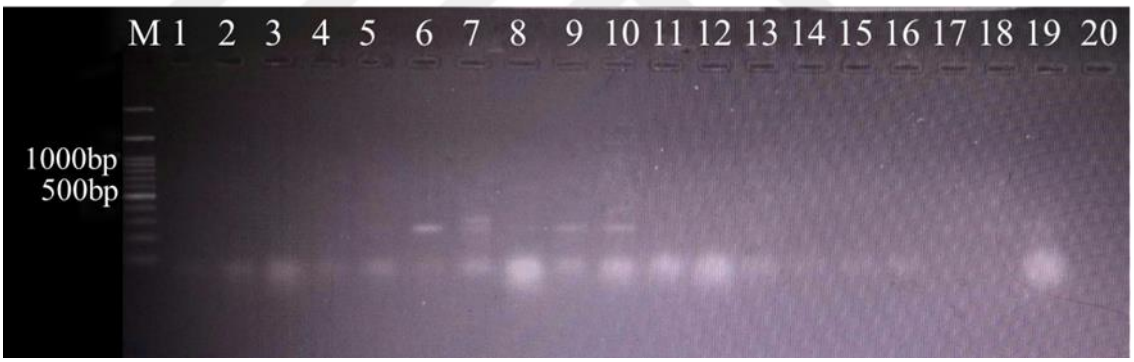


Figure 3.8. Agarose gel electrophoresis of RT-PCR products of 5 selected plants harboring 4 hairpin constructs showing resistance. Lane 1 to 5: Plants with *UTR/P1* hairpin construct, Lane 6 to 10: Plants with *HCPro* hairpin construct, Lane 11 to 15: Plants with *HCPro/P3* hairpin construct, Lane 16 to 20: Plants with *CP* hairpin construct. M: 100bp DNA ladder.

3.9 Small RNA sequencing

In order to investigate the small RNA profiles in transgenic plants, small RNA sequencing was performed for plants harboring five different hairpin constructs and control plants, separately.

3.9.1 The small RNA profiles of sequenced samples

After filtering out the low quality reads, adaptor dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA), repeats and retaining reads of 18-25 nt in length, (4,897,746) clean reads of total reads in *UTR/P1* l, (10,503,221) in *P1*/*HCPro*, (4,085,612) in *HCPro*, (4,594,829) for *HCPro*/*P3*, (4,364,345) in *CP*, (3,309,238) in uninfected wild-type and (12,955,705) in infected wild-type plant libraries were obtained (Table 3.2). Higher number of clean reads were seen in susceptible transgenic and the infected wild-type plants. While, almost similar number of clean reads were observed in resistant transgenic plants and uninfected wild-type plants.

The small RNA profiles represent the miRNAs and siRNAs in each of these libraries. The profile of produced miRNAs in each library was also determined after alignment of clean reads to the miRNA database (miRBase). In addition, the siRNA population and their distribution along the PPV genome in each library was detected by alignment of reads to the PPV genome.

Table 3.2. Overview of reads from raw data to cleaned sequences

RNA class	UTR/*P1*	*P1*/*HCPro*	*HCPro*	*HCPro*/*P3*	*CP*	WT-	WT+
Raw reads	11727939	14008687	12345962	13434494	13776048	7764101	16091996
3′ADT and length filter	6389699	3330697	7803334	8405750	8834878	4156314	2974702
Junkfilter	26750	29085	20185	26798	25309	22267	28003
Rfam	292436	72114	308299	312215	403801	203502	55509
mRNA	142521	78915	171039	111169	187184	89710	80922
rRNA	215496	51302	206405	229885	285290	146950	37421
tRNA	65810	18263	90477	67984	101533	48194	14668
snoRNA	2160	466	1770	2071	2393	1730	910
Repeats	4333	2691	4145	3724	6113	4502	3935
Clean sequences	4897746	10503221	4085612	4594829	4364345	3309238	12955705

Note: Reads lacking 3′ ADTs or length with <18 and >25 nt were removed. 3′ ADT: reads with null 3′ adapter.Junk: >=2N, >=7A, >=8C, >=6G, >=7T, >=10Dimer, >=6Trimer, or >=5Tetramer .Rfam: Collection of many common non-coding RNA families except micro RNA; http://rfam.janelia.org.snoRNA: small nucleolar RNA

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3.9.2 Detected miRNAs in different libraries

In total, 241 conserved miRNAs, member of 68 plant miRNA families, were detected in all seven libraries. In (UTR/*P1*) sample 200 known miRNAs, 184 in (*HCPro*), 194 in (*HCPro*/*P3*), 200 in (*CP*), 190 in (*P1*/*HCPro*), 185 in (WT-) and 173 in (WT+) were detected. 140 out of 241 miRNAs were simultaneously expressed in all seven samples.

A total of 77 novel 5p or 3p derived miRNA candidates were screened in all 7 libraries. Among these novel miRNAs, 63 in UTR/P, 52 in *HCPro*, 57 in *HCPro/P3*, 60 in *CP*, 74 in *P1*/*HCPro*, 53 in WT- and 72 in WT+ were detected. A total of 77 novel 5p or 3p derived miRNA candidates were screened in all seven libraries

3.9.3 Gene ontology (GO) enrichment analysis of target genes

To predict the genes targeted by most abundant miRNAs, computational target prediction algorithms TargetFinder (https://github.com/carringtonlab/TargetFinder/) were used to identify miRNA binding sites. Subsequently, in order to understand the functions of the expressed miRNAs in each library, the predicted target genes were subjected to GO analysis. GO is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species and it has been known as a promising method for uncovering the miRNA gene regulatory network.

The distribution of enriched GO terms in three pathways in different libraries is shown in Figure 3.9 to Figure 3.15. As it is shown in Figure 3.9 and Figure 3.10, the percentage of genes involved in biological-process, regulation of transcription (DNA-templated), transcription (DNA-templated), protein phosphorylation and defense response in infected wild-type showed a significant decrease upon virus infection in comparison with un-infected wild-type plants. Among transgenic virus inoculated plants, the percentage of genes involved in above mentioned processes in three resistant transgenic plants harboring UTR/*P1*, *HCPro/P3* and *CP* hairpin constructs were very close to the un-infected wild type plants (Figure 3.13, Figure 3.14 and Figure 3.15).

Whereas, in *HCPro* and *P1/HCPro* containing hairpin constructs the percentage of these genes are very close to infected wild type plants (Figure 3.11 and Figure 3.12).

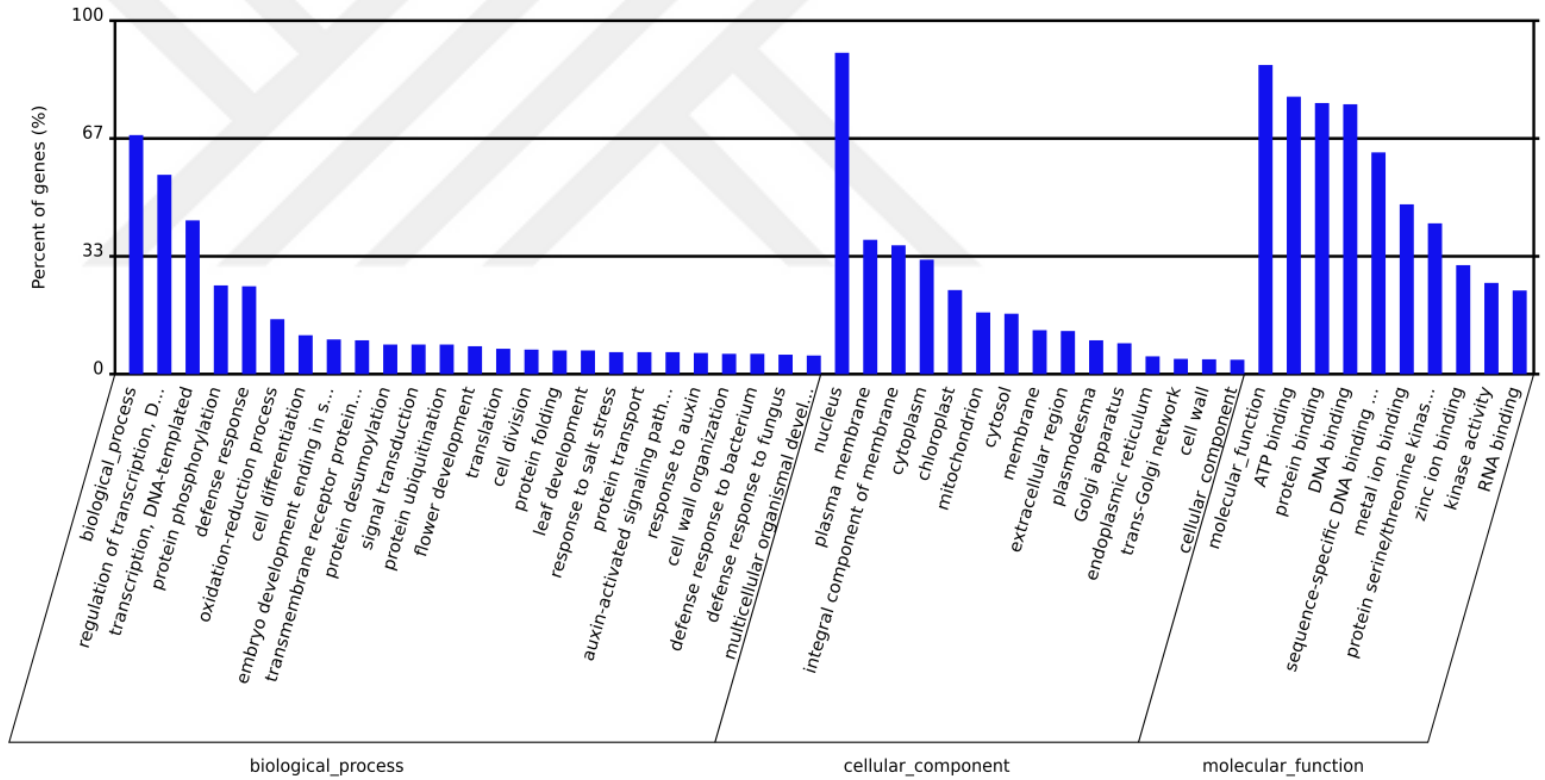

Figure 3.9. The distribution of enriched GO terms in biology process, cellular component and molecular function in PPV-infected wild-type library.

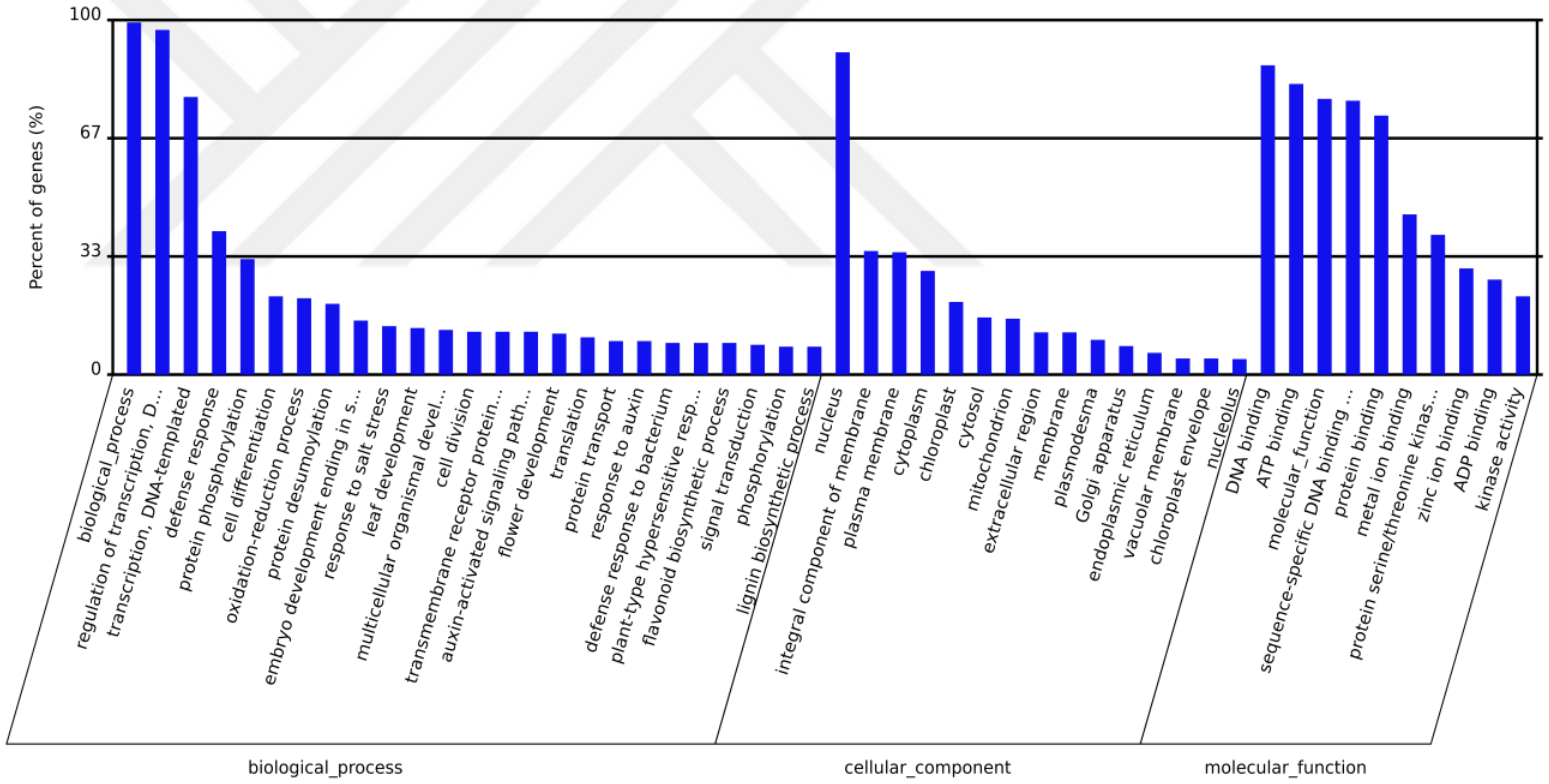

Figure 3.10. The distribution of enriched GO terms in biology process, cellular component and molecular function in un-infected wild-type library.

