TRANSIENT EXPRESSION OF POTATO VIRUS X COAT PROTEIN GENE IN *Nicotiana benthamiana*

Yonghui Huang

A Thesis Submitted in Partial Fulfillment of the Requirement for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2010

การแสดงออกแบบไม่ถาวรของยีนโปรตีนห่อหุ้มไวรัส X ของมันฝรั่ง ในยาสูบ (*Nicotiana bentamiana*)

นางยงหุ้ย หวง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2553

TRANSIENT EXPRESSION OF POTATO VIRUS X COAT PROTEIN GENE IN *Nicotiana benthamiana*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master ’s Degree.

Thesis Examining Committee

_____

(Assoc. Prof. Dr. Montarop Yamabhai)

Chairperson

_____

(Asst. Prof. Dr. Mariena Ketudat-Cairns)

Member (Thesis Advisor)

_____

(Asst. Prof. Dr. Chokchai Wanapu)

Member

_____

(Assoc. Prof. Dr. James R. Ketudat-Cairns)

Member

_____

(Prof. Dr. Sukit Limpijumnong)

Vice Rector for Academic Affairs

_____

(Asst. Prof. Dr. Suwayd Ningsanond)

Dean of Institute of Agricultural Technooology

ยงหุ้ย หวง : การแสดงออกแบบไม่ถาวรของยีนโปรตีนห่อหุ้มไวรัส X ของมันฝรั่งในยาสูบ (*Nicotiana benthamiana*) (TRANSIENT EXPRESSION OF POTATO VIRUS X COAT PROTEIN GENE IN *Nicotiana benthamiana*) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 82 หน้า.

โรคไวรัสในมันฝรั่งชนิด X (Potato virus X; PVX) เป็นโรคที่มีการระบาดทั่วไปในเขตที่มีการปลูกมันฝรั่ง ไวรัสนี้ทำให้เกิดความเสียหายต่อผลผลิตและท่อนพันธุ์ การทดลองนี้ได้ทำการศึกษาการสร้าง RNAi เพื่อรบกวนต่อการแสดงออกของยีนที่ใช้สร้างโปรตีนห่อหุ้มอนุภาคไวรัส (coat protein; CP) PVX โดยวิธี transient expression ในใบยาสูบ (*Nicotiana bentamiana*) PVX CP cDNA ถูกโคลนมาจากใบมันฝรั่งที่เป็นโรค PVX ในแปลงปลูก และทำการโคลนยีน PVX CP ส่วนที่เป็น conserved fragment เข้าสู่ RNAi vector, pHellsgate12 และถ่ายเข้าสู่พืชเพื่อสร้างความต้านทานต่อ PVX ในพืช การศึกษา transient expression ของ hairpin CP นี้ใช้เทคนิคการถ่ายยีนเข้าสู่พืชผ่านเชื้อ *Agrobacterium* (Agroinfiltration) และทดสอบความต้านทานต่อการเข้าทำลายของไวรัสโดยวิธีสังเกตลักษณะการแสดงออกภายนอก และการตรวจสอบด้วยเทคนิค DAS-ELISA ผลการทดสอบพบว่า มันฝรั่งที่ถูกถ่ายยีนด้วยวิธี Agroinfiltration สามารถต้านทานต่อ PVX ได้ 100% นอกจากนี้ยังได้ทำการพัฒนาหาเทคนิคที่เหมาะสมสำหรับการถ่ายยีนนี้เข้าสู่เนื้อเยื่อของมันฝรั่ง ซึ่งพบว่าเนื้อเยื่อของมันฝรั่งถูกดัดแปลงยีนสามารถตรวจสอบการถ่ายยีนโดยเทคนิค PCR ได้ ในอนาคตงาน ทดลองที่เกี่ยวข้องกับการดัดแปลงยีนในมันฝรั่งเพื่อสร้างความต้านทานต่อ PVX สามารถใช้เทคนิคที่กล่าวมาข้างต้นได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

YONGHUI HUANG : TRANSIENT EXPRESSION OF POTATO VIRUS X COAT PROTEIN GENE IN *Nicotiana benthamiana*. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 82 PP.

POTATO VIRUS X/TRANSIENT EXPRESSION/HAIRPIN RNA/RESISTANCE

Potato virus X (PVX) is widespread in the world, causing yield losses and seed degeneration of potato. The interference effect of a PVX coat protein (CP) based construct was studied by transient expession in *Nicotiana bentamiana*. Potato leaves with PVX infection symptoms in the fields were collected to obtain the PVX CP cDNA. A conserved fragment of the PVX CP gene was subcloned into pHellsgate12, an RNAi vector, with the aim of achieving the resistance to PVX. Transient expression of the hairpin CP via agroinfiltration was performed in model plant, *Nicotiana bentamiana*. Virus challenging assay followed by phenotype observation and DAS-ELISA detection demonstrated that 100% of the tobacco plants infiltrated with *Agrobacterium* harboring the construct were resistant to PVX. Furthermore, optimization of the system for *Agrobacterium* mediated potato transformation led to detection of transgenic potato calli by PCR. This work revealed the possibility of achieving transgenic potato plants resistant to PVX.

School of Biotechnology Student's Signature _____

Academic Year 2010 Advisor's Signature _____

ACKNOWLEDGEMENT

My deepest gratitude goes first to my supervisor, Asst. Prof. Dr. Mariena Ketudat-Cairns, for her valuable instructions and suggestions with her profound knowledge in plant molecular biology, as well as her constant patience and kindness. I have learned from her a lot about research and writing. I am very grateful for her effort to help me complete this thesis.

I wish to express my gratitude to Prof. Dr. Zuoyi Liu for his guidance and suggestions on my research, for his encouragement and push on my graduate study. I am grateful to Assoc. Prof. Dr. Montarop Yamaphai for her guidance and instruction on my study. I am also grateful to Assoc. Prof. Dr. James R. Ketudat-Cairns and Asst. Prof. Dr. Wanapu Chokchai for their concern and precious suggestions on my thesis work. Thanks go to all other teachers in School of Biotechnology and School of Biochemistry who have given instruction to me. Special thanks go to Assoc. Prof. Dr. Jutharat Attajarusit for her kindhearted care in many ways during my study in Suranaree University of Technology.

I owe my thanks to my colleagues and friends in Guizhou Key lab for Agricultural Sciences. I would like to thank Dr. Gang Tao for his guidance on molecular biology techniques, Dr. Guosheng Zhu and Dr. Yongxiang Liu for their suggestions on general study and research techniques. Thanks are also due to my friends Ying Zhu, Ren Qiu, Youbin Qiu and Qike Li for pleasant cooperation in the transgenic potato project and Zhenyu Zhu for helping with primer design. I would like

to thank Guizhou Academy of Agricultural Sciences and Guizhou Biotechnology Institute for financial support.

I am also indebted to my classmates and friends in Suranaree University of Technology, Wanwisa Suphap, Sasiprapa Kanchanawattana, Onladda Juachan and Chonticha Yasaro, for their kindly help on my study and my living in Thailand. I would also like to thank my senior, Dr. Wipaporn Wanthanaleart, for her suggestions on vector construction and plant transformation.

Finally I would like to express my special thanks to my family for their endless support on my study with their love, understanding and encouragement.

Yonghui Huang

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

AS	=	Acetosyringone
Amp	=	Ampicillin
6-BA	=	6-Benzylaminopurine
bp	=	Base pairs
BSA	=	Bovine serum albumin
Carb	=	Carbenicillin
CP	=	Coat protein
DAS-ELISA	=	Double antibody sandwiched enzyme linked immumosorbent assay
DEPC	=	Diethyl Pyrocarbonate
DMSO	=	Dimethylsulfoxide
DTT	=	DL-Dithiothreitol
dsRNA	=	Double stranded RNA
2,4-D	=	2,4-dichlorophenoxy acetic acid
EB	=	Ethidium bromide
EDTA	=	Ethylene diamine tetra acetic acid
GA3	=	Gibberellic acid 3
hpRNA	=	Hairpin RNA
IPTG	=	Isopropyl-β-D-thiogalactopyranoside
IR	=	Inverted repeat

LIST OF ABBREVIATIONS (Continued)

Kb	=	Kilobase
Km	=	Kanamycin
MES	=	2- (N-Morpholino) ethanesulfonic acid hydrate
M-MLV	=	Moloney murine leukemia virus
NAA	=	Naphthalene acetic acid
NPT	=	Neomycin phosphotransferase
nt	=	Nucleotide
ORF	=	Open reading frame
pNPP	=	p-Nitrophenyl phosphate disodium salt
PVP	=	Polyvinyl pyrrolidone
PVX	=	Potato virus X
PVY	=	Potato virus Y
RdRp	=	RNA-dependent RNA polymerase
Rif	=	Rifampicin
RISC	=	RNA induced silencing complex
RNA	=	Ribonucleic acid
RNAi	=	RNA interference
RNasin	=	Ribonulease Inhibitor
siRNA	=	Small interfering RNA
ShRNA	=	Small hairpin RNA
Spec	=	Spectinomycin

LIST OF ABBREVIATIONS (Continued)

TC-RT-PCR	=	Tube capture reverse transcription PCR
TGS	=	Transcriptional gene silencing
U	=	Unit, μmol/min
X-gal	=	5-Bromo-4-chloro-3-indolyl β-D-galactoside
ZT	=	Zeatin

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Potato，*Solanum tuberosum*, is the fourth most important food crop in the world, being surpassed in total production only by rice, wheat and corn. Due to high productivity, as well as high starch, vitamin and protein content, potato is very important for human and animal nutrition and for industrial processing. China is now the world's largest potato producing country. Having an output of 72 million tones and a harvested area of 5 million hectares, China accounts for more than 20 percent of both the global potato-growing area and the world potato harvest in 2007 (FAOSTAT, http://faostat.fao.org/).

Viruses are widespread in potato and cause degeneration of potato seeds and severe yield losses. Potato is particularly susceptible to viral diseases, in part because vegetative propagation allows viruses to accumulate several years over successive generations. Potato leafroll virus (PLRV), potato virus Y (PVY) and potato virus X (PVX) are the most important viruses.

PVX is the most widespread of all the potato viruses. It is found worldwide wherever potato is grown. In potato, yield losses from PVX are generally 15% or more. Mixed infections with other potato viruses cause particular damage in production (Barker and Dale, 2006). Traditional ways to control potato virus X include selecting certified seeds, traditional breeding, producing virus-free seeds and

restriction of its spread. Genetic engineering of potato has also been an alternative measure to effectively control viral disease in recent years.

RNA interference (RNAi), which was found from the study of transgene overexpression, is a way to knock down the homologous transgene and endogenous genes simultaneously. RNAi is a disease-resistance mechanism of plant and can be used as an antiviral strategy. The Waterhouse Lab (CSIRO) designed a series of RNAi vectors, thus making it easier to generate high efficiency constructs to be used in antiviral genetic engineering.

1.2 Objectives of the study

The objectives of this study are to obtain the cDNA of PVX CP (coat protein) from potato leaves in the field of Guizhou, which is a main potato producing province, and then to construct a hairpin RNA- encoding vector with a conserved fragment of the viral CP cDNA. The second aim was to test the interference effeciency of the construct by transient expression via agroinfiltration. Furthermore, *Agrobacterium* mediated plant transformation of the hairpin construct to obtain transgenic antivirus potato materials.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of Potato virus X

Potato virus X (PVX) is the most widespread of all the potato viruses. It is found worldwide wherever potato is grown. PVX infects and causes systemic infections in several *Solanaceae* crops, including potato, (*S. tuberosum*), tobacco (*Nicotiana tabacum*), tomato (*Lycoperscion*), and pepper (*Capsicum. annuum*). The virus can also infect members of the *Chenopodiaceae*, *Amaranthaceae*, *Euphorbia helioscopia*, *Trifolium pretense* and *Vitis vinifera* (Sutic et al., 1999). In potato, yield losses from PVX are generally 15% or more. Mixed infections with potato virus A (PVA) and potato virus Y (PVY) are particularly damaging in production. The viruses are preserved and transmitted by infected tubers. PVX is mechanically transmitted by contact between infected plant and healthy plant by machineries and animals. There must be wounding and an exchange of plant sap for infection to occur.

PVX is the member of the *Potexvirus* group. Four strain groups of PVX, X1, X2, X3 and X4, differ in their reactions with the plant resistance genes, Nb, Nx and Rx in cultivated varieties of potato. Nb and Nx determine a hypersensitive response in cultivated varieties of potato and Rx determines an extreme resistance (Solomon-Bloackbum RM, 2001). Group 1 strains, the X1 cause a hypersensitive response in the presence of Nb or Nx. Group 2, the X2 cause a hypersensitive response only with Nb. Group 3, the X3 only with Nx and group 4, the X4 with neither, but fails to infect plants carrying an Rx gene (Cockerham, 1955, 1970).

Symptoms produced by PVX are variable, depending on the PVX strain and potato cultivar. In general, no visible symptoms are produced, hence the name latent virus. The mild mosaic symptom is the main clear visible symptom on young plants at the early infection stage, but this symptom becomes less apparent when the temperature increases. Cloudy weather and temperatures of 16 to 20 ^{o}C favor symptom development (Sutic et al., 1999). Figure 2.1 shows the comparison of normal potato leaves with PVX infected potato leaves. A mild mosaic can be discerned in the infected leaves.

Figure 2.1 Potato leaves in the field. Nomal potato leaves (a). PVX infected potato leaves (b).

PVX virions have a size of about 515×13 nm^2. They are non-enveloped, flexuous, and filamentous. PVX virion contains a plus single -stranded RNA of 6435 nucleotides excluding the poly (A) tail at the 3' end. It also contains a m^{7}GpppG cap structure at the 5' end. There are 5 ORFs on the genomic strand (Huisman et al., 1988, Bercks, 1970). ORF1 encodes a 166kDa RNA-dependent RNA polymerase (RdRp). The overlap three ORF called Triple-gene block (TGB) follows ORF1. It encodes some kind of movement proteins associated with cell-to-cell movement of the viral genome in infected plants (Agell et al., 1996, Batten, 2003). ORF5 encodes coat

protein (CP). PVX virions contain only the genomic RNA, but the protein synthesis factory in eukaryotic host can only recognize the first ORF in the viral single RNA strand, so in infected tissue the virions use a subgenomic RNA strategy to translate themselves (Mandahar, 2000). Figure 2.2 shows the genome of PVX and its expression.

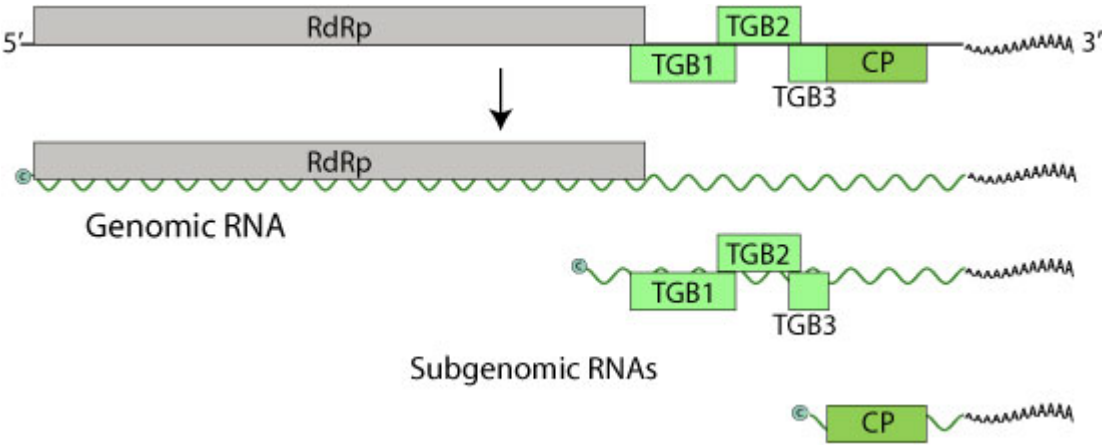


Figure 2.2 Potato virus X (PVX) genome structure and expression (http://education.expasy.org/images/Potexvirus_genome.jpg).

2.2 Viral Coat protein gene is used in engineered virus resistance

Genetic engineering of potato plants is considered as an effective measure to control viral disease and potato yield losses. Several kinds of viral proteins can mediate viral resistance and thus the nucleic acids encoding these proteins can be used in the genetic engineering. The proteins used include: a) the viral coat protein (CP), with which many plants have been engineered, some of which had been released to the fields; b) the movement protein (MP), which is required for the movement of most viruses; c) the RNA-dependent RNA polymerase (RdRp), which is the vital enzyme for RNA virus propagation; and d) replication associated protein (Rep), which is

important in single stranded DNA viruses that do not encode polymerase (reviewed by Prins et al., 2008).

