SCREENING FOR *Papaya ringspot virus* (PRSV) RESISTANCE AND ANALYSIS OF TRANSGENES IN TRANSGENIC PAPAYAS

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the most economic fruit grown wildly in different geographical areas in Thailand. It is largely consumed as fresh dessert fruit and green fruit as salad, called Somtum, a popular food of rural communities in Thailand. However, production of papaya in many cultivated areas have been limited by the destructive disease caused by *Papaya ringspot virus* (PRSV). Papaya ringspot disease is rapidly transmitted by numerous species of aphid in a non-persistent manner. PRSV infected plants develop various symptoms such as mosaic and chlorosis of leaf lamina, water-soaked oily streaks on the petiole and upper part of the trunk, and distortion of young leaves (Gonsalves, 1998). Spreading of PRSV is difficult to control by conventional methods, such as rouging, chemical spray for aphid control, planting with naturally resistant papaya and cross protection with a mild strain of PRSV. Consequently, efforts to develop virus resistant papaya have been focusing on genetic manipulation via genetic transformation techniques to insert part of viral genome into papaya genome. This practice was developed from concept of pathogen-derived resistance (Sanford and Johnston, 1985) that exploited PRSV-derived gene as a transgene for papaya transformation. PRSV-derived genes used for papaya transformation were coat protein (*CP*) gene (Gonsalves, 1998; Ferreira *et al*., 2002) and nuclear inclusion b (*NIb*) gene (Chen *et al*., 2001). Gene delivery techniques have been successfully applied via particle bombardment (Gonsalves, 1998; Ferreira *et al*., 2002) and *Agrobacterium*-mediated transformation (Chen *et al*., 2001; Davis and Ying, 2004) to produce transgenic papaya resistant to PRSV. In Thailand, Chowpongpang *et al*. (2002) successfully transformed Thai papaya, Khak Nual cultivar via particle bombardment and subsequently obtained transgenic papaya with CP gene of PRSV, Chiang Mai (PRSV-CM) isolate. The resistant lines KN1.2.3, KN13.2.3 and KN49 were resistant to PRSV-CM isolate and other isolates from different geographic regions in Thailand.

Biosafety concerning the release of genetically modified crops requires comprehensive analysis of inserted genes in plant genome in order to confirm that they do not encode any harmful substances and safe to environment and living organisms. Molecular characterization of the inserted genes must be documented, including size, number of insertion, T-DNA flanking sequences, stability and expression of inserted gene. In addition, studies of T-DNA flanking sequences have been investigated in other transgenic plants, such as rice (Kim *et al*., 2003; Zhai *et al*., 2004), *Arabidopsis thaliana* (Liu *et al*., 1995; Graaff *et al*., 1996) potato (Cottage *et al*., 2001) and tobacco (Zhou *et al*., 1997), but it has not been reported in transgenic papaya. The finding is necessary to study of T-DNA integration (Wang *et al*., 2005), insertion mutation (Sha *et al*., 2004), location of inserted genes in plant cells (Cottage *et al*., 2001) and beneficial in selecting plants for breeding program (Zhai *et al*., 2004). Recently, Cottage and colleagues (2001) successfully determined the T-DNA flanking sequences in transgenic potato and transgenic tobacco. The result of blast analysis showed that the insertional event was into a chromosomal locus and did not disrupt any known genes or ORFs. T-DNA flanking sequences can be analyzed by PCR-based methods, such as Thermal Asymmetric Interlaced PCR (TAIL-PCR) (Liu *et al*., 1995), High-efficiency Thermal Asymmetric Interlaced (HE-TAIL) PCR (Michiels *et al*., 2003), Inverse PCR (IPCR) (Kim *et al*., 2003) and Adaptor Ligation-mediated PCR (Cottage *et al*., 2001).

The aim of this study was to analyze PRSV-derived transgenes in transgenic papaya resistant to PRSV. The following condition; number of insertion, transgene expression and T-DNA flanking sequences was evaluated to the PRSV resistance of transformed papaya in molecular level.

OBJECTIVE

1. To screen PRSV resistance in transgenic papaya, R_0 and R_1 generations, under glasshouse condition.
2. To conduct transgene analysis for the integration of PRSV-derived genes in papaya genome.
3. To identify T-DNA flanking sequences in PRSV resistant transgenic papaya.

LITERATURE REVIEW

1. Papaya

Papaya (*Carica papaya*), called Pawpaw in Australia, belongs to the *Caricaceae*, a family mainly inhabiting in South and Central America. It is a small, unbranched soft-wooded tree, dioecious plant depending on varieties or cultivars, some cultivars are monoecious, but hermaphrodite (bisexual) flowers and trees also occur. Different types of hermaphrodite flowers may occur on the same tree, depending on the seasons or on the age of tree. Fruits formed from female flowers are oblong to nearly spherical, while fruits derived from bisexual flowers are pear-shaped, cylindrical or grooved (Samson, 1986). Papaya is diploid with 9 pairs of chromosomes. It has a small genome of 372 Mb and a generation time as short as 9 months (Arumuganathan and Earle, 1991).

Ripe papayas are largely consumed as a fresh dessert and green fruit is used as Thai salad, called Somtum. The fruit contains about 85% water, 10-13% total sugar, 0.6 % protein, much vitamin A and fair amounts of vitamins B1, B2 and C (Samson, 1986). Papain from latex of unripe fruits is used as a meat tenderizer and a hydrolytic enzyme for medical and industrial purposes (Foyet, 1972). Moreover, methanol sub-fraction of seeds of *C. papaya* has been used as a putative male contraceptive in rats (Lohiya *et al*., 2006).

Papayas are classified into various cultivars, depending on geographical regions, such as Kamiya (Solo type) releasing from the University of Hawaii, Sunset (Sunset Solo) originated from the University of Hawaii and Vista Solo originated from Vista, California (Samson, 1986). In Thailand, Khak Dum and Khak Nual cultivars are preferred fruit in the markets of rural communities. They are hermaphrodite fruit with elongated shape and deep yellow-orange flesh in flavorful fruit and sweet.

For many years, production of papaya in Thailand has been lost by the destructive diseases especially viral disease. Papaya ringspot disease is a severe disease affecting papaya cultivation, due to a reduction on plant growth. Plants become stunting and drastic reductions of the fruit size and yield.

2. Potyviruses

Potyviruses are the largest of plant viral genus in the *Potyviridae* family, containing over 100 species which have flexuous particles, 680-900 nm long and 11-15 nm wide, non-envelope (Urcuqui-Inchima *et al*., 2001). Viral particles are transmitted by aphids in a non persistent manner. They infect a broad range of host plants, both monocots and dicots, resulting in a severe damage to economic crops. Their genomes compose of a positive sense single stranded RNA about 10 kb long, carrying a viral genome linked protein (VPg) covalently bound to its 5' UTR and a poly (A) tail at its 3' end. The genome contains a single long ORF translated into a large 340-370 kDa polyprotein that is subsequently cleaved by viral proteinases, P1, HC-Pro and NIa into at least seven functional proteins (Figure 1 and Table 1). The best known members of potyvirus group are *Tobacco etch virus* (TEV), *Papaya ringspot virus* (PRSV) and *Potato virus Y* (PVY) (Adams *et al*., 2005).

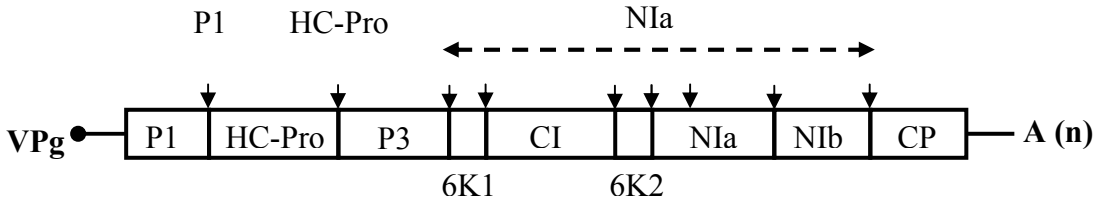


Figure 1 Organization of *Potyviru*s genome. The functional proteins are boxed. The position of cleave sites of three proteinases, P1, HC-Pro and NIa are indicated at the arrows.

Source: Urcuqui-Inchima *et al*. (2001)

Table 1 Properties of the different *Potyvirus* proteins

Proteins	Properties
P1 (32-64 K)	Trypsin-like serine proteinase, C-terminal autocleavage, Symptomatology
HC-Pro (56-58 K)	Aphid transmission, Self-interaction, Systemic movement, Suppression of gene silencing, Synergism and symptom development, Papain-like cysteine proteinase, C-terminal autocleavage
P3 (37 K)	Plant pathogenicity
6K1	?
CI (70 K)	ATPase/RNA helicase, Cell-to-cell movement
6K2	Anchoring the viral replication complex to membranes
NIa (49 K)	Cellular localization, VPg involved in genome replication, Trypsin-like serine proteinase, acts in *cis* and in *trans*, Protein-protein interaction
NIb (58 K)	RNA-dependent RNA polymerase (RdRp), Involved in genome replication
CP (28-40 K)	Aphid transmission, Cell-to-cell and systemic movement, Virus assembly

Source: Urcuqui-Inchima *et al*. (2001)

3. *Papaya ringspot virus* (PRSV)

Papaya ringspot virus (PRSV) was first detected in Thailand in 1975 and is now widespread throughout all papaya plantations in many countries. PRSV is a member of *Potyvirus* genus in the family of *Potyviridae* which has flexuous and filamentous particle of 780 × 12 nm (Figure 2D). The virion contains a positive-sense of single-stranded RNA that encapsulated by a coat protein (CP), approximately 36 kDa in size (Gonsalves and Ishii, 1980). The viral genome contains only one open

reading frame (ORF), which encodes a polyprotein of about 330 kDa that is subsequently cleaved into at least seven functional proteins by three virus-encoded proteinases, P1, HC-Pro and NIa (Figure 1). The properties of these functional proteins are summarized in Table 1 (Urcuqui-Inchima *et al*., 2001). PRSV infected papayas developed various symptoms such as ringspot on fruit (Figure 2A), mottling and distortion of leaves (Figure 2B) and water-soaked oily streaks on stem (Figure 2C). PRSV is transmitted by numerous species of aphid such as *Myzus persicae*, *Aphis gossypii* and *Aphis craccivora* in a non-persistent manner. PRSV is grouped into two biotypes, PRSV-P infects cucurbits and papaya whereas PRSV-W infects only cucurbits (Gonsalves, 1998).

PRSV-P causes serious problems in papaya industry throughout geographical regions in Thailand such as Khon Kaen, Yasothorn, Suphan Buri, Nakhon Pathom and Surat Thani. In the past, efforts to control the viral disease were rouging, chemical spray for aphid control and breeding for resistant cultivars. However, these efforts could not solve the problems because aphid transmission was rapidly widespread (30 sec for transmission) and there were not any insecticides for aphid elimination. In 1986, research efforts were began to overcome the viral disease by cross protection, the phenomenon that plants were systemically infected with a mild strain of PRSV and were protected against the second infection by the virulent related strains (Kositratana *et al*., 1991). This practice has been long known and used to control citrus tristeza, tobacco mosaic virus and zucchini yellow mosaic virus. There were two mild strains of PRSV, PRSV HA5-1 and PRSV HA6-1, were selected, following nitrous acid treatment of leaf extracts of squash infected with PRSV-HA, a severe strain from Hawaii that was previously characterized. These strains were mild on papaya and showed the protection against PRSV-HA under greenhouse condition. However, the cross protection technique has not been successfully used for control of PRSV in other geographical areas. For several reasons, cross protection requires extra cultural management and care, limitation of related PRSV mild strains and mild strain can cause symptoms and mutate to a severe strain (Gonsalves, 1998).

Molecular basis of PRSV-P originally isolated from different geographic locations in Thailand have been reported. Genetic organization of PRSV-P is similar to other potyviruses with exception that P1 protein is 18 kDa to 34 kDa larger than those of all reported potyviruses (Wang and Yeh, 1997). The complete nucleotide sequence of PRSV has highly conserved region in the 5′ leader sequence, a region as the binding of VPg, the assembly initiation site of viral particle, or the polymerase recognition signal for RNA replication. Among ten putative proteins, P1 protein was the most variable (73.9% similarity) when compared to the other full PRSV sequences, while CI protein was the most conserved protein (99.1% similarity) (Charoensilp *et al*., 2003).

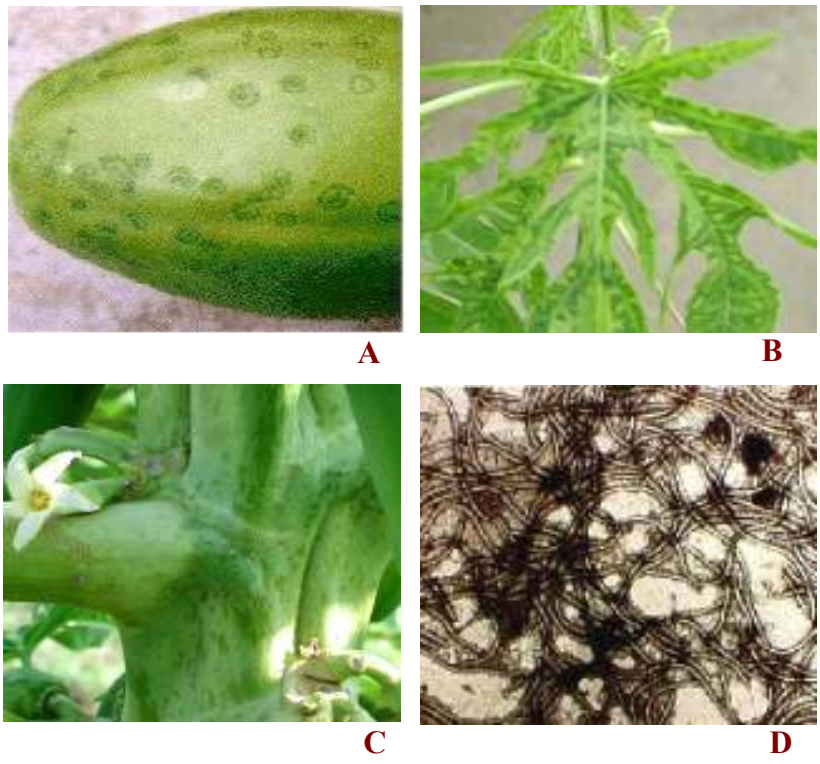


Figure 2 PRSV infected papaya developed various symptoms. Ringspot and streaking on fruit (A), mottling and distortion of leaves (B), water-soaked oily streaks on stem (C) and flexuous viral particles (D).