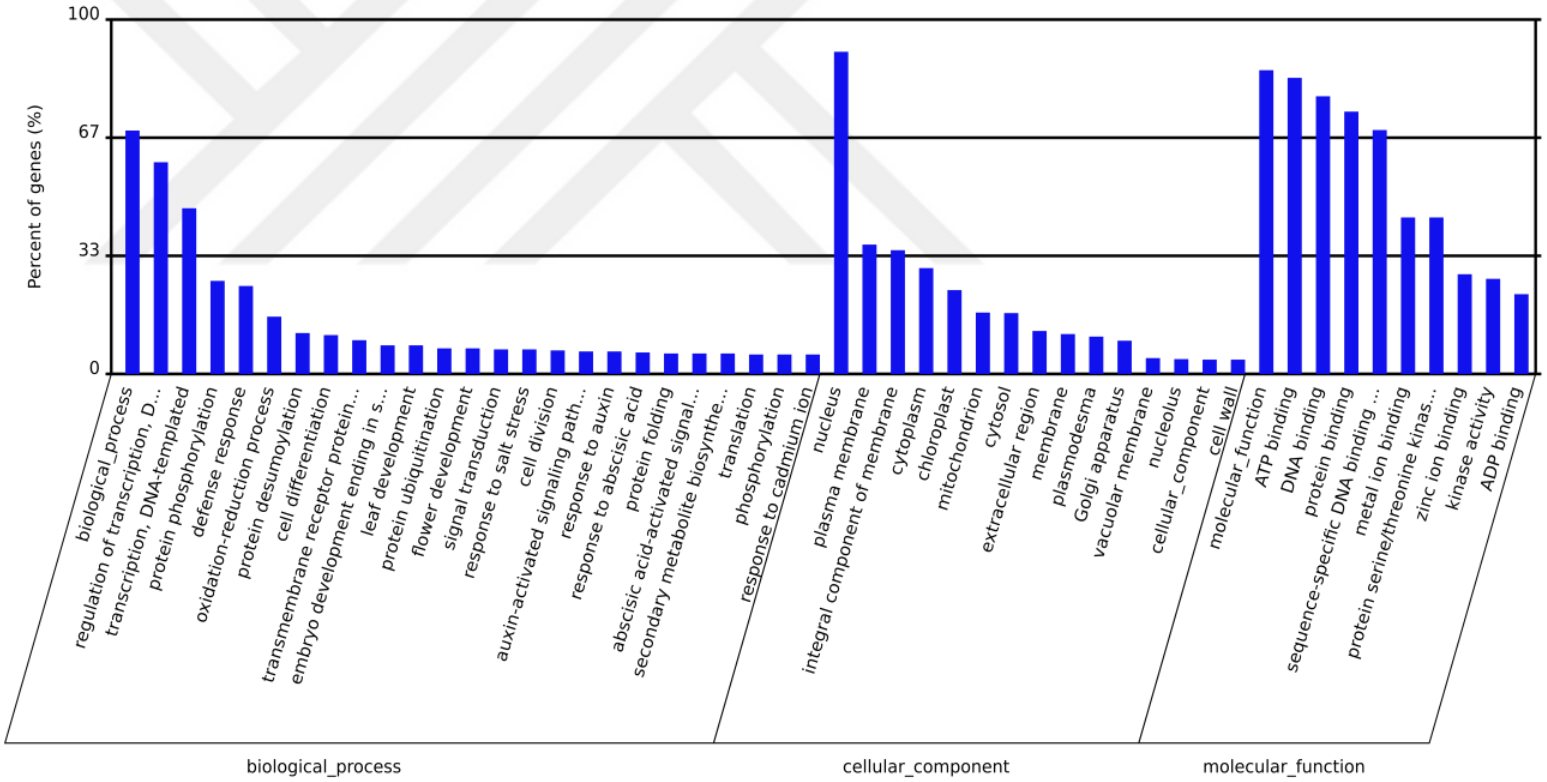

Figure 3.11. The distribution of enriched GO terms in biology process, cellular component and molecular function in *P1/HCPro* hairpin construct containing library.

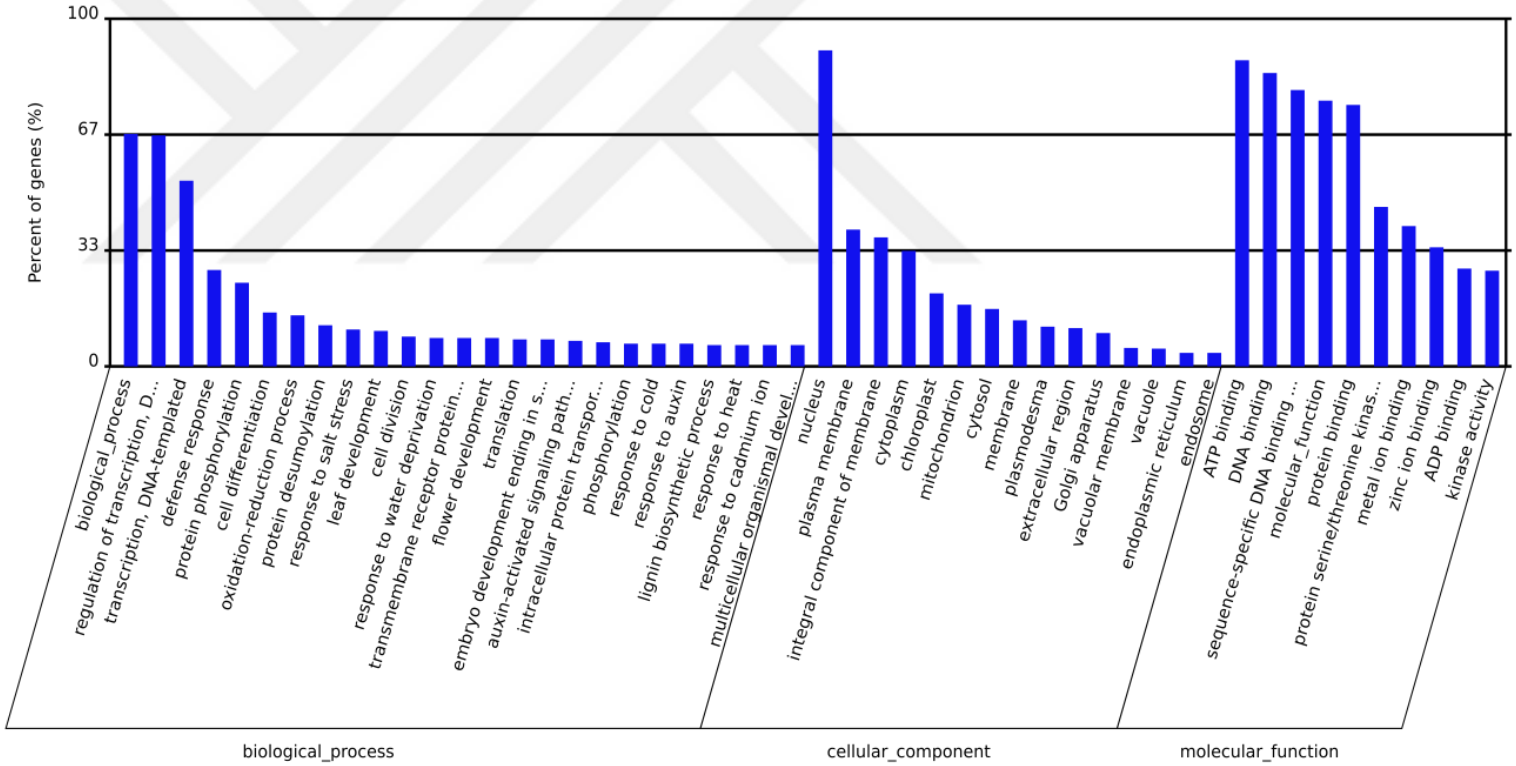

Figure 3.12. The distribution of enriched GO terms in biology process, cellular component and molecular function in *HCPro* hairpin construct containing library.

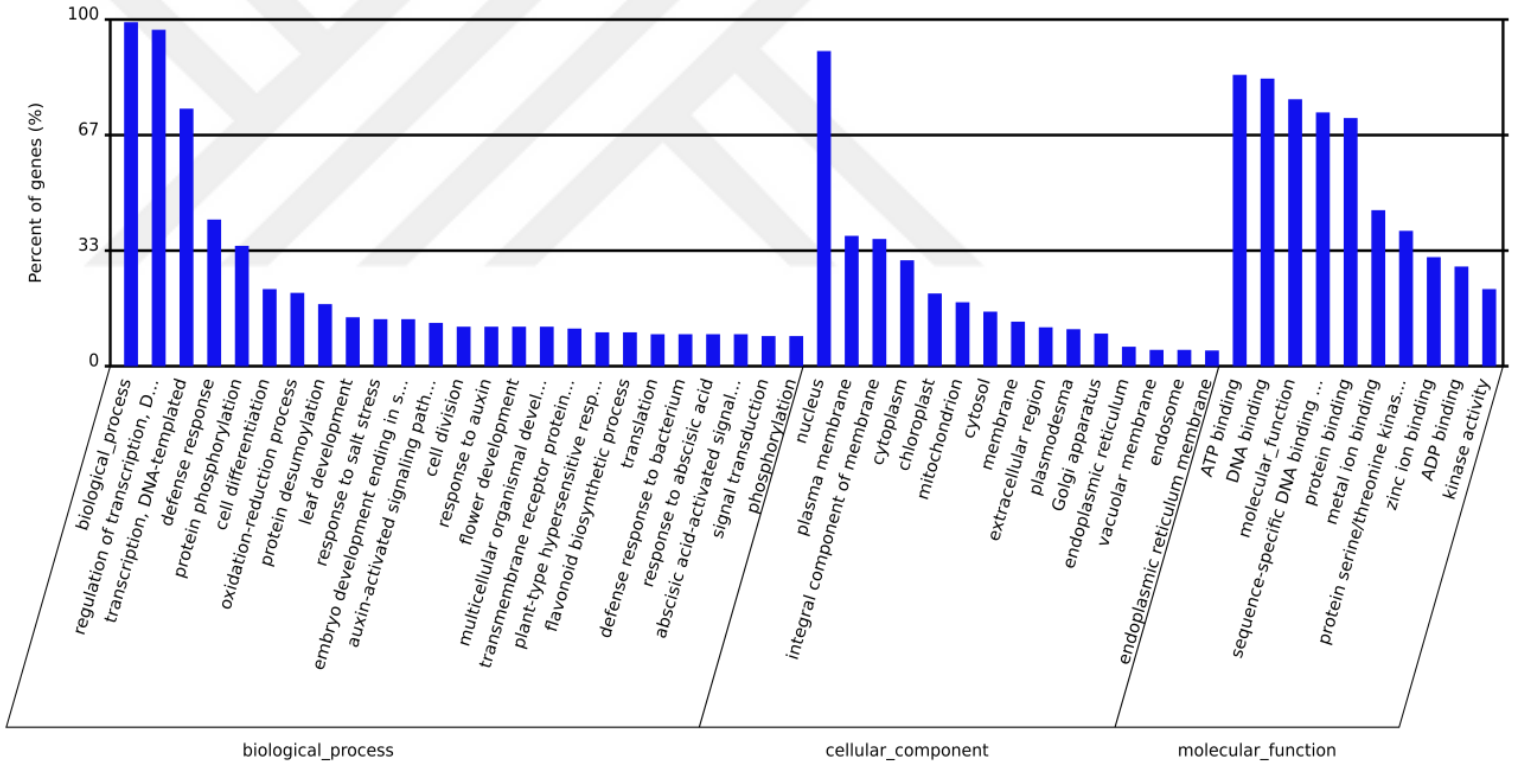

Figure 3.13. The distribution of enriched GO terms in biology process, cellular component and molecular function in *UTR/P1* hairpin construct containing library.

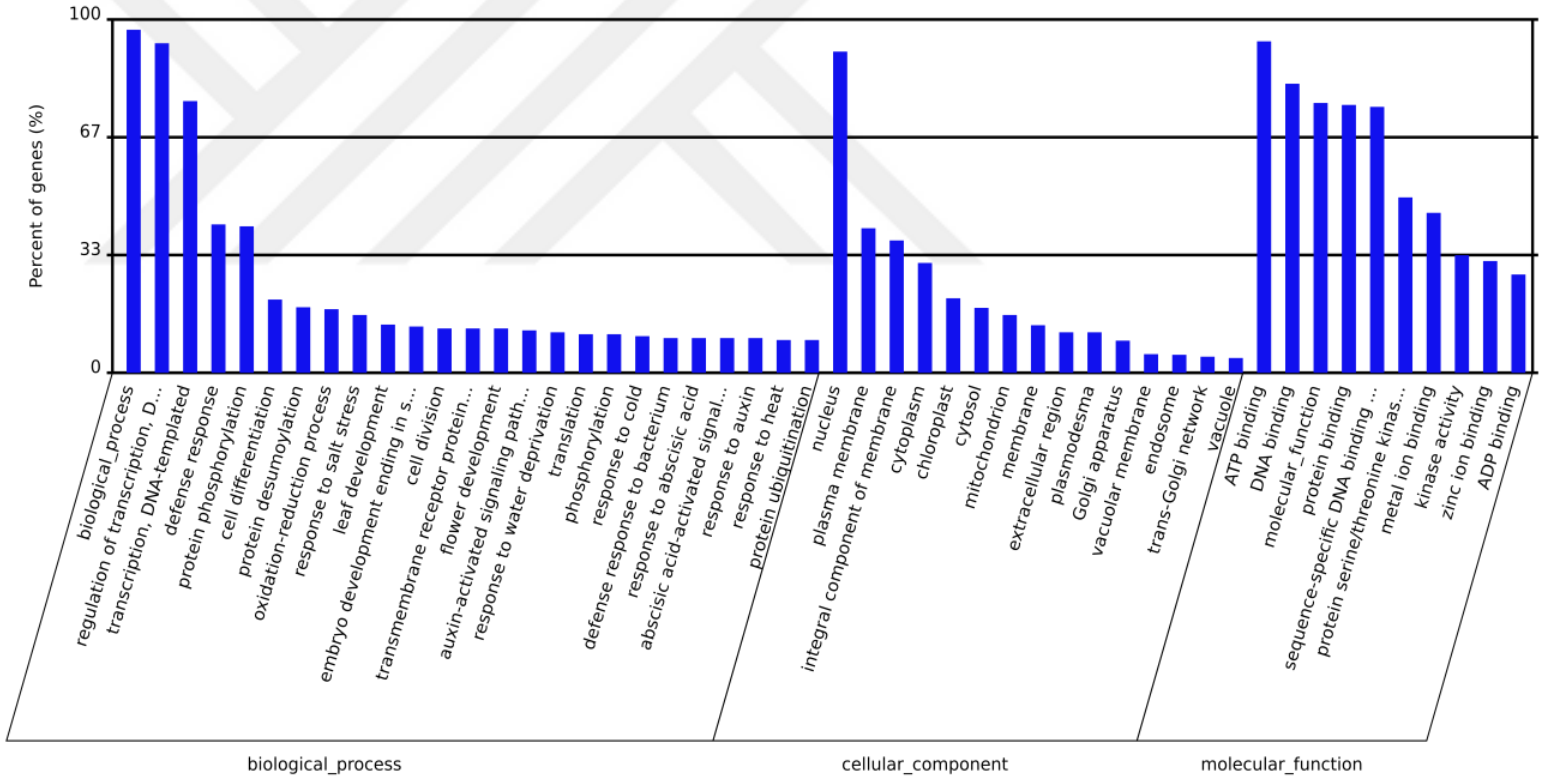

Figure 3.14. The distribution of enriched GO terms in biology process, cellular component and molecular function in *HCPro/P3*hairpin construct containing library.