The primary function of the CP is defined by their structural role in encapsidation of viral genomic nucleic acids, which is that it protects the genetic information from degradation. CPs also play an essential role in viral spread throughout the infected plants, both in long-distance transport through the vasculature and cell-to-cell movement (Carrington et al., 1996, Fedorkin et al., 2001). Moreover, CP may participate in other stages of the viral life cycle, such as replication, vector transmission, and silencing suppression (Callaway et al., 2001; Tomas et al., 2003; Qu et al., 2003).

Abel et al. (1986) first demonstrated engineered resistance against plant virus utilizing the CP gene. In this experiment, when challenged with Tobacco mosaic virus (TMV), the transgenic tobacco plants expressing the coat protein gene of TMV either did not display symptoms of infection or exhibited a delay in the appearance of symptoms. Since then, many successful examples of CP-mediated resistance (CPMR) from different plant virus families have been reported, with different constructs in different hosts (Beachy et al., 1990, Fitchen et al., 1993). The CPMR is effective for many different virus groups. Tricoll et al (1995) tested virus resistance of transgenic squash transformed with constructs containing single or multiple CP gene derived from cucumber mosaic virus (CMV), watermelon mosaic virus 2 (WMV2) and zucchini yellow mosaic virus (ZYMV). In the field, most transgenic lines remained nonsymptomatic throughout the growing seasons and produced marketable fruits, while other lines showed a delay in the onset of symptoms and/or a reduction in symptom severity. Forty five percent to 95% of the transgenic lines containing single

CP constructs of either CMV, WMV2 or ZYMV were resistant to the virus from which the CP gene was derived. Similar resistance to virus was also demonstrated in papaya (Ferreira et al 2002). In this study, 100% of the non-transgenic control and 91% of the matrix plants were infected by *papaya ringspot virus* (PRSV), while PRSV infection was not observed on any of the transgenic plants. Fruit production increased significantly and was three times higher than industry average.

Mechanisms of CPMR are not clearly explored though a lot of studies have been done. Cuozzo et al. (1988) generated transgenic tobacco plant with sense and antisense cucumber mosaic virus (CMV) CP gene and found that the efficiency of resistance was related to the amount of CP transgenically expressed. This was further proven by expression of TMV CP gene in transgenic tobacco plants (Osbourn et al., 1989). Ingelbrecht (1999) and Bendahmane (2002) proposed that the expression of the transgene interferes or blocks different steps of the viral life cycle resulting in attenuated symptoms of the disease or resistance to the virus.

2.3 RNA interference (RNAi)

2.3.1 Discovery of RNA silencing

RNA silencing was first found by Napoli's group (Napoli et al, 1990). They conducted research on the transgenic petunia plants. In their research, they attempted to overexpress the chalcone synthase (*CHS*) gene, which controls anthocyanin biosynthesis, in order to enhance the pigmentation of petunia flowers. The purple petunia flowers were transformed with chalcone synthase gene, but unexpectedly, 42% of the transgenic petunia plants showed white flowers or irregular-colored flowers. Then, it was proven by isolated nuclear run on transcription assay that the

loss of *chsA* mRNA in the cytoplasm was not caused by a decrease of transcription in the nucleus (Van Blokland et al., 1994). Jorgensen called this phenomenon co-suppression, indicating that the expression of *chsA* transgene and the endogenous *chsA* gene were both suppressed. Almost at the same time, two other labs reported that introducing a sense transcriptional transgene could negatively mediate the expression of the endogenous gene (Van der Krol et al., 1990; Ingelbrecht et al., 1994).

The subsequent study revealed that expression of the transgene led to formation of double stranded RNA, which initiated post transcriptional gene silencing (PTGS) in petunia petal, as the coding region locating in *chsA* and its 3' untranslated region had an inter-complementary region (Metzlaff et al., 1997). The *chsA* mRNA can form a segment of double stranded RNA and the co-suppression occurred. Hamilton and his group introduced a p35S-ACC (1-aminocyclopropane-1-carboxylate [ACC] oxidase) gene carrying a 5' untranslated region of reverse repeated sense transgene, and the results validated the role of dsRNA structure to trigger PTGS (Hamilton et al., 1998).

Although PTGS was first discovered in plants, it is not limited to plants. In the past ten years, PTGS relevant events were described in almost all kinds of eukaryotes, including protozoa, nematodes, insects, parasites, mouse and human.

In fungi, during attempts to enhance the production of an orange pigment by the mold *Neurospora crassa*, gene silencing was observed. Cogoni et al (1997) introduced extra copies of a gene involved in making a carotenoid pigment into mold cells. In the result, rather than turning a deeper orange, a third of the engineered mold appeared yellow or white. It seemed that something had suppressed the pigment genes. They called this observed phenomenon gene quelling.

In *Caenorhabditis elegans*, antisense RNA experiments showed strange results. In this approach, an RNA sequence complementary to the mRNA transcribed from a target gene was introduced into the research organism. Hybridization of the antisense RNA and sense mRNA arrested translation of the target gene. But in a control, in which a sense strand was injected, gene silencing also happened (Guo, et al., 1995). Later the presence of very small amounts of the corresponding sense strand in the antisense RNA preparation explained this result. The presence of dsRNA duplex led to an RNAi effect (Parish, S et al., 2000). It is supposed that these small contaminants would have no effect on gene expression.

Then, Fire and his group (1998) demonstrated that injection of double stranded RNA was more effective in gene silencing than injection of either sense or antisense strands alone. This great discovery showed that only a few molecules of injected double stranded RNA were required to block expression of protein in a cell. The dsRNA gene-silencing mechanism is highly gene-specific. They termed this phenomenon of gene silencing using dsRNA "RNA interference" (RNAi).

2.3.2 The mechanism of PTGS

In the past years, research on RNAi mechanism has clarified the RNAi mechanism. Combining studies *in vivo* and *in vitro*, the two step mechanism of RNAi/PTGS came out. The first step includes the binding of RNase to a long double stranded RNA and cutting it down into 21-25 nt RNA fragments, called small interfering RNA (siRNA). Second, the siRNA join in the endonuclease-containing, RNA-induced silencing complex (RISC), and target the homologous single stranded mRNA for degradation.

Post transcriptional RNA silencing is triggered by dsRNA, which comes from the transcription of inverted repeated regions and the replication of exogenous RNA in nature. Viruses and transgenes are the dominant exogenous cause of dsRNA formation in plants. Most single stranded RNA viruses generate dsRNA intermediates by replication of the virus by the virus encoded RNA dependent RNA polymerase (RdRp). These intermediates contain sequence-specific information that guide the plant to degrade viral RNA to protect itself (Sijen, et al., 2001). Double stranded RNA generated by trangenes has different mechanisms according to the transgene structure and the way of integration into the plant genome. If the transgene is designed to have a dsRNA structure or multiple copies of the transgene are inserted into the plant genome in a reverse repeat manner, then the need for RNA-dependent RNA polymerase can be avoided (Waterhouse et al., 1998). Therefore, the use of dsRNA designed to induce RNA silencing is a conventional tool in reverse genetics.

Hamilton and Baulcombe (1999) first revealed that the appearance of 21~25 nt dsRNA (si RNA) was vital in transgene induced gene silencing in their research in plant. The siRNA was first detected in plants undergoing co-suppression or virus-induced RNA silencing and was not detectable in control plants.

Using a *Drosophila in vitro* system, Tuschl et al. (1999) examined the molecular mechanism underlying RNAi. They found that during the RNAi reaction, both strands of the dsRNA were processed to RNA fractions of 21~23 nucleotides in length. Targeted mRNA was not required in this process, the mRNA was cleaved to 21~23 nt only within the region of identity with the dsRNA, which suggested that the 21~23 nt fragments from the dsRNA were guiding mRNA cleavage. Later they proved the role of siRNA by adding synthesized 21~23 nt siRNA into *Drosophila* free

cell culture, which led to the degradation of homologous mRNA (Elbashir et al., 2001a). Zamore et al. (2000) isolated non-processed and processed dsRNAs from *Renillaluc* dsRNA treated *Drosophila* cell lines and they demonstrated that the homologous mRNA was degraded only when natural siRNAs were included. When the fragments were heated to 95 ^{o}C, they lost their ability to degrade RNA, these *in vitro* and *in vivo* studies built the foundation to establish the fact that siRNA was an intermediate of RNAi. Based on the cut and ligation characteristics of *E.coli* RNaseIII, Bass et al. (2000) first put forward that an RNaseIII type endonuclease is involved in the degradation of dsRNA to siRNA. RNaseIII cleaves the dsRNAs disymmetrically, leaving a 2 nt 3' overhanging end. Elbashir and Tuschl's group provided the evidence of RNaseIII being involved in the RNAi process. When they chemically analyzed the sequence of 21~23 nt RNA that were generated after dsRNA treatment in the *Drosophila in vitro* system, and found that these 21~23 nt RNAs had a 5' phosphate group, a 3' hydroxyl group, a 2 nt 3' overhanging, and a unmodified sugar phosphate frame (Elbashir, et al., 2001b). The research of Bernstein (2001) indicated that the dicer gene in *Drosophila* encodes a RNA binding enzyme that cleaves the dsRNAs into 22 nt fragments *in vitro*. The dicer protein family is conserved in fungi, plants and animals (Tijsterman et al., 2002).

One of the characteristics of RNAi is the obvious catalytic property. Only a few dsRNA molecules are enough to degrade the target mRNA produced in continuous long term transcription. Although the change of long dsRNAs to many siRNAs leads to a limited amplification, it is not enough to explain the continuous mRNA degradation. Since mutants of the RdRp encoding gene influence RNAi, it is conferred that this kind of polymerase may replicate siRNAs as an exogenous

mediator, allowing siRNAs to spread in plants and in nematode generations. Lipardi et al. (2001) revealed from their study on the dsRNA-dependent degradation of targeted mRNA in a *Drosophila* embryo cell extract that dsRNAs were produced from single strand labeled siRNAs in the early instant, and both ssRNA homologous to the targeted mRNA and dsRNA can be the template for RdRp replication, new full-length dsRNAs formed quickly and then were cleaved. They also demonstrated that the 3' hydroxyl and 5' phosphate groups on the siRNA were necessary in the RdRp mediated reaction. Sijen et al. (2001) further revealed the role of RdRp activity in RNAi. They noticed that several new siRNAs were homologous to targeted mRNAs, but differed from trigger dsRNAs. Formation of secondary siRNA showed distinct polarity (antisense 5'-3'), implying that circulative replication used the present siRNAs as the primers.

In the first step of RNAi, the double stranded siRNA formed in this step is supposed to bind to the RNAi specific protein complex to form the RISC. Activation of the complex needs ATP, so the antisense component of unbound siRNA is exposed and the RISC performs the latter reactions. In the activated RISC, the loose antisense strand of the siRNA base pairs with homologous mRNA and then this complex cleaves mRNA near the double stranded region (Hannon，2002). In the second step, part of the cleaved mRNA fragments are transformed to the double stranded form by RdRp activity, this form has functions similar to siRNA and enters the amplification reaction, so amplification of RNAi can probably occur in both the first step and the second step. Figure 2 shows the general two steps in RNAi. Another model suggests that siRNA do not act as primers for RdRp. It is likely to assemble along target RNA and then form complementary RNA by RNA ligase, the complementary RNA

hybridizes with target RNA and is later degraded by dicer protein (Schwartz et al., 2002).

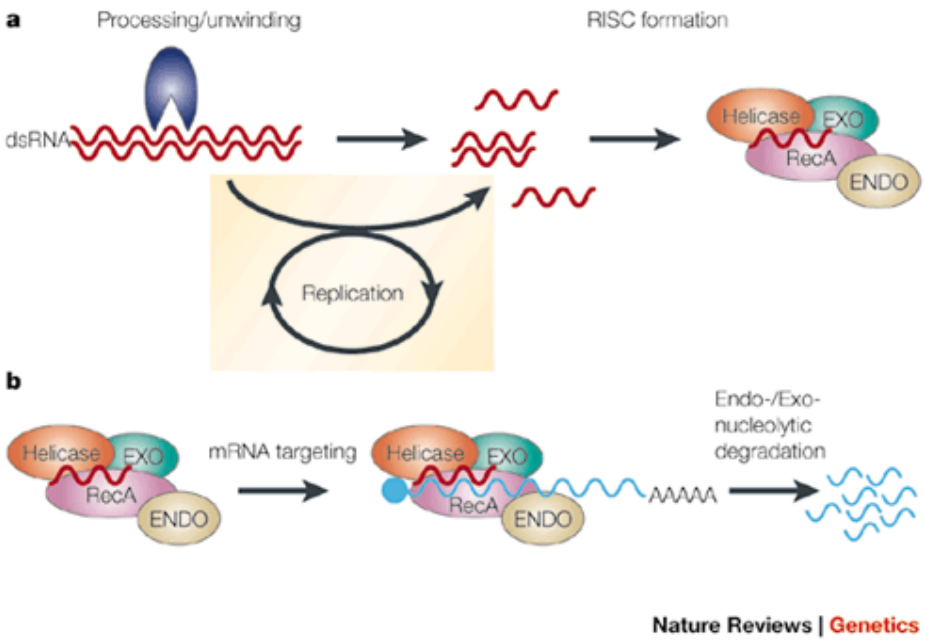


Figure 2.3 Two steps of RNAi (Scott et al., 2001). a) In the first step, input double-stranded (ds) RNA is processed into 21–23-nucleotide 'guide sequences'. b)The guide RNAs are incorporated into a nuclease complex, called the RNA-induced silencing complex (RISC), which acts in the second effector step to destroy mRNAs that are recognized by the guide RNAs through base-pairing interactions. (Endo, endonucleolytic nuclease; exo, exonucleolytic nuclease; recA, homology-searching activity related to *E. coli* recA.)

2.4 PTGS is an antiviral mechanism in plants

One kind of PTGS induced by virus was the recovery phenotype that occurred after virus invasion. In recovery plants, PTGS was not induced until the plant was infected with virus carrying sequences homologous to the transgene (Vance et al., 2001). This virus-induced PTGS presumably occurred as a consequence of the production of high levels of the genomic RNA as well as double-stranded RNA replication intermediates. Once PTGS was triggered, both the viral RNA and the virus-derived transgene RNA would be the targets, bringing on knock down of the virus and recovery of the plant.

The study of PTGS facilitated genetic modification of plants to obtain resistance to virus. Since dsRNA is the precursor of siRNA, construction of inverted repeat (IR) transgenes has been explored. The generation of tobacco lines resistant to PVY with constructs containing IR transgenes normally yielded more than 50% of all transgenic plants resistant to PVY, with gene constructs encoding intron spliced RNA capable of inducing PTGS with almost 100% efficiency, but single stranded sense or antisense approaches yielded resistance in only 5~20% of the transgenic plants (Smith et al., 2000).

Furthermore, hairpin RNA-encoding constructs containing inverted repeat sequences were proven to be able to induce transgenic resistance against cucumber mosaic virus at levels as high as 75% at the R_0 stage (Chen et al., 2004). However, resistance efficiencies reached with single transgene constructs containing the same sequence only reached 10-20% at the S1 level. A significant improvement was shown for the hairpin RNA constructs compared to the single transgene constructs.

Kalantidis (2002) reported the engineering of transgenic oriental tobacco lines that expressed dsRNA homologous to the 3’ region of the CMV RNA3 genome. This

manipulation made several transgenic lines completely resistant to CMV. They demonstrated that the presence of homologous CMV siRNAs in the plants prior to infection can be used as a molecular marker to predict resistance. Plant lines that failed to produce siRNAs at a detectable level were susceptible to CMV infection, whereas plants producing siRNAs were resistant to CMV. The correlation between the detection of short RNAs and virus resistance provided a molecular marker, which made possible the prediction of success in attempts to engineer virus resistance by dsRNA.

Nicola-Negri (2005) reported the hairpin-mediated RNA silencing for resistance to *Plum pox virus* (PPV) infection in *N. benthamiana* plants. They utilized the genes for the P1 and HC-Pro protein, which were considered to act as RNAi suppressor, to construct hairpin RNA encoded vector. In the result, 38 out of 40 T_0 transgenic plants were resistant to PPV infection and 248 out of 253 T_1 transgenic plants were resistant to local and systemic PPV infection. These data indicated that the hairpin RNA construct with RNAi suppressor was an efficient way to obtain virus resistant plants.