Source: Chowpongpang *et al*. (2002)

4. Replication of PRSV genome

Replication of (+) single stranded RNA viruses was separated into four overlapping steps: (i) uncoating of the virus, which exposes nucleic acid to the replication processes. (ii) translation, with the RNA of PRSV serves as a messenger RNA (mRNA), produces structural and non-structural proteins such as the viral proteinases (P1, HC-Pro, NIa), RNA dependent RNA polymerase (RdRp) for genome replication and coat protein for encapsidation. In this step, PRSV has a long ORF which is translated and then cleaved into smaller functional proteins by the viral proteinase. (iii) replication of the genome which yields progeny RNA molecules takes place in two stages, both catalysed by an RdRp: (1) synthesis of a full length complementary (negative) RNA strand using genomic (positive) RNA strand as a template. (2) synthesis of progeny genomic RNA using the negative strand RNA as a template. Finally (iv) the progeny genomic strands are encapsidated (Bustamante and Hull., 1998) (Figure 3).

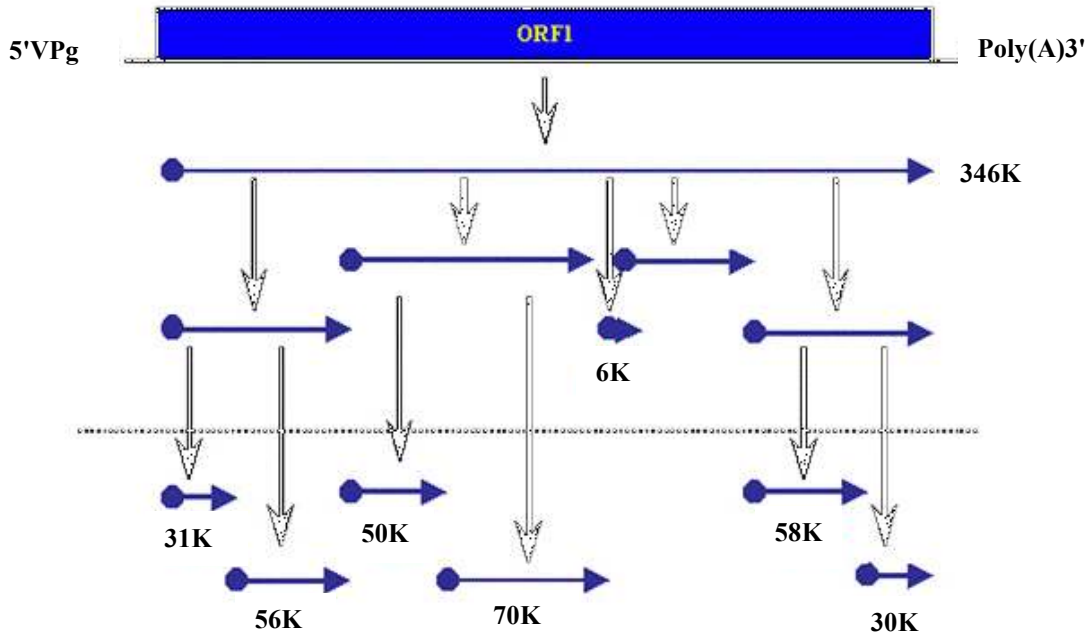


Figure 3 Organization and expression of a *Potyvirus* gemone (Polyprotein strategy). The functional proteins: 31K, cell to cell movement; 58K, proteinase and insect transmission; 50K, proteinase; 70K, replication; 6K?; 49K, proteinase; 58K, polymerase; 30K, coat protein.

Source: Bustamante and Hull (1998)

5. Generation of transgenic plants

Generation of transgenic plants have been successfully carried out via *Agrobacterium tumefaciens* and particle bombardment. In this study, we focused on the use of *A. tumefaciens* to transfer foreign genes into plant genomes. It was evident that low copy number of inserted genes, especially one copy, was found in most of *Agrobacterium*-transformed plants (Sha *et al*., 2004).

Agrobacterium is a species of phytopathogens, gram negative bacteria, and cause crowngall and hairy root diseases. There are two strains of *Agrobacterium*, *A. tumefaciens* and *A. rhizogenes* which cause crowngall and hairy root diseases, respectively. The molecular biology of infection by *A. tumefaciens* is the best understood which are preferably used as a gene delivery for plant transformation. During infection, the bacterium transfer a plasmid DNA of its own genetic material (T-DNA) into the genome of the host plant's cell. *A. tumefaciens* carries tumor-inducing or Ti plasmid whereas *A. rhizogenes* contains root-inducing or Ri plasmid, both plasmids are about 200 kb in size (Webb and Morris, 1994). The Ti plasmid carries two components needed for genetic transformation, *vir* gene and T-DNA regions (Figure 4a). The T-DNA region is flanked by 25 bp repeated sequences which are recognized by the endonucleases encoded by the *vir* genes (Figure 4b). The bacterial endonucleases are VirD1 and VirD2, both proteins act together as an site-specific endonuclease which nick between the third and the fourth nucleotides of each of the border sequences and subsequently released as single-stranded (ss) DNA molecule (T-strand) with a single VirD2 molecule attached to its 5′-end (Figure 4c). This mobile complex containing the residual sequences of the T-DNA borders can be imported into the plant cell and integrated into the plant's DNA. The residual sequences of the T-DNA borders are often used as reference points for T-DNA orientation and integrity in the plant cell. The integration of T-DNA in the plant DNA are classified in three type: (i) a single full-length T-DNA integration (Figure 4d) (ii) truncated T-DNA integration (Figure 4e) and (iii) multiple T-DNAs integration (Figure 4f) (Tzfira *et al*., 2004).

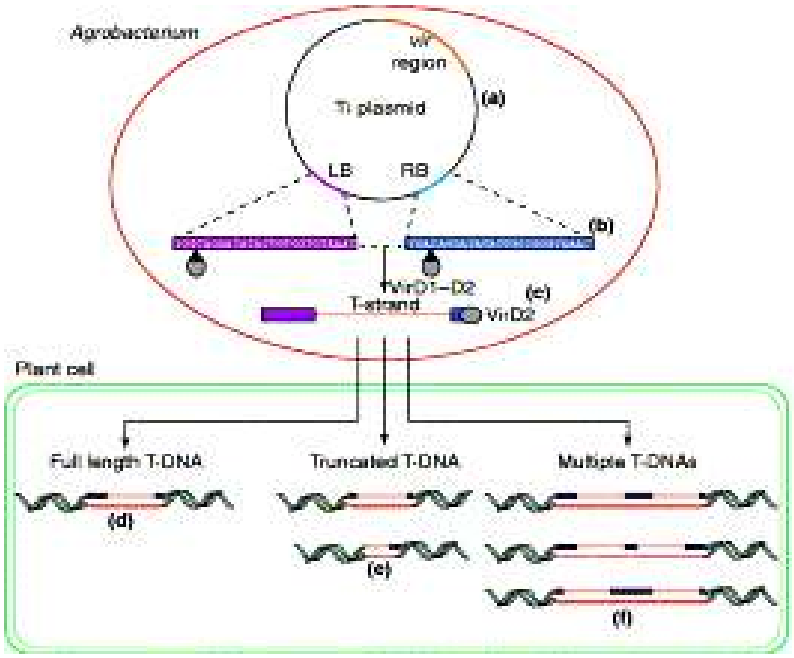


Figure 4 Schematic structure of the *Agrobacterium* Ti-plasmid and orientation of T-DNA integration in plant DNA. The structure of Ti plasmid (a), 25 bp repeated sequences of T-DNA borders (LB: left border, RB: right border) (b), the mobile complex of single-stranded (ss) DNA molecule (T-strand) with a single VirD2 protein attached to its 5′-end (c). The typical T-DNA integration in plant DNA: a single full length T-DNA integration (d), truncated T-DNA integration (e), multiple T-DNAs integration (f).

Source: Tzfira *et al*. (2004)

5.1 T-DNA integration in transgenic plants

Genetically modified crops, including transgenic papaya require the analysis of inserted DNA in plant's genomes. Molecular characterization of the inserted genes have been documented, such as number of insertion, T-DNA integration mechanism and T-DNA flanking sequences (Wang *et al*., 2005). These findings are considered as biosafety concerns in order to release commercialized transgenic crops. Zhai and colleague (2004) reported the T-DNA flanking sequences in *Agrobacterium*-transformed rice with the transgene *Xa21*, a bacterial blight (BB) resistance gene. They classified these sequences into three types on the basis of the nature of their sequences, type A sequences were integrated with the definite left (LB) and right borders (RB), type B sequences were linked with the adjacent vector backbone sequences and type C sequences were complex recombination. Recently, Wang *et al*. (2005) reported the T-DNA flanking sequences in *Agrobacterium*-transformed rice in order to study mechanism of T-DNA integration. They found that the integrated ends of the T-DNA right border mainly occurred on five nucleotides "TGACA" in inverse repeat (IR) sequence of 25 bp, especially on the third base "A". For PRSV resistant transgenic papaya lines, T-DNA flanking sequences have not been reported because the whole plant genome is currently under the process of sequencing.

The characterization of T-DNA integration in *Arabidopsis thaliana* was investigated by Brunaud *et al*. (2002). They analyzed the statistic of 9,000 flanking sequence tags (FST) characterizing T-DNA transformants in *A*. *thaliana* in order to determine the sequence specificity of T-DNA integration sites (IS) and integration process. The result revealed that the FST distribution in the genome of *A*. *thaliana* was even throughout the five chromosomes, especially about 40% of integrations were in genes with more frequently found in introns than exons. Moreover, T-DNA integration was favoured in plant DNA regions with an A-T rich content. However, the T-DNA insertions within gene sequence were more frequently occurred in the

position of 5'-upstream region while insertions in coding sequences (exons and introns) were less frequently found (Forsbach *et al*., 2003).

5.2 Strategies for analysis of T-DNA flanking sequences

T-DNA flanking sequences can be analyzed by using PCR base method, such as Thermal Asymmetric Interlaced PCR (TAIL-PCR) (Liu *et al*., 1995), High-Efficiency Thermal Asymmetric Interlaced (HE-TAIL) PCR (Michiels *et al*., 2003), Inverse PCR (IPCR) (Kim *et al*., 2003) and Adaptor Ligation- mediated PCR (Cottage *et al*., 2001).

5.2.1 Thermal Asymmetric Interlaced (TAIL) PCR

TAIL-PCR strategy was developed by Liu and Whittier for amplifying insert ends from yeast artificial chromosome (YAC) and P1 clone or for mapping *Arabidopsis thaliana* T-DNA insert junctions (Liu *et al*., 1995). This strategy uses nested sequence-specific primers together with 16-bp degenerated primers in a multi-step thermal cycling program. The procedure consists of an alternation of low-stringency and high-stringency cycling to allow amplification of gene-specific flanking regions. Most of the amplified products range from 300 bp to 2 kb (Sha *et al*., 2004). This technique can be applied to highly complex genomes, such as rice (Sha *et al*., 2004; Zhai *et al*., 2004), but amplification efficiency is lower, and amplified fragment lengths rarely exceed 700 bp.

5.2.2 High-Efficiency Thermal Asymmetric Interlaced (HE-TAIL) PCR

HE-TAIL PCR strategy is a modified Thermal Asymmetric Interlaced (TAIL) PCR which uses the design of long gene specific primers. This method was developed by Michiels *et al*. (2003) to determine the unknown genomic DNA sequences adjacent to known sequences in GC-rich plant DNA, such as *C. intybus* L. and isolating the FEH IIa gene promoter. Moreover, it is suitable for sequence

walking from short known sequences, such as sequence-tagged site (STS), expressed sequence tags (EST), or short exon sequences, and enable to clone full-length open reading frames (ORFs) without library screening (Michiels *et al*., 2003).

5.2.3 Inverse PCR (IPCR)

Inverse PCR is an efficient method for determining unknown genomic DNA sequences adjacent to known sequences (Kim *et al*., 2003). Genomic DNA is digested with restriction enzymes yielding sticky-ended fragments that are religated to be circularized and served as a template for PCR amplification. The specific back to back primers are designed from the known sequences of the ligated region. This method can be applied to determine T-DNA flanking sequence in *Agrobacterium*-transformed rice (Kim *et al*., 2003).

5.2.4 Adaptor Ligation-mediated PCR

Adaptor Ligation-mediated PCR strategy was modified by Cottage *et al*. (2001). This method requires the digestion with restriction enzymes yielding blunt-ended fragments that are ligated to asymmetric adaptors. The specific primers are designed from the known sequence of an adaptor that combined with specific primers designed for the T-DNA. Amplified fragments are high yielding which ranged from 500 bp to 2 kb. However, this method requires various restriction enzymes to create the largest fragment.

In this study, we applied an efficient method base on the strategies of inverse PCR and long-PCR for amplification of T-DNA flanking sequence in PRSV resistant transgenic papaya.