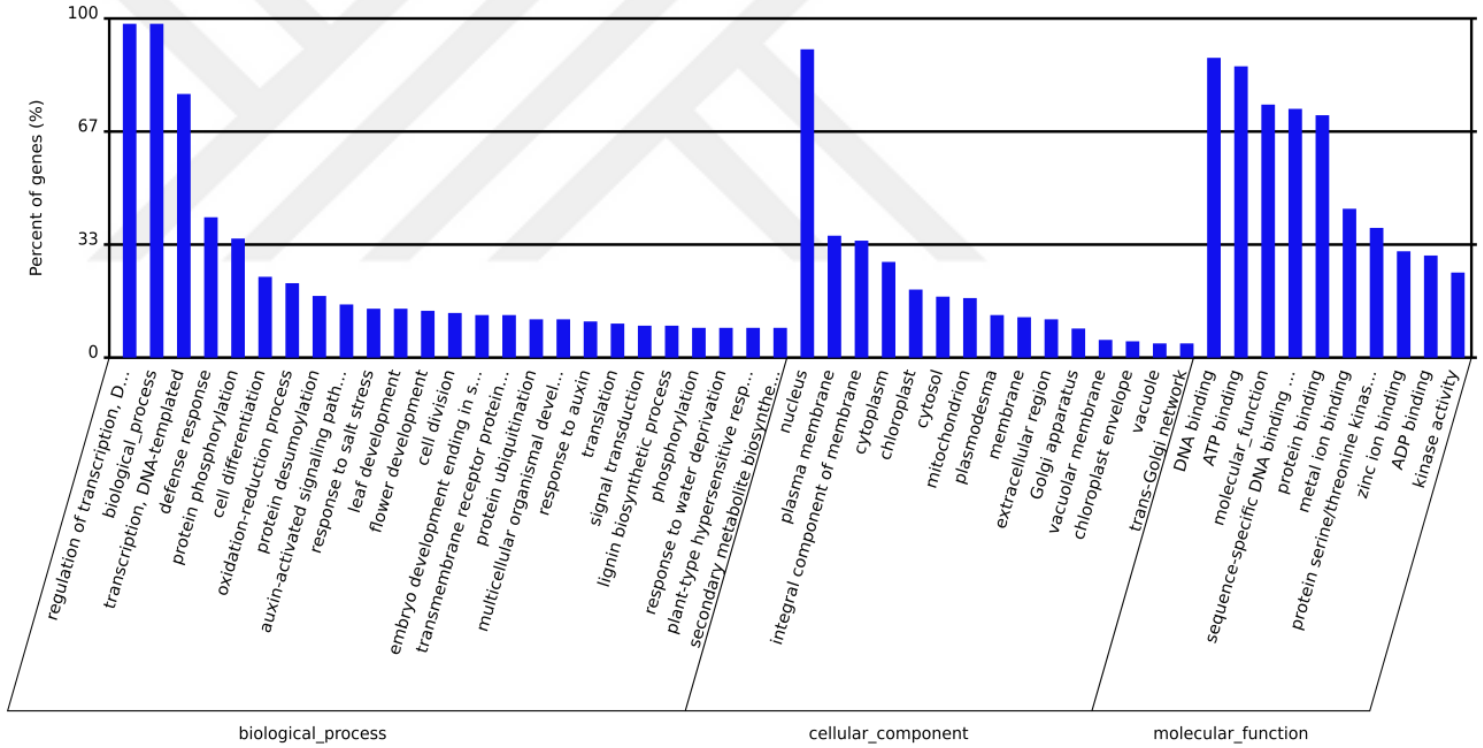

Figure 3.15. The distribution of enriched GO terms in biology process, cellular component and molecular function in *CP* hairpin construct containing library.

3.9.4 Identification of PPV responsive miRNAs

To identify plant miRNAs responsive to PPV infection, the expression profiles of miRNAs in infected wild type and un-infected wild type plants were compared based on the read counts obtained from high-throughput sequencing. After normalization of the raw sequence read numbers, the reads meeting the stringent criteria ($p<0.05$, fold change >2 and more than 100 reads in at least one library after normalization) were selected for further analysis of differential expression levels miRNAs. Based on these criteria, 91 miRNAs, consisting of both known and novel miRNAs, were differentially expressed after PPV infection (Table 3.3 and Table 3.4). Of those, 67 miRNAs were highly up-regulated, while 22 were extremely down-regulated upon virus infection.

Among differentially expressed miRNAs upon virus infection, some miRNAs (nta-miR482d, sly-miR6026, nta-miR6020b) which have been shown to target resistance genes (R-Genes) were present. In plant genome R-genes are responsible for plant disease resistance against pathogens by producing R proteins. sly-miR6026 and nta-miR6020b showed up-regulation in PPV infected wild-type plants whereas, nta-miR482d showed down-regulation. Also, in transgenic susceptible plant (sly-miR6026, nta-miR6020b) showed statistically significant different expression level and like infected wild-type plants these miRNAs were up-regulated. However, in transgenic resistant plants the expression level of the above mentioned miRNAs did not show statistically significant different expression level compared with un-infected wild type plants (data not shown).

Table 3.3. Profiles of differentially expressed known miRNAs responsive to PPV infection

miR_name	Up/down reulation	Fold changes	Log_2 fold	P-value (chi_square_2x2)	P-value (fisher_test)
ath-miR8175_L-2	Down	0.04	-4.68	8.93E-81	1.28E-94
osa-miR5077_L-1_1ss5GA	Down	0.12	-3.07	1.91E-51	1.04E-55
gma-MIR6300-*P3*_1	Down	0.13	-2.90	1.06E-27	1.77E-29
ptc-miR164a_R+3	Down	0.21	-2.23	2.53E-33	2.07E-34
zma-miR171b-3p_L+3R+2	Down	0.23	-2.10	6.85E-86	3.80E-89
gma-miR403a_1ss21GT	Down	0.32	-1.66	8.81E-19	5.19E-19
gma-miR6300	Down	0.32	-1.63	1.54E-33	5.36E-34
nta-miR482d_R+1	Down	0.35	-1.52	2.39E-14	1.84E-14
nta-miR6149a_R+4	Down	0.36	-1.47	0.00E+00	7.76E-199
nta-miR394	Down	0.36	-1.46	0.00E+00	3.42E-222
nta-miR6149a	Down	0.38	-1.40	0.00E+00	0.00E+00
csi-miR159a-3p_R+3	Down	0.38	-1.38	0.00E+00	3.54E-103
sly-miR159_R+1	Down	0.41	0.41	7.73E-76	3.59E-76
nta-miR827	Down	0.41	-1.29	6.03E-86	2.80E-86
sly-miR166c-3p_R+4	Down	0.42	-1.25	0.00E+00	5.12E-103
bdi-miR167a_R+4	Down	0.43	-1.21	4.56E-13	8.26E-13
gma-miR6300_R+5_1	Down	0.47	-1.10	1.17E-11	1.43E-11
gma-miR6300_R+5_2	Down	0.47	-1.10	1.17E-11	1.43E-11
ptc-miR6478_R+4_1ss21GA	Down	0.48	-1.07	7.66E-13	1.06E-12
sly-miR166c-3p	Down	0.49	-1.04	0.00E+00	0.00E+00
ata-miR167a-5p_R+3	Down	0.49	-1.02	2.52E-15	2.13E-15
nta-miR6155	Down	0.49	-1.02	2.07E-71	3.64E-71
stu-miR391-3p_L+1	Up	inf	inf	0.00E+00	2.06E-162
stu-miR162a-5p_1ss14CT	Up	inf	inf	0.00E+00	3.66E-148
stu-miR393-5p	Up	2.01	1.01	5.81E-19	2.83E-19
sly-miR398a_R+1	Up	2.06	1.04	1.14E-03	9.99E-04
bra-miR168b-5p_R+4	Up	2.22	1.15	4.39E-10	3.24E-10
sly-miR6026_2ss3CT18AC	Up	2.42	1.27	9.09E-07	6.74E-07
rgl-miR7972_1ss14TG	Up	2.43	1.28	1.80E-27	1.65E-28
stu-miR8036-3p_1ss2AT	Up	2.66	1.41	1.86E-34	4.24E-36
rgl-miR7972_L+3_1ss17TG	Up	3.09	1.63	1.95E-08	1.17E-08
nta-miR6161a_L+1R-4	Up	3.40	1.76	1.30E-14	1.44E-15
nta-miR408	Up	4.00	2.00	0.00E+00	6.46E-120
nta-miR390a	Up	4.14	2.05	1.54E-40	4.23E-44
ata-miR169h-5p_L+2R-1_1ss22GC	Up	4.49	2.17	2.10E-39	4.35E-43
tcc-miR162	Up	4.87	2.28	0.00E+00	1.94E-170
sly-miR398a_L+2R-2	Up	5.55	2.47	1.62E-75	1.35E-84
nta-miR1919_1ss2AT	Up	6.69	2.74	3.01E-80	3.36E-92
aly-miR390b-3p_R-1_1ss20CT	Up	9.97	3.32	5.46E-43	2.35E-50
aly-miR167b-3p_1ss10CT	Up	11.15	3.48	2.92E-19	2.80E-22
nta-miR168a_R+1	Up	11.55	3.53	0.00E+00	0.00E+00
sly-miR396a-3p	Up	13.38	3.74	0.00E+00	0.00E+00
sly-miR403-5p_2ss14AT18AG	Up	13.39	3.74	0.00E+00	7.37E-220
nta-miR168a_1ss21CA	Up	14.11	3.82	0.00E+00	1.00E-306
sly-miR171f	Up	18.29	4.19	0.00E+00	2.42E-263
sly-miR168b-3p_R+1	Up	18.79	4.23	0.00E+00	3.76E-154
sly-miR398a	Up	19.98	4.32	0.00E+00	0.00E+00
sly-miR171b-5p_L+2R-2	Up	21.21	4.41	1.73E-19	7.26E-24
stu-miR160a-3p	Up	21.43	4.42	4.56E-72	4.39E-92
sly-miR391_L+1R-1_1ss5CT	Up	22.88	4.52	0.00E+00	2.23E-177
nta-miR390b	Up	26.66	4.74	6.90E-25	3.61E-31
sly-miR166c-5p_1ss10TG	Up	32.54	5.02	0.00E+00	7.15E-139
aly-miR393a-3p_2ss12TC20TC	Up	273.68	8.10	0.00E+00	0.00E+00

Table 3.4. Profiles of differentially expressed novel miRNAs responsive to PPV infection

miR_name	Up/down reulation	Fold changes	Log_2 fold	P-value (chi_square_2x2)	P-value (fisher_test)
aly-MIR166d-p5_2ss2AC17GC	Up	inf	inf	0.00E+00	1.81E-203
PC-3p-18324_266	Up	inf	inf	3.36E-22	9.28E-31
nta-MIR166h-p5	Up	2.33	1.22	2.57E-04	2.03E-04
nta-MIR172d-p5_1ss5CT	Up	2.39	1.26	6.37E-10	3.79E-10
nta-MIR172e-p5_1ss5CT	Up	2.39	1.26	6.37E-10	3.79E-10
nta-MIR172i-p5_1ss5CT	Up	2.39	1.26	6.37E-10	3.79E-10
nta-MIR172c-p5_1ss5CT	Up	2.39	1.26	6.37E-10	3.79E-10
nta-MIR172f-*P3*_1ss5CT	Up	2.39	1.26	6.37E-10	3.79E-10
PC-3p-8146_611	Up	2.65	1.41	1.15E-07	5.22E-08
PC-5p-4420_949	Up	2.75	1.46	4.36E-10	1.91E-10
nta-MIR482a-p5_1ss3GT	Up	2.81	1.49	1.62E-21	1.73E-22
PC-5p-674_2522	Up	2.83	1.50	2.77E-25	2.06E-26
PC-3p-205_4860	Up	3.19	1.67	5.58E-65	4.88E-69
PC-3p-7530_651	Up	3.20	1.68	1.75E-18	2.32E-19
nta-MIR167d-*P3*	Up	3.53	1.82	0.00E+00	2.18E-112
nta-MIR166b-p5	Up	3.78	1.92	1.63E-18	1.04E-19
nta-MIR166a-p5	Up	4.29	2.10	3.74E-88	2.34E-96
nta-MIR159-p5	Up	6.21	2.64	7.44E-58	8.82E-66
PC-3p-4344_960	Up	7.58	2.92	3.41E-45	2.77E-52
nta-MIR398-*P3*_1ss4GA	Up	8.66	3.11	0.00E+00	0.00E+00
nta-MIR397-*P3*_1ss6GA	Up	10.14	3.34	1.81E-35	1.14E-41
nta-MIR408-p5_1ss2CT	Up	11.92	3.58	0.00E+00	3.87E-170
PC-5p-1760_1591	Up	12.90	3.69	0.00E+00	1.88E-128
nta-MIR6155-*P3*	Up	19.07	4.25	3.83E-33	1.18E-40
nta-MIR168c-*P3*	Up	21.28	4.41	0.00E+00	0.00E+00
nta-MIR160a-*P3*	Up	21.43	4.42	4.56E-72	4.39E-92
PC-3p-24596_182	Up	21.53	4.43	1.02E-30	9.59E-39
nta-MIR6019a-*P3*	Up	22.58	4.50	7.75E-21	1.34E-25
nta-MIR6019b-*P3*	Up	22.58	4.50	7.75E-21	1.34E-25
nta-MIR171a-p5_1ss5AG	Up	29.05	4.86	0.00E+00	3.20E-142
nta-MIR166c-p5	Up	41.26	5.37	0.00E+00	3.96E-153
nta-MIR6025b-p5	Up	54.09	5.76	0.00E+00	9.00E-304
nta-MIR6025a-*P3*	Up	54.09	5.76	0.00E+00	9.00E-304
nta-MIR164a-*P3*	Up	57.69	5.85	2.99E-77	1.68E-104
nta-MIR398-p5_2ss8TC18CT	Up	57.90	57.90	0.00E+00	9.77E-239
nta-MIR6147-*P3*	Up	58.55	5.87	0.00E+00	1.46E-180