An inverted repeat construct was also applied to induce Grapevine Fanleaf virus (GFLV)-specific silencing. Jardak-Jamoussi (2009) developed an inverted repeat (IR) constructs consisting of a conserved region of the GFLV movement protein (MP) gene and transformed it into grapevine. They got some transgenic virus resistant grapevine lines after challenge inoculation with the relevant virus.

In addition to dsRNA, several novel strategies were developed in recent years. The microRNA (miRNA) pathway, which was shown to down-regulate endogenous gene expression in plants, has been used to design artificial miRNAs (amiRNAs) (Niu, 2006). Expression of the amiRNAs in transgenic plants conferred resistance against plant viruses. Lopez-Ochoa et al (2006) showed that expression of a peptide targeting a

replicase of tomato golden mosaic virus (TGMV) can confer resistance to geminivirus. They suggested that this strategy should be applied to engineer virus resistant crops.

2.5 Agroinfiltration

2.5.1 Agroinfiltration is a versatile tool

Agroinfiltration is a transient expression mediated by infiltration (by vacuum infiltration or injection) of *A. tumefaciens* that harbor vectors or chimeric constructs into intact plant leaves. It provides a rapid and simple way of exogenous expression assay. Therefore it has been applied in several areas of research, including gene silencing (Schob et al., 1997), avirulence gene and resistence (R) gene interaction (Van der Hoorn et al., 2000), promoter and transcription factor function assay (Yang et al., 2000) and protein production (Sawers et al., 2006).

Several means of transient expression assay have been performed, such as electroporation of protoplast, particle bombardment, microinjection and viral vector-mediated infection. Agroinfiltration in plant leaves was first developed from the study of T-DNA transfer processes and optimization of stable *Agrobacterium* mediated transformation (Rossi et al., 1993). It was Kapila et al. (1997) who put forward the infiltration of *Agrobacterium* into intact leaves as a suitable way for functional analysis of different promoters or identification of genes via functional complementation. The study also revealed that this system was also applicable to different plant species. Popular plant species for infiltration are tobacco, tomato, lettuce and *Arabidopsis*, although some other species were also applicable, such as grapevine (Zottini et al., 2008) and potato (Bhaskar et al., 2009). In addition to leaves, fleshly fruit (Spolaore et al., 2001) and living hydroponic root (Levy et al., 2005) were studied with histochemical assays.

Transient expression via agroinfiltraion is achieved by the proximity of agrobacteria cells and plant cells. When *Agrobacterium* suspension was introduced into plant leaf and spread evenly in the intercellular spaces, the *Agrobacterium* transforms the T-DNA gene to a portion of the plant cells (Kapila, 1997). The genes remain transiently present in the nucleus, leading to the transient expression. The plant can be monitored for a possible effect in the phenotype or harvested and used for purification of protein of interest. In contrast to stable expression, T-DNA copies are transiently present in the nucleus and transcribed rather than integrated into the plant genome.

2.5.2 Comparison of agroinfiltration and stable transgenic expression

Agroinfiltration is based on the *Agrobacterium* Ti plasmid mediated transformation thus is also called agrobacterium mediated transient expression. When compared with transgenic expression, agroinfiltration has higher expression efficiency and is a simpler procedure.

First, transgene expression in transgenic plants often varies significantly due to insert position and other effects (Kapila et al., 1997). Therefore, the data analysis is complicated. Second, regeneration of transgenic plants is time consuming. The expressed proteins can be analyzed whereas gene expression can be measured only several days after the agroinfiltration in transient expression. For species recalcitrant to regeneration, transient expression is especially an ideal alternative. Furthermore, agroinfiltration targets many more cells in a leaf, which means higher transformation efficiency than stable transgene expression.

However, agroinfiltration based transient expression is of limited use in the study of tissue-specific or developmentally regulated promoter activity. Stable transformation is preferred for study of these promoters (Baum, 1997).

CHAPTER III

MATERIALS AND METHODS

3.1 Cloning of potato virus X (PVX) coat protein (CP) gene

3.1.1 Materials

3.1.1.1 Samples for cDNA extraction

Potato leaves with PVX infected symptoms were collected from fields of Guizhou Academy of Agricultural Sciences (GZAAS), Weng'an county and Weining county in Guizhou province.

Virus-free potato plantlets provided by Guizhou Engineering Research Center for Potatoes were used as the negative control. All leaf samples were kept at -70 ^{o}C.

3.1.1.2 Reagents

Reagents for tube capture reverse transcriptase polymerase chain reaction (TC-RT-PCR) consisted of 200 U/μl Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, 40 U/μl RNase inhibitor, 0.1 M DL-Dithiothreitol (DTT), 5 × RT-PCR buffer and 0.1% Diethyl Pyrocarbonate (DEPC) water. PCR amplification reagents consisted of 5 U/μl *Taq* DNA polymerase, 10×PCR buffer, 10 μM primers and 10 mM dNTPs. Agarose gel electrophoresis reagents consisted of 0.5% TAE buffer, agarose, 1.2 kb DNA Marker and 6 × loading buffer. A Tiangen gel purification kit was used to extract the PCR products from the agarose gel. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) and Isopropyl-β-D-thiogalacto-

pyranoside (IPTG) were used for blue/white screening of *E. coli* transformation. M-MLV reverse transcriptase, 5×RT-PCR buffer and DEPC were purchased from Promega Company. RNase inhibitor, *Taq* DNA polymerase and dNTPs were purchased from Takara Company. DTT solution and agarose were purchased from Sangon Company. X-gal and IPTG were purchased from MBI Company. Tiangen gel purification kit and DNA Marker were purchased from Tiangen Company. All chemicals used were analytical grade.

3.1.1.3 Primers

Primers were designed from the PVX genome sequence number EF063709 in GeneBank by using the Primer Premier 5.0 software (Dieffenbach, 1995). The oligonucleotides were synthesized by Beijing Sunbiotech Company. The PVX CP primer sequences and other primer sequences are listed in table 3.1. The italic bases were added to protect the 5' end of the primers.

Table 3.1 Primers for PCR detection of transgenic potato callus.

Name	Sequence	Primer size (bp)	Expected size (bp)
PVXf	5'-*GA*ATGTCAGCACCAGCTAGCAC-3'	22	714
PVXr	5'-*GC*TTATGGTGGTGGGAGAGTGAC-3'	23	
X302f	5'-GGGGACAAGTTTGTACAAAAAAGCA GGCTATGAAGTATGCCCCAGTG- 3'	47	360
X302r	5'-GGGGACCACTTTGTACAAGAAAGCT GGGTGCGGTTGTTGTTCCAGTA- 3'	47	
NPTIIf	5' ATGGCAATTACCTTATCCGC 3'	20	822
NPTIIr	5' TCAGAAGAACTCGTCAAGAAGG 3'	22	

Note: underlined sequences are the attB sites.

3.1.1.4 Plasmid and bacteria

pMD18-T vector provided with high efficient ligation solution I was purchased from Takara Company and *E. coli* strain $DH5_{\alpha}$ was obtained from Guizhou Key Laboratory for Agricultural Biotechnology.

For centrifugation referred in this thesis, an Eppendorf 5424 centrifuge was used for tubes smaller than 2 ml, Beckman Allegre 21R centrifuge was used for 10 ml tubes and Beckman AVanti J-25 centrifuge was used for 50 ml tubes.

3.1.2 Methods

3.1.2.1 Cloning PVX CP gene by TC-RT-PCR

Potato leaf samples were collected during late May to late June, 2008. The potato plants were in the flowering stage or seed setting period, depending on the altitude of the area. Different areas in Guizhou province were chosen to collect potato leaf samples, including Guiyang, Weining and Weng'an. Samples consisting of four to five pieces of leaves were separated in labeled plastic bags after cutting from the potato plant and then directly put into an ice box. The samples were kept in a -70 ^{o}C freezer until use.

For preparation of RT-PCR, the tips and tubes were soaked in 0.1% DEPC water for 8 h before autoclave sterilization with other instruments for three times. About 10 mg leaves were ground very quickly with 1 ml grinding buffer (2% polyvinyl pyrrolidone (PVP) in PBS, pH 7.0) in a pre-chilled mortar. The materials were then transferred into a 1.5 ml centrifuge tube and spun at 4 ^{o}C, 9000 rpm for 10 min in an Eppendorf 5424 centrifuge. Two hundred microliters of supernatant was transferred to a new 0.5 ml tube and incubated at 37 ^{o}C for 3 h. The liquid was then removed. The tube was rinsed with phosphate-buffered saline with 0.05% Tween-20

(PBST) 3 times and 0.1% DEPC water once. Finally the tube was spun again to remove all the liquid from the bottom of it.

Reverse transcriptase PCR components were added to the tube, including 11.5 µl DEPC water, 4 µl 5×RT-PCR buffer, 1 µl of 10 mM dNTPs, 1 µl of 10 µM reverse primer PVXr and 1 µl of 0.1 M DTT. After that, the reaction was mixed well by pippeting and incubated at 70 ^{o}C for 5 min, followed by placing it on ice for 5 min. One microliter of 200 U/µl reverse transcriptase M-MLV and 0.5 µl of 40 U/µl RNase inhibitor were added to the tube that was then incubated at 42 ^{o}C for 1 h for the reaction of reverse transcription. Afterwards the tube was transferred to a 90 ^{o}C bath and incubated for 15 min to stop the reaction.

For the second cDNA strand amplification, PCR components were mixed as follows, 2.5 µl 10×PCR buffer, 0.5 µl of 10 mM dNTP，0.5 µl of 10 µM forward primer PVXf, 0.5 µl of 10 µM reverse primer, 0.125 µl of 5 U/µl *Taq* DNA polymerase, 5 µl first strand cDNA and ddH_2O were added to reach the volume of 25 µl. These components were mixed well in a 0.2 ml tube and the PCR reaction was performed in a Biorad Thermocycler. The PCR was done at 94 ^{o}C for 4 min as the initial denaturation step, followed by 35 cycles of denaturation at 94 ^{o}C for 45 sec, annealing at 55 ^{o}C for 45 sec and extension at 72 ^{o}C for 60 sec, ending with final extension at 72 ^{o}C for 5 min.

The PCR product was loaded onto a 1.2% (w/v) agarose gel to check by electrophoresis. The expected band on the gel was excised and purified with TIANgel Mini/Midi Purification Kit.

3.1.2.2 Ligation of PCR product to plasmid

In a 0.2 ml tube, 0.5 µl plasmid pMD18-T DNA (50 ng/µl) and 2.5 µl PCR purification product were added and mixed thoroughly. Five microliters of

Solution I (provided with pMD18-T) and 2 μl ddH_2O were sequentially added into the reaction tube and also mixed thoroughly. The reaction was incubated at 16 ^oC overnight and then directly used for bacterial transformation.

3.1.2.3 Preparation of competent *E. coli* cells

E. coli strain DH5α was streaked on an LB agar plate and incubated at 37 ^oC overnight. A single colony was picked and inoculated in 20 ml LB medium, followed by shaking at 37 ^oC, 250 rpm overnight. Five hundred microliters of the overnight culture was diluted to 100 ml with fresh new LB medium in a flask and incubated at 37 ^oC with shaking at 250 rpm until it reached early log phase (OD_{600} =0.2~0.4). The final culture was chilled on ice for 10 min and then transferred into 50 ml centrifuge tubes to spin at 4 ^oC, 4000 rpm for 10 min to collect the cells. The supernatant was removed, followed by resuspending the cells in 10 ml ice-cold 0.1 M $CaCl_2$. The cells were chilled on ice for 20 min and then spun at 4 ^oC, 4000 rpm for 10 min to remove supernatant again. The cell pellets were resuspended in 5 ml ice-cold 0.1 M $CaCl_2$ and chilled on ice for 30 min. Then cell pellets were collected as before and the supernatant was removed. Two milliliters of ice-cold 0.1 M $CaCl_2$ containing 20% glycerol was added to the pellets and mixed gently. Aliquots of 100 μl competent cells were added into 1.5 ml tubes and dipped into a liquid nitrogen bath. The frozen cell aliquots were transferred immediately into a -70 ^oC freezer.

3.1.2.4 *E. coli* transformation (heat-shock method)

One microliter of plasmid DNA (10-20 ng) or 10 μl ligation reaction and 100 μl competent cells were added in a 1.5 ml tube and mixed by pipetting. The reaction was heat shocked for 90 sec at exact 42 ^oC (a thermocycler or a water bath) after being incubated on ice for 30 min. The tube was held on ice for 2

min and then prewarmed 800 µl LB medium was added to it. After that, the mixture was shaken at 220 rpm on a horizontal shaker at 37 ^{o}C for 1 h. The culture was placed on ice for 2 min and 200 µl aliquots were spread on LB agar plates containing 100 mg/l Ampicilin (Amp), 40 µl 2% (w/v) X-gal and 4 µl 20% (w/v) IPTG (Appendix B). After air drying for about 20 minutes, the plates were then incubated inverted at 37 ^{o}C overnight untill the bacterial colonies were able to be picked (about 15-18 h).

3.1.2.5 Colony PCR detection

Colony PCR consisted of 2.5 µl 10×PCR buffer, 0.5 µl 2 mM dNTPs, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 0.25 µl 5 U/µl Taq polymerase and 19.75 µl ddH_2O in a 25 µl system. The mixture was added to 0.2 ml PCR tube on ice before colonies were added. The colonies were selected and numbered on the bottom side of the plate. By using sterile pipette tips or toothpicks, colonies were picked to touch on a new agar plate and also resuspended in the correspondingly labeled PCR tubes for the colony PCR amplification. The PCR was done at 94 ^{o}C for 5 min as the initial denaturation step, followed by 35 cycles of denaturation at 94 ^{o}C for 45 sec, annealing at 55 ^{o}C for 45 sec, extension at 72 ^{o}C for 60 sec, ending with a final extension at 72 ^{o}C for 5 min.

The PCR product was loaded onto a 1.2% agarose gel to detect whether it exhibited the expected band. The positive clone was cultured in LB broth containing 100 mg/l ampicillin (Amp) overnight to extract the plasmid and send for sequencing at Sangon Company.

3.1.2.6 Sequence alignment and phylogenetic tree construction

Sequences were analyzed and stored with EditSeq program (Lasergene ver. 5.0, DNASTAR Inc.). The sequences were aligned with Clustal X

(Thompson J.D. et al., 1997) program and the alignment was transferred to Mega 4.0 software (Tamura K. et al., 2007) to generate an N-J tree. Bootstrap percentages based on 1,000 resamplings were calculated to evaluate internal branch support. Genetic distances were estimated from sequence data by Kimura two-parameter methods (Kimura, 1980).

3.2 Vector construction

3.2.1 Materials

3.2.1.1 Template

The PVX CP cDNA cloned in section 3.1 was used as template to amplify the PVX302 fragments for vector construction.

3.2.1.2 Bacteria and plasmid

E. coli strain DB3.1, used to maintain pDONR221 and pHellsgate12, was purchased from Invitrogen.

Entry vector pDONR221, purchased from Invitrogen, contains attP sites that were used to clone interest PCR products flanked by attB sites and so as to generate the entry clone (see appendix). The destination vector pHellsgate12 was provided by Waterhouse lab (Commonwealth Scientific and Industry Research Organization (CSIRO), Austrilia). The pHellsgate12 vector contains two attR cassettes that are in reverse orientation and thus result in hairpin RNA structure after two identical fragments were inserted and transcribed. The vector was also designed with dual introns such that all the clones contain an intron in the proper orientation (see Appendix).

3.2.1.3 Reagents

Reagents for regular PCR amplification, agarose gel electrophoresis and gel purification were the same as in section 3.1.1.2. BP Clonase™

II Enzyme Mix, LR Clonase™ II Enzyme mix and M13 Primer were purchased from Invitrogen Company. Plasmid DNA extraction kit and 1.0 kb Marker were purchased from Tiangen Company. The restriction enzymes *Xho*I provided with 1×M-buffer, *Xba*I provided with 1×H-buffer and DL15000 DNA Marker were purchased from Takara Company.

3.2.1.4 Primers

The 3' coding region of the PVX CP gene sequence was used to design primers. The attB1 and attB2 sites were added to the 5' end of the primers to clone into pDONR221 (table 3.1). The oligonucleotides were synthesized by Invitrogen Company.