6. Development of transgenic papaya with viral resistance

Since Powell-Abel *et al*. (1986) provided the first demonstration that transgenic plants expressing the viral coat protein (*CP*) gene exhibit resistance to infection with that virus. Attempts to achieve viral resistance in transgenic papayas have been focused on genetic transformation with PRSV derived transgenes. The PRSV derived transgenes successfully used for papaya transformation were *CP* gene (Gonsalves, 1998; Ferreira *et al*., 2002) and nuclear inclusion b (*NIb*) gene (Chen *et al*., 2001).

6.1 Coat protein (CP)

The *CP* gene of PRSV isolates from different geographic areas in Thailand have been sequenced and published in GenBank database (NCBI). It can be roughly divided into three domains, the variable N- and C- terminal domains that are exposed on surface of particle and are sensitive to mild trypsin treatment, and the more conserved central or core domain (Figure 5). The N-terminal domain contains DAG motif that highly conserved among *Potyviruses*. The DAG motif involved in aphid transmission process, based on the result of point mutation changing amino acids which cause a reduction on transmissibility. The core domain of CP is responsible for viral RNA encapsidation. The CP closely interacts with RNA interior of virion and forms intersubunit contacts necessary for assembly and stability of particle (Urcuqui-Inchima *et al*., 2001). In addition, the CP can be interact with other proteins: (i) interacts with movement protein (MP) to assist cell-to-cell spread and systemic movement of the virus (Dolja *et al*., 1994). (ii) interacts with the NIb to regulation of viral RNA synthesis (Hong *et al*., 1995).

For genetically engineered viral resistant plants, the *CP* gene has been cloned and subsequently constructed into plant expression vectors for papaya transformation. The use of *CP* gene as a transgens have been clearly shown in many research groups (Chiang *et al*., 2001; Ferreira *et al*., 2002; Davis *et al*., 2004). Davis *et al*. (2004)

investigated the efficiency of PRSV resistance in transgenic papaya harboring either unmodified or modified *CP* gene of PRSV Florida isolate (PRSV H1K). The *CP* genes used in this group were in the sense orientation (S-CP), anti-sense orientation (AS-CP), sense orientation with a frame-shift mutation (FS-CP), or sense orientation mutated with three-in-frame stop codons (SC-CP). Transgenic papaya lines derived from each *CP* transgene showed highly resistance to PRSV H1K. In fact, the virus coat protein is linked to a strong promoter (35S CaMV promoter) and is stably integrated into the genome of transgenic plants by *Agrobacterium*-mediated transformation. Consequently, transgenic plants express the viral coat protein which confer viral resistance, that is called coat protein-mediated resistance (CPMR) (Reavy and Mayo, 1994) (Table 2). In this mechanism suggested that the CP of PRSV may play an important role for inhibiting viral infection processes, such as uncoating of viral RNA or inhibition of the process of viral replication (Urcuqui-Inchima *et al*., 2001). However, transgenic susceptible lines also expressed the CP, but the viral resistance did not correlate with the expression level (Bau *et al*., 2003).

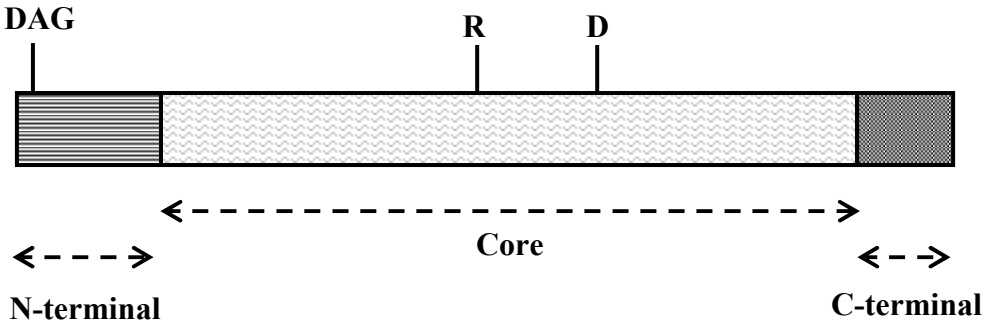


Figure 5 Schematic representation of CP structure and the position of the conserved DAG motif. R and D are conserved amino acids of the core region.

Source: Urcuqui-Inchima *et al*. (2001)

Table 2 Examples of coat protein-mediated resistance to virus infection

Source of coat protein gene	Plant transformed	Virus resistance is exhibited to
TMV	Tobacco	TMV
TMV	Tobacco	ToMV, TMGMV
TMV	Tobacco	PVX, CMV, AIMV, SHMV
TMV	Tobacco	TMV, ToMV
AIMV	Tobacco	AIMV
AIMV	Tobacco	PVX, CMV
AIMV	Tomato	AIMV
AIMV	Alfalfa	AIMV
TRV	Tobacco	TRV
TRV	Tobacco	PEBV
TSV	Tobacco	TSV
CMV	Tobacco	CMV
SMV	Tobacco	PVY, TEV
BNYVV	Sugarbeet	BNYVV
PVX	Tobacco	PVX
PVX	Potato	PVX
PVX + PVY	Potato	PVX, PVY

Table 2 (continued)

Source of coat protein gene	Plant transformed	Virus resistance is exhibited to
PVS	Potato	PVS
PLRV	Potato	PLRV
GCMV	Tobacco	TBRV
PaRSV	Tobacco	TEV
TVMV	Tobacco	TVMV, TEV

Abbreviations for virus names: AIMV = alfalfa mosaic virus; BNYVV = beet necrotic yellow vein virus; CMV = cucumber mosaic virus; GCMV = grapevine chrome mosaic virus; PaRSV = papaya ringspot virus; PLRV = potato leafroll virus; PVS = potato virus S; PVX = potato virus X; PVY = potato virus Y; SHMV = sunn-hemp mosaic virus; SMV = soyabean mosaic virus; TBRV = tomato black ring virus; TEV = tobacco etch virus; TMV = tobacco mosaic virus; ToMV = tomato mosaic virus; TRV = tobacco rattle virus; TSV = tobacco streak virus; TVMV = tobacco vein mottling virus.

Source: Reavy and Mayo (1994)

6.2 Nuclear inclusion b (*NIb*) gene

The nuclear inclusion b (*NIb*) gene of *Potyvirus*es encodes the RdRp, as it carries the GDD sequence, the hallmark of RdRp and is responsible for replicase activity. It can interact with the *CP* gene to stimulate genome replication, based on the observations that mutations in the highly conserved GDD motif of NIb confer regulation of viral RNA synthesis (Hong *et al*., 1995). The *NIb* gene is one of the most applicable transgene for production of virus resistant plants. In this case, virus resistant plant was first shown in transgenic tobacco against TMV which contained the 54 kDa putative *rep* gene (Dasgupta *et al*., 2003). Similar resistances have been developed for other viruses, such as pea seed-borne mosaic potyvirus (PSbMV) in transgenic pea (Jones *et al*., 1998), PRSV in transgenic papaya (Chen *et al*., 2001) and

Potato virus Y (PVY) in transgenic potato (Simon-Mateo *et al*., 2003). Chen *et al*. (2001) first reported the PRSV resistance in transgenic papaya lines containing the *NIb* gene of PRSV AL. They used a modified *NIb* gene, 3' truncated and 5' extended fragment, which contains ATG start and TAA stop codons for gene construction. This result was consistent to the resistance mediated by the *NIb* gene or partial *NIb* gene of Potato virus Y, which belongs to the same potyvirus group as PRSV. However, the expression of *NIb* transgene and the mechanism of resistance remains unknowed (Chen *et al.*, 2001).

MATERIALS AND METHODS

1. Transgenic plants

Transgenic papayas, Khak Dum cultivar used in this study, were obtained from *Agrobacterium*-mediated transformation with chimeric constructs of PRSV derived genes, conducted by Dr. Kanokwan Romyanon (BIOTEC, Thailand). For the production of transgenic papaya, a protocol developed by Horarung *et al*. (2006) was carried out. The plant expression vectors were obtained by cloning of *CP* and *NIb* genes of PRSV, Suphan Buri (PRSV-SB) and Nakhon Pathom (PRSV-NP) isolates into a binary vector for *Agrobacterium*-mediated papaya transformation, kindly provided by Monsanto company, USA. Each vector contains PRSV-derived genes, either *CP* or *NIb* genes, neomycin phosphotransferase II (*npt*II) gene that used for selection of transformants and 35S CaMV promoter for regulation of gene expression (Figure 6). Four constructs were *CP* gene of PRSV-SB (pMON65301); *NIb* gene of PRSV-SB (pMON65302); *CP* gene linked with 250 bp inverted repeat sequence of the *CP* gene (*CP-IR*) of PRSV-SB (pMON65303), and *NIb* gene of PRSV-NP (pMON59366). Forty-one putative transgenic papaya lines were regenerated on kanamycin containing medium and grown in a temperature-controlled glasshouse (25°C) for evaluation of PRSV resistance (Table 3).

Table 3 Number of putative transgenic papaya lines derived from four transgene constructs

Construct	Transgene	No. of regenerated lines
pMON65301	CP	15
pMON65302	NIb-SB	2
pMON65303	CP-IR	20
pMON59366	NIb-NP	4
Total		**41**

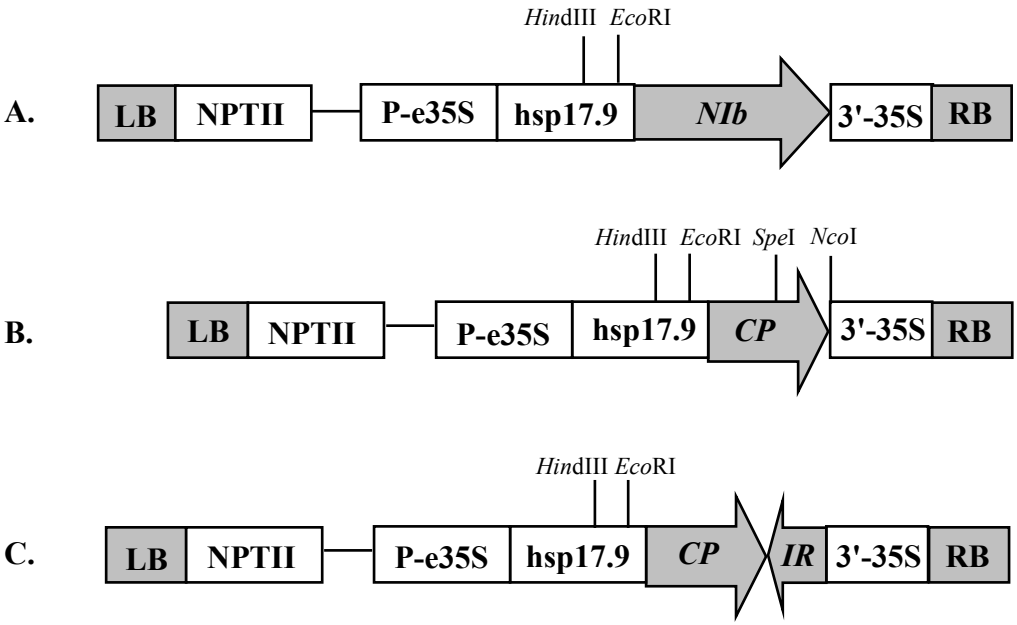


Figure 6 Schematic representation of the orientation of genes in the chimeric constructs for papaya transformation. The NIb construct (A), the CP construct (B) and the CP-IR construct (C). The sites for restriction enzymes are indicated by the arrows.

Source: Flasinski *et al*. (2002)

Abbreviations:

LB = left border sequence

NPTII = neomycin phosphotransferase II gene

P-e35S = 35S CaMV promoter

hsp1 = heat shock protein 17.9 from soybean, called leader sequence, function as enhancer

NIb = nuclear inclusion b gene

CP = coat protein gene

CP-IR = coat protein linked with 250 bp inverted repeat sequence of the *CP* gene

3'-35S = untranslated sequence from CaMV used as terminator

RB = right border sequence

2. Molecular analysis of transformed papaya lines

2.1 Genomic DNA extraction from papaya

Genomic DNA was extracted from young fresh leaves of papaya using modified protocol from CTAB method (Rogers *et al*., 1996). Two capped of leaves were ground with plastic pestle in a 1.5 ml microcentrifuge tube containing 700 µl of CTAB buffer (0.1 M Tris-HCl, pH 8.0, 0.02 M EDTA, pH 8.0, 1.4 M NaCl, 2% CTAB). The mixture was followed by extraction with an equal volume of phenol : chloroform : isoamyl alcohol; PCI (25 : 24 : 1). Supernatant was harvested by centrifugation at 12,000 rpm for 15 min. DNA pellet was precipitated with an equal volume of cold isopropanol, centrifuged at 12,000 rpm for 15 min and then rinsed with cold 70% ethanol. Ethanol residues were removed as much as possible by a vacuum for 15 min to ensure that ethanol did not remain in microcentrifuge tube, because it affected to PCR and restriction enzyme digestion reactions. Dry pellet was resuspended in distilled water containing 20 µg/ml of RNaseA. Total DNA concentration was determined by spectrophotometer at OD 260 nm and calculated the purity of DNA with the ratio of OD 260/280 nm.

2.2 PCR analysis of transgenes in transformed papaya lines

Genomic DNA from papaya was used as a template for PCR reaction. PCR mixture was composed of 1X PCR buffer (200 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.3 mM dNTPs, 10 pmol of each primer, kindly provided by Dr. Srimek Chowpongpang, Kasetsart University (Table 4), 1 unit of *Taq* DNA polymerase (Invitrogen), 100 ng of genomic DNA and followed by adding distilled water to adjust the total volume in 20 µl. PCR reaction was started with initial step of DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 1 min, extension at 72°C for 1.30 min and 1 cycle of final extension at 72°C for 10 min. PCR product was separated on 0.8% agarose gel in 1X TAE buffer and visualized under UV transluminator.