3.9.5 Characterization of siRNAs targeting PPV

The profile of the filtered reads and aligned to PPV-M isolate isMrPl220 genome (Acc. Number MF370984), revealed accumulation of 21- to 25-nt siRNA species targeting the PPV-M genome, in all transgenic and infected wild type plants. In wild-type and susceptible transgenic plants, where PPV was successfully multiplied and established, 153,839 and 143,287 reads of 21-25-nt siRNA species were detected, respectively. In both libraries the 22-nt, 21-nt, 23-nt, 24-nt and 25-nt siRNAs were the most frequent siRNAs, respectively (Figure 3.16 and Figure 3.17).

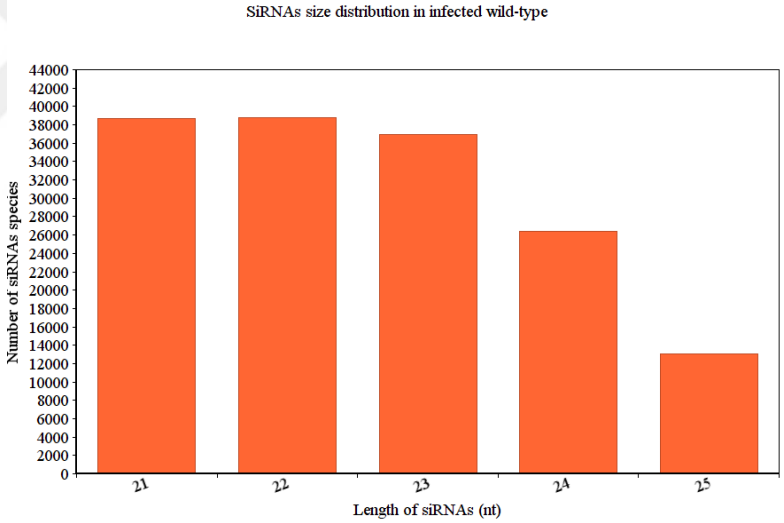

Figure 3.16. Size distribution of the 21-25-nucleotide siRNAs in infected wild-type plants.

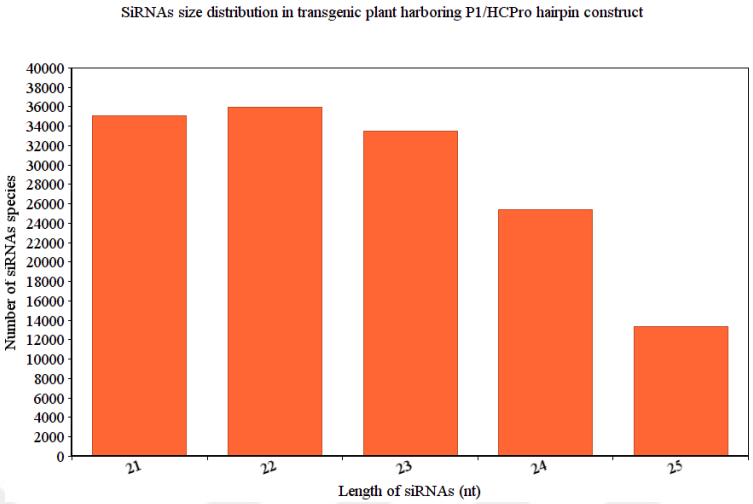

Figure 3.17. Size distribution of the 21-25-nucleotide siRNAs in transgenic plant harboring *P1/HCPro* hairpin construct.

In resistant transgenic plants the expression pattern and the number of siRNAs were totally different. Among resistant transgenic plants, in terms of the number of 21-25-nt in length siRNAs, *UTR/P1* and *CP* ihpRNA constructs with 5,251 and 5,205 reads produced higher number of total reads in comparison with *HCPro* and *HCPro/P3* ihpRNA constructs with 2,971 and 2,637 reads, respectively. In addition, transgenic plants harboring *UTR/P1* and *CP* hairpin constructs shared another feature. In transgenic plants harboring the *UTR/P1* and *CP* hairpin constructs, 24-nt siRNAs outnumbered the 23-nt siRNAs. This is while, in transgenic plants harboring *HCPro* and *HCPro/P3* hairpin constructs, the frequency of 24-nt class of siRNAs was less than 23-nt siRNAs. Despite the difference in frequency of 24-nt siRNAs between two groups of resistant transgenic plants, all resistant transgenic plants showed a similar response marked by significant accumulation of 21-nt siRNAs (Figure 3.18).

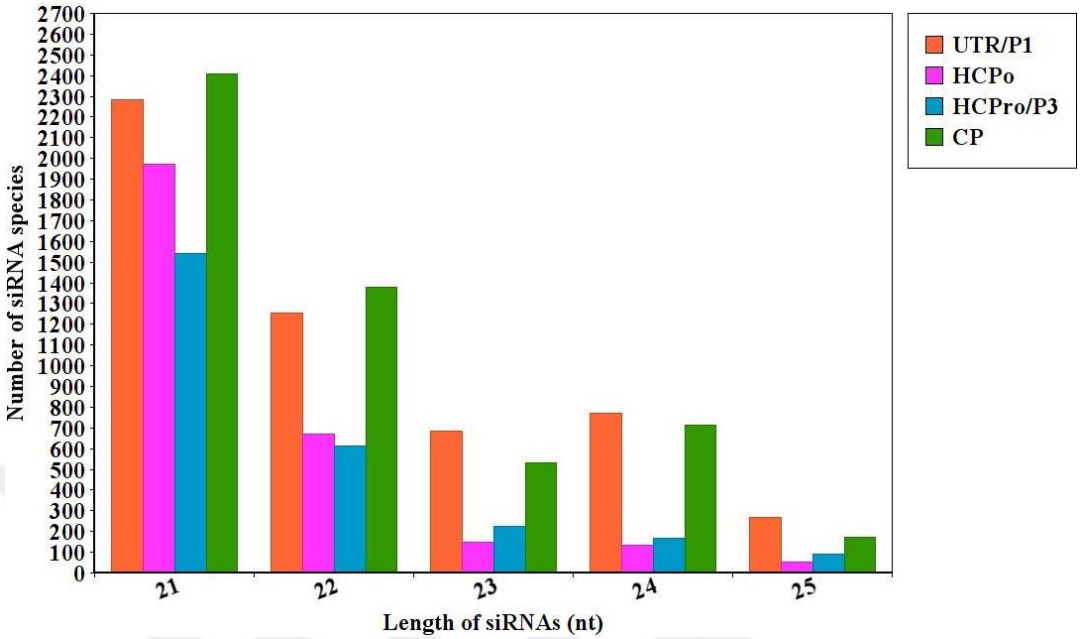

Figure 3.18. Size distribution of the 21-25-nucleotide siRNAs in transgenic plant harboring *UTR/P1*, *HCPro*, *HCPro/P3 and CP* hairpin constructs.

3.9.6 Distribution of siRNAs targeting PPV

In infected wild-type and susceptible transgenic plants the siRNAs distributed throughout the whole genome targeting the full-length of virus genome (Figure 3.19 and Figure 3.20). In contrast, in resistant transgenic plants, accumulation of siRNAs on hairpin origin regions of genome was seen. However, the concentration of siRNAs on these target rigions were different among these resistant transgenic plants. In resistant transgenic plants harboring the *UTR/P1* and *CP* hairpin constructs dense accumulation of siRNAs on the target regions was observed (Figure 3.21 and Figure 3.22). Accumulation of siRNAs on corresponding regions in resistant transgenic plants harboring *HCPro* and *HCPro/P3* hairpin constructs was much less than transgenic plants harboring *UTR/P1* and *CP* hairpin constructs (Figure 3.23 and Figure 3.24).

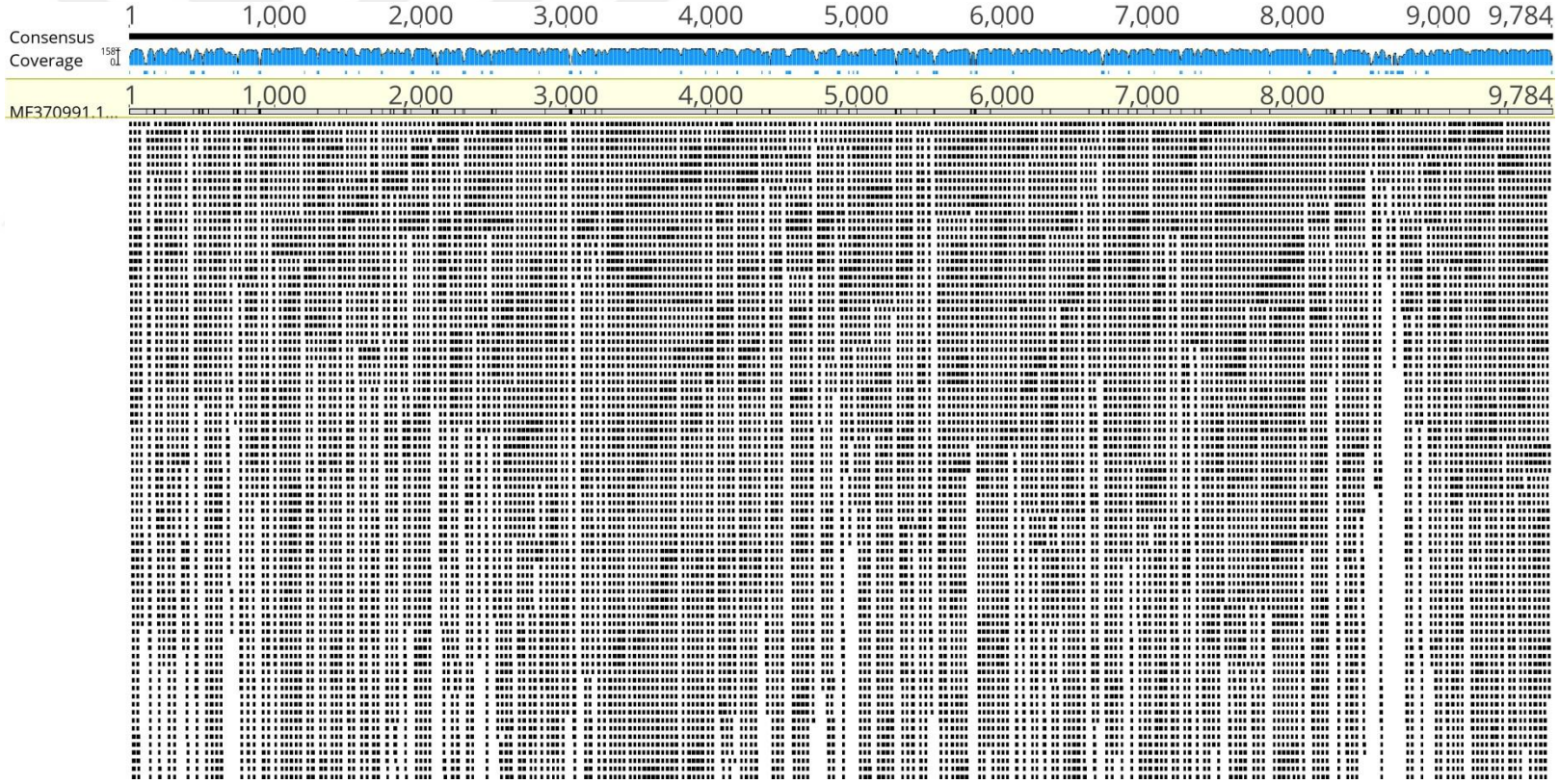

Figure 3.19. Distribution of siRNAs along PPV genome in infected wild type plants.