3.2.2 Methods

3.2.2.1 PCR amplification of PVX302

A 50 µl reaction was used for PCR amplification of PVX302. It consists of 2 µl PVX CP DNA , 5 µl of 10×PCR buffer (Mg^{2+}), 1µl of 2 mM dNTPs, 2 µl of 10 µM forward primer, 2 µl of 10 µM reverse primer, 0.25 µl of 5 U/µl *Taq* polymerase and 37.75 µl ddH_2O. The PCR was done at 94 ^oC for 2 min as the initial denaturation step, followed by 35 cycles of denaturation at 94 ^oC for 30 sec, annealing at 55 ^oC for 30 sec and extension at 72 ^oC for 60 sec, ending with final extension at 72 ^oC for 5 min.

PCR and electrophoresis were performed as described above. The PCR product on the agarose gel was excised and purified by Tiangen gel purification kit.

3.2.2.2 BP reaction

The BP clonase was used to subclone the PVX CP segment into pDONR221 with attP and attB sites. The BP reaction consisted of 5 µl purified PCR

product containing attB sites, 1 µl of 150 ng/µl pDONR221, 2 µl of BP Clonase^{TM} II enzyme mix and adequate distilled water added to give a volume of 10 µl. The reaction was incubated at 25 ^{o}C for 8 h and stopped with adding of 1 µl proteinase K. Afterwards the reaction mixture was transformed into *E. coli* strain DH5$_{\alpha}$ by heat shock and then plated on LB agar containing 100 mg/l ampicillin. Positive clones were selected by colony PCR and after cultured in LB liquid medium containing 100 mg/l Amp, the entry clone vector was extracted with Plasmid DNA Extraction Kit.

3.2.2.3 LR reaction

The LR reaction contained 7 µl entry clone vector, 1 µl pHellsgate12 destination vector and 2 µl LR Clonase^{TM}II enzyme mix. The reaction was incubated at 25 ^{o}C overnight and stopped with adding of 1 µl proteinase K. One microliter of the reaction was then used to transform *E. coli* strain DH5α. Positive clones were grown on an LB agar plate containing 100 mg/l Spectinomycin (Spec), selected by colony PCR, and then cultured in LB medium for plasmid extraction with a Plasmid DNA Extraction Kit.

3.2.2.4 Restriction enzyme digestion

The recombinant plasmid was digested by *Xba*I and *Xho*I. *Xba*I digestion reaction consisted of 2 µl 1×M-buffer, 2 µl BSA, 1 µl *Xba*I (8-20 U/µl), 8 µl DNA and 7 µl ddH_2O. The *Xho*I digestion reaction consisted of 2 µl 1×H-buffer, 1 µl *Xho*I (4-12 U/µl), 8 µl DNA and 9 µl ddH_2O. Both reactions were done with incubation at 37 ^{o}C for 1 h. After digestion was finished, the reaction was loaded on 1.2% agarose gel to check the results. The correct constructs were named as pHellsX302.

3.3 Transient expression

3.3.1 Materials

3.3.1.1 Virus resource, antiserum and acceptor plant

PVX isolate, PVX antiserum (Wu and Zhou, 2005) and tobacco *Nicotiana benthamiana* seeds were kindly provided by Professor Xueping Zhou at Zhejiang University.

3.3.1.2 Bacteria

A. tumefaciens strain *EHA*105 was obtained from Guizhou Key Laboratary for Agricultural Biotechnology.

3.3.1.3 Reagents

2-(N-Morpholino) ethanesulfonic acid hydrate (MES) was purchased from Sangon Company and acetosyringone (AS) was purchased from Solabio Company. Enzyme conjugate was purchased from Adgen Company. Both BSA and p-nitrophenyl phosphate (pNPP) [Sigma 104-105] were purchased from Sigma Company. All chemicals were analytical grade.

3.3.2 Methods

3.3.2.1 Preparation of Agrobacterium competent cells for electroporation

A. tumefaciens strain EHA105 was streaked on a YEP agar plate and incubated at 28 ^{o}C for 48 hr. A single colony was picked and inoculated in 20 ml YEP medium containing 50 mg/l rifampicin (Rif), followed by shaking at 28 ^{o}C, 220 rpm overnight. One milliliter of the culture was inoculated in 200 ml YEP medium containing 50 mg/L Rif and shaken at 28 ^{o}C, 220 rpm overnight until log phase (OD_{600} = 0.5). The final culture was spun at 4 ^{o}C, 5000 rpm for 10 min to collect the cells. The cell pellets were rinsed with cold sterile ddH_2O three times followed by

10% glycerol once and resuspended 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 then preserved in a -70 ^{o}C freezer.

3.3.2.2 Transformation of *Agrobacterium* by electroporation

Fifty microliters of competent EHA105 cells were thawed on ice and mixed with 5µl pHellsX302 plasmid DNA. The mixture was then transferred to the cuvette on ice, avoiding air bubbles by gently shaking, and electroporated at 2500 V. One hundred microliters of cold YEP was added in the DNA/*Agrobacterium* mixture and mixed gently by pipetting. After that, the mixture was added to a 10 ml tube containing 900 µl YEP and then shaken at 28 ^{o}C, 220 rpm for 3 h. One hundred microliters of the culture was spread on a YEP agar plate containing 50 µg/ml Rif and 100 µg/ml Spec and incubated inverted at 28 ^{o}C for 2 d until colonies were available for PCR detection.

3.3.2.3 Preparation of *Agrobacterium* broth

*A. tumefaciens EHA*105 containing pHellsX302 was streaked on a YEP agar plate containing 100 µg/ml Spec and 50 µg /ml Rif and incubated at 28 ^{o}C for 24-48 h until colonies were seen. A colony was selected to inoculate into 3 ml YEP medium containing 100 µg/ml Spec and 50 µg/ml Rif, then it was shaken at 28 ^{o}C, 220 rpm for 24 h. Fifty microliters of the culture was inoculated in 50 ml YEP medium containing 100 µg/ml Spec and 50 µg/ml Rif and shaken at 28 ^{o}C, 220 rpm untill log phase (OD_{600} = 1.2-1.8). The culture was spun at 8000 rpm for 10 min to collect the cells that were then resuspended in sterile ddH_2O containing 10 mM $MgCl_2$, 10 mM MES and 200 µM AS. The suspension was then adjusted to $OD_{600} \approx$ 0.8 and placed at room temperature for 3 h before use.

3.3.2.4 Infiltration

Fifteen individual plants of *N. benthamiana* that grew in a greenhouse for 6-7 weeks were used for pHellsX302 and 15 for pHellsgate12 transient expression assays. Another one individual plant infiltrated with *Agrobacterium* containing pHellsX302 was prepared as negative control (not inoculated later). Two expanding leaves in the top middle of the plant were selected for infiltration. A needle was used to stab from the back of the selected leaves and prepared *Agrobacterium* suspension (10 µl or more was used until the whole leaf was soaked) was injected into the leaves through the hole by a syringe without needle.

3.3.2.5 Inoculation

Four days after infiltration by *Agrobacterium*, inoculi was made by grinding 1/10 (w/v) PVX infected wild type tobacco leaves in 0.05 mM 10×PBS buffer (pH 7.0). The liquid was collected to inoculate the agroinfiltrated tobacco leaves with sterile quartz sand by scrub. Generally 50 µl inoculum was used on one leaf. No inoculation was performed for the negative control.

3.3.2.6 DAS-ELISA

Ten days after virus inoculation, the leaves of the tested plants were taken for DAS-ELISA analysis. The PVX antiserum was diluted 1:1000 in 0.1 M coating buffer, pH 9.6, and 100 ml were added into each well of a microtitre plate. The plate was then incubated at 37 °C for 4 h with parafilm sealing. After incubation, the plate was taken out to be washed with PBS-Tween 20 (0.05%) three times. The plate was soaked for a few minutes and then blotted dry by tapping upside down on tissue paper every time.

One hundred milligrams of the test leaf was ground with 1 ml PBS to make the sample for DAS-ELISA analysis. The volume of PBS can be adjusted

according to the infection degree of the tobacco leaf. Supernatant was collected by centrifugation at 4000 rpm for 5 min. The supernatant was dispensed into 100 µl aliquots and added to duplicate wells. The plate was incubation at 4 ^{o}C overnight and then washed and blotted three times as before.

One hundred microliters of enzyme conjugate diluted 1: 1000 in conjugate buffer was added to each well. The plate was then incubated at 37 ^{o}C for 1 h and washed four times. One hundred µl freshly prepared substrate, 10 mg pNPP dissolved in 10 ml of substrate buffer (see Appendix B), was added to each well. The plate was incubated at room temperature in the dark for 60 min.

Results of DAS-ELISA were assessed by visual observation of the color in test wells and spectrophotometric measurement of absorbance at 405nm after reaction. Development of yellow color in test wells indicated positive results. Wells in which no significant color developed indicated negative results. Comparison of the OD_{405} value between samples and the negative control was used to decide whether the test sample was positive or negative. If OD_{405} value of the sample was higher than twice that of NCx (average OD_{405} value of the negative control), the sample would be designated as positive, otherwise it was negative (Clark and Adams, 1977).

3.4 Potato transformation

3.4.1 Materials

3.4.1.1 Acceptor potato plants and bacterium

Virus free potato plants of the varieties Russet Burbank, Favorita and Shepody were provided by Guizhou Engineering Research Center for Potatoes. *A. tumeficiens* strain LBA4404 harboring pHellsX302 were used for potato transformation.

3.4.1.2 Media

Basic medium MS for plant tissue culture was used for transgenic plant regeneration and YEP medium was used for *Agrobacterium* culture.

Table 3.2 Components of media used for potato regeneration

Medium	**Hormone (mg/L)**					**Sucrose (g/L)**	**Km (mg/L)**	**Carb (mg/L)**
	6-BA	**NAA**	**GA_3**	**2,4-D**	**ZT**			
A	2	0.5	0.5	-	-	30	75	400
B	-	0.1	0.5	-	2	30	75	400
C	-	-	-	2	0.8	30	75	500

Note: 6-BA is 6-benzylaminopurine, NAA is naphthalene acetic acid, GA_3 is gibberellic acid 3, 2,4-D is 2,4-dichlorophenoxy acetic acid and ZT is zeatin. Km is kanamycin and Carb is carbenicillin. Basic medium is MS.

3.4.1.4 Primers

Primers for the NPTII gene in pHellsgate12 vector were designed according to accession No. AJ311874 in Genbank. The primer sequences are shown in table 3.1.

3.4.1.5 Reagents

The antibiotics kanamycin (Km) and carbenicillin (Carb), and hormones 6-BA, NAA, ZT, GA3 and 2,4-D were purchased from Sanland Company. RNase was purchased from Tiangen Company. All chemicals were analytical grade.

3.4.2 Methods

3.4.2.1 Potato transformation

A. tumeficiens LBA4404 containing pHellsX302 was streaked on a YEP agar plate containing 50 mg/l Rif and 100 mg/l Spec and incubated at 28 ^{o}C for

2-3 d until colonies were available. Colonies were checked with the specific primers for the insert fragment by colony PCR and a positive clone was inoculated into YEP medium containing 50 mg/l Rif and 100 mg/l Spec and shaken at 28 ^{o}C overnight. Fifty microliters of the culture was inoculated into 50 ml new YEP medium containing antibiotics and incubated at 28 ^{o}C until OD_{600} was about 0.5.

Potato leaves were cut into pieces of 0.5×0.5 cm^2, internodes were cut into segments of 0.5 cm and they were precultured on MS differentiation medium without antibiotics at 25 ^{o}C, light 16 h/d, illumination density 2000 lx for 2-3 days. *Agrobacterium* culture was spun at 9000 rpm for 5 min and supernatant was removed, basic MS medium was added to dilute the cells to reach OD_{600}=0.5. The precultured internodes or leave pieces were soaked in *Agrobacterium* broth for 5-10 min and residual broth was blotted up by sterile filter paper before the materials were transferred onto MS medium. The explants were co-cultured at 28 ^{o}C for 2 d in the dark.

After co-cultivation, the explants were blotted up with sterile filter paper, transferred onto callus induction medium containing 75 mg/l Km and 400 mg/l Carb, and cultured under the same conditions as in the preculture step. The callus induction media was changed every two weeks. About 2 months later, calli were able to be separated from explant and transferred onto shoot inducing MS medium containing 75 mg/L Km and 400 mg/L Carb.

3.4.2.2 DNA extraction and PCR detection

DNA of transgenic potato callus was extracted with modified CTAB methods. Each callus was cut into small pieces and transferred into a 1.5 ml eppendorf tube. A little quartzite and prewarmed (65 ^{o}C) 200 μl extraction buffer were

added to the tube before the tissue was ground with a sterilized cuspidal stick thoroughly. Four hundred microliters of extraction buffer was added to the tube and mixed thoroughly by pipeting, the tube was then incubated at 65 ^{o}C for 10 min with a few occasional gentle inversions during the time.

Six hundred microliters of 24:1 chloroform/isoamyl alcohol was added to the tube, the mixture was mixed thoroughly and then spun at 12,000 rpm for 10 min. Three hundred microliters of the top layer was transferred to a new tube, into which 400 µl chloroform/isoamyl alcohol was then added. The extraction step was repeated once again. After that, 300-400 µl of the top layer liquid was transferred to a new tube and 600 µl ice-cold ethanol was added to the tube and mixed thoroughly. Then, the mixture was placed at room temperature for 5 min and spun at 12,000 rpm for 10 min.

The supernatant was removed and 500 µl of 70% alcohol was added to the tube to wash the sediment. The sediment was flipped up in the liquid and the tube was placed at room temperature for a few minutes. The tube was washed again and then air dried. Finally, 30-80 µl TE buffer was added to the sediment to dissolve it. One microliter of 50 µg/ml RNase was added to the DNA solution that was then kept at room temperature for 2 h. The DNA solution was kept at -20 ^{o}C for long time preservation.

One microliter of DNA solution was used for a 25 µl PCR reaction, as described above. PCR products were loaded on 1.2% agarose gel to run electrophoresis to check the result.

CHAPTER IV

RESULTS AND DISCISSION

4.1 PVX CP cDNA

In Guiyang, a total of 18 leaf samples were obtained from Guizhou Academy of Agricultural Sciences (GZAAS), with 8 samples from a greenhouse and 10 samples from planting fields. In Weining, samples were taken from planting fields of three different villages, Xiaoshan, Maza and Caohai with 7, 13 and 8 samples, respectively. In Weng'an, 17 leaf samples were collected from planting fields. All together, 63 samples were collected for PVX CP cDNA extraction. Mosaic potato leaves were hypothesized to indicate PVX infected samples (figure 4.1).

Figure 4.1 Potato leaf sample in field.

The specific primers PVX-f and PVX-r were used to amplify the PVX CP cDNA by the TC-RT-PCR technique. PVX CP cDNA was obtained from only 1 sample collected from Xiaoshan village, Weining County. The PCR product was about 700 bp (figure 4.2). The band was excised from an agarose gel, purified for cloning and sequenced.

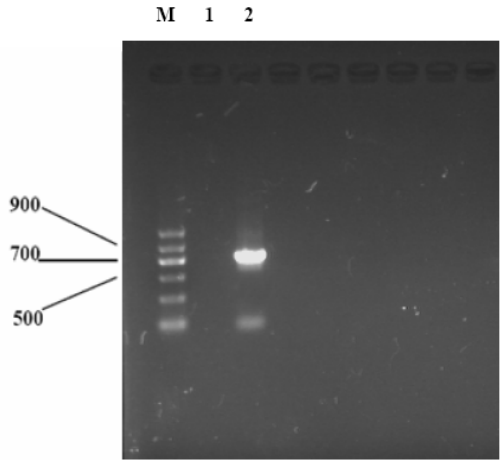


Figure 4.2 Agarose gel electrophoresis of purified PVX CP cDNA. Lane M: MarkerII，lane 1: Negative control, lane 2: sample from Xiaoshan village.

The purified PVX CP cDNA was ligated to the pMD18-T vector and sequenced with M13 primers in Sangong Company. The sequencing result indicated that the PVX CP cDNA obtained was 714 bp. The nucleotide sequence and its deduced amino acid sequence are shown in figure 4.3. It is designated as XSH2, according to the sample collecting place.