Table 4 Primers used for genes amplification by PCR

Primer Name	Sequence (5'-3')	Specific gene
SC113	AAG CTG TGG ATG CTG GTC TTA ATG	CP
SC104	ATT GCG CAT ACC TAG GAG AGA GTG	
NIb-F	CCT TGG CGA GTG GAA GTC GAT	NIb
NIb-R	GGA TCC TCA TTC GTG ATA CAC	

2.3 Preparation of PCR DIG-labeling DNA probe

The DIG-labelling DNA probes were used for *in vitro* hybridization with complementary DNA in order to confirm the integration of PRSV genes in transformed papaya genome. They were synthesized by PCR using DIG-11-dUTP-labelling mixture (Roche). PCR mixture was composed of 1X PCR buffer (200 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 1X DIG-PCR labeling mix, 20 pmol of each primer (Table 4), 2 unit of *Taq* DNA polymerase (Invitrogen), 50 ng of plasmid DNA harboring PRSV gene that used as a template for PCR reaction and followed by adding distilled water to adjusted the total volume in 50 μl. The PCR reaction was done as described above (2.2). The DIG-labelled DNA product was investigated by comparing with non-labelled DNA product on 0.8% agarose gel, the band of labeled DNA was larger than the non-labelled DNA and chemiluminescent detection on positive charge nylon membrane. The DNA probes were denatured by boiling for 10 min and immediately chilled on ice for 10 min before hybridized with complementary DNA.

2.4 Southern blot hybridization of transformed papaya lines

Genomic DNA was digested with either *Eco*RI or *Hin*dIII that each cut inside the T-DNA region of all transgene constructs once (Figure 6), but they did not cut inside the PRSV-derived transgenes. Reaction for digestion was composed of 1X

buffer (BioLabs), 80 units of each enzyme, 20 μg of genomic DNA and followed by adding distilled water to adjust the total volume in 150 μl. The reaction was incubated at 37°C for 16-18 h. A five microlitter of digestion was separated on 0.8% agarose gel in 1X TAE buffer in order to confirm complete digestion, DNA bands smeared in many small sizes. Digested DNA was purified by phenol chloroform extraction to remove the buffer, precipitated with 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate, pH 5.4 and incubated at -20°C for 3 h. DNA pellet was harvested by centrifugation at 12,000 rpm for 15 min and rinsed with 70% ethanol. The DNA pellet was resuspended in distilled water and kept at -20°C until use.

Southern blot hybridization was described by Southern (1975) for determination of number of inserted DNA in papaya genome. The digested DNA was separated on 1% agarose gel in 1X TAE buffer at 60 V for 120 min. Then, the gel was depurinated in 0.25 N HCl for 10 min, rinsed with distilled water and denatured in 0.4 N NaOH for 10 min. The DNA from gel was transferred to the positive charge nylon membrane (Roche) by capillary transfer in 0.4 N NaOH at room temperature for 16-18 h. The digested DNA was fixed on the membrane by baking at 120°C for 30 min. The membrane was incubated in hybridization buffer [5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 0.1% N-lauroylsarcosine, 1% blocking reagent (Roche)] at 65°C for 2 h. Then, the pre-hybridization buffer was removed, added a new hybridization buffer containing 2 μg of denatured DIG-labelled DNA probe and incubated at 65°C for 16-18 h. After hybridization, the expected bands of hybridized DNA were detected by chemiluminescent procedure. The membrane was soaked twice in 2X washing buffer (2X SSC, 0.1% SDS) at room temperature for 15 min and then soaked twice in 0.1X washing buffer (0.1X SSC, 0.1% SDS) at 65°C for 15 min. The membrane was soaked briefly in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20) at room temperature and then incubated in blocking buffer [1% blocking reagent (Roche), 100 mM Maleic acid, 150 mM NaCl, pH 7.5] with shaking at room temperature for 30 min. The membrane was incubated in

blocking buffer containing anti-digoxiginin-AP at room temperature for 30 min. Ratio of anti-digoxiginin-AP to blocking buffer was 1/10,000. Then, the membrane was washed twice in washing buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20) at room temperature for 15 min and then soaked briefly in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl). The membrane was incubated briefly in CDP-*Star* substrate (Roche) that was diluted to 1/200 in detection buffer. The hybridized DNA bands were visualized by exposing membrane on X-ray film (KODAK) for at least 10 min. After exposure, the X-ray film was soaked in developing solution for 3 min, rinsed with water, soaked in fixing solution for 3 min and washed with water.

3. Evaluation of PRSV resistance under glasshouse condition

3.1 Mechanical inoculation of PRSV

Plantlets from transgenic papaya lines (R_0 generation) with 5-6 leaves stage were evaluated for PRSV resistance. Transgenic papaya lines were inoculated with either PRSV-SB or PRSV-NP isolates. PRSV infected young fresh leaves were ground in a cold mortar and pestle containing 0.1 M phosphate buffer (NaH_2PO_4, Na_2HPO_4, pH 7.0). Ratio of leaf to buffer was 1:10. The homogenate was mixed with 0.5% w/v of Celite (diatomaceous earth) in order to assist the viral infection into papaya cells. The plants were inoculated by rubbing onto 3 leaves of papaya plants very gently. Non-transformed papayas were used as positive control to confirm symptom development in every inoculation. After inoculation, celite was rinsed off with water to prevent shading. The inoculated plants were kept in glasshouse at 25°C for symptom observation. Inoculation was repeated once after 14 days of the first inoculation.

3.2 Detection of viral protein by ELISA

After 14 days of the last inoculation, the inoculated plants were evaluated symptom development by detection of viral protein with polyclonal antibody. A one gram of inoculated plants were ground in plastic bag containing 1 ml of coating buffer (0.05 M carbonate buffer, pH 9.6). A 100 μl of homogenate was loaded into 96-well microplate and incubated at 37°C for 3 h. The microplate was washed with PBST 3 times, each time for 3 min, to remove uncoated proteins. Coated proteins were incubated with polyclonal antibody against the CP of PRSV diluted 1/10,000 in bovine albumin serum (BSA) solution (1% BSA in PBS) at 37°C for 2 h. The microplate was washed as above to remove uninteracted proteins and then incubated with goat anti-mouse IgG (ZYMED) that conjugated with alkaline phosphatase diluted 1/5,000 in 1% BSA solution for 1 h, it was used as a secondary antibody for reducing substrate of the alkaline phosphatase. The microplate was washed as above to remove the anti-mouse IgG residues. The interaction between antigens and antibodies was visualized by adding PNPP (para-nitrophenyl phosphate) substrate (ZYMED) (50 μl of 100X PNPP stock in 10 ml of diethanolamine buffer, 0.1 mg/ml) of alkaline phosphatase, incubated at 37°C for 30 min and measured by ELISA microplate reader at absorbent 405 nm (A_{405}).

3.3 Detection of viral gene by RT-PCR

Total RNA was extracted from leaves of inoculated papayas as described in part 4.1. The total RNA was treated with DNase to remove contaminated genomic DNA. The reaction was composed of 1X RQI DNase buffer (Promega), 2 μg of total RNA, 0.5 units of RQI DNase, followed by adding distilled water to adjusted the total volume in 20 μl, incubated at 37°C for 20 min, then stopped the enzyme activity by adding 1 μl of stop solution, incubated at 65°C for 10 min, immediately placed on ice for 5 min and then used as a template for RT-PCR. RT-PCR reaction was performed with one step RT-PCR kit (Qiagen). The reaction was composed of 1X RT-PCR buffer, 0.3 mM dNTPs, 10 pmol of each primer for NIb gene (Table 4), 1 units of

enzyme mix, 2 μg of total RNA and RNase-free water to adjust the total volume in 20 μl. The mixture was started with initial step of reverse transcription to synthesize cDNA at 50°C for 30 min and then cDNA was denatured at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1.30 min and 1 cycle of final extension at 72°C for 10 min. PCR product was analyzed as describe above (2.2).

4 Analysis of transgene expression in transgenic resistant lines

4.1 RNA extraction from papaya

Total RNA was extracted from young fresh leaves of papaya using modified protocol from Verwoerd *et al*. (1989). Approximately 1 g of young fresh leaves were frozen in liquid nitrogen and ground to fine power in a cold mortar and pestle. The powder was transferred into a 1.5 ml microcentrifuge tube containing 750 μl of TLES buffer (Chowpongpang *et al*., 2002) (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, pH 8.0, 1% SDS) and mixed by inverting. The mixture was followed by mixing with an equal volume of PCI. Supernatant was harvested by centrifugation at 12,000 rpm at 4°C for 15 min and transferred into a new microcentrifuge tube. RNA pellet was precipitated to final concentration of 2 M LiCl and kept at 4°C for 16-18 h. The RNA pellet was harvested by centrifugation at 12,000 rpm at 4°C for 15 min, resuspended in 100 μl of DMPC treated distilled water, concentrated by adding 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate, pH 5.4 and kept at -20°C for 2 h. Then, the RNA pellet was harvested by centrifugation at 12,000 rpm at 4°C for 15 min, rinsed with 70% ethanol and resuspended in DMPC treated distilled water. Total RNA concentration was determined by spectrophotometer at OD 260 nm and calculated the purity of RNA with the ratio of OD 260/280 nm.

4.2 Northern blot hybridization of PRSV resistant lines

Total RNAs extracted from papayas as describes above (4.1) were used for determination of mRNA accumulation in papaya cells. A 20 μg of total RNA was size-fractionated on 1% formaldehyde agarose gel electrophoresis (Sambrook *et al*., 1989). The formaldehyde agarose gel was prepared by the following ingredients: melting 1% agarose gel in 62 ml of DMPC-treated distilled water, cooling to 60°C, adding 20 ml of 5X formaldehyde gel-running buffer (0.1 M MOPS, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0) and adding 18 ml of 37% formaldehyde to give the final concentration of 2.2 M. RNA samples were mixed with an equal volume of RNA loading buffer (5X formaldehyde gel running buffer, 37% formaldehyde, deionized-formamide, formaldehyde gel-loading buffer), incubated at 65°C for 10 min and immediately chilled on ice for 5 min. Before loading the samples, the gel was prerun at 60 V for 5 min, immediately loaded the samples into the lanes of the gel and running at 60 V for 120 min in 1X formaldehyde gel-running buffer. After electrophoresis, the gel was rinsed with DMPC-treated distilled water and soaked in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) twice at room temperature for 15 min. RNA from gel was transferred to the positive charge nylon membrane (Roche) by capillary transfer in 20X SSC for 16-18 h. Transferred RNA was fixed on the membrane by baking at 120°C for 30 min. The membrane was incubated in high SDS concentration hybridization buffer (7% SDS, 50% formamide, 5X SSC, 2% blocking reagent, 50 mM sodium-phosphate, 0.1% N-lauroylsarcosine) and then hybridized with DIG-labelled DNA probe in high SDS concentration hybridization buffer at 50°C for 16-18 h. The quantity of RNA in each lane was determined by reprobing with 18S rRNA probe, kindly provide by Plant Research Group (BIOTEC). The membrane was striped in boiling 0.1% SDS-solution with shaking for 10 min, washed in washing buffer at room temperature for 5 min and then proceed to the prehybridization. The procedure for hybridization, washing and chemiluminescent detection of DIG-labelled membrane as described above (2.4).

4.3 Total protein extraction from papaya

Total protein was extracted from fresh leaves of papaya using modified protocol from Macintosh *et al* (1992). Approximately 1 g of fresh leaves was ground in 2.5 ml of protein extraction buffer (0.05 M Tris-HCl, 0.06 M sodium sulphite, pH 8.5) and transferred the homogenate into a 1.5 ml microcentrifuge tube. The homogenate was centrifuged at 10,000 rpm for 5 min. The supernatant was transferred into a new microcentrifuge tube that used as total protein for further analysis and kept at -20°C until use. Total protein concentration was estimated by Bradford method (Bradford MM, 1976). The total protein was diluted to the final concentration of 1/200, 1 µl of total protein in 199 µl of distilled water, added 1 ml of Comassie Brilliant Blue solution and mixed by pipetting. Then, protein concentration was measured by spectrophotometer at 595 nm and calculated protein concentration by comparing with the concentration of BSA standard.

4.4 Western blot analysis of PRSV resistant lines

Western blot analysis used for detection of CP translated protein using polyclonal antibody against the CP of PRSV, kindly provide by Dr. Srimek Chowpongpang, Kasetsart University. Twenty micrograms of total protein was separated on SDS-PAGE (5% staking and 12% separating polyacrylamide gels) (Sambrook *et al*., 1989). Protein samples were mixed with 1X loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% bromophenol blue, 8% glycerol, 2.5% 2x mercapto ethanol), boiled for 10 min and cooled at room temperature for 15 min. Then the protein samples were loaded into the lanes of staking gel and run at 50 V for 30 min in 1X running buffer and then increasing to 100 V for 90 min. After electrophoresis, protein from gel was transferred to the nitrocellulose membrane (Amersham) in cold transfer buffer at 100 V for 100 min. Then, the membrane was soaked in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) with shaking at room temperature for 5 min and then incubated in 4% blocking buffer (4% BSA in TBST buffer) with shaking for 16-18 h. The membrane was incubated in 1%

BSA (1% BSA in TBST buffer) containing diluted 1/10,000 polyclonal antibody to the CP of PRSV at room temperature with shaking for 1 h. Then, the membrane was washed in TBST buffer 3 times each time for 5 min and then incubated in 1% BSA containing diluted 1/5000 goat anti-mouse IgG conjugated with alkaline phosphatase (ZYMED) at room temperature with shaking for 1 h. The membrane was washed as described above. The expected band of the CP was visualized by adding NBT/BCIP substrate (ZYMED) for alkaline phosphatase.