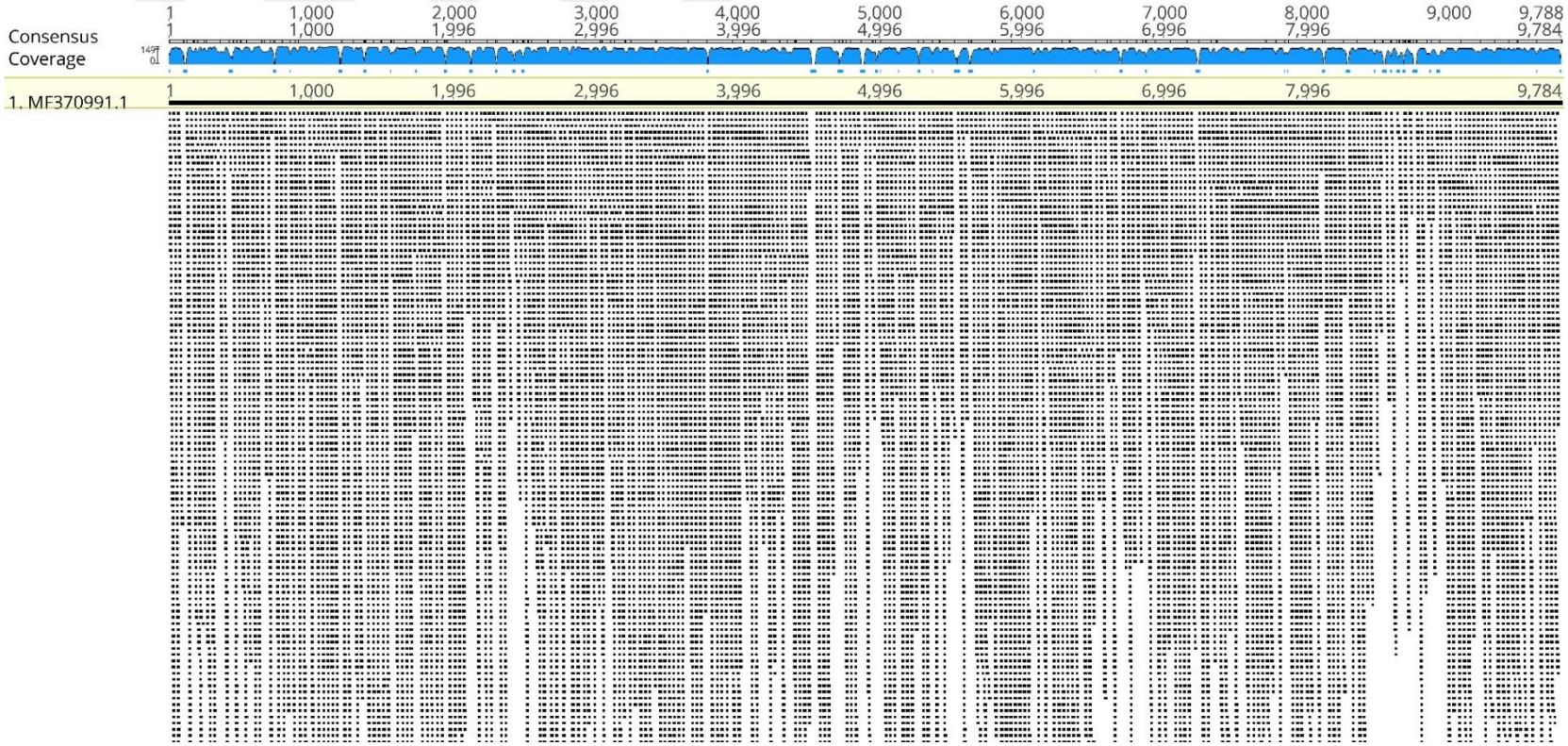

Figure 3.20. Distribution of siRNAs along PPV genome in transgenic plant harboring *P1/HCPro* hairpin construct.

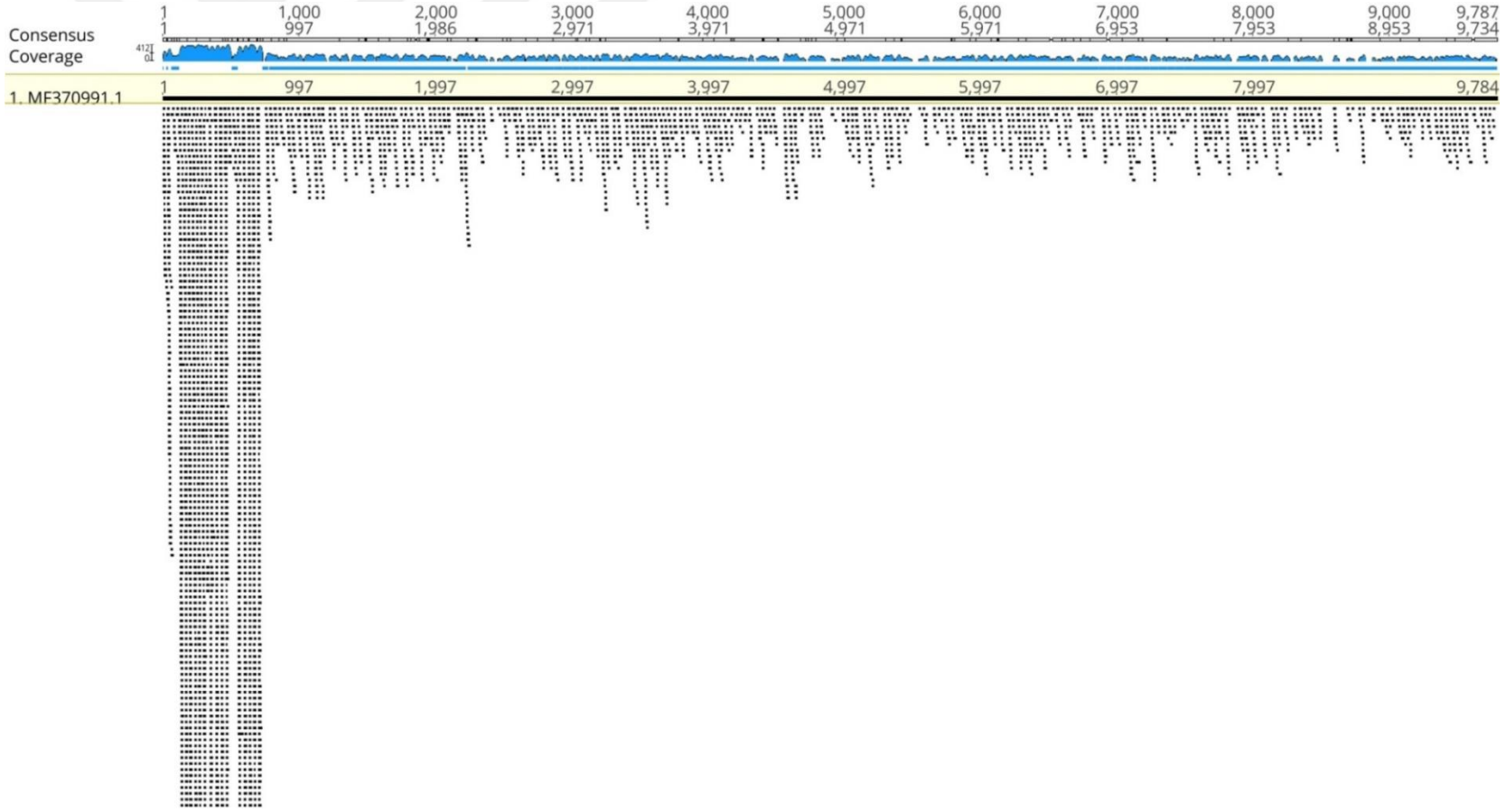

Figure 3.21. Distribution of siRNAs in transgenic plants harboring *UTR/P1* hairpin construct and accumulation of them on target region (nt 2-733).

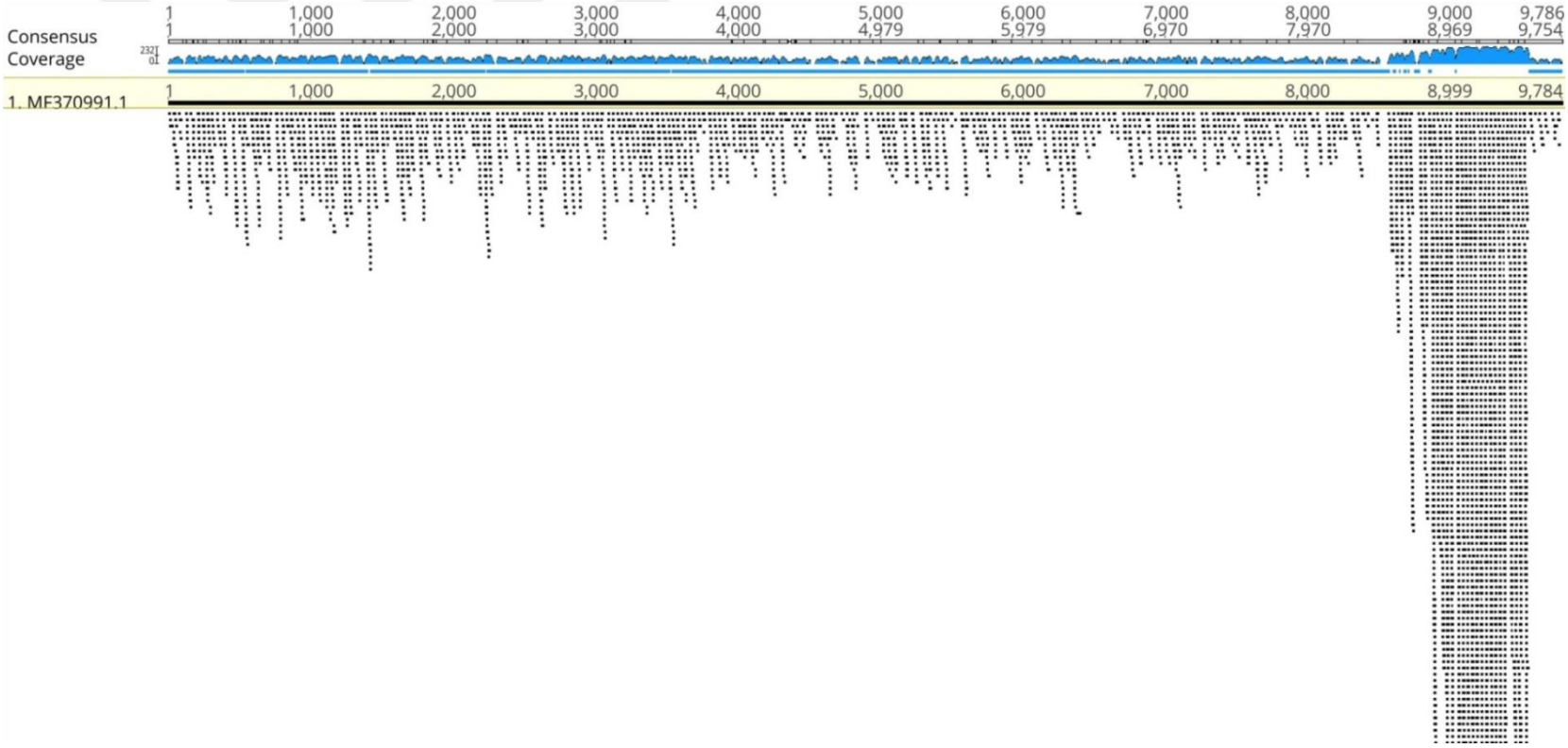

Figure 3.22. Distribution of siRNAs in transgenic plants harboring *CP* hairpin construct and accumulation of them on target region (nt 8577-9564).

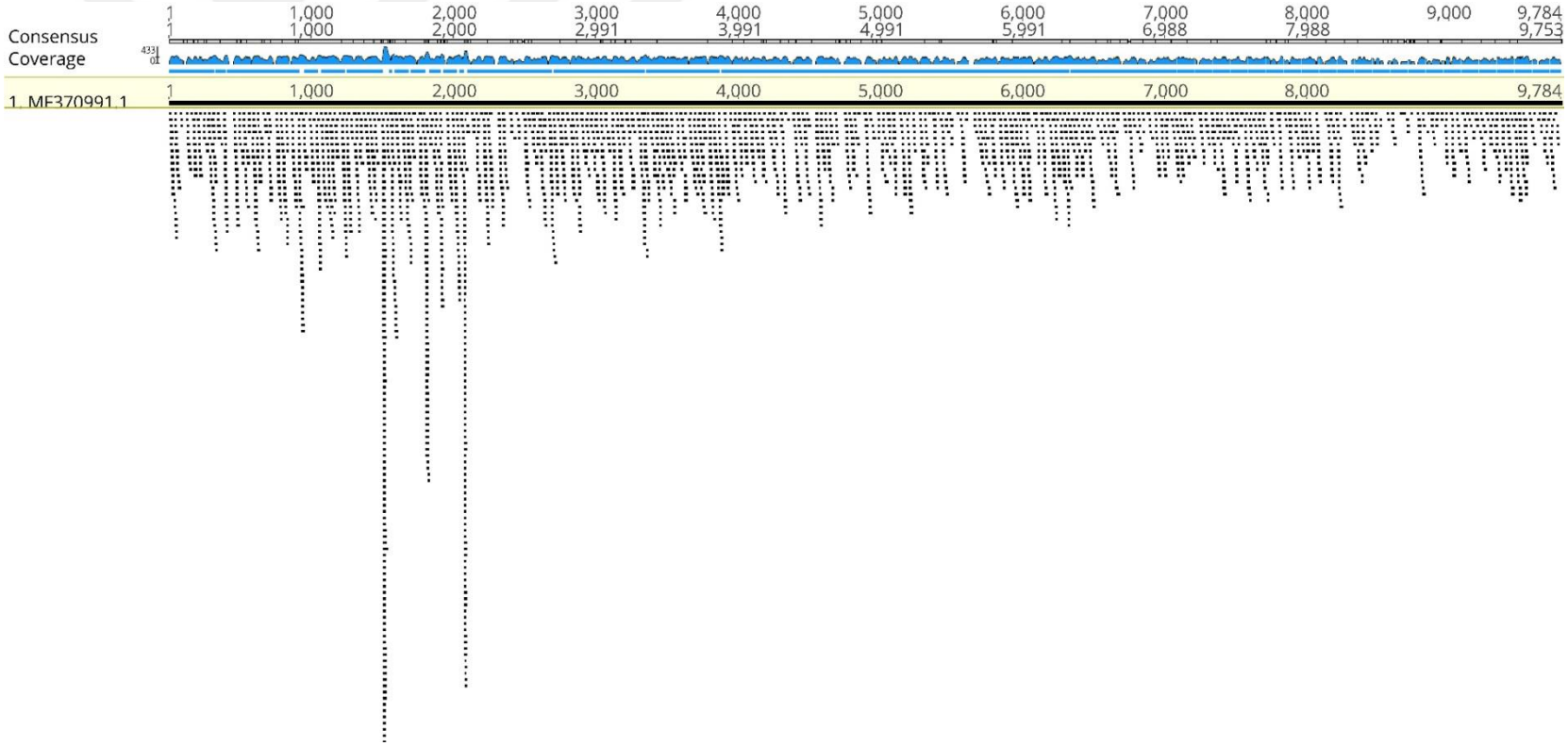

Figure 3.23. Distribution of siRNAs in transgenic plants harboring *HCPro* hairpin construct and accumulation of them on target region (nt 1505-097).