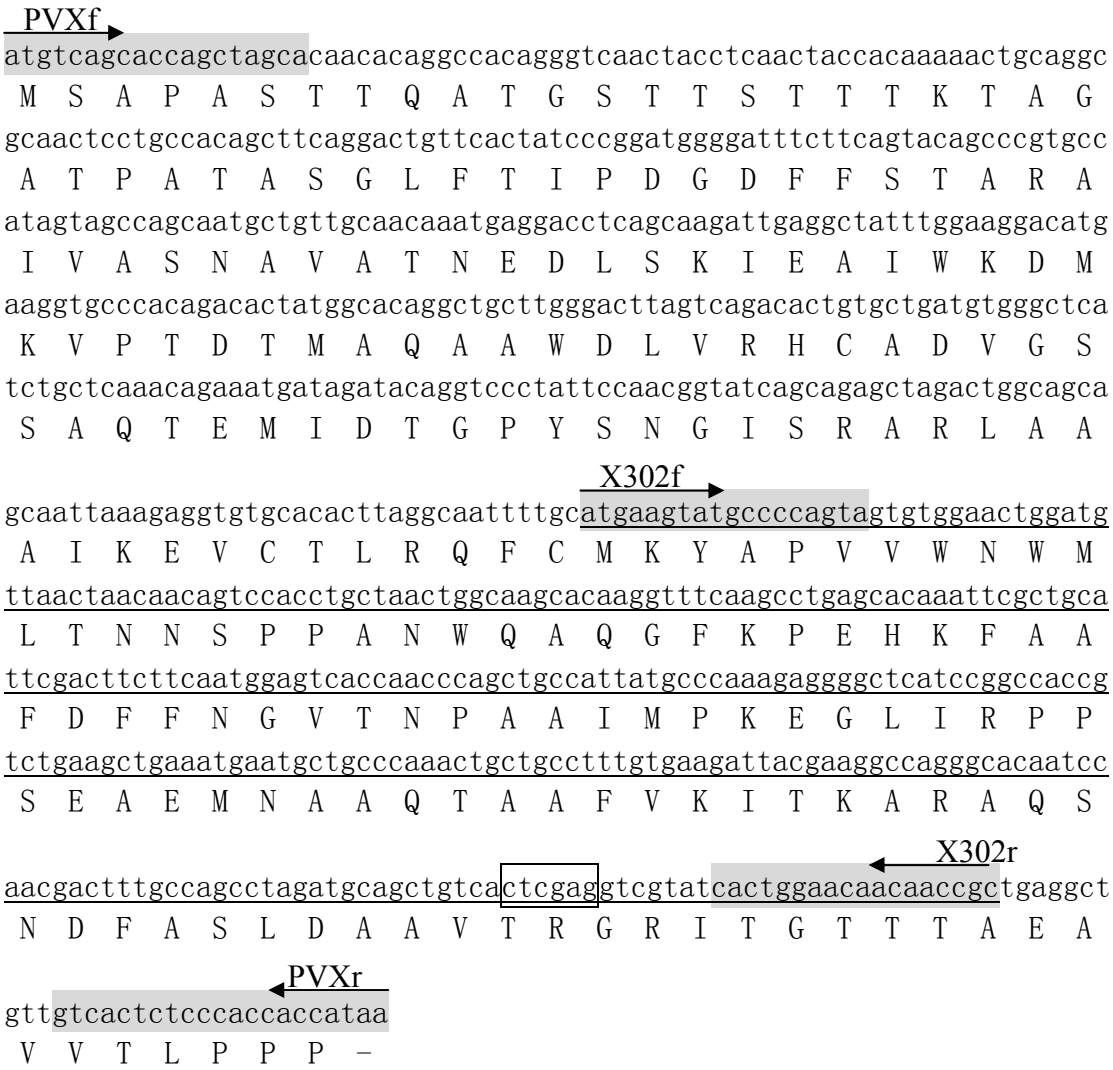


Figure 4.3 Sequence of PVX CP XSH2 and its amino acid sequence. The sequence was translated with the Expasy Translation Tool (http: //www.expasy.org/ tools/dna.html). The gray boxes are the location of the PVX CP and PVX302 forward and reverse primers. The small box is an *Xho*I recognition site. The underlined nucleotides are the conserved sequences of X302 for RNAi vector construction.

The XSH2 sequence was aligned with published nucleotide sequences in NCBI Genbank database using the BLASTn program, and the result showed that it had

maximum identity of 99% to the PVX CP gene (data not shown), which indicated that the XSH2 cDNA is from the PVX CP gene.

There are more than 80 PVX CP genes reported in the Genbank database, but papers about PVX infection symptoms correlated with sample collection were not found. Collection of the leaf samples was difficult to conduct because the description of the symptoms in the literature was vague. Zheng (2006) reported that PVX infection rarely happened in Guizhou province, as determined by checking with DAS-ELISA. Leaf samples here were collected by the appearance of mild mosaic. However, the sample from which the PVX was detected and the cDNA was cloned appered to be a severe mosaic by chance. It can be speculated that because of the co-infection of PVY and PVX, the sample was seen to be severe mosaic, which was considered to be typical symptom of PVY.

TC-RT-PCR utilizes the nonspecific attachment between virion particles and the wall of an Eppendorf tube to capture virion and their RNA is reverse transcribed to cDNA (Qiu et al., 2009). After PCR amplification of the corresponding virus, specific PCR product can be obtained. Although immuno-capture RT-PCR (IC-RT-PCR) is very specific because it captures the virus by antibody, the detection is influenced by the serum strains. TC-RT-PCR is a simple method avoiding RNA extraction and antibody preparation. It decreases the cost and keeps its advantage of simple, quick and sensitive. It may have broader application than RT-PCR and IC-RT-PCR.

From the BLAST result, 17 PVX CP nucleotide sequences from different areas around the world were chosen to be aligned with XSH2 by DNAMAN software (www.lynnon.com). Identities at nucleotide and amino acid levels were calculated with the program (table 4.1).

Table 4.1 Similarity of nucleotide (nt) and amino acid (aa) sequences between XSH2 and other PVX CP

Accession	Origin	nt Identity (%)	aa Identity(%)
D87962	Japan	98.8	99.6
EU031437	Beijing, China	98.3	99.2
FJ461343	Iran	97.7	99.2
AJ505748	Spain	97.6	100
D00344	Netherlands	97.6	100
AY512654	Zhejiang, China	97.5	99.2
Z29333	Estonia	97.3	98.7
AF272736	Taiwan, China	97.3	100
X88784	UK	97.2	100
Z34261	Argentina	96.9	100
EF063709	Guizhou, China	96.8	99.6
FJ643623	India	96.5	97.9
GQ496608	Latvia	96.1	99.2
AF260641	Korea	95.9	99.2
AF202462	Canada	95.9	100
EU021215	Russia	95.3	99.6
AY763582	Egypt	94.9	97.9

XSH2 had very high nucleotide (nt) identity and amino acid (aa) identity with the 17 PVX CP sequences. It had the highest nt identity with D87926 from Japan, with the value of 98.8% and also a relatively high aa identity value 99.6%. The three

other isolates from China had higher than 96% nt identity and higher than 99% aa identity with XSH2. Another isolate from Guizhou, accession number EF063709, had high aa identity to XSH2, although it had relatively low nt identity (96.8%). Isolates from Spain, the Netherlands, Taiwan, UK, Argentina and Canada share the same highest aa identity of 100% with XSH2.

To estimate the phylogenetic relationship of PVX CP genes in the world, including XSH2, a series of PVX CP genes were retrieved from the NCBI database. Fifty seven isolates from different geographical origins were used to construct a phylogenetic tree (Figure 4.4). The white clover mosaic virus (WCMV) CP gene, accession X16636, was used as the outgroup.

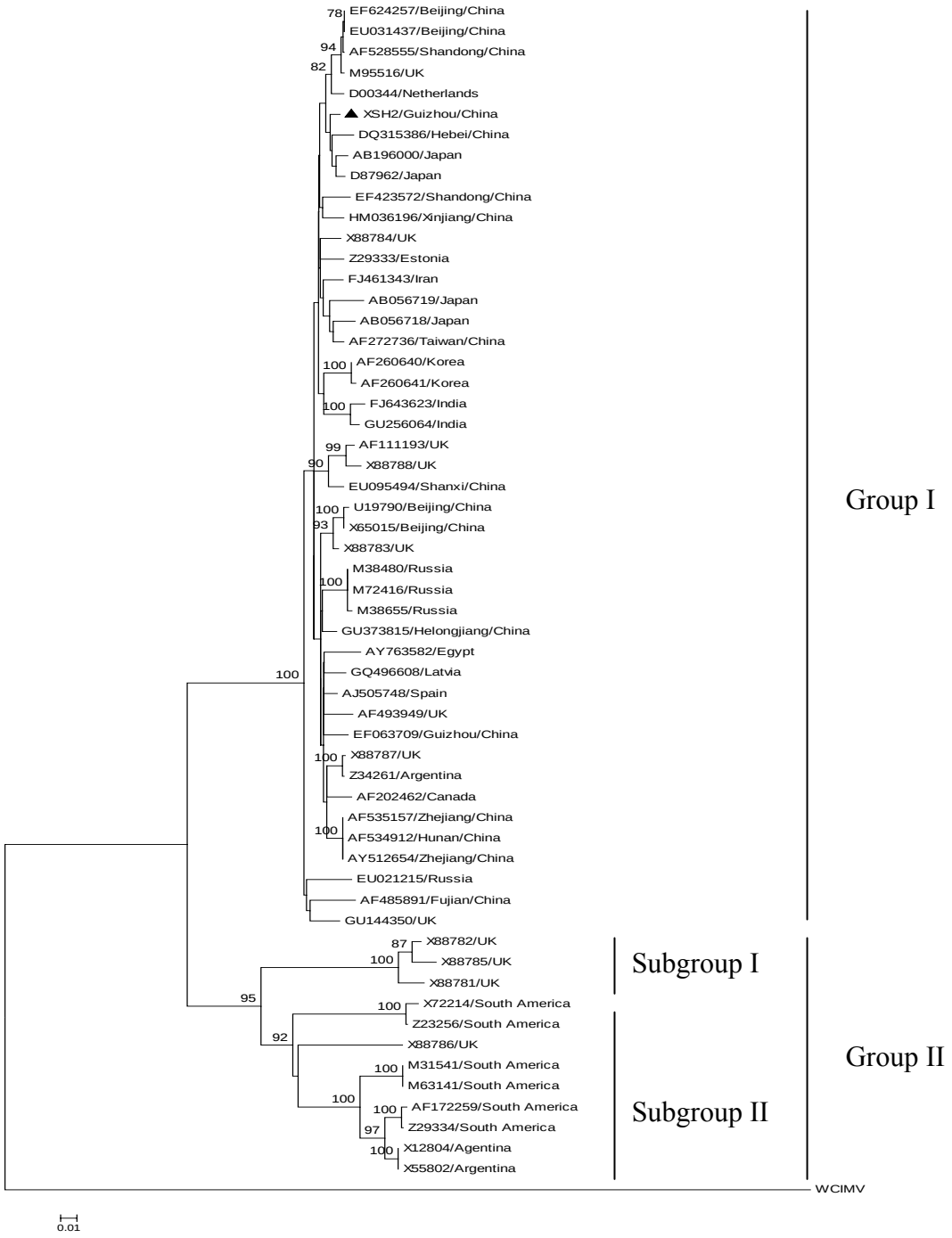


Figure 4.4 Phylogenic tree of PVX CP gene. Isolates are indicated in the tree by accession number and the origin. Numbers at nodes indicate bootstrap values higher than 70%. The scale bar represents a distance of 0.01 substitutions per nucleotide site. WCIMV: white clover mosaic virus.

The PVX CP genes accessions X88781, X88782 and X88785 from the UK had 744 nucleotides, AF 172559, M31541 and several other accessions had only 711 nucleotides. All other PVX CP genes had 714 nucleotides and encoded 237 amino acids. These PVX CP isolates were clearly divided into two groups, the bigger group I and the smaller group II. Forty-five isolates that originated from all over the world clustered in group I and 12 isolates mainly from America clustered in group II. There were definitely two subgroups in group II, subgroup I was composed of three isolates from the UK and subgroup II was mainly composed of American isolates. Yu et al (2010) analyzed the phylogeny of 16 PVX complete genomes, as well as 37 PVX CP genes and obtained the same result that PVX can be generally divided into two groups, the Eurasia group (group I) and America group (group II).

Two isolates from Korea and two from India were clearly clustered into their small subgroups in group I, while isolates from UK dispersed into two groups. Isolate XSH2 belonged to group I and was closest to DQ315386 from Hebei in China and another two Japanese isolates. Chinese isolates gathered in group I and dispersed in different subgroups. In China, some isolates from the same province seemed to have little or no phylogenic distance with each other, such as isolates in Beijing and Zhejiang. However, isolates from the same province may also differ from each other, such as isolates in Guizhou, Shandong, Beijing and Zhejiang. It can be explained by the widespread occurrence of Potatoes in the country and transfer between provinces.

Potato was originally found in South America and then cultivated in Europe, North America and some countries of the former Soviet Union, but its production and demand in Asia, Africa and Latin America has been dramatically increased since 1990s (FAOSTAT). The phylogenic tree of the PVX CP gene

indicated that due to the virus transmission by planting infected tubers, in the meantime of potato spread, its reproduction by tuber made its virus disease also widespread in the world.

As described in the literature review, grouping of PVX was based on their reactions with the resistance genes Nb, Nx and Rx in potato. Research on the interaction of these genes and PVX showed that the PVX CP gene was vital for virus-host interaction, the 121st and 127th codon in the CP revealed major determinants for resistance breaking in potato (Goulden et al., 1993). Mutations in CP were revealed as an important factor to render resistance-breaking ability (Kavanagh et al., 1992). PVX CP triggered hypersensitive resistance (HR) or extreme resistance (ER) in the virus-host interaction. In other words, PVX CP plays an important role in PVX strain grouping. As seen in table 4.1, 6 isolates had 100% aa identity with XSH2. However, only the isolate with accession number D00344 from the Netherland was reported to be in the X3 group (Huisman et al., 1988). Considering the 100% aa similarity between XSH2 and D00344, XSH2 may also belong to the group X3.

Bai et al (2008) compared the PVX CP genes and PVX whole genome sequences and revealed that PVX CP gene was more conserved than the whole genome, and the 3' end is more conserved than the 5' end. Therefor, a short fragment of the 3' end of PVX CP gene was used to construct an RNAi vector and generate efficient PVX resistant potato plants.

4.2 Construction of the hairpin cp vector

PVX CP gene was used as template to obtain a conserved fragment for insertion into the vector pDONR221. A 302 bp fragment at the 3' end of the PVX CP gene was amplified by PCR with the specific primer X302f and X302r containing attB sites

(figure 4.5).

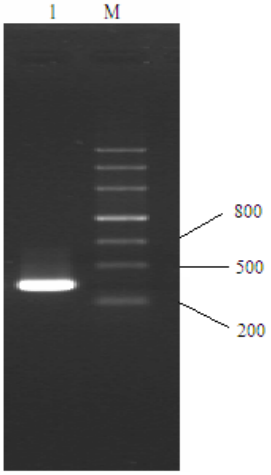


Figure 4.5 Agarose gel electrophoresis of PVX CP 302 fragment (including attB site). Lane 1: PVX 302, lane M: Marker.

The PCR product was excised and purified. The purified PCR product was then used to conduct BP reaction with pDONR221 to generate an entry clone. The entry clone, designated as pENTRX302, was transformed into *E. coli* strain DH5a and the colonies were checked by colony PCR (figure 4.6). The transformation efficiency was high and positive clones were almost 90%.

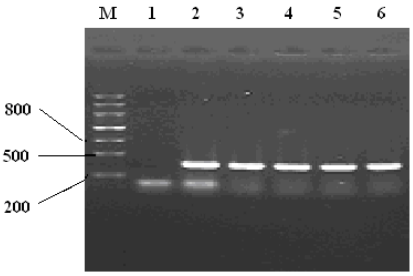


Figure 4.6 Agarose gel electrophoresis of colony PCR product for entry clone pENTRX302. Lane M: Marker, lane 1: Negative control, lane 2 to lane 6: colony PCR results of PVX302 in pENTRX302. Negative control was a PCR product of the 15µl PCR reaction without adding colony.

The entry clones were purified (figure 4.7) and sent to sequence. The result was as expected that the gene fragment X302was inserted into the clones (data not shown).

Figure 4.7 Agarose gel eletrophoresis of purified entry clone. Lane M: DNA Marker, lane 1: pENTRX302.