5 Study of T-DNA flanking sequences in PRSV resistant transgenic papaya

5.1 Preparation of DNA fragments

Genomic DNAs from PRSV resistant transgenic papaya containing one insertion of transgene were used as template for amplification of T-DNA flanking sequences. Ten micrograms of genomic DNA was digested with *Spe*I, *Nco*I, and *Eco*RI that each cut in the T-DNA region once (Figure 6). Reaction for digestion was composed of 1X buffer for each enzyme (BioLabs), 60 units of each enzyme, 10 μg of genomic DNA and followed by adding distilled water to adjust the total volume in 200 μl. The reactions were incubated at 37°C for 16-18 h. Digested genomic DNA was precipitated as described above (2.1). DNA fragments were religated to circularize using T4 DNA ligase. Reaction for ligation was composed of 1X T4 buffer (50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin), 40 units of T4 DNA ligase (BioLabs), 5 μg of digested DNA and followed by adding distilled water to adjust the total volume in 200 μl. The ligated DNA was purified by phenol chloroform extraction and precipitated as described above (2.1). The ligated DNA was resuspended in distilled water and kept at -20°C until use.

5.2 Amplification of T-DNA flanking sequence

T-DNA flanking sequences were amplified by inverse PCR (IPCR) together with long PCR strategies, using specific primers which were designed from the known sequences of the pMON65301 construct (Figure 7). PCR reaction was performed with long PCR to amplify unknown size of the fragments. The reaction for long PCR was composed of 1X long PCR buffer (Fermentas), 1.5 mM $MgCl_2$, 200 μM each of dNTP, 0.5 μM each of primers (Table 5), 4% DMSO, 1 units of Long PCR Enzyme Mix (Fermentas), *Taq* DNA polymerase together with 3'→5' exonuclease (proof reading activity), 1 μg of ligated DNA fragments and followed by adding distilled water to adjust the volume in 25 μl. The PCR reaction was started with initial step of DNA denaturation at 94°C for 5 min, followed by 2 steps of long PCR: (i) 10 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 68°C for 4 min (ii) 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 68°C for 4 min and 1 cycle of final extension at 68°C for 10 min. PCR product was analyzed by 0.8% agarose gel electrophoresis and followed the protocol for Southern blot hybridization in order to detect the positive bands by chemiluminescent procedure as describe above (2.4). The positive bands were extracted from the gel or purified using DNA purification kit (Promega) and cloned into cloning vector for sequencing.

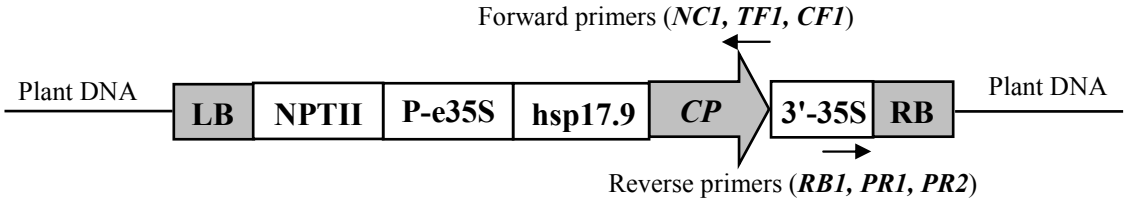


Figure 7 Location of specific primers designed from the known sequences of construct. The forward primers (*NC1, TF1, CF1*) were designed from both *CP* transgene and promoter regions, whereas the reverse primers (*RB1, PR1, PR2*) were designed from the promoter region.

Table 5 List of primers used for amplification of T-DNA flanking sequence

Primer Name	Sequence (5'-3')	Specific site
RB1	GGC ATT TGT AGG AGC CAC CTT CTT	35S promoter
NC1	GGA GAG GAC ACA GAA ACA TTC GCA	Leader sequence
PR1	GAT AGC TGG GCA ATG GAA TCC GAG GAG	35S promoter
TF1	GCA ATT GAG GAT CCT CTA GAG TCC GCA	Terminator
CF1	GCG TCG AGA AGC ATC GAC GAT TA	*CP*
PR2	GGA AGG GTC TTG CGA AGG ATA GT	Leader sequence

5.3 Cloning PCR product of flanking sequence into cloning vector

5.3.1 Preparation of competent cells of *E. coli* strain DH5α

Transformation-competent cells were used for DNA uptake by heat-shock transformation. The single fresh colony of *E. coli* strain DH5α was cultured in 5 ml of 2xYT medium at 37°C with shaking for 16-18 h which used as an inoculum. A 0.4% of inoculum was grown in SOB medium containing 10 mM $MgCl_2$ and 10 mM $MgSO_4$ at 37°C with shaking for 4 h (until OD_{600} = 0.2-0.4). Bacterial cell culture was inactivated on ice for 15 min and then centrifuged at 3,500 rpm for 15 min at 4°C. Cell pellet was resuspended very gently in cold RF1 buffer (100 mM KCl, 50 mM $MnCl_2.4H_2O$, 30 mM K-acetate, 10 mM $CaCl_2.2H_2O$, 15% glycerol, pH 5.8) and incubated on ice for 15 min. Then, the cell suspension was centrifuged at 3,500 rpm for 15 min at 4°C. The cell pellet was resuspended very gently in cold RF2 buffer (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% glycerol, pH 6.8). A 100 µl aliquot of cell suspension was transferred into a new microcentrifuge tube, immediately frozen in liquid nitrogen and stored at -80°C until use.

5.3.2 Ligation of PCR product into plasmid cloning vector

PCR product was purified by PCR purification kit (Promega) which used for ligation with cloning vector. Reaction of ligation was composed of 1X buffer, 2 µl of purified PCR product, 1 unit of DNA blunting enzyme and followed by adding distilled water to adjust the total volume in 18 µl. The mixture was incubated at 70°C for 5 min to remove 3' A overhanged of PCR product for getting blunt end, and then immediately placed on ice for 5 min. The mixture was mixed with 1 µl of pJET1/blunt (Fermentas) and T4 DNA ligase and then incubated at 22°C for 1 h. Then, the mixture was transformed into competent cells by heat-shock transformation.

5.3.3 Heat-shock transformation of the ligation mixture into competent cells

The ligation mixture from step 5.3.2 was mixed with the competent cells on ice for 30 min, incubated at 42°C for 1 min and immediately placed on ice for 5 min. The competent cells were grown in 1 ml of LB liquid medium at 37°C with shaking for 1 h. Cell pellet was harvested by centrifugation at 8,000 rpm for 1 min, resuspended in 100 µl of LB liquid medium, spread on solid LB medium containing 100 mg/ml of ampicilin and incubated at 37°C for 16-18 h. The colonies were picked and cultured in LB liquid medium for isolation of plasmid DNA.

5.3.4 Isolation of plasmid DNA from *E. coli* by Alkaline Lysis method

Plasmid DNAs were extracted from bacterial transformants by alkaline lysis method (Sambrook *et al*., 1989). Bacterial colonies were grown in LB liquid medium containing 100 mg/ml of ampicilin at 37°C with shaking for 16-18 h. One millilitter of bacterial culture was centrifuged at 8,000 rpm for 5 min. Cell pellet was resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) and mixed by vortexing. Bacterial cells were lysed by adding 200 µl of fresh solution II (0.2 N NaOH, 1% SDS) and mixed by inverting. This step showed that the solution should become viscous resulting from cell lysis. A 150 µl aliquot of solution III (3 M K-Potassium acetate, pH 4.8) was added to the mixture, mixed by inverting and placed on ice for 10 min, a thick clot was formed, plasmid DNA renatured during this step and remained soluble. The mixture was centrifuged at 12,000 rpm for 10 min and then transferred supernatant into a new microcentrifuge tube. The supernatant was purified by adding an equal volume of PCI (25 : 24 : 1) and mixed by vortexing. The supernatant was harvested by centrifugation at 12,000 rpm for 10 min and transferred into a new microcentrifuge tube. The plasmid DNA pellet was precipitated by adding an equal volume of cold absolute ethanol and centrifuged at 12,000 rpm for 10 min. Then, the pellet was rinsed with 70% ethanol and removed the ethanol residues by a vacuum for 10 min. Dry pellet was

resuspended in distilled water containing 20 μg/ml of RNase and frozen until the next procedure was started.

5.3.5 Restriction enzyme digestion of plasmid DNA

Plasmid DNA was double digested with *Xho*I and *Xba*I (BioLabs) to separate the inserted DNA from the cloning vector (pJET1/bulnt). Reaction for digestion was performed with 1X NEB buffer1 (BioLabs), 5 units of each enzymes, 5 μg of plasmid DNA and followed by adding distilled water to adjust the total volume in 20 μl. The mixture was incubated at 37°C for 3 h and then the digested plasmids were separated by 0.8% agarose gel eletrophoresis to confirm the presence of expected bands of insert DNA.

5.3.6 Sequencing and sequence analysis

The positive recombinant clones from 5.3.5 were grown in 2XYT media containing 100 mg/ml of ampicilin at 37°C for 16-18 h and then used as a template for sequencing by automatic sequencer (ABI377 and ABI3110, Applied Biosystem) at Bio Service Unit, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Bankok. The sequences were analyzed using DNASTAR™ program (Lasergene) and compared to sequences in GenBank database. DNASTAR™ contained the following program subunits; EditSeq, SeqMan, MegAlign, PrimerSelect, GeneQuest, MapDraw and Protein.

RESULTS

1. PCR and Southern blot analysis of putative transgenic papaya lines R_0

Forty one putative transgenic papaya lines were successfully regenerated on kanamycin containing medium which were named as A1 to A41 (Table 3). Genomic DNA from leaves of these lines were extracted and used for analysis of the presence of transgenes in their genomes by PCR and Southern hybridization (Figure 8A). PCR product of expected band, 868 bp, was amplified from the genomic DNA of 33 putative transgenic lines, including 13 lines derived from *CP* transgene and 20 lines derived from *CP-IR* transgene (Figure 9A and Table 6). For *NIb* transgene, 1.6 kb fragments were amplified from the genomic DNA of five transgenic papaya lines, including A11, A12, A14, A16 and A17 (Figure 9B and Table 6). Both transgenes were not detected in non-transformed papaya and distilled water, using as the negative controls (Figure 9A and 9B).

Number of insertion event of transgenes in papaya genomes were determined by Southern hybridization (Southern, 1975). Genomic DNA was completely digested with either *Eco*RI or *Hin*dIII and the small size DNA bands were generated (Figure 7B). DNA probes were labelled with the DIG-11-dUTP-labelling mixture (Roche), approximately 1 kb and 1.7 kb of DNA labelled products were carried out and hybridized with complementary DNA, according to the protocol of Southern hybridization (Figure 10). Result showed that the insertion number of transgene was ranged from 1 to 3 copies, presented one band as one insertion (Table 6). The insertion fragments were exhibited in different sizes and up to 3 kb in size was found in each transgenic papaya line (Figure 11).

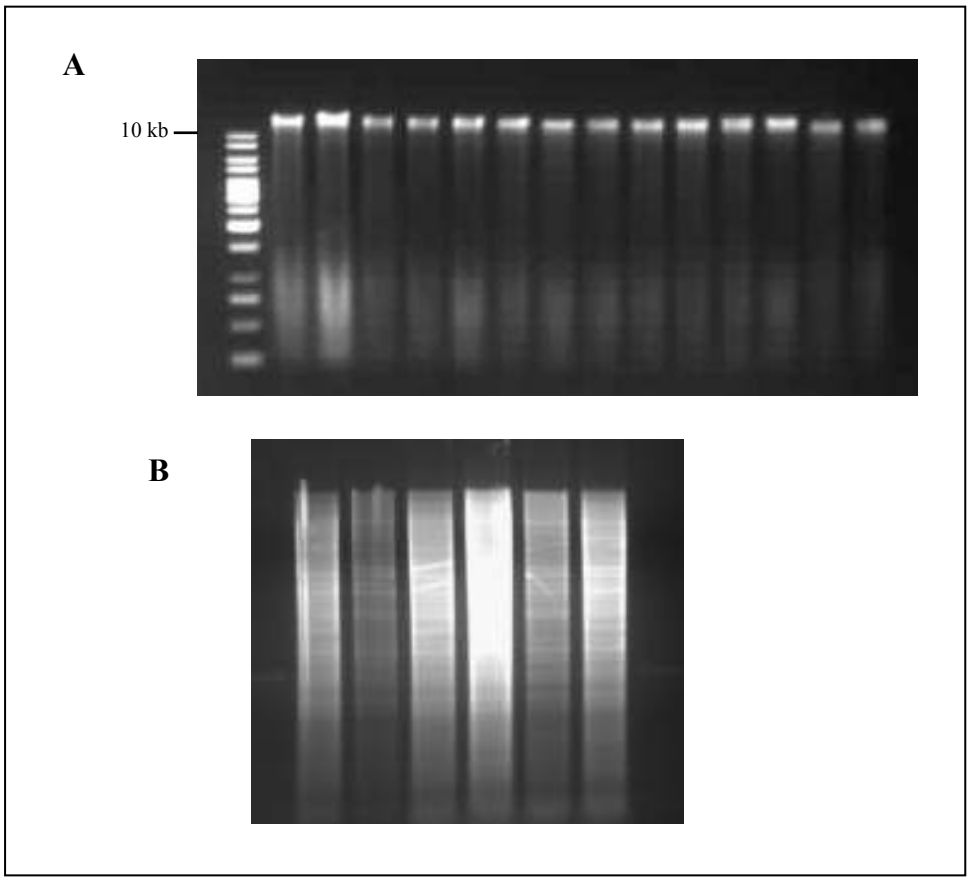


Figure 8 Analysis of genomic DNA extracted from young fresh leaves of papayas by agarose gel electrophoresis. Genomic DNAs were separated on 0.8% agarose gel in 1XTAE buffer at 100 V for 30 min (A). Twenty microgram of genomic DNAs were completely digested with 80 units of *Eco*RI at 37°C for 16 h and separated on 1% agarose gel in 1XTAE buffer at 60 V for 120 min (B).