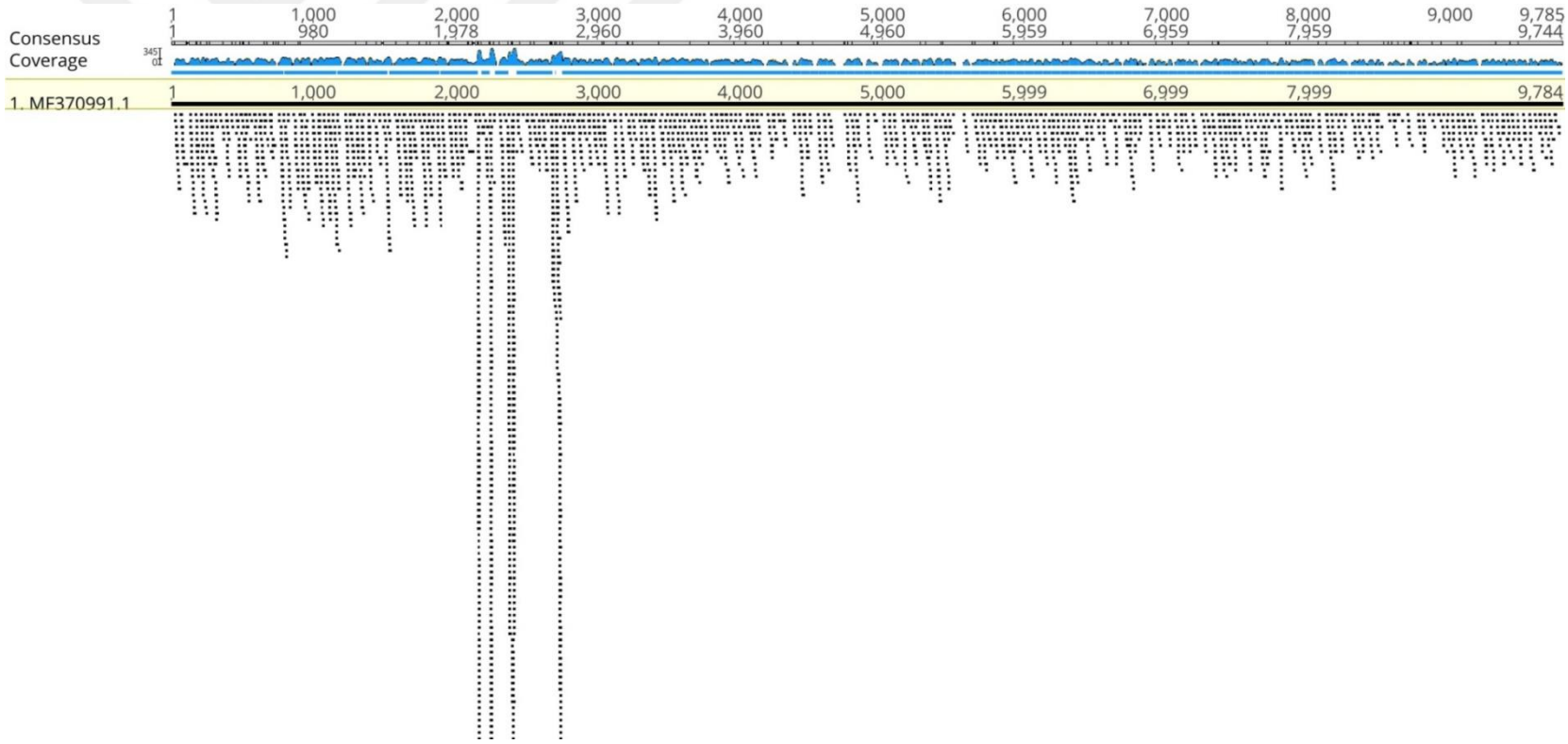

Figure 3.24. Distribution of siRNAs in transgenic plants harboring *HCPro/P3* hairpin construct and accumulation of them on target region (nt 2159-2754).

4. CHAPTER
DISCUSSION

Expression of pathogen-derived sequences encoding hairpin RNAs in transgenic plants is a suitable startegy to obtain resistance against respective pathogens. This strategy has been widely used for different plant viruses including PPV (Pandolfini et al., 2003; Di Nicola-Negrietal., 2005; Zhang et al., 2006; Lu et al., 2007; Hily et al., 2007; Wang et al., 2009; Monticelli et al., 2012; García-Almodóvaretal., 2015; Wang et al., 2013). In previous studies, the hairpin consructs were derived from conserved genomic regions of isolates belonging to PPV-D or PPV-M strains. In the present study, in order to evaluate the efficiency of PPV-T strain as the source for construction of hairpin structures, an isolate from PPV-T strain was chosen for hairpin construction.

In this study, four hairpin constructs were derived from *HC-Pro* and *P1* regions of the genome which were shown to be involved in the suppression of the RNA silencing (Pruss et al., 1997; Kasschau and Carrington, 1998), and previously were used for hairpin construction using PPV-M as the source (Di Nicola-Negri et al., 2005). In addition, the whole coat protein of the genome was used for hairpin construction due to its high capacity in induction of resistance against PPV (Hily et al., 2007). After transformation of *N. benthamiana* with these five genomic regions, resistance assay and deep sequencing analysis were performed to compare the efficiency of these five different regions of the PPV-T strain in inducing of RNA silencing.

The obtained T_1 progeny of transgenic lines harboring five different hairpin structures showed various susceptible/resistant responses to mechanical PPV inoculation.

Different reactions of lines to infection can be due to their various potentials in production of siRNAs. In transgenic plants containing *UTR/P1* hairpin construct, 10 different lines were obtained. Except two individual plants of two lines in PPV-T inoculation, none of T1 progenies of these lines in three resistance tests with three PPV strains showed virus symptoms and no virus was detected in ELISA test. In transgenic plants containing *CP* hairpin construct, replicates of none of each two lines (4-3 and 21-5) showed virus symptoms; besides, in ELISA test, the five selected plants in inoculation with three strains were virus-free.

Among five lines obtained in transgenic plants containing *HCPro* hairpin construct, one line (8-1) with one infected plant in PPV-D inoculation test was the most resistant one. In transgenic plants harboring *HCPro/P3* hairpin construct, two lines (5-4 and 5-6) were the most resistant lines with no symptoms and no virus detection in ELISA tests. In RT-PCR of selected resistant plants performed at 60 dpi, no virus was detected at molecular level in transgenic plants harboring UTR/*P1*, *HCPro/P3*and *CP* hairpin constructs. However, despite no observation of virus symptoms in plants harboring *HCPro* hairpin construct, virus was detected at molecular level in tested plants. Therefore, transgenic plants containing *HCPro* construct considered as moderate resistant plants. The ability of the same regions of genome in triggering gene silencing mechanism and resistance had been shown in previous studies using PPV-M and PPV-D as the source for hairpin construction. However, Our resistance assay results for above mentioned constructs suggest that the PPV-T derived UTR/*P1*, *HCPro*, *HCPro/P3* and *CP* (ihpRNA) constructs have the same ability in inducing of RNA silencing and resistance against PPV as PPV-D or PPV-M derived constructs. However, among five constructed hairpin structures, in performed resistance assay, the transgenic plants containing *P1/HCPro* hairpin construct did not show resistance in any of three virus inoculation trials. This result was in contrast with the resistance capacity of the same region derived from PPV-M strain of the virus. (Di Nicola-Negri et al., 2005)

The above mentioned results showed successful induction of resistance by four different hairpin constructs. However, in order to have a more precise and better assessment of the abundance, complexity and diversity of the produced siRNA population in resistant transgenic plants containing UTR/*P1*, *HCPro*, *HCPro*/*P3*, *CP* hairpin constructs and the

susceptible transgenic plant harboring *P1/HCPro* construct, deep sequencing of PPV infected transgenic plants and control plants (un-infected and infected wild-type plants) was performed. The results showed considerable accumulation of siRNAs with length of 21- to 25-nt in infected wild-type plants. The accumulation of virus derived siRNAs in non-transgenic infected plants indicated activation and involvement of the host RNAi mechanism and antiviral defense response to viral infection. In this mechanism, viral-derived dsRNA is cleaved by DCL to produce 21- to 24-nt siRNAs which are subsequently incorporated into RISC containing Argonuate (AGO) to perform sequence-specific viral degradation (Aliyari and Ding, 2009).

Alignment of siRNAs to the PPV genome revealed that the 21-25-nt siRNAs from both polarities were continuously distributed throughout the whole genome. As this result shows, despite production of large set of siRNAs which targeted the virus genome, virus multiplication successfully occurred and infection symptoms appeared in infected wild-type plants indicating that PPV overcame plant defense mechanism. Successful establishment of the virus despite the presence of large number of siRNAs targeting the PPV genome is an expected phenomenon and can be interpreted by different strategies that viruses have evolved to overcome the host defensive responses. Virus proteins that block the RNA silencing machinery are the most common ones which are known as RNA silencing suppressors (RSSs) (Roth et al., 2004). In *potyviruses* including PPV, the helper component–protease (*HCPro*) was the first RSS to be described (Anandalakshmi et al., 1998; Brigneti et al., 1998).

In susceptible transgenic plant harboring *P1/HCPro* construct, like infected wild-type plants, a large set of 21-nt to 25-nt in length siRNAs were detected. Alignment of theses siRNAs to the PPV genome revealed that like infected wild-type plants, these siRNAs were distributed throughout the whole genome. In addition to approximately the same number of siRNA accumulation in susceptible transgenic and infected wild-type plants, they showed similar abundance of each of 21-nt to 25-nt siRNA species. Antiviral defense response in plants mostly consists of accumulation of 21-nt and 22-nt species which are produced by DCL4 and DCL2, respectively (Akbergenov et al., 2006; Bounche et al., 2006). High accumulation of these to class of siRNAs were also observed in infected wild-type and susceptible transgenic plants and in both plants, 22-

nt siRNAs with a slight difference compared with 21-nt siRNAs were the most abundant class of siRNAs. Although generally 21-nt siRNAs are the most frequent siRNA species in antiviral defense response, in some cases higher or the same abundance of 22-nt siRNAs have been reported (Silva et al., 2011; Qiao et al., 2018; Donaire et al., 2008) and this can be considered as an evidence of various reactions of hosts to different viruses. Similar expression pattern of siRNAs in susceptible transgenic *P1/HCPro* and infected wild-type plant, and more importantly no accumulation of produced siRNAs at target region (*P1*/*HCPro*) and successful establishment of virus in susceptible transgenic plant, showed that the *P1/HCPro* ihpRNA construct failed to induce RNA silencing against the virus. This result is not in accordance with the results of Di Nicola-Negri et al. 2005 study in which the resistance assay of transgenic plants harboring PPV-M-derived *P1/HCPro* hairpin construct revealed that only one out of 46 plants of line 79 and four out of 41 plants of line 87 were infected. In addition, in this study, two out of 4 infected transgenic plants of line 87 recovered at 27 dpi. By contrast, in the present study, the plant lines containing *P1/HCPro* hairpin construct were not resistant and no recovery was seen at 30 dpi. Considering the fact that sequencing analysis after transformation procedure confirmed the presence of inserts in correct orientation in hairpin structures, it is assumed that inefficiency of *P1/HCPro* ihpRNA construct in induction of resistance must have another reason. The *P1/HCPro* region in PPV-T strain contains the recombination site. However, further investigations is required to find out if there is any association between failure of this hairpin construct in induction of resistance and the recombination site.

In resistant transgenic plants harboring the *UTR/P1*, *HCPro*, *HCPro/P3* and *CP* hairpin constructs, among produced siRNA species, higher abundance of 21-nt siRNA species (34%, 59%, 50%, 38%) was seen compared with other siRNA species. It has been demonstrated that DCL4 is necessary for RNA interference mechanism and involvement of this enzyme in production of 21-nt siRNA species (Dunoyer et al., 2005) shows that 21-nt class of siRNAs play an important role in this mechanism. The second most abundant class of resistant transgenic siRNAs is the 22-nt class. The *CP* construct with 22% of 22-nt siRNA showed the highest level of accumulation of this

class of siRNAs compared with *UTR/P1*(19%), *HCPro* (20%), *HCPro/P3*(20%) constructs. The dominant frequency of 21-nt and 22-nt among siRNAs classes of transgenic and virus infected plants in this study, is in accordance with previous findings indicating that in siRNA-directed RNA degradation pathway, endogenous or exogenous long dsRNA or hrpRNA are mostly processed by DCL4 and DCL2 into 21- and 22-nt siRNAs, respectively (Dunoyer et al., 2010; Garcia-Ruiz et al., 2010; Qi et al., 2009). In addition, in many virus-derived transgene plants considerable accumulation of 21-nt siRNA had been reported (Leibman et al., 2011; Qiao et al., 2018; Montes et al., 2014; Lu et al., 2015). Therefore, it seems that predominant accumulation of 21-nt and 22-nt siRNAs in transgenic plants is necessary for inducing of RNAi and in present study this pattern in production of siRNA species was observed in resistant transgenic plants.