An LR clonase recombination reaction was conducted with pENTRX302 and pHellsgate12 to generate the RNAi clone. After the LR reaction, positive colonies were selected and cultured to extract the plasmid. The restriction enzymes *Xba*I and

*Xho*I were used to digest the expression vector and the result is shown in figure 4.8.

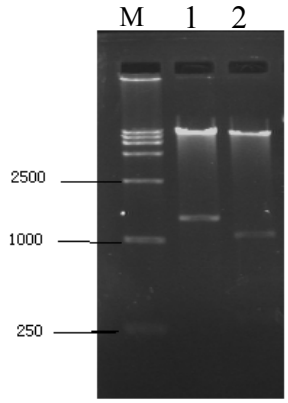


Figure 4.8 Agarose gel electrophoresis of digested pHellsX302. Lane M: DNA Marker, lane 1: pHellsX302 digested by *Xba*I, lane 2: pHellsX302 digested by *Xho*I.

After digestion of pHellsX302 by *Xba*I, a band of about 1500 bp was observed. It was because during the recombination with pHellsgate vectors, two products were possible to generate (figure 4.9) and the pHellsgate 12 vector contained two introns in opposite orientation to ensure that after LR recombination either of the introns was effective. In this recombination, it happened to generate product B, which consisted of the insert fragment, attB sites and the two introns (figure 4.10). This was proven by sequencing of the digested fragment. The second point was noticed that the digestion of *Xho*I did not generate the fragment with that of *Xba*I. The reason was retrieved by restriction analysis of the PVX302 fragment by DNAMAN that there was an *Xho*I recognition site CTCGAG in the PVX302 fragment (figure 4.3). Due to inert digestion of the X302, digestion of *Xho*I released a fragment smaller than that of *Xba*I and another small fragment. The big fragment consisted of part of X302, attB sites and the two introns and the small fragment was the other part of X302 fragment.

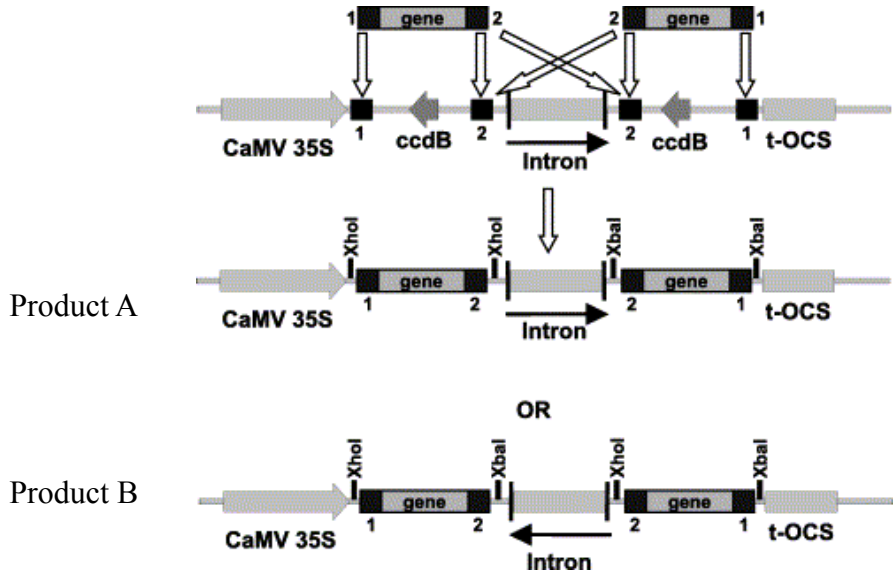


Figure 4.9 Recombination products with pHellsgate vectors. The attL2 sites flanking the gene fragment can interact with the attR2 sites on either side of the intron giving rise to two possible constructs with the intron in either the sense or the antisense orientation with respect to the promoter (Helliwell and Waterhouse, 2003).

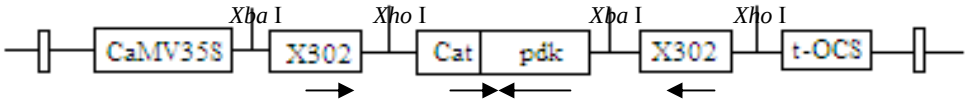


Figure 4.10 Schematic representation of pHellsX302. pHellsgate 12 was designed with dual introns such that all the clones contain an intron in the proper orientation.

4.3 Transient expression of pHellsX302

Each 15 individual *Nicotiana benthamiana* plants grown for 6 weeks were infiltrated with *Agrobacterium* strain *EHA*105 harboring the RNAi vector pHellsX302 or empty vector pHellsgate12. The infiltrated *N. bentamiana* plants were inoculated

with PVX suspension 4 days later. Four days post-inoculation (dpi) of PVX, individual plants infiltrated with pHellsX302 remained symptomless while individual plants infiltrated with pHellsgate12 started to display mosaic symptoms on leaves (figure 4.10).

Figure 4.11 Typical symptom of PVX inoculation

The phenotype of all tested tobacco plants were observed and recorded at 10 dpi (table 4.2). The typical mosaic on the leaves was determined as susceptible and no symptom was determined as resistance.

Table 4.2 Phenotype of infiltrated tobacco plants at 10 dpi.

Vectors for infiltration	No. of infiltrated plants	No. of Susceptible plants	No. of Resistant plants
pHellsgate12	15	15	0
pHellsX302	15	0	15

All tobacco plants infiltrated with *Agrobacterium* harboring pHellsX302 showed no symptoms indicating that they were resistant to PVX infection. In contrast, all tobacco plants infiltrated with *Agrobacterium* harboring the empty pHellsgate12 vector were susceptible to PVX and phenotypes apparent mosaic were observed on the leaves.

Double antibody sandwiched ELISA (DAS-ELISA) was also performed at 10 dpi. Yellow or light yellow was observed in the wells of pHellsgate12 while pHellsX302 wells remained clear, as did the wells of the negative control. Measurement of the reaction absorbance revealed high OD_{405} values for plants infiltrated with pHellsgate12 and very low values for those with pHellsX302 (Table 4.3). The results indicated that plants infiltrated with *Agrobacterium* harboring the empty vector pHellsgate12 had a high accumulation of virus particles but low number of virus particles was detected for plants infiltrated with *Agrobacterium* harboring pHellsX302.

Table 4.3 ELISA determination of the virus load in the infiltrated tobacco samples.

pHellsgate12			**pHellsX302**		
Plant	**OD_{405}**		**Plant**	**OD_{405}**	
	Individual	**Average**		**Individual**	**Average**
1	0.841	0.839±0.003	1	0.074	0.072±0.003
	0.837			0.070	
2	0.821	0.789±0.045	2	0.070	0.071±0.001
	0.757			0.071	
3	0.743	0.733±0.014	3	0.074	0.074±0.000
	0.722			0.074	
4	0.104	0.107±0.004	4	0.079	0.082±0.004
	0.109			0.085	
5	0.841	0.826±0.022	5	0.065	0.064±0.002
	0.810			0.062	
6	0.823	0.849±0.037	6	0.063	0.062±0.001
	0.875			0.061	
7	0.797	0.777±0.028	7	0.069	0.073±0.005
	0.756			0.076	
8	0.857	0.865±0.011	8	0.080	0.077±0.004
	0.873			0.074	
9	0.747	0.765±0.025	9	0.069	0.070±0.001
	0.783			0.070	
10	0.864	0.868±0.006	10	0.084	0.078±0.009
	0.872			0.071	
11	0.893	0.907±0.020	11	0.055	0.056±0.001
	0.921			0.057	
12	0.759	0.760±0.001	12	0.063	0.064±0.001
	0.761			0.064	
13	0.849	0.855±0.008	13	0.065	0.064±0.002
	0.861			0.062	
14	0.763	0.745±0.026	14	0.063	0.062±0.001
	0.726			0.061	
15	0.954	0.958±0.006	15	0.069	0.073±0.005
	0.962			0.076	
Average	0.776±0.013		Average	0.069±0.007	

The average OD_{405} value of the negative control (NCx) was 0.046 and twice this value is 0.092. Therefore, all pHellsgate12 plants were positive and all pHellsX302 plants were negative, even though plant No. 4 of pHellsgate 12 showed a comparable low OD_{405} value, not much higher than plants of pHellsX302 (Figure 4.11). This supported the result of phenotype observation. Together, the results indicated that the PVX CP fragment containing hairpin construct may efficiently triggered RNA silencing in tested plants and protect them from PVX infection. The resistance of the transgenic potato after efficient transformation of potato plant with the *Agrobacterium* harboring this vector can be predicted.

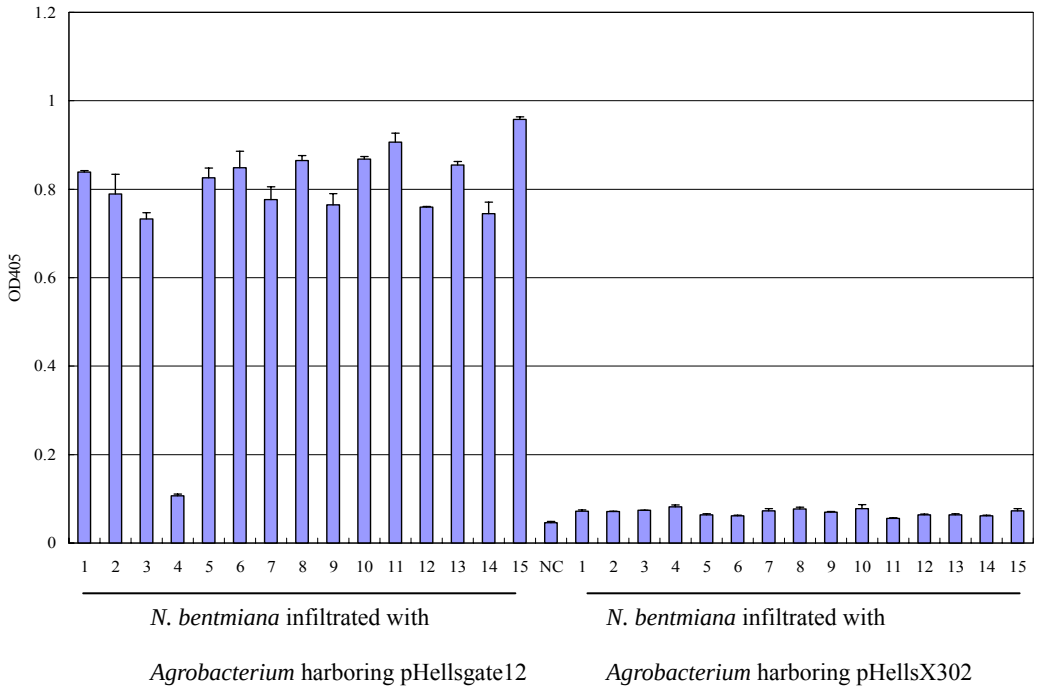


Figure 4.12 Detection of potato virus X by DAS-ELISA in infiltrated *N. bentamiana* plants at 10 days post-inoculation (dpi). NC: negative control. *Bars* represent standard error mean values from two replicas of each extract.

For inverted repeats, there are many factors in the construct affecting RNA silencing. These factors include the sequence of the repeats, the orientation of the repeats; the size of the gene fragments, the size of the spacer, and so on. It is suggested that the size of the gene sequence in the inverted repeat can obviously influence the silencing efficiency. Akashi et al. (2001) tested length dependence of the RNAi effect in tobacco BY-2 cells by co-transformation of a luciferase gene construct and a luciferase dsRNA expression plasmid and concluded that the presence of 300 bp of dsRNA was sufficient to obtain efficient silencing and the presence of a longer dsRNA did not increase silencing efficiency. Heilersig et al (2006) tested a large number of constructs harboring granule-bound starch synthase (GBSSI) cDNA and obtained the result that the small inverted repeat constructs with a repeat size of 500-600 bp and a spacer of about 150 bp were more efficient than the large inverted repeat constructs with repeat size of 1.1 or 1.3 kb and a spacer of 1.3 or 1.1 kb. Based on the result and their research, they drew the conclusion that the optimal size for an inverted repeat was 300-500 bp.

In this study, a hairpin RNA vector with a conserved region of the PVX CP gene of 302 bp in size was constructed. Transient expression assay via agroinfiltration revealed that when introduced the construct into plant, protection against PVX infection occurred. For pHellsgate12, Helliwell and Waterhouse (2003) have recommended that insertion of a 300-600 bp gene fragment would maximize the efficiency of silencing, but other sizes were also applicable.

Agroinfiltration has been employed in several studies on virus interference by hp RNA-encoding constructs. Zhu et al (2007) constructed a vector with the PVX CP gene and designed an inverted repeat. After transient expression and challenge

inoculation, 19 individual plants in a total of 25 test tobacco plants displayed resistance to PVX infection. In 2008, Vargas et al has shown that chimeric construct containing the potato virus Y (PVY) CP gene, IRCPPVY, in a transient assay, resulted in a complete block of PVY transmission by aphids. Here we demonstrated that transient expression of an hp CP construct induced high resistance to PVX inoculation. The efficiency of the construct was obtained by directly cloning a fragment of the gene of interest into an RNAi vector, which was more convenient than vectors with only multiple restriction sites for cloning. These studies suggested that transient expression via agroinfiltration may provide a simple way for screening of DNA constructs before stable plant transformation.

4.4 Agro bacterium mediated potato transformation

Potato transformation of pHellsX302 was conducted to get transgenic potato plants resistant to PVX infection. Although the silencing efficiency was tested via the transient expression assay, the real transgenic potato plants were desired in the potato production to decrease the damage by potato virus X.

Both potato leaves and internodes were applied to generate transgenic potato plants. In general, internodes had much higher callus inducing rate and lower browning rates, although the callus generated from leaves were able to induce more shoots. The internodes swelled in the preculture period and calli were induced one month after infection with *Agrobacterium* (figure 4.12). Green calli were seen one month later.

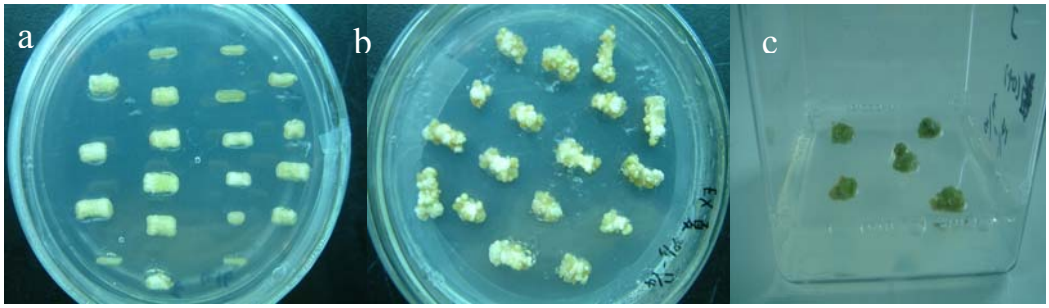


Figure 4.13 Generation of transgenic potato calli. a: internodes after preculture. b: one month calli, c: two month calli.

In potato transformation, the basic medium for regeneration is Murashiki and Skook (MS). Transformation efficiency of potato depends on many factors. For example, the variety of potato, the *Agrobacterium* concentration, infection time and also the plant growth hormones. Different potato genotypes need different plant hormones. Here we performed an experiment screening the hormone type and concentration effect in callus induction. In the experiment, potato variety Favorita and Shepody were tested with varied hormone types and concentrations to select the best combination for each variety. According to previous reports and the pilot experiment, three kinds of media composed of different hormones and different concentrations were tested for callus induction and growth (table 4.3). For each treatment, 100 internodes were used to transform with *Agrobacterium* containing pHellsX302.

Table 4.4 Responses of two potato varieties to three types of media.

Variety	**Favorita**			**Shepody**		
Medium	**A**	**B**	**C**	**A**	**B**	**C**
Internodes Transformed	**100**	**100**	**100**	**100**	**100**	**100**
Induced Calli	47±5.7	59.3±4.2	52.7±6.6	76.7±3.7	65.3±4.6	60±8.9
Browning calli	16.3±2.8	22±4.2	19.3±1.7	17±3.1	15±2.4	19.7±2.6

Note: Medium A: 6-BA 2.0 mg/L + NAA 0.5 mg/L + GA_3 0.5 mg/L, Medium B: NAA 0.1mg/L + GA_3 0.5 mg/L + ZT2.0 mg/L, Medium C: 2, 4-D 2.0 mg/L + ZT 0.8 mg/L (Table 3.2).