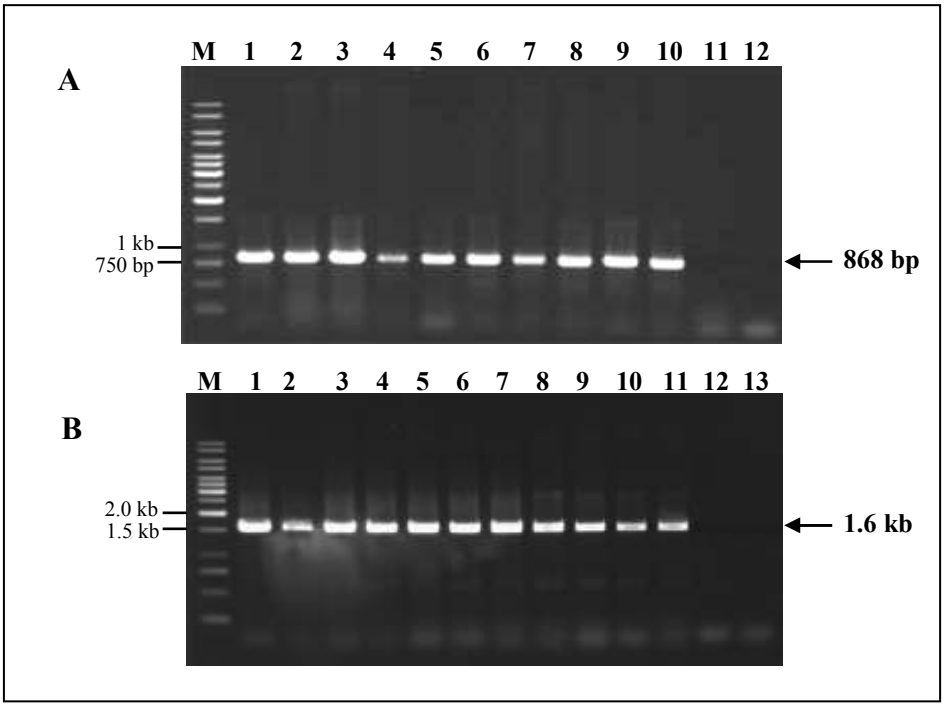


Figure 9 PCR analysis of PRSV-derived transgenes in putative transgenic papaya lines.

A: PCR products of *CP* gene. Lane M: 1 kb DNA ladder (Fermentas), lane 1: plasmid construct harboring *CP* gene (pMON65301), lane 2-5: transgenic papaya lines derived from pMON65301 construct (A3, A8, A9, A19), lane 6-10: transgenic papaya lines derived from pMON65303 construct (A6, A25, A27, A28), lane 11: non-transformed papaya, lane 12: distilled water

B: PCR products of *NIb* gene. Lane M: 1 kb DNA ladder (Fermentas), lane 1: plasmid construct harboring the *NIb* gene (pMON65302), lane 2-7: transgenic papaya lines derived from pMON59366 construct (A11, A14, A16, A17), lane 8-11: transgenic papaya lines derived from pMON65302 construct (A7, A12), lane 12: non-transformed papaya, lane 13: distilled water

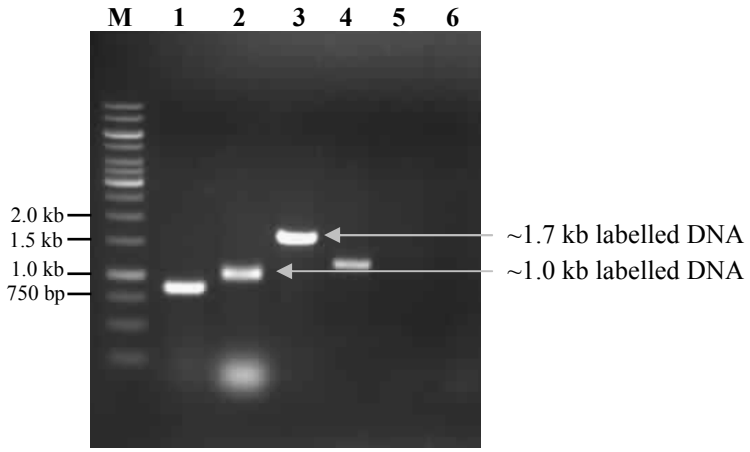


Figure 10 Synthesis of the DIG-labelled DNA probes using the DIG-11-dUTP-labelling mixture (Roche).

Lane M : 1 kb DNA ladder (Fermentas)
Lane 1 : PCR product of non-labelled CP gene
Lane 2 : PCR product of the CP-DIG-labelled probe
Lane 3 : PCR product of the NIb-DIG-labelled probe
Lane 4 : PCR product of non-labelled NIb gene
Lane 5 : distilled water
Lane 6 : non-transformed papaya

Figure 11 Analysis of number of transgene in transgenic papaya genome by Southern hybridization.

A, B and C: The number of *CP* gene insertion. Lane 1: PCR product of 868 bp fragment of *CP* gene, lane 2-14: transgenic papaya lines derived from CP-IR construct (A26, A29, A30, A31, A33, A35, A36, A38, A39, A40, A42, A43 and A44, respectively), lane 15: non-transformed papaya, lane 16-20: transgenic papaya lines derived from CP construct (A9), lane 21: non-transformed papaya, lane 22-26: transgenic papaya lines derived from CP construct (A21), lane 27-31: transgenic papaya lines derived from CP-IR construct (A30), lane 32-36: transgenic papaya lines derived from CP construct (A45)

D: The number of *NIb* gene insertion. Lane 1: plasmid construct harboring *NIb* gene (pMON65302), lane 2-6: transgenic papaya lines derived from NIb construct (A12), lane 7: non-transformed papaya

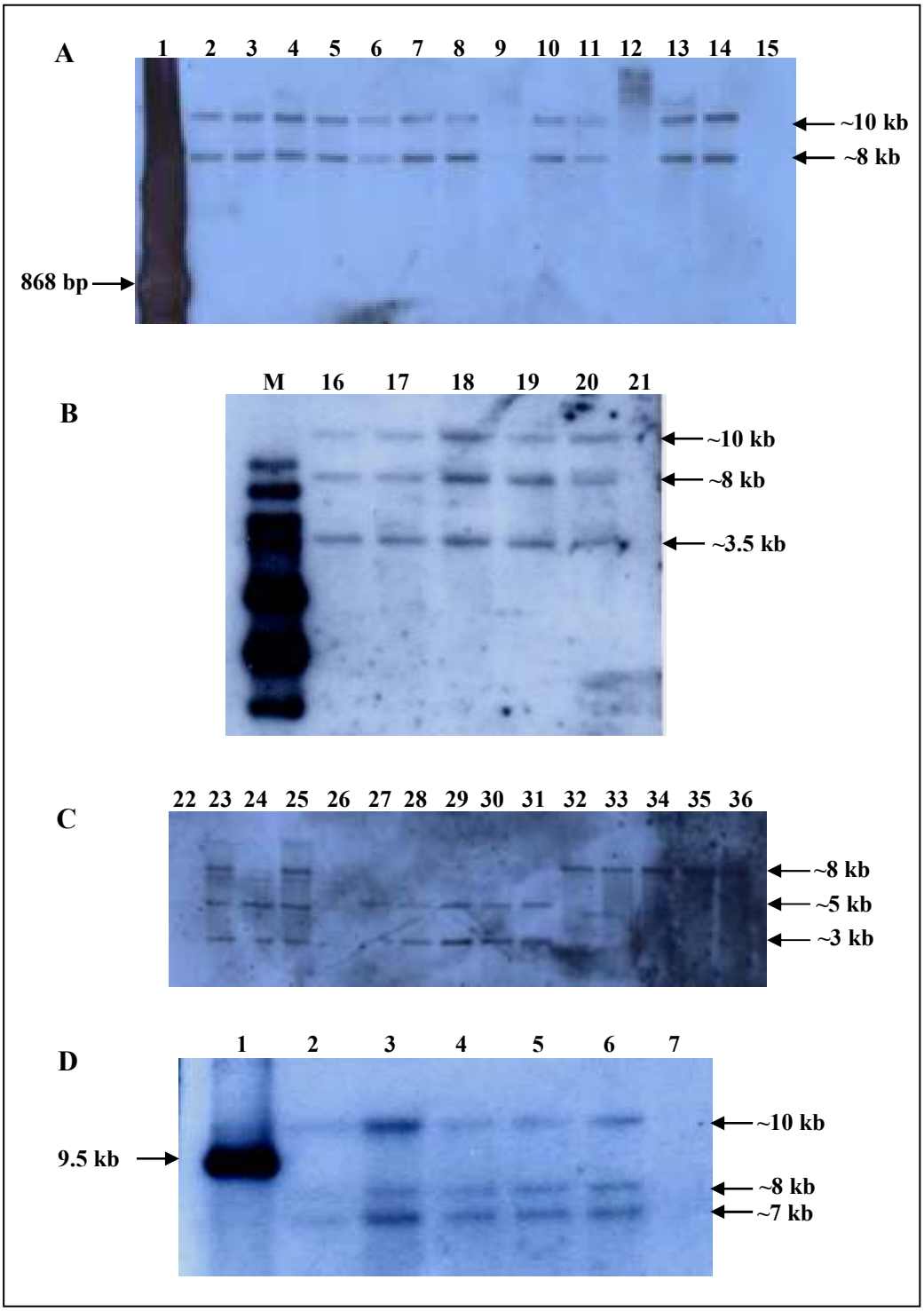

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
~10 kb
~8 kb
868 bp
B
M 16 17 18 19 20 21
~10 kb
~8 kb
~3.5 kb
C
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
~8 kb
~5 kb
~3 kb
D
1 2 3 4 5 6 7
~10 kb
~8 kb
~7 kb
9.5 kb

Table 6 Molecular analysis of transgenic papaya R_0 lines for the presence of *CP* and *NIb* transgenes.

construct	transgenic papaya R_0 lines	PCR analysis	Southern hybridization (*Eco*RI digestion)
1. pMON65301[a]	A1	+	2
	A2	+	1
	A3	+	1
	A4	+	-
	A5	+	3
	A8	+	2
	A9	+	3
	A10	+	-
	A18	+	2
	A19	+	2
	A20	+	3
	A21	+	3
	A23	+	3
	A24	+	2
	A45	+	1
2. pMON65302[b]	A7	+	-
	A12	+	3
3. pMON65303[c]	A6	+	2
	A13	+	1
	A25	+	1*
	A26	+	2
	A27	+	2
	A28	+	1
	A29	+	2
	A30	+	2
	A31	+	2
	A32	+	2
	A33	+	2
	A35	+	2
	A36	+	2
	A37	+	2
	A38	+	3
	A39	+	2
	A40	+	2
	A42	+	2
	A43	+	2
	A44	+	2
4. pMON59366[d]	A11	+	3
	A14	+	3
	A16	+	3
	A17	+	3

* *Hin*dIII digestion
+ = presence of transgene, - = absence of transgene
[a]pMON65301, the construct containing *CP* transgene of PRSV-SB
[b]pMON65302, the construct containing *NIb* transgene of PRSV-SB
[c]pMON65303, the construct containing *CP-IR* transgene of PRSV-SB
[d]pMON59366, the construct containing *NIb* transgene of PRSV-NP

2. Evaluation of PRSV resistance in transgenic papaya lines R_0

Plantlets from putative transgenic papaya lines (41 lines) were challenged with either PRSV-SB or PRSV-NP isolates under glasshouse condition (Figure 12A). Symptom observation was done after 14 days of every inoculation and confirmed the PRSV resistance by detection of coat protein of PRSV using ELISA technique. Result showed that non-transformed papayas and susceptible lines, A1, A2, A3, A5 and A14 were systemic infection after 14 days of the first inoculation. They showed yellow mottling and distortion on young leaves and water-soaked oily streak on stem (Figure 12C and 12D). In contrast, 34 resistant lines derived from four constructs did not show any PRSV symptoms and were completely resistant to the challenged isolate after 14 days of the last inoculation (Figure 12B). A_{405} values of ELISA test of the susceptible lines were greater than 0.3 (about 3 times of the A_{405} value of healthy plants), while the A_{405} values of the transgenic resistant lines were less than 0.3, nearly to that value of coating buffer and the healthy plants (Table 7). The replicase gene of PRSV (NIb) was determined by RT-PCR in order to confirm the virus could not replicate in transgenic resistant lines after inoculation. In this purpose, 2 selected inoculated resistant transgenic lines, A43 and A30 derived from the CP transgene were detected for the presence of viral replicase gene by RT-PCR at 14 days after inoculation. Result indicated that the virus could not replicate in both transgenic resistant lines, while the replication of PRSV was found in inoculated non-transformed papaya, based on the presence of 1.6 kb fragment of *NIb* gene (Figure 13). However, CP gene was still expressed in both resistant lines during PRSV infection (Figure 12).