In contrast with 21-nt and 22-nt siRNAs which were the most abundant siRNAs and showed the same pattern of accumulation in all resistant transgenic plants, the accumulation of 24-nt siRNA species in transgenic plants containing *UTR/P1* and *CP* hairpin constructs was different compared with transgenic plants harboring *HCPro* and *HCPro/P3* constructs. In transgenic plants harboring *UTR/P1* and *CP* ihpRNA, 24-nt siRNA had higher frequency compared with 23-nt siRNA. Whereas, in resistance transgenic plants harboring *HCPro* and *HCPro/P3* ihpRNAs, the 24-nt species frequency was lower than 23-nt species. 24-nt siRNAs are associated with transcriptional gene silencing via DNA methylation and some modifications which lead to chromatin repression. DCL3 is involved in biogenesis of this class of siRNAs which are known as heterochromatic siRNAs (Zilberman et al., 2003; Law and Jacobsen, 2010). In DNA virus infections of plants, 24-nt siRNA is the most common class of produced siRNAs, although 21-nt and 22-nt siRNAs are also generated (Poogin, 2013). Whereas, in RNA virus infection, accumulation of 21-nt and 22-nt viral siRNAs is more common and in some rare cases 24-nt siRNAs are also detectable. The siRNAs of PPV infected wild-type plants in this study, as RNA virus infection situation, showed the routine pattern of siRNA accumulation. This pattern was also seen in transgenic plants. However, compared with other two resistant transgenic plants, the different pattern of 24-nt siRNA accumulation in two resistant transgenic plants raises the question whether

the different regions of the virus genome that have been utilized for hairpin construction in this study have various capacities in production of siRNAs, especially the 24-nt species siRNAs? Different studies have reported accumulation of virus-derived 24-nt siRNAs in some transgenic plants transformed and subsequently inoculated with RNA viruses; although in these studies massive accumulation of siRNAs derived from the inverted repeat of corresponding virus genes has been proposed as the major reason for successful resistance of transgenic plants against the viruses, proposing no special role for produced 24-nt siRNAs. Accumulation of 24-nt siRNA in one line of transgenic cucumber harboring a hairpin construct derived from *HCPro* region of a RNA virus, *Zucchini yellow mosaic potyvirus* (ZYMV) (Leibman et al., 2011) and detection of 24-nt (17.74%) in addition to predominant accumulation of 21-nt (34.90%) and 22-nt (32.45%) siRNAs in rice transformed with the *CP* gene of another RNA virus, *Rice stripe virus* (RSV) (Li et al., 2016) are examples of reported accumulation of 24-nt siRNA in transgenic plants inoculated with RNA viruses.

Interestingly, in cucumber line transformed with hairpin construct derived from ZYMV in Leibman et al., (2011) study, high level of resistance to different related viruses was reported. Qiao et al. (2018) detected greater proportion of 24-nt transgene-derived siRNAs in *N. benthamiana* plants which were immune to a (single-stranded RNA) *Crinivirus, Lettuce infectious yellows virus* (LIYV) and in transgenic melon plants with immunity to *Cucurbit yellow stunting disorder virus* (CYSDV). In contrast with other studies, the predominant accumulation of 24-nt class of siRNAs in this study has been considered as an indicator for involvement of this class of siRNAs in *crinivirus* immunity. On the other hand, in another survey, transgenic *N. benthamiana* plants transformed with PPV coat protein hairpin construct and the wild-type plants were inoculated with PPV for characterization and comparison of produced siRNA population by large-scale small RNA sequencing. siRNA data processing of a transgenic-susceptible (TG-S) line and untransformed wild-type plant showed high level of 21- and 22-nt siRNAs whereas 24-nt siRNA was scarce in these individuals. Decreased level of 24-nt siRNAs in these plants which were susceptible compared with transgenic-resistant plants made the authors to raise the question that if there was any relevance between DNA methylation processes and plant defense mechanism in RNA

virus infected plants (Montes et al., 2013). To date, the role of 24-nt siRNAs in defense against RNA viruses is not clear and regulation of their expression and their hypothetical roles in RNA silencing-mediated viral immunity need to be further investigated.

In this study, the siRNAs in each of resistant transgenic plants containing the UTR/*P1*, *HCPro*, *HCPro/P3* and *CP* hairpin constructs were aligned to the PPV genome to investigate their distribution patterns. The sequence distribution profile of transgenic siRNAs on the PPV genome, shown in Figure 3.21 to Figure 3.24, revealed that the transgenic siRNAs targeted different regions throughout the PPV genome, but an outstanding accumulation of transgenic siRNAs in hotspot regions was observed. These hotspots in each of transgenic plants were the regions which were utilized for hairpin construction for induction of RNA silencing. *UTR/P1* region started at the 5′end of PPV genome and included a part of the *P1* gene (nt 2–733), *P1/HCPro* covered the 3′end of *P1* gene and partially the *HCPro* gene (nt 736–1465), *HCPro* covered the central portion of *HCPro* (nt 1505–2097), the *HCPro/P3* covered the 3′end of *HCPro* gene and a part of *P3* gene (nt 2159–2754) and the last part was *CP* gene (nt 8577-9564). Interestingly, the amount of transgene-derived siRNA accumulation on the corresponding regions is different among these 4 resistant transgenic plants. In terms of the amount of siRNA accumulation on transgene regions, the *UTR/P1* and *CP* ihpRNA containing transgenic plants with higher accumulation of transgene-derived siRNAs on target regions were similar. On the other hand, the *HCPro* and *HCPro/P3* ihpRNA containing transgenic plants with lower amount of transgene-derived siRNAs on transgene regions were similar. Higher accumulation of siRNAs on virus targeted regions in resistant transgenic plants harboring *UTR/P1* and *CP* hairpin constructs compared with transgenic plants containing *HCPro* and *HCPro/P3* hairpin constructs is due to higher capacity of *UTR/P1* and *CP* regions in production of 21-25nt siRNAs. The number of 21-25 nt PPV-derived siRNAs in *UTR/P1* and *CP* ihpRNA-containing transgenic plants were 6,647 and 6,316 reads, respectively. Whereas, the transgenic plants harboring *HCPro* and *HCPro/P3* hairpin constructs with 3,351 and 3,094 reads, respectively, produced almost half amount of produced siRNAs in transgenic plants harboring *UTR/P1* and *CP* hairpin constructs. This result indicates that the *UTR/P1* and

CP regions with higher potential in production of siRNAs which can perfectly accumulate on targeted regions are better choices for hairpin construction compared with *HCPro* and *HCPro/P3* genomic regions. In order to obtain optimum resistance using virus-derived ihpRNA constructs, the selection of best candidate target sites from virus genome is crucially important. RNA interference-mediated resistance has been used to confer resistance against different plant viruses. However, the level of introduced resistance in plant hosts to viruses varies widely depending on genomic regions used for hairpin construction. There have been several reports of imperfect or moderate resistance to some viruses in plants harboring different genomic regions of virus to induce RNAi (Kalantidis et al., 2002; Kamachi et al., 2007; Bonfim et al., 2007). As the results of different studies have showed, the produced siRNAs in transgenic plants plays an important role and, as several studies illustrate, direct correlation between transgene siRNA population and resistance has been described (Wu et al., 2009; Yu et al., 2011; Patil et al., 2011; Qu et al., 2007; Kalantidis et al., 2002; Chen et al., 2004; Missiou et al., 2004; leibman et al., 2011).

The high capacity of *UTR/P1* region of PPV genome in inducing resistance against the virus had been also reported previously in Di Nicola-Negri et al. (2010) study in which the *UTR/P1* ihpRNA construct was produced using PPV-M strain as the source. In this study, the transgenic plants containing hairpin *UTR/P1* construct not only showed complete and long-lasting resistance to seven PPV isolates of D, M and REC strains, they were fully resistant to PPV-C and -EA isolates which are phylogenetically distantly related to D, M and T strains. In this study, siRNAs prediction tools were utilized to investigate that whether the hairpin constructs used in this study were also sites for production of potentially effective siRNAs. Interestingly, six out of eight siRNA patterns identified the *UTR/P1* as a construct capable of producing siRNAs homologous to PPV Swc. In another study, the abilitiy of PPV *CP* in successful resistance induction was also shown when ihpRNA constructs containing the full-length and the second half of the PPV *CP* gene were used for *N. benthamiana* transformation. The results indicated that the full-length *CP* construct was a better choice (Hily et al., 2007). In accordance with previous studies, the present study, supported by evidences gained by deep sequencing analysis such as siRNA population and high accumulation of these siRNAs

on target regions, the PPV-T derived *UTR/P1* and *CP* ihpRNA constructs can be considered as the best candidates to induce of RNA silencing against PPV.

In this study, in addition to assessment of PPV-derived siRNAs in transgenic, the lateral purpose of small RNA sequencing was the determination and comparison of the expression profile of miRNAs in transgenic, infected and un-infected wild-type plants. In order to have a general view of the functions of expressed miRNAs in each plant, the predicted target genes by most aboundant miRNAs were subjected to gene ontology (GO) enrichment analysis which is a bioinformatics initiative for interpreting sets of genes. It identifies the processes that are enriched in relative sets of genes and assesses the coherent functional signals of those sets of genes. Therefore, GO can provide some data about miRNA-gene regulatory network. This information can give us a view of possible interference of PPV in plant miRNA expression which will lead to changes in expression of different genes. In addition, the GO analysis in transgenic plants harboring the ihpRNA constructs, will show the reaction of these plants against virus infection and possible effects of transformation on miRNA expression profile. The comparsion of distribution of enriched GO pathways of predicted traget genes in PPV-infected wild-type and uninfected wild-type libraries revealed that the frequency (total number of target genes) of biological-processing, transcription regulation, transcription, protein phosphorylation and defense response-related GO terms dercreased upon PPV infection. It means that the number of expressed genes related to above mentioned processes reduced after PPV infection. Generally, viruses are capable of reprograming and altering plant gene expression which will lead to different cellular stress responses and developmental defects in plants. In compatible plant-virus intranctions, where viral particle is not recognized by the host plant, the virus finds the opportunity to interfere with accumulation and function of host vital elements such as proteins and nucleic acids as well as important reactions such as host defense responses (Whitham et al., 2006).

A phenomenon known as host gene shut-off which is down-regulation of the expression of host genes, has been reported in vius infected plants (Escaler et al., 2000a,b; Havelda and Maule, 2000; Wang and Maule, 1995). The observed changes in genes involved in above mentioned processes upon PPV infection, can be explained by the fact that viral RNA silencing suppressor (RSS) usually promotes infection by interfering with

miRNAs and trans-acting small interfering RNAs expression and since miRNAs are involved in gene expression regulation this interference might lead to changes in expression of some plant's genes. In PPV the *HCPro* and *P1* genes have been demonstrated to be involved in suppression of RNA silencing (Pruss et al., 1997; Kasschau and Carrington, 1998). On the other hand, altering plant gene expression, plant viruses can cause biological and developmental defects by perturbing or interfering with signaling pathways and thus affect plant growth and development.

Virus infection in plants can cause defense-like responses by induction of pathogenesis-related (PR) genes and genes which are involved in plant disease defense. These genes encode resistance (R) proteins which are responsible for defense responses. Various defense-related proteins have been found in plants with sharing common characterized domains, including NBS-LRR (nucleotide-binding site-leucine-rich repeat) (Galvez et al., 2014). Based on the results of different studies it has been shown that miRNAs are involved in regulation of these disease resistance genes (Fei et al., 2016). On the other hand, RNA viruses from different genera or families are capable of altering miRNA levels and regulating host miRNA expression (Boa et al., 2018). In a study the NBS-LRR proteins, as an important disease resistance family, was shown to be down-regulated upon *Soybean mosaic virus* (SMV) infection in susceptible soybean plants which facilitates successful infection of SMV. This down-regulation of R genes was observed to be the consequence of up-regulation of miR1507a, miR1507c and miR482a which are targeting NBS-LRR gene family. In our study, the observed reduced defense response upon PPV infection can be the result of successful establishment of virus and its interference with expression of R genes. In order to detect the miRNAs responsive to PPV infection, the expression profiles of miRNAs in PPV-infected and un-infected wild plants were compared. Among differentially expressed miRNAs, there were some miRNAs such as nta-miR6020b and sly-miR6026 which are shown to be involved in targeting the resistance genes which encode the NBS-LRR protein family. These miRNAs were up-regulated in PPV infected plants. Determination of expression level of the target genes of these miRNAs was not the scope of this study; however, as the GO function analysis of predicted targets indicated in this study the targets of these miRNAs were R genes and their up-regulation may negatively regulate these R genes.

Among transgenic plants, the distribution of enriched GO terms of resistant transgenic plants harboring UTR/*P1*, *HCPro/P3* and *CP* constructs showed a similar pattern to enriched GO terms of un-infected plants, while in susceptible transgenic and moderate resistant plants, the pattern of enriched GO terms were more similar to infected wild-type plants. The similarity of three resistance transgenic plants to enriched GO terms of un-infected plants can be interpreted by inhibition of establishment of the virus in transgenic plants and prevention of their interference in host gene expression. In susceptible and moderately resistant transgenic plants harboring *P1/HCPro* and *HCPro* constructs, respectively, the similar pattern of enriched GO terms to infected wild-type plant can be explained by the ability of the virus in successful establishment and its subsequent interference in host gene expression.

Based on ELISA result in resistance assay which was performed 14dpi, in examined resistant transgenic plants harboring *HCPro* construct, except for one individual plant in line 8-1 (the line which was selected as resistant line for deep sequencing analysis), the virus was not detected in other plants. However, in RT-PCR of selected resistant plants which was carried out 60 dpi, despite no observation of virus symptoms in *HCPro* hairpin construct containing transgenic plants, the virus was detected at molecular level in tested plants. Similar enriched GO terms of this transgenic plant with the susceptible transgenic and infected wild-type plants might be the result of presence of virus in this transgenic plant.

In conclusion, in the present study as the first attempt for introduction of resistance to PPV using RNAi mechanism in Turkey, five different PPV-T-derived regions of virus genome utilized and transgenic plants were obtained successfully. This is the first step on the route to obtaining resistant plants via RNA silencing hairpin-mediate technology. Therefore, in order to have a better view of RNA-mediated gene silencing defense process in obtained transgenic plants, profiling of siRNAs and miRNAs in genomic scale was carried out using small RNA high-throughput sequencing technology. The expression profile of miRNAs in infected and un-infected wild-type plants and also in transgenic plants and gene ontology (GO) categorization of the predicted targets of expressed miRNAs in each library, revealed that PPV infection caused considerable changes in expression of genes which are involved in different biological and

transcriptional regulation processes and defense responses in non-transgenic host plant. However, in transgenic plants showing resistance to virus infection, due to preventive role of RNAi-mediated resistance and inhibition of virus establishment, these changes in plant gene expression were not seen. This indicated that induced RNAi against the virus with no dramatic changes in host plant was achieved.