The results indicated that the highest callus inducing percentage was produced by medium A (6-BA 2.0 mg/L+ NAA 0.5 mg/L+ GA_3 0.5 mg/L for Shepody and medium B (NAA 0.1 mg/L+ GA_3 0.5 mg/L+ ZT 2.0 mg/L) for Favorita, respectively. The lowest browning percentage was generated by 6-BA 2.0 mg/L+ NAA 0.5 mg/L+ GA_3 0.5 mg/L for both Shepody and Favorita. Considering both the callus inducing percentage and browning percentage, medium A was chosen for Shepody and medium B was chosen for Favorita in later callus inducition. Analysis of the average percentages also revealed that although the two varieties had different responses to different medium, Shepody induced callus easier and gave less brown callus. The varieties that had strong plantlets in tissue culture would be preferred to generate transgenic plants because they had less contamination and callus browning.

4.5 Detection of the transgene and NPTII gene

The calli of pHellsX302 grown for 2 month were used to extract the DNA. They were rinsed with distilled water to remove attached medium and blotted up with filter paper to remove the water. Although the method was simplified for PCR detection,

the quality of the DNA was good, as shown in figure 4.13. DNA from the same individual transgenic callus was used for both the PVX302 and NPTII gene detection by PCR.

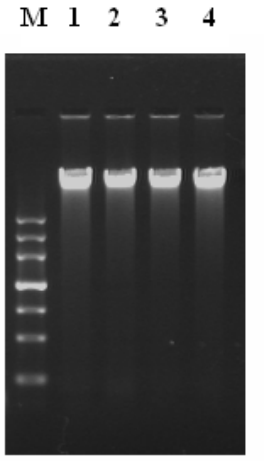


Figure 4.14 Agrose gel Electrophoresis of callus DNA. Lane M: DNA Marker, lane 1- 4: callus DNA.

PCR amplification was done using the specific primers for X302 and NPTII gene (table 3.1). *Agrobacterium* conferring the corresponding construct was used as positive control and non-transgenic calli from tissue culture were used as negative controls. For the callus generated with *Agrobacterium* harboring pHellsX302, 9 lines of Favorita and 10 lines of Shepody were tested with primers for X302 (figure 4.14) and NPTII gene (figure 4.15).

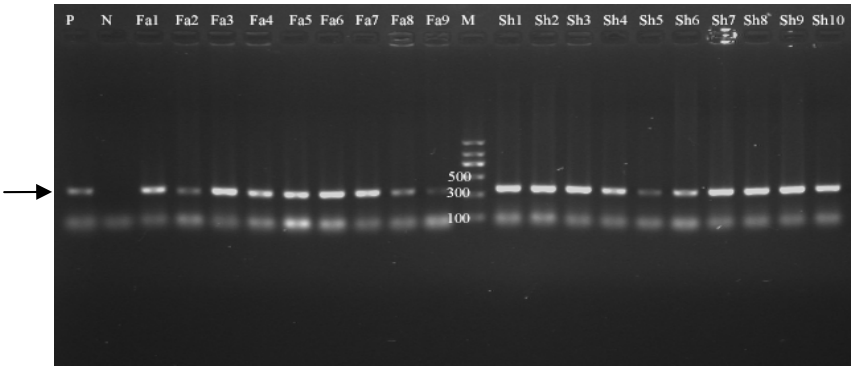


Figure 4.15 Agarose gel electrophoresis of X302 in transgenic calli. Lane p: Positive control (*Agrobacterium* harboring pHELS12X302). Lane N: negative control (nontransgenic potato callus). Lane Fa1-Fa9: individual transgenic calli of Favorita. Lane M: DNA marker. Lane Sh1- lane Sh10: individual transgenic calli of Shepody.

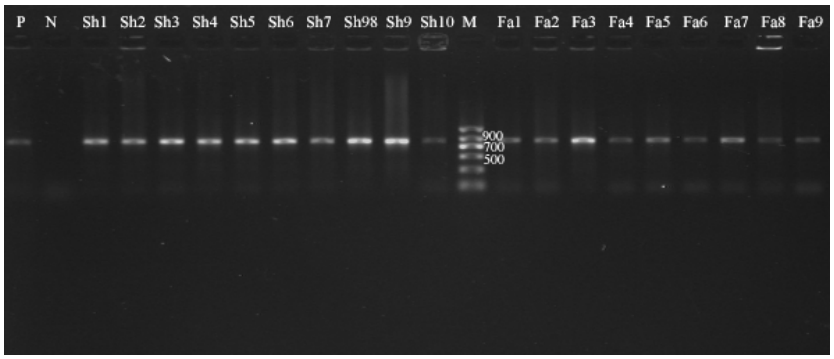


Figure 4.16 Agarose gel electrophoresis of NPTII in transgenic calli. Lane p: Positive control (*Agrobacterium* horboring pHellsX302). Lane N: negative control (nontransgenic potato callus). Lane Sh1-lane Sh10: individual transgenic calli of Shepody. Lane M: DNA marker. Lane Fa1-Fa9: individual transgenic calli of Favorita.

Total 19 individual calli from Favorita and Shepody were tested and all showed positive results for detection of both the PVX CP insert fragment and the NPTII gene . The result indicated that the simplified DNA extraction method was sufficient for PCR detection of the transgenic calli, the transformation system we used was efficient and the T-DNA was transformed into the calli. Efficient potato transformation result should be achieved with careful manipulation of the transformation steps and strict control of the experiment conditions. Although the regeneration of the transgenic potato has not been reached because of time limitation, these results laid a foundation for the future reseach.

CHAPTER V

CONCLUSIONS

The PVX CP gene was cloned from potato leaves in the fields, using TC-RT-PCR. This PVX CP gene was 714 bp long and its deduced protein sequence consisted of 237 amino acids. Alignment of the PVX CP gene and protein by DNAMAN showed that the isolate XSH2 belongs to the X3 group. Further phylogenic analyses indicated that the PVX CP genes was mainly divided into two groups and XSH2, along with other Chinese isolates belonged to the Asian group.

A small conserved fragment (X302) of PVX CP was cloned with specific primers containing attB sites, which were added to the fragment for Gateway cloning. The X302 was cloned into pDONR221 with high efficiency and then transferred to pHellsgate12 by LR recombination reaction, resulting in pHellsX302. Restriction analysis of the expression vector pHellsX302 demonstrated that X302 was inserted into the plasmid.

The construct pHellsX302 was electroporated into *Agrobacterium* strain *EHA*105, which was used to infiltrate tobacco leaves and check the resistant effect. The result of challenging inoculation was evaluated by phenotype observation and DAS-ELISA. Fifteen tobacco plants infiltrated with *Agrobacterium* harboring pHellsX302 showed resistance to PVX inoculation while those with the control pHellsgate12 showed the susceptible phenotype.

The construct pHellsX302 was also transformed into *Agrobacterium* strain *LBA*4404 to transform it into potato. Optimization of medium for the two potato varieties was performed and a hormone combination of 6-BA 2.0 mg/L + NAA 0.5 mg/L + GA_3 0.5 mg/L in MS medium was the best for Shepody and 6-BA 2.0 mg/L + NAA 0.5 mg/L + GA_3 0.5 mg/L in MS medium was the best for Favorita. Following this system, early potato transformation was successful and PCR detection of the NPTII gene and the PVX CP gene fragment showed positive results for all tested putative transgenic callus samples of both Shepody and Favorita varieties.

The study suggested that transient expression of a hairpin RNA-encoding vector constructed with a PVX CP conserved gene fragment induced high resistance to PVX inoculation. It was assumed that high gene silencing efficiency was triggered in the plants. It is predictable that transgenic potato plants transformed with this vector should also have high resistance to PVX. The construction strategy might also be applied in many crops to achieve the transgenic plants resistant to viral diseases.

REFERENCES

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T. and Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. **Science.** 232 (4751): 738-743.

Akashi, H., Miyagishi, M. and Taira, K. (2001). Suppression of gene expression by RNA interference in cultured plant cells. **Antisense Nucleic Acid Drug Dev**. 11: 359-367.

Angell, S. M., Davies, C. and Baulcombe, D. C. (1996). Cell-to-cell movement of Potato virus X is associated with a change in the size-exclusion limit of plasmodesmata in trichome cells of *Nicotiana clevelandii*. **Virology.** 216 (1): 197-201.

Bai, Y. F., Yang, H. C., Qu, L., Zheng, J., Zhang, J. P., Wang, M. Y., Xie, W., Zhou, X. M. and Wang, G. Y. (2007). Inverted repeat transgenic maize plants resistant to sugarcane mosaic virus. **Acta Agronomica Sinica.** 33 (6): 973-978.

Bai, Y. F.，Bai, D. M.，Zhang, W. F. and Tian, F. R. (2008). Cloning and sequencing of cDNA encoding coat protein for potato virus X. **Chin J Appl Environ Bio**. 14 (5): 599-603.

Barker, H., and Dale, M. F. B. (2006). Resistance to viruses in potato. Pages 341-366 in: *Natural Resistance Mechanisms of Plants to Viruses*. G. Loebenstein and J. P. Carr, eds. Springer, Dordrecht, the Netherlands.

Bass, B. L. (2000). Double-stranded RNA as a template for gene silencing. **Cell.** 101: 235-238.

Batten, J. S., Yoshinari, S. and Hemenway, C. (2003). Potato virus X: a model system for virus replication, movement and gene expression. **Mol Plant Pathol.** 4 (2): 125-131.

Baulcombe, D. C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. **Plant Mol Biol.** 32: 79-88.

Baulcombe, D.C. (2002). RNA silencing. **C Biol.** 12: 82-84.

Baum, K., Gröning, B. and Meier, I. (1997). Improved ballistic transient transformation conditions for tomato fruit allow identification of organ-specific contributions of I-box and G-box to the RBCS2 promoter activity. **Plant J**. 12 (2): 463-469.

Beachy, R. N., Loesch-Fries, S. and Tumer, N. E. (1990). Coat protein-mediated resistance against virus infection. **Annu Rev Phytopathol.** 28: 451-472.

Bendahmane, M., Szecsi, J., Chen, I., Berg, R. H., Beachy, R. N. (2002) Characterization of mutant tobacco mosaic virus coat protein that interferes with virus cell-to-cell movement. **Proc Natl Acad Sci USA.** 99 (6): 3645-3650.

Bercks, R. *CMI/AAB* (1972). Potato virus X, *Descriptions of Plant Viruses* **No. 4**, Commonwealth Agricultural Bureaux.

Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. **Nature**. 409: 363-366.

Bhaskar, P. B., Venkateshwaran M., Wu, L., Ané, J. M. and Jiang J. M. (2009). *Agrobacterium*-mediated transient gene expression and silencing: a rapid tool for functional gene assay in potato. **PLoS ONE**. 4 (6): e5812.

Blokland, R. V., Geest, V. D., Mol, J. M. and Kooter, J. M. (1994). Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. **Plant J**. 6: 861-877.

Bornke, F., U. S. and Biemelt, S. (2007). Potato. *Biotechnology in Agriculture and Forestry*. Springer. 59: 297.

Callaway, A., Giesman-Cookmeyer, D., Gillock, E. T., Sit, T. L. and Lommel, S. A. (2001). The multifunctional capsid proteins of plant RNA viruses. **Annu. Rev. Phytopathol.** 39: 419-460.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K., and Schaad, M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. **Plant Physiol.** 8: 1669-1681.

Chen, Y. K., Lohuis, D., Goldbach, R. and Prins, M. (2004). High frequency induction of RNA-mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs. **Mol. Breed.** 14 (3): 215-226.

Clark, M. F. and. Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. **J. Gen Virol.** 34: 475-483.

Cockerham, G. (1954). Strains of potato virus X. *Proceedings of the Second Conference of Potato Virus Disease*. Lisse-Wageningen. 89-92.

Cockerham G. (1970). Genetical studies on resistance to potato virus X and Y. **Heredity.** 25: 309-348.

Cogoni, C., Irelan, J. T., Schumacher, M., Schmidhauser, T. J., Selker, E. U., and Macino, G. (1996). Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. **EMBO J**. 15 (12): 3153-3163.

Cogoni, C. and Macino, G. (1997). Isolation of quelling-defective (*qde*) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. **Proc Natl Acad Sci USA**. 94 (19): 10233-10238.

Cruz, S. S. and Baulcombe, D. C. (1995). Analysis of potato virus X coat protein genes in relation to resistance conferred by the genes Nx, Nb and Rx1 of potato. **J Gen Virol.** 76: 2057-2061.

Cuozzo, M., O'Conell, M. K., Kaniewski, W., Fang, R. X., Chua, N. H. and Tumer, N. E. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. **Nature Biotech.** 6: 549-557.

Dieffenbach, C. W. and Dveksler, G. S. (1995). PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview. 287-292.

Elbashir, S. M., Lendeckel, W. and Tuschl, T. (2001a). RNA interference is mediated by 21- and 22-nucleotide RNAs. **Genes & Dev.** 15: 188-200.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001b). Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture. **Nature.** 411: 494-498.

Fedorkin, O. N., Solovyev, A. G., Yelina, N. E., Zamyatnin, A. A., Jr, Zinovkin, R. A., Makinen, K., Schiemann, J. and Morozov, S. Y. (2001). Cell-to-cell movement of potato virus X involves distinct functions of the coat protein. **J Gen Virol.** 82: 449-458.

Ferreira, S. A., Pitz, K. Y., Manshardt, R. Zee F., and Fitch, M., Gonsalves, D. (2002). Virus coat protein transgenic papaya provides practical control of papaya ringspot virus in Hawaii. **Plant Dis**. 86: 101-105.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. **Nature.** 391: 806-811.

Fitch, M. M. M., Manshardt, R. M., Gonsalves, D., Slightom, J. L. and Sanford, J. C. (1992). Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. **Nature Biotech.** 10: 1466-1472.

Fitchen, J. and Beachy, R. N. (1993). Genetically engineered protection against viruses in transgenic plants. **Annu Rev Microbiol**. 47: 739-763.

Goulden, M, Kohm, B., Cruz, S. S.， Kavanagh, T. and Baulcombe, D. C. (1993). A feature of the coat protein of potato virus X affects both induced virus resistance in potato and viral fitness. **Virology.** 197: 293-302.

Guo, S. and Kemphues, J. K. (1995). *par-1*, a gene required for establishing polarity in embryos, encodes a putative Ser/Thr kinase that is symmetrically disrupted. **Cell.** 81 (4): 611-620.

Hamilton, A. J., Brown, S., Han, Y. H., Ishizuka, M., Lowe, A., Solis, A. A. and Grierson, D. (1998). A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. **Plant J**. 15: 737-746.

Hamilton, A. J. and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. **Science**. 286: 950-952.

Hammond, S. M., Caudy A. A. and Hannon, G. J. (2001). Post-transcriptional gene silencing by double-stranded RNA. **Nature Rev Gen.** 2: 110-119.

Hannon, G. J. (2002). RNA Interference. **Nature.** 418: 244-251.

Heilersig, H. J. B., Loonen, A., Bergervoet, M., Wolters, A. M. A. and Visser, R. G. F. (2006). Post-transcriptional gene silencing of GBSSI in potato: effects of size and sequence of the inverted repeats. **Plant Mol Biol**. 60: 647-662.

Helliwell, C. and Waterhouse, P. (2003). Constructs and methods for high-throughput gene silencing in plants. **Methods.** 30: 289-295.

Howard, A. R., Heppler, M. L., Ju, H. J., Krishnamurthy, K., Payton. M. E. and Verchot-Lubicz, J. (2004). Potato virus X TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. **Virology.** 328 (2): 185-197.

Huisman, M. J., Linthorst, H. J. M., Bol, J. F. and Cornelissen, B. J. C. (1988). The complete nucleotide sequence of *Potato virus X* and its homologies at the amino acid level with various plus-stranded RNA viruses. **J Gen Virol**. 69: 1789-1798.

Ingelbrecht, I. L., Houdt, V. H., Montagu, M. V. and Depicker, A. (1994). Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. **Proc Natl Acad Sci USA.** 91(22): 10502-10506.

Ingelbrecht, V. I., Irvine, J. E., Mirkov, T. E. (1999). Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. **Plant Physiol.** 119: 1187-1198.

Jardak-Jamoussi, R., Winterhagen, P., Bouamama, B., Dubois, C., Mliki, A., Wetzel, T., Ghorbel, A. and Reustle, G. M. (2009). Development and evaluation of a GFLV inverted repeat construct for genetic transformation of grapevine. **Plant Cell Tiss Organ Cult.** 97: 187-196.