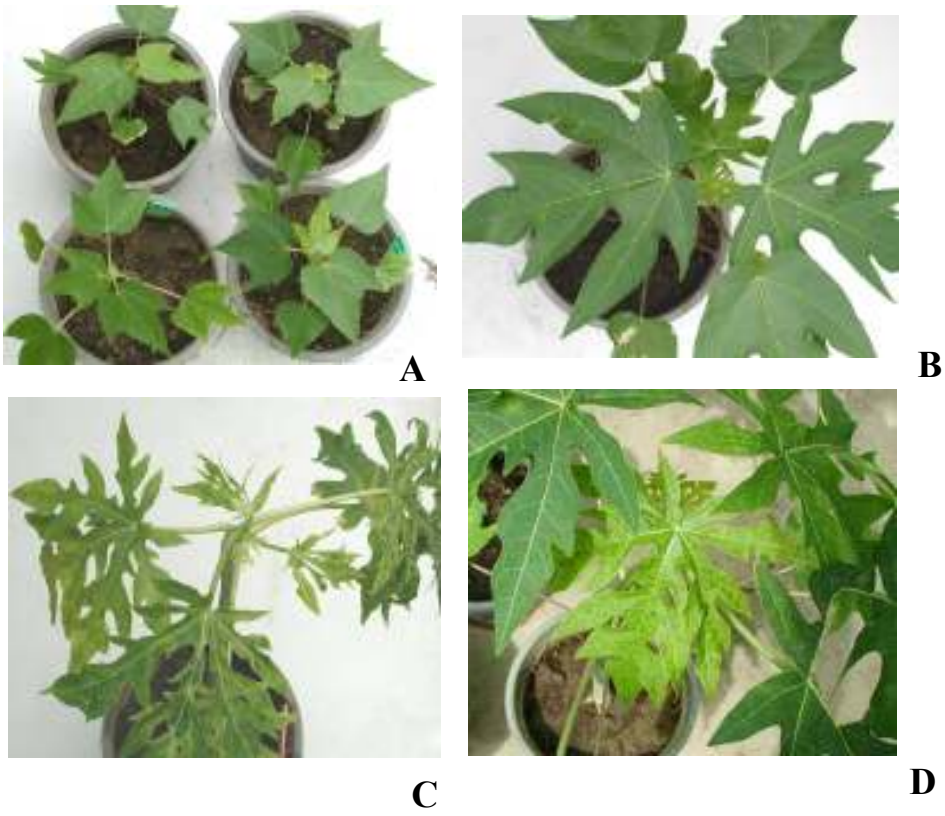


Figure 12 Symptom observations of the inoculated transgenic papaya lines containing PRSV-derived transgenes after 14 days of the last inoculation.

A: Transgenic papaya containing PRSV transgene with 5-6 leaves stage used for inoculation

B: The resistant line (A6)

C: Non-transformed papaya showed yellow mottling and distortion on young leaves

D: The susceptible lines showed yellow mottling on young leaves

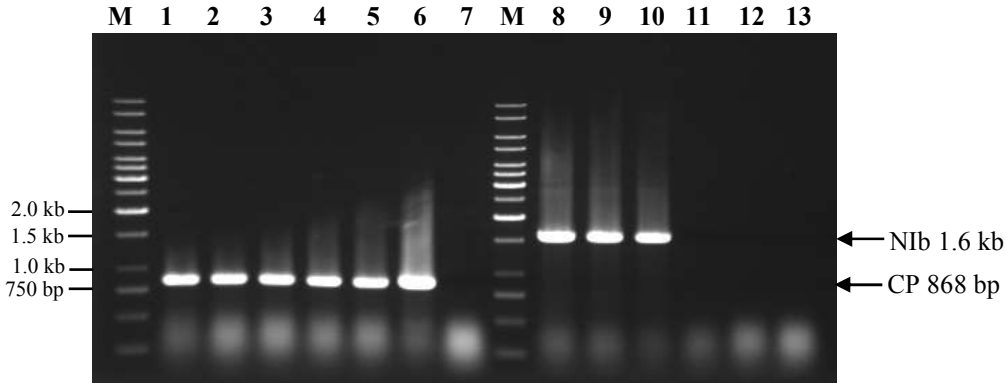


Figure 13 Detection of *NIb* and *CP* transgenes in the transgenic resistant lines by RT-PCR after inoculation.

Lane M: 1 kb DNA ladder (Fermentas), lane 1-2: RT-PCR product of the CP gene of the transgenic resistant lines, A43 derived from 14 days after inoculation, lane 3-5 : RT-PCR product of *CP* gene of the transgenic resistant lines, A30 derived from 14 days after inoculation, lane 6: RT-PCR product of *CP* gene of inoculated non-transformed papaya derived from 14 days after inoculation, lane 7: RT-PCR product of the *CP* gene of the healthy plant, non-transformed papaya, lane 8-10: RT-PCR product of *NIb* gene of inoculated non-transformed papaya derived from 14 days after inoculation, lane 11-12: RT-PCR product of *NIb* gene of the transgenic resistant lines, A43 derived from 14 days after inoculation, Lane 13: RT-PCR product of *NIb* gene of the inoculated transgenic resistant line, A30 derived from 14 days after inoculation

Table 7 Evaluation of PRSV resistance in transgenic papaya R_0 lines containing PRSV transgenes under glasshouse condition.

Transgenic papaya lines[a]	PRSV genes[b]	PRSV isolates	PRSV resistance
A1	CP	PRSV-SB	-
A2	CP	PRSV-SB	-
A3	CP	PRSV-SB	-
A5	CP	PRSV-SB	-
A6	CP-IR	PRSV-SB	+
A8	CP	PRSV-SB	+
A9	CP	PRSV-SB	+
A11	NIb-NP	PRSV-NP	+
A12	NIb-SB	PRSV-SB	+
A13	CP-IR	PRSV-SB	+
A14	NIb-NP	PRSV-NP	+
A16	NIb-NP	PRSV-NP	+
A17	NIb-NP	PRSV-NP	+
A18	CP	PRSV-SB	+
A19	CP	PRSV-SB	+
A20	CP	PRSV-SB	+
A21	CP	PRSV-SB	+
A23	CP	PRSV-SB	+
A24	CP	PRSV-SB	+
A25	CP-IR	PRSV-SB	+
A26	CP-IR	PRSV-SB	+
A27	CP-IR	PRSV-SB	+
A28	CP-IR	PRSV-SB	+
A29	CP-IR	PRSV-SB	+
A30	CP-IR	PRSV-SB	+
A31	CP-IR	PRSV-SB	+
A32	CP-IR	PRSV-SB	+
A33	CP-IR	PRSV-SB	+
A35	CP-IR	PRSV-SB	+
A36	CP-IR	PRSV-SB	+
A37	CP-IR	PRSV-SB	+
A38	CP-IR	PRSV-SB	+
A39	CP-IR	PRSV-SB	+
A40	CP-IR	PRSV-SB	+
A42	CP-IR	PRSV-SB	+
A43	CP-IR	PRSV-SB	+
A44	CP-IR	PRSV-SB	+
A45	CP	PRSV-SB	+
PRSV infected non-transformed papaya (A_{405} = 1.589)		PRSV-SB and PRSV-NP	-
non-infected papaya (Healthy) (A_{405} = 0.087)			
coating buffer (A_{405} = 0.098)			

[a]Transgenic papaya from independent lines (R_0 generation) with 5-6 leaves stage, at least 5 trees were inoculated with PRSV.
[b]Transgene analysis using PCR and Southern hybridization
+ = A_{405} value of ELISA test was nearly to that value of coating buffer and healthy plants.
- =A_{405} value of ELISA test was greater than three times of that value of coating buffer and healthy plant.

3. Transgene expression in PRSV resistant lines R_0

Transgene expression analysis was focused on the accumulation of CP transcripts in papaya cells. Among transgenic-resistant lines, eight of uninoculated lines containing *CP* transgene (A18, A19, A20, A23) and *CP-IR* transgene (A25, A28, A35, A37) were detected the presence of CP transcripts by Northern hybridization (Figure 14A). From our result, it was showed that CP transcripts were highly expressed in four resistant lines, A18, A19, A28 and A37 which revealed the strong signal of exposed bands of CP transcript and 18S rRNA represented an equal amount of RNA in each lane (Figure 14B). In contrast, the transgenic resistant lines, A20, A23, A25 and A35, showed less signal of exposed bands which could be indicated that CP genes were lower transcriptionally in these plants (Figure 14C). However, the CP transcript was also highly expressed in the susceptible lines (A3) (Figure 14B).

To compare the accumulation of CP transcript in papaya cells before and after PRSV inoculations. We selected the transgenic resistant lines, A20 and A23 containing the *CP* transgene, which were extracted the total RNAs before PRSV inoculation, 12 and 24 days after PRSV inoculation. Result indicated that the accumulation of CP transcripts did not differ during PRSV infection and the level of CP expression was low in both lines (Figure 15A). Moreover, the replicase gene of PRSV (*NIb*) was not found in both lines after PRSV inoculation, based on the result of viral replicase gene detection by RT-PCR (Figure 15B).

Four selected transgenic resistant lines, A18, A19, A28 and A37 that highly expressed the CP transcripts from the result of Northern hybridization were determined the CP of PRSV by Western blot analysis with polyclonal antibody against the CP of Thai-isolate PRSV. The predicted band of the CP, approximately 32 kDa in size, was found in PRSV infected non-transformed papaya and the susceptible line (A3), but it was not found in the transgenic resistant lines, A18, A19,

A28 and A37 (Figure 16). This result suggested that the expression of CP transgene in the transgenic resistant lines was occurred in transcriptional level.

For the *NIb* transgene, the uninoculated transgenic resistant lines, A11, A12, A14, A16, and A17 were also determined the accumulation of NIb transcripts in papaya cells by Northern hybridization. From our result, it was noticed that NIb transcripts were highly expressed in A11, A16 and A17, while A12 and A14 showed the lowest expression of this gene among these plants (Figure 17). These resistant lines were not detected the NIb protein expression by Western blot because the production of antibody to the NIb of Thai-isolate PRSV was not successful obtained.

Figure 14 Analysis of the accumulation of CP transcripts in the uninoculated transgenic resistant lines by Northern blot hybridization.

A: Total RNA was extracted from young fresh leaves of papayas and separated on 1% agarose gel in 1X TAE buffer. Lane 1: transgenic papaya A20, lane 2: transgenic papaya A23, lane 3-5: transgenic papaya A25, lane 6-8: transgenic papaya A35, lane 9-10: transgenic papaya A37, lane 11: non-transformed papaya

B: High expression of the CP transcripts in PRSV resistant lines. Lane 1 and 2: the susceptible lines (A3), lane 3: transgenic resistant lines derived from *CP* transgene (A18), lane 4-5: transgenic resistant lines derived from *CP* transgene (A19), lane 6-7: transgenic resistant lines derived from *CP-IR* transgene (A28), lane 8-9: transgenic resistant lines derived from *CP-IR* transgene (A37), lane 10: non-transformed papaya, lane 11: PRSV infected non-transformed papaya used as a positive control

C: Low expression of the CP transcripts in PRSV resistant lines. Lane 1: PRSV infected non-transformed papaya used as a positive control, lane 2: transgenic resistant lines derived from *CP* transgene (A20), lane 3: transgenic resistant lines derived from *CP* transgene (A23), lane 4-6: transgenic resistant lines derived from *CP-IR* transgene (A25), lane 7-11: transgenic resistant lines derived from *CP-IR* transgene (A35), lane 12: non-transformed papaya