On the other hand, the results of small RNA sequencing provided important information about the potential of each hairpin construct in producing siRNAs and more importantly, their ability in targeting the virus genome which is a critical stage in RNAi-mediated resistance. Considering the fact that hairpin constructs with different origins confer various degrees of resistance, the results of small RNA sequencing helped us to be able to compare the five different transgenic plants harboring different ihpRNA constructs by investigation of the abundance of produced transgenic siRNAs and their accumulation in target regions in each of transgenic plants.

Based on findings of this study, hairpin constructs of UTR/*P1*, *HCPro*, *HCPro/P3* and *CP* were able to induce resistance against virus infection in resistance assay while the *P1/HCPro* hairpin construct failed to induce resistance against PPV. Failure of PPV-T derived *P1/HCPro* construct in inducing of resistance against PPV was in contrast with Di Nicola-Negri et al. (2005) findings in which the *P1/HCPro* hairpin construct from PPV-M isolate induced full resistance in transgenic plants. Although this result makes this region of virus genome not a good candidate for hairpin construction, decision about the credibility of this region for RNAi induction requires more studies and evidences. Therefore, further studies on the ability of this region of genome derived from different virus strains for hairpin construction is suggested.

Except for *P1/HCPro* hairpin construct, different transgenic lines derived from other four constructs showed resistance in resistance assay. However, the results of siRNA sequencing revealed that the *UTR/P1* and *CP* hairpin constructs managed to produce higher number of siRNAs. In addition, these produced siRNAs showed more concentrated patterns of accumulation in targeted regions compared with the pattern of siRNA accumulation in *HCPro* and *HCPro/P3* hairpin constructs. Moreover, in transgenic plants containing *UTR/P1* and *CP*, 24-nt class siRNAs outnumber the 23-nt

and 25-nt siRNAs. Although the role of 24-nt siRNAs in antiviral defense against RNA viruses in plants is not clear, some evidences indicated existence of association between these species of siRNAs and the immunity in related transgenic plants (Qiao et al., 2018).

Introduction of persistent and durable resistance is the main goal and considering the laborious process of stone fruit plants transformation, choosing the best hairpin construct is crucially important. Based on the results of this study, it is concluded that despite observed resistance in transgenic plants containing *HCPro* and *HCPro/P3* hairpin constructs, *UTR/P1* and *CP* hairpin constructs with supporting evidences of deep sequencing analysis are better candidates for inducing resistance by RNAi mechanism. This result can be important for future attempts in induction of resistance against PPV by RNAi mechanism in stone fruit plants in Turkey.

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APPENDIX

1. Media preparation

LB medium

Dissolve 10 g Bacto-Tryptone, 5 g Yeast Extract and 5 g NaCl in 900 ml distlled water. Adjust the pH to 7.4 with NaOH and the make the volume up to 1 L with distilled water. Autoclave the solution at 121^{o}C for 20 min. Allow the medium cool down before adding antibiotics with concentration recommended. To prepare plates, add 15 g agar to 1 L liquid medium before autoclave. Store the medium at 4^{o}C.

YEP medium

Dissolve 10 g Bacto-Tryptone, 10 g Yeast Extract powder and 5 g NaCl in 900 ml distilled water. Adjust the pH to 7.0 with 1N NaOH and make up the volume to 1 L with distilled water. Autoclave the solution at 121^{o}C for 20 min. Allow the medium cool down before adding antibiotics with concentration recommended. To prepare plates, add 15 g agar to 1 L liquid medium before autoclave. Store the medium at 4^{o}C.

MS medium

Mix 50 ml Stock solution (Macro/MS), 5 ml Stock solution 2 (Micro/MS), 5 ml Stock solution 3 (Fe-EDTA), 5 ml Stock solution 4 (table 1) and 30 g sucrose. Adjust the volume to 1L with distilled water and pH to 5.8 with NaOH. Autoclave sterilize the solution at 121oC for 20 min. For solid medium, add 15 g agar to 1 L liquid medium and sterilize by autoclaving for 20 min. Store the medium at 4^{o}C.

2. Solution preparation

Antibiotics

Antibiotics referred in this thesis are Kanamycin, Spectinomycin, Cefotaxime and Rifampicin.

The antibiotics stock and working concentration.

Antibiotic	Stock concentration	Working concentration
Kanamycin	50 mg/ml in sterile water	50 µg/ml
Spectinomycin	100 mg/ml in sterile water	100 µg/ml
Cefotaxime	100mg/ml in sterile water	500 µg/ml
Rifampicin	Rifampicin 50 mg/ml in Methanol	50 µg/ml

Filter sterilize all antibiotic solutions and store them at -20^{o}C.

Hormone and AS

BAP

Dissolve100 mg 6-BA in 10 ml 1 M NaOH and adjust the volume to 100 ml with distilled water. Store the solution at 4^{o}C.

AS

Dissolve 19.6 g AS (Acetosyringone) in 5 ml DMSO. Add distilled water to the volume of 10 ml. Filter sterilize the solution. Dispense the solution into aliquots and store them

Solutions for DAS-ELISA

Grinding buffer (PBS+2% PVP)

Dissolve 8.0 g NaCl, 0.2 g KH2PO4, 1.15 g Na2HPO4, 0.2 g KCl and 20 g PVP in 900 ml distilled water. Adjust the pH to 7.4 with NaOH and the volume to 1 L with distilled water. Store the solution at 4^{o}C.

Coating buffer

Dissolve 1.59 g Na2CO3, 2.93 g NaHCO3 and 0.2 g NaN3 in 900 ml distilled water. Adjust the pH to 7.4 with NaOH and the volume to 1 L with distilled water. Store the solution at 4^{o}C.

Rinse buffer (PBST)

Dissolve 8.0 g NaCl, 0.2 g KH2PO4, 1.15 g Na2HPO4, 0.2 g KCl and 0.5 ml Tween-20 in 900 ml distilled water. Adjust the pH to 7.4 with NaOH and the volume to 1 L with distilled water. Store the solution at 4^{o}C.

Conjugate buffer

Dissolve 0.2 g BSA in 100 ml sterile PBST buffer. Store the solution at 4^{o}C.

Substrate buffer (DEA buffer)

Dissolve 9.039 g diethanolamine, 0.01 g MgCl2·6H2O and 1.982 g diethanolamine-HCl in 80 ml distilled water, Adjust the pH to 9.8 with NaOH and the volume to 100 ml with distilled water. Store the solution at 4 oC. Add 10 mg pNPP (p-Nitrophenyl phosphate disodium) to 10 ml diethonalamine buffer directly before use.

Solution for Agarose gel electrophoresis

50×TAE buffer

Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water. Add 40 ml 0.5 M Na2EDTA (pH 8.0) (alternatively use 9.3 g Na2EDTA). Adjust volume to 1 Liter. Store at room temperature

Solution for DNA extraction

CTAB buffer

Mix 10 ml 1 M Tris-HCl (pH 8.0), 4.0 ml 0.5 M EDTA (pH 8.0) and 28.0 ml 5 M NaCl. Add distilled water to the volume of 100 ml and sterilize the solution by autoclave at 121 oC for 20 min. Dissolve 2.0 g CTAB and 1 g PVP in the solution at 65^{o}C and adjust the pH to 5.0.

Chloroform/isoamyl alcohol (24:1)

Add 96 ml chloroform and 4 ml isoamyl alcohol in a brown bottle. Mix the solution thoroughly and store it at 4^{o}C.

0.5 M EDTA

Dissolve Na2EDTA·H2O 18.61 g in 90 ml distilled water. Adjust pH to 8.0 and volume to 100 ml with distilled water. Store the solution at room temperature.

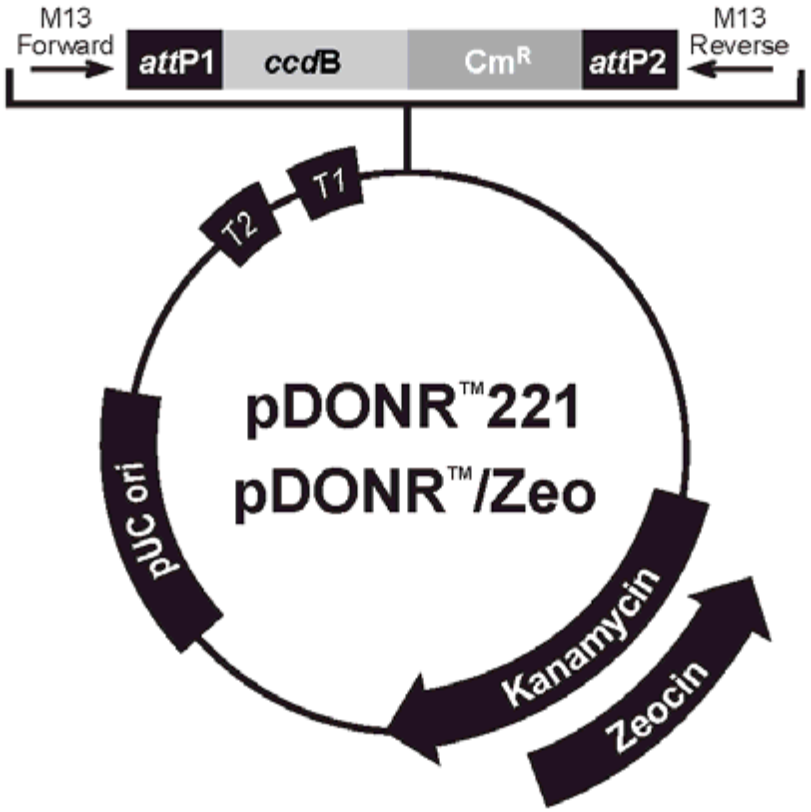
M Tris (pH 7.4/8.0)

Dissolve 121.1 g Tris Base in 800 ml distilled water. Adjust the volume to 1 L with distilled water and the pH to 7.4/8.0 with HCl. Sterilize the solution by autoclave and store it at room temperature.

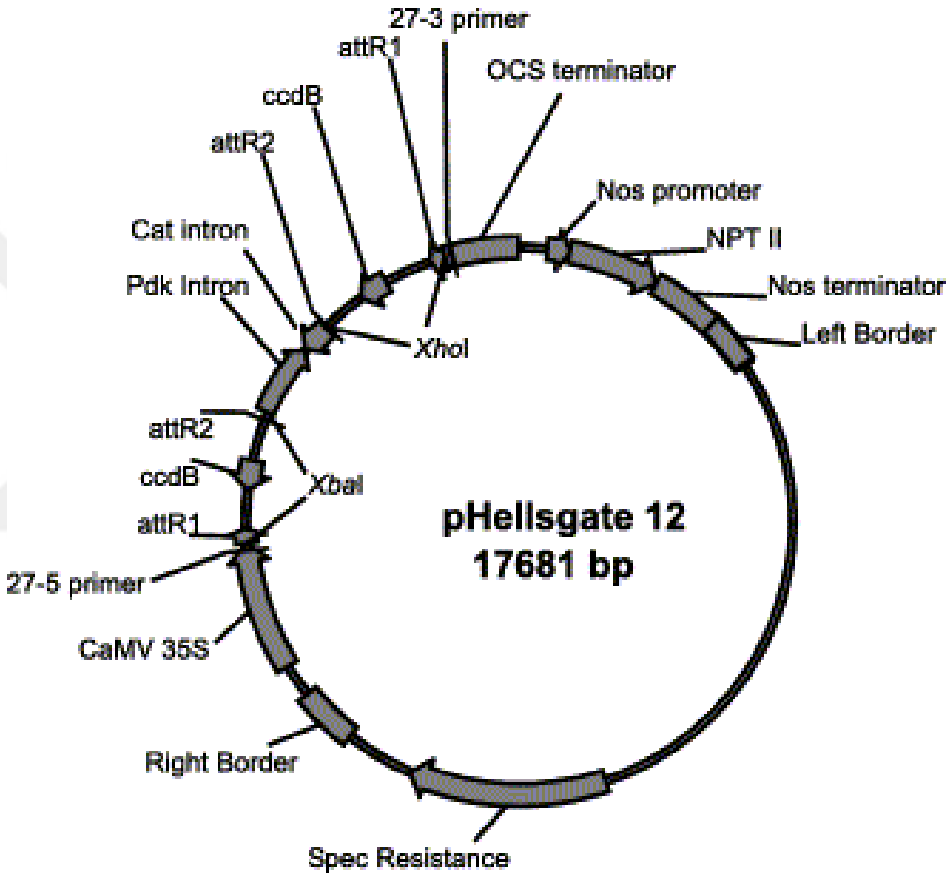
TE (1×)

Mix 5ml 1M Tris-HCl buffer (pH8.0) and 1ml 0.5M EDTA (pH8.0) in 400 ml distilled water. Adjust the volume to 500 ml with distilled water. Sterilize the solution by autoclave and store it at room temperature.

3. Plasmids



Comments for:	pDONR™221 4762 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
*att*P1:	570-801	570-801
*ccd*B gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1847-2506	1847-2506
*att*P2 (c):	2754-2985	2754-2985
T7 Promoter/priming site (c):	3000-3019	3003-3022
M13 Reverse priming site:	3027-3043	3027-3043
Kanamycin resistance gene:	3156-3965	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4086-4759	3615-4288


27-3 primer
attR1
OCS terminator
ccdB
attR2
Nos promoter
Cat intron
NPT II
Pdk Intron
Nos terminator
Left Border
XhoI
attR2
ccdB
XbaI
attR1
pHellsgate 12
17681 bp
27-5 primer
CaMV 35S
Right Border
Spec Resistance

CURRICULUM VITAE

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PUBLICATIONS

1. Ghaderi Sohi, M., Shahraeen, N., Rakhshandehroo, F. 2013. Biological and phylogenetic study of Cauliflower mosaic virus on Canola in Iran. Iranian Journal of Plant Pathology. Vol. 49(1). 93-99.

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CONFERENCE PRESENTATIONS

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