Kalantidis, K., Psaradakis, S., Tabler, M. and Tsagris, M. (2002). The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. **Mol Plant-Microbe Interact**. 15 (8): 826-833.

Kapila, J., Ryckea, R. D. and Montagu, M. V. (1997). An *Agrobacterium*-mediated transient gene expression system for intact leaves. **Plant Sci**. 124 (2): 227

Kavanagh, T., Goulden, M., Cruz, S. S., Chapman, S., Barker, I. and Baulcombe, D. (1992). Molecular analysis of a resistance breaking strain of potato virus X. **Virology.** 189: 609-617.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. **J Mol Evol**. 16 (2): 111-120.

Krol, A. R. V., Mur, L. A., Beld, M., Mol, J. N. I. and Stuitje, A. R. (1990). Flavanoid genes in petunia: addition of limited number of gene copies may lead to a suppression of gene expression. **Plant Cell.** 2: 291-299.

Levy, M., Rachmilevitch, S. and Abel, S. (2005). Transient *Agrobacterium*-mediated gene expression in the *Arabidopsis* hydroponics root system for subcellular localization studies. **Plant Mol Biol Rep**. 23: 179-184.

Lindbo, J. A. and Dougherty, W. G. (2005). Plant pathology and RNAi: a brief history. **Annu Rev Phytopathol.** 43:191-204.

Lipardi, C., Wei, Q. and Paterrson, B. M. (2001). RNAi as random degradation PCR: siRNA primers convert mRNA into dsRNA that are degraded to generate new siRNAs. **Cell.** 101: 297-307.

Lopez-Ochoa, L., Ramirez-Prado, J., and Hanley-Bowdoin, L (2006). Peptide aptamers that bind to a geminivirus replication protein interfere with viral replication in plant cells. **J Virol.** 80: 5841-5853.

Mandahar C. L. (2000). Plant viruses volume I structure and replication. CRC Press. 188-189.

Metzlaff, M., O'dell, M., Cluster, P. D. and Flavell, R. B. (1997). RNA-mediated RNA degradation and chalcone synthase A silencing in Petunia. **Cell.** 88: 845-854.

Napoli, C., Lemieux, C. and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans. **Plant Cell.** 2 (4): 279-289.

Nicola-Negri, E. D., Brunetti, A., Tavazza, M. and Ilardi, V. (2005). Hairpin RNA - mediated silencing of *Plum pox virus* P1 and HC-Pro genes for efficient and predictable resistance to the virus. **Transgenic Research.** 14: 989-994.

Niu, Q. W., Lin, S. S., Reyes, J. L., Chen, K. C., Wu, H. W. Yeh, S. D. and Chua, N. H. (2006). Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. **Nature Biotech.** 24: 1420-1428.

Osbourn, J. K., Watts, J. W., Beachy, R. N., and Wilson, T. M. (1989). Evidence that nucleocapsid disassembly and a later step in virus replication are inhibited in transgenic tobacco protoplasts expressing TMV coat protein. **Virology.** 172: 370-373.

Parish, S., Fleenor, J., Xu, S.Q., Mello, C. and Fire, A. (2000). Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. **Mol Cell.** 6: 1077-1087.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M. and Tepfer, M. (2008). Strategies for antiviral resistance in transgenic plants. **Mol Plant Path.** 9 (1): 73-83.

Qiu, Y. B., Tao, G., Huang, Y. H., Li, Q. K., Liu, Z. Y., Zhu, Y. and Zheng, S. L. (2009). Application of TC-RT-PCR in potato viruses identification and cloning of their coat protein genes. **Sw Chin J Agric Sci**. 22: 329-331.

Qu, F., Ren, T. and Morris, T. J. (2003). The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. **J Virol.** 77 (1): 511-522.

Rossi, L., Escudero, J., Hohn, B. and Tinland, B. (1993). Efficient and sensitive assay for T-DNA-dependent transient gene expression. **Plant Mol Biol Rep**. 11(3): 220-229.

Sawers, R. J., Farmer, P. R., Moffett, P., Brutnell, T. P. (2006). In planta transient expression as a system for genetic and biochemical analyses of chlorophyll biosynthesis. **Plant Methods.** 2: 15.

Schöb, H., Kunz, C. and Meins, J. F. (1997). Silencing of transgenes introduced into leaves by agroinfiltration: a simple, rapid method for investigating sequence requirements for gene silencing. **Mol and Gen Genet**. 256 (5): 581-585.

Schwartz, D. S., Hutvagner, G., Haley, B. and Zamore, P. D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human pathways. **Mol Cell.** 10: 537-548.

Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G. and Waterhouse, P. M. (2000). Total silencing by intron spliced hairpin RNA. **Nature.** 407: 319-320.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. **Cell.** 107: 465-476.

Solomon-Bloackbum, R. M. and Barker, H. (2001). A review of host major-gene resistance to potato virus X, Y, A and V in potato: genes, genetics and mapped locations. **Heredity**. 86: 8-16.

Spolaore, S., Trainotti, L. and Casadoro, G. (2001). A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. **J Experi Bot**. 52 (357): 845-850.

Sutic, D. D., Ford, R. E. and Tosic, M. T. (1999). Handbook of plant virus diseases. **CRC Press**. 255.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. **Mol Biol Evol.** 24: 1596-1599.

Thomas, C. L., Leh, V., Lederer, C. and Maule, A. J. (2003). Turnip crinkle virus coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana.* **Virology.** 306: 33-41.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. **Nucleic Acids Research.** 25: 4876-4882.

Tijsterman, M., Ketting, R. F., Okihara, K. L., Sijen, T. and Plasterk, R. H. A. (2002). RNA helicase MUT-14-dependent gene silencing triggered in *C. elegans* by short antisense RNAs. **Science.** 295: 694–697.

Tricoll, D. M., Carney, K. J., Russell, P. F., Groff, D. W., Hadden, K. C., Himmel, P. T., Hubbard, J. P., Boeshore, M. L. and Quemada, H. D. (1995). Field evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to Cucumber Mosaic Virus, Watermelon Mosaic Virus, and Zucchini Yellow Mosaic Virus. **Nature Biotech.** 13: 1458-1465.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. and Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. **Genes Dev.** 13: 3191-3197.

Van der Hoorn, R. A. L., Laurent, F., Roth, R., and De Wit, P. J. G. M. (2000). Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. **Mol Plant-Microbe Interactions**. 13 (4): 439-446.

Vance, V. and Vaucheret, H. (2001). RNA Silencing in plants-defense and counterdefense. **Science.** 292: 2277-2280.

Vargas, M., Martinez-Garcia, B., Diaz-Ruiz, J. R. and Tenllado, F. (2008). Transient expression of homologous hairpin RNA interferes with PVY transmission by aphids. **Virol J**. 5, 42.

Verchot-Lubicz, J., Ye, C. M. and Bamunusinghe, D. (2007). Molecular biology of potexviruses: recent advances. **J Gen Virol.** 88: 1643-1655.

Wang, M. B., Abbott, D. C., Waterhouse, P. M. (2000). A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. **Mol Plant Pathol.** 1 (6): 347-356.

Waterhouse, P. M., Graham, M. and Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. **PNAS.** 95 (23): 13959-13964.

Weigel, D. and Glazebrook, J. (2002). *Arabidopsis*: a laboratory manual. **Cold Spring Harbor Laboratory Press**.

Wu, J. X. and Zhou, X. P. (2005). Production and application of monoclonal antibodies against Potato virus X. **J Zhejing Univ (Agric and Life Sci)**. 31 (5): 608-612.

Yang, Y. N., Li, R. G., and Qi, M. (2000). In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. **Plant J**. 22 (6): 543-551

Yu, X. Q., Jia, J. L., Zhang, C. L., Li, X. D. and Wang, Y. J. (2010). Phylogenetic analyses of an isolate obtained from potato in 1985 revealed potato virus X was introduced to China via multiple events. **Virus Gene.** 3 (40): 447-451.

Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA 21- to 23-nucleotide intervals. **Cell.** 101: 25-33.

Zamore, P. D. (2001). Thirty-three years later, a glimpse at the ribonuclease III active site. **Mol. Cell.** 8: 1158-1160.

Zheng, S. L., and Liu, Z. Y. (2006). DAS-ELISA detection for three potato viruses of Guizhou. **J Guizhou Agric Sci**. 6 (34): 42-44.

Zhu, X. P., Liu, J. L., Tian, Y. P., Yu, X. Q., Li, X. D. and Liu, H. M. (2007). Comparison of RNA silencing inducing efficiency of three PVX CP gene constructs via transient Expression. **Chin J Appl Environ Bio**. 13 (1): 1-4.

Zottini, M., Barizza, E., Costa, A., Formentin E., Ruberti, C., Carimi F. and Schiavo F. L. (2008). Agroinfiltration of grapevine leaves for fast transient assays of gene expression and for long-term production of stable transformed cells. **Plant Cell Rep**. 27 (5): 845-853.

APPENDICES

APPENDIX A

Media

1. 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.

2. 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.

3. 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 121^{o}C 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.

Table 1. Stock solution in MS.

Stock solution 1 （100×）：	**Stock concentration (mg/L)**	**Work concentration (mg/L)**
NH_4NO_3	82,500	1,650
KNO_3	95,000	1,900
$MgSO_4·7H_2O$	18,500	370
$CaCl_2·2H_2O$	88,000	880
KH_2PO_4	17,000	170
Stock solution 2 （100×）： Na_2-EDTA	3,730	37.3
$FeSO_4·7H_2O$	2,780	27.8
Stock solution 3 (100×)		
H_3BO_3	620	6.2
$MnSO_4·H_2O$	1,690	16.9
$ZnSO_4·7H_2O$	860	8.6
KI	83	0.83
$Na_2MoO_4·2H_2O$	25	0.25
$CuSO_4·5H_2O$	0.25	0.025
$CoCl_2·6H_2O$	0.25	0.025
Stock solution 4 （100×）：		
Inosotol	10,000	100
Glycine	200	2
Nicotinic Acid	50	0.5
Pyridoxine HCl	50	0.5
Thiamine HCl	10	0.1

APPENDIX B

Solution preparation

1. Antibiotics

Antibiotics referred in this thesis are Kanamycin, Spectinomycin, Carbenicillin and Rifampicin. The concentration is shown in table 2.

Table 2. The antibiotics stock and working concentration.

Antibiotic	Stock concentration	Working concentration
Kanamycin	100 mg/L in sterile water	75 μg/L
Spectinomycin	100 mg/L in sterile water	100 μg/L
Carbenicilin	400 mg/L in sterile water	400 μg/L
Rifampicin	50 mg/L in Methanol	50 μg /L

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

2. Hormones and AS

2.1 NAA, 2,4-D and GA_3

Dissolve 100 mg NAA, 2, 4-D or GA_3 in 20 ml 95% ethanol. Add distilled water to the volume of 100 ml and store the solution at 4^{o}C.

2.2 6-BA

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.

2.3 ZT

Dissolve 100 mg ZT (Zeatin) in 20 ml ethanol. Add distilled water to the volume of 100 ml. Filter sterilize the solution. Dispense the solution into aliquots and store them at -20 ^{o}C.

2.4 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 at -20 ^{o}C.

3. Solution for bacteria transformation

3.1 IPTG stock solution

Dissolve 0.12 g IPTG in 5 ml distilled water. Filter sterilize the solution. Dispense the solution into aliquots and store them at -20 ^{o}C.

3.2 X-gal stock solution

Dissolve 50 mg X-gal in 250 ml dimethylformamide. Dispense the solution into aliquots. Wrap the tubes with aluminum foil and store them at -20°C.

4. Solution for TC-RT-PCR

4.1 DEPC treated water

Add 1 ml DEPC in distilled water and adjust the volume to 1L with more distilled water. Shake the solution vigorously to mix thoroughly. Keep it overnight and autoclave for 15 min. Store the solution at room temperature.

4.2 Grinding buffer (PBS+2% PVP)

Dissolve 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 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.

4.3 Coating buffer

Dissolve 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3 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 ^oC.

5. Solution for DAS-ELISA

5.1 Rinse buffer (PBST)

Dissolve 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 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 ^oC.

5.2 Conjugate buffer

Dissolve 0.2 g BSA in 100 ml sterile PBST buffer. Store the solution at 4 ^oC.

5.3 Substrate buffer (DEA buffer)

Dissolve 9.039 g diethanolamine, 0.01 g $MgCl_2 \cdot 6H_2O$ and 1.982 g diethano-lamine-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.

6. Solution for Agarose gel electrophoresis

50×TAE buffer

Mix 242 g Tris base, 57.1 ml glacial acetic acid and 0.5 M EDTA (pH 8.0). Make up to 1 L with distilled water. Store the solution at room temperature.

7. Solution for DNA extraction

7.1 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 ^{o}C 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.

7.2 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.

7.3 RNase A

Dissolve 50 μg RNase A in 1 ml 10 mM Tris-HCl (pH 7.4), 15 mM NaCl buffer (sterile). Store the aliquots at -20 ^{o}C.

7.4 0.5 M EDTA

Dissolve $Na_2EDTA{\cdot}H_2O$ 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.

7.5 1 M Tris (pH 7.4/8.0)

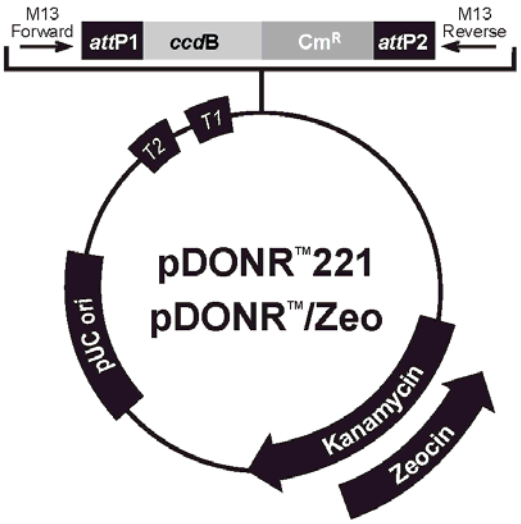
Dissolve 121.1 g Tris Base in 800 ml distilled water. Adjust the volume to 1 L with distilled water and the pH to 7.4/8.0 with HCl. Sterilize the solution by autoclave and store it at room temperature.

7.6 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.

APPENDIX C

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

Figure 1. The map of pDONR221 (Invitrogen).

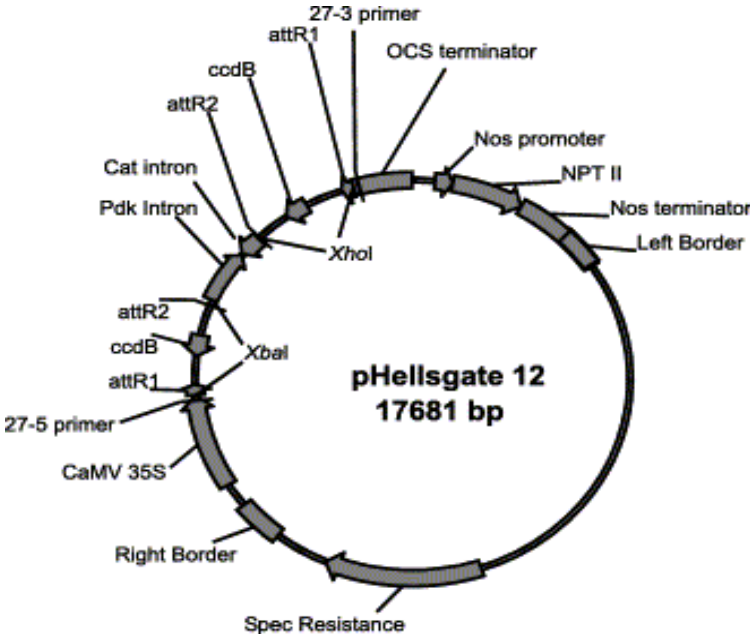


Figure 2. The map of pHellsgate 12 (CSIRO).

BIOGRAPHY

Yonghui Huang was born on May 8th, 1981 in Guizhou province, the People's Republic of China. In 2004, she graduated with a bachelor degree in Agriculture from Institute of Food Science, Southwest Agricultural University (presently Southwest University), Chongqing, China. Since graduation she had been working in Institute of Biotechnology, Guizhou Academy of Agricultural Sciences, Guizhou, China. In 2006, she got the opportunity to study master degree under the supervision of Asst. Prof. Dr. Mariena Ketudat-Cairns in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.