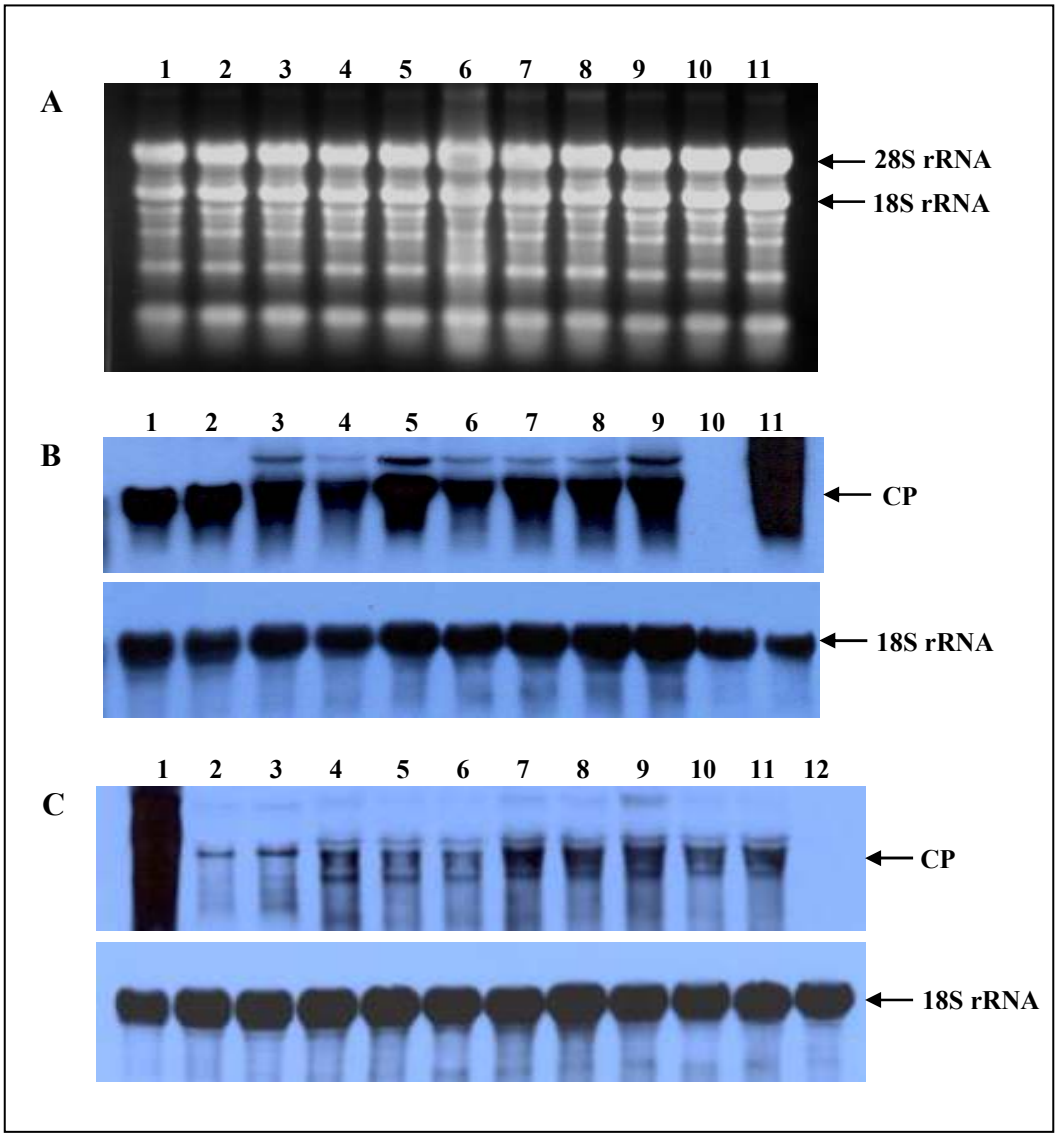
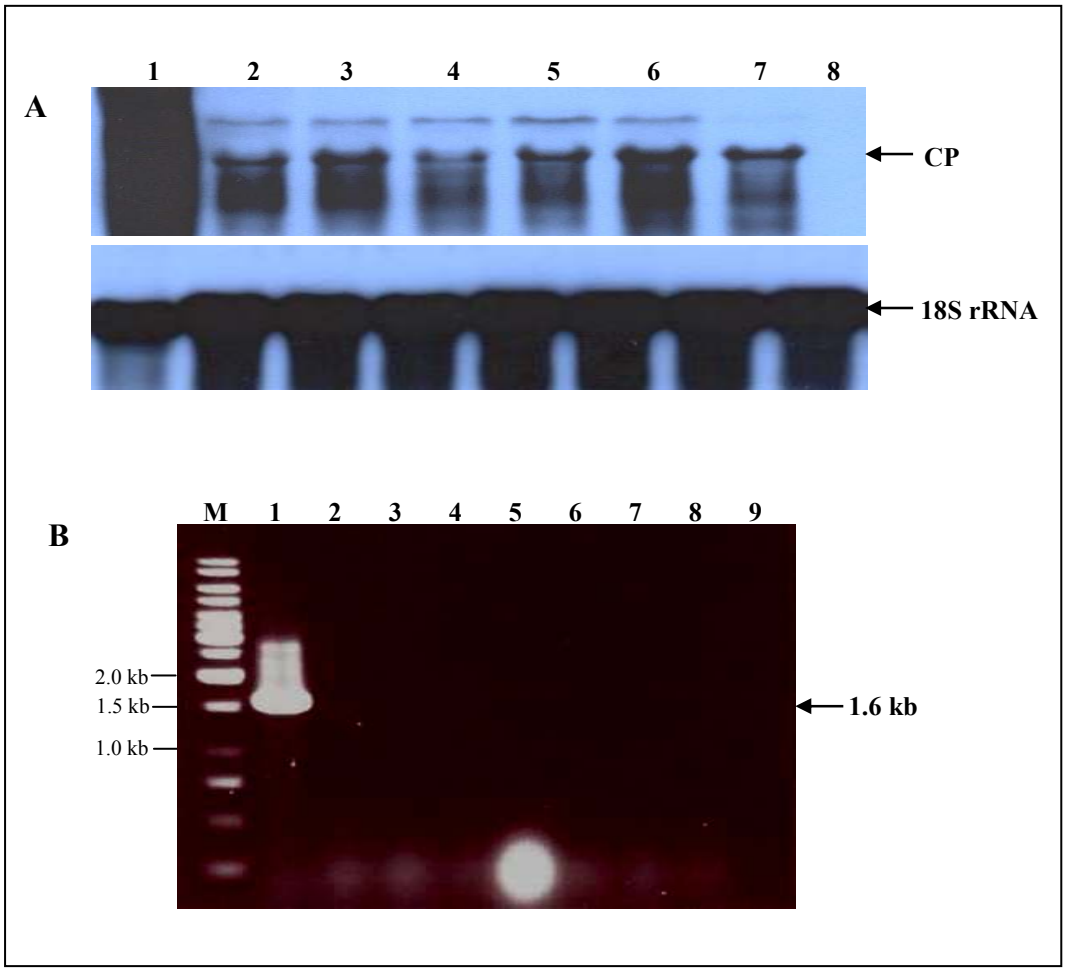

A
1 2 3 4 5 6 7 8 9 10 11
28S rRNA
18S rRNA
B
1 2 3 4 5 6 7 8 9 10 11
CP
18S rRNA
C
1 2 3 4 5 6 7 8 9 10 11 12
CP
18S rRNA

Figure 15 Analysis of the accumulation of CP transcripts in the transgenic resistant lines during PRSV pre-inoculation and post-inoculation.

A: Northern blot hybridization of the transgenic resistant lines before and after PRSV inoculations. Lane 1: PRSV infected non-transformed papaya used as a positive control, lane 2-3: the uninoculated resistant lines, A20 and A23, respectively, derived from CP transgene, lane 4-5: the inoculated resistant lines, A20 and A23, respectively, obtained from 12 days after inoculation, lane 6-7: the inoculated resistant lines, A20 and A23, respectively, obtained from 24 days after inoculation, lane 8: uninoculated non-transformed papaya

B: RT-PCR product of *NIb* gene from transgenic resistant lines after PRSV inoculation. Lane M: 1 kb DNA ladder (Fermentas), lane 1: PRSV infected non-transformed papaya, lane 2-3: the transgenic resistant line, A20 obtained from 12 days after inoculation, lane 4: the transgenic resistant line, A23 obtained from 12 days after inoculation, lane 5: uninoculated transgenic resistant A23, lane 6-7: the transgenic resistant line, A20 obtained from 24 days after inoculation, lane 8: the transgenic resistant A23 obtained from 24 days after inoculation, lane 9: uninoculated non-transformed papaya


A
1 2 3 4 5 6 7 8
CP
18S rRNA
B
M 1 2 3 4 5 6 7 8 9
2.0 kb
1.5 kb
1.0 kb
1.6 kb

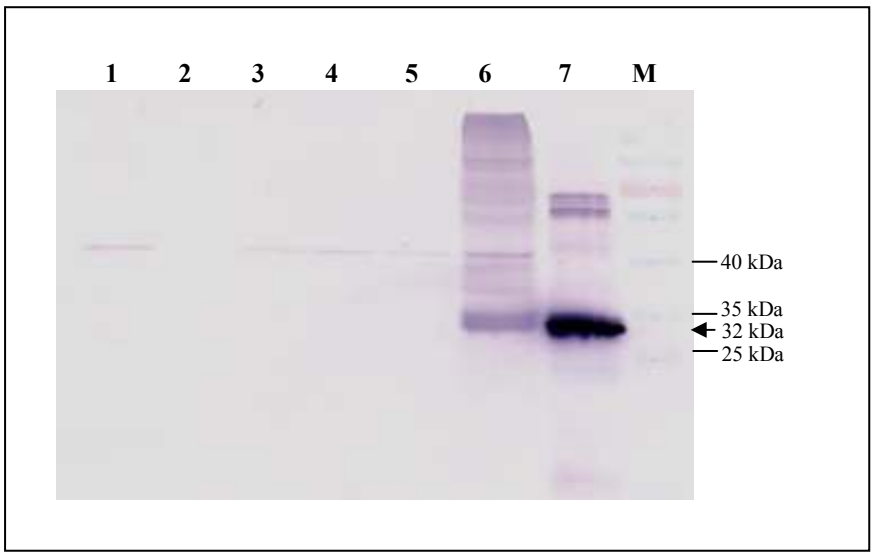


Figure 16 Analysis of PRSV-CP in the uninoculated transgenic resistant lines by Western blot analysis.

Lane M : Protein ladder (Fermentas)
Lane 1 : Non-transformed papaya
Lane 2 : Transgenic resistant line, A37, derived from *CP-IR* transgene
Lane 3 : Transgenic resistant line, A28, derived from *CP-IR* transgene
Lane 4 : Transgenic resistant line, A18, derived from *CP* transgene
Lane 5 : Transgenic resistant line, A19, derived from *CP* transgene
Lane 6 : The susceptible line, A3, derived from *CP* transgene
Lane 7 : PRSV infected non-transformed papaya

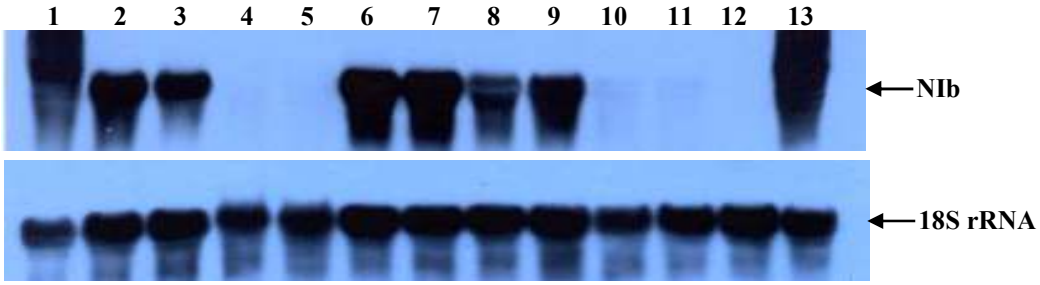


Figure 17 Analysis of the accumulation of NIb transcripts in the uninoculated transgenic resistant lines by Northern hybridization.

Lane 1	: PRSV infected non-transformed papaya
Lane 2-3	: Transgenic papaya line, A11, derived from pMON59366 construct
Lane 4-5	: Transgenic papaya line, A12, derived from pMON65302 construct
Lane 6-7	: Transgenic papaya line, A16, derived from pMON59366 construct
Lane 8-9	: Transgenic papaya line, A17, derived from pMON59366 construct
Lane 10-11	: Transgenic papaya line, A14, derived from pMON59366 construct
Lane 12	: Non-transformed papaya
Lane 13	: PRSV infected non-transformed papaya

4. Evaluation of PRSV resistance in transgenic papaya lines R_1

The transgenic resistant lines were planted and grown in screenhouse condition, transgenic resistant line, A44, exposed hermaphrodite flowers, while transgenic resistant line, A6, exposed female flowers which they were maintained for production of fruit. Seeds from A6 were obtained by pollination with non-transformed papaya, Khake Dum cultivar. The progenies (R_1 generation) from both lines were grown in glasshouse at 25°C for PRSV resistance evaluation. Forty-six R_1 plants from A44 were inoculated with PRSV-SB, 34 plants were resistance but 12 plants were systemic infected after 14 days of the first inoculation as they showed high value of A_{405} from ELISA test (Table 8). For the transgenic resistant A6, 231 of R_1 plants were also inoculated with PRSV-SB. Result showed that 193 plants were resistant with low value of A_{405} while 38 plants were susceptible to PRSV that they showed high value of A_{405} (Table 8). All of the resistant R_1 plants from both lines contained *CP* gene when they were analyzed by PCR. A44 and A6 progenies (R1) revealed 74% and 83% of transgene segregation, respectively. It was noticed that the *CP* gene could be inheritable to their progenies and conferred PRSV resistance in R_1 generation.

5. Study of T-DNA flanking sequence in the transgenic resistant line

T-DNA flanking sequence was determined in the transgenic resistant A45 which contained one insertion of transgene in its genome, based on the result of Southern hybridization (Table 6). Genomic DNA from this line was digested with *Spe*I, *Nco*I and *Eco*RI to give sticky end fragments. All fragments derived from individual restriction enzyme digestion were religated to circularize and used as a template for inverse PCR (IPCR). The reaction was performed with long PCR which contained the proof reading *Taq* DNA polymerase, 3'→5' exonuclease activity and the designed primers specific to the location of each enzyme. PCR products from each digested fragment were confirmed for the correction of band by PCR blot hybridization with the CP gene or 35S CaMV probes. Interestingly, approximately

800 bp fragment of PCR product, namely called SL2 was amplified using the fragment of *Spe*I digestion and the pair of primers CF1 and PR2 that could hybridize with the CP gene probe (Figure 18A and 18B). Therefore, this fragment was purified and cloned into pJET1 blunt cloning vector for nucleotide sequencing. However, the fragments of *Spe*I digestion and the pair of primers TF1 and PR1, RB1 and NC1 were also amplified variable fragments, shown in lane 1, 2, 3 and 4 (Figure 18A). These fragments did not represent the positive band and occurred non specific bands in non-transformed papaya and undigested DNA (Figure 18A and 18B).

SL2 sequence was compared to sequences in GenBank database using BLASTN program (NCBI). Result revealed that the SL2 sequence was part of cloning vector pRGK336 (AY739898), especially in the regions of 25 bp repetitive sequences of RB to the enhanced 35S CaMV promoter (Figure 19). Sequence analysis using SeqMan program (Lasergene) was done in order to investigate sequence overlapping between pMON65301 sequence [25 bp repetitive sequences of RB through the *Spe*I site (5'-ACTAGT-3')] and SL2 sequence. Result showed that SL2 sequence was overlapped with pMON65301 sequence in the position of 25 bp repetitive sequences of RB and obtained mismatch base pairing in this region while the other region of both sequences were the most identical base pairing (Figure 20).

Actually, the purpose of this part is to find the transferred DNA's (T-DNA) sequence was flanked in the transgenic papaya's genome. From our finding, the SL2 sequence that contained the 35S CaMV promoter region and part of the CP gene at position from the ATG start codon to the *Spe*I site, both regions could link to the 25 bp repetitive sequences of RB (Figure 19). In summary, the plant's sequence that flanked with the pMON65301's sequence was not successfully obtained. However, this result suggested that pMON65301's sequence, especially from the enhanced 35S CaMV promoter region through the *CP* gene was completely integrated into the plant's genome.

Table 8 Evaluation of PRSV resistance in transgenic papaya R_1 lines

No. of transgenic papaya R_1 line	PCR analysis	No. of tested plants	Percentage of PRSV resistance[a]
1-12#A44	-	12	-
13-46#A44	+	**34 (~74%*)**	**100**
1-38#A6	-	38	-
39-231#A6	+	**193 (~83%*)**	**100**
A_{405} of coating buffer = 0.050			
A_{405} of healthy plant = 0.053			
A_{405} of PRSV infected plant = 0.927			

The resistant plants were shown in bold.

* Percentage of transgene segregation.

[a]The PRSV resistance was evaluated by comparing with A_{405} value of coating buffer and healthy plant. A_{405} value of ELISA test that was nearly to that value of coating buffer and healthy plant, showing PRSV resistance and A_{405} value was greater than three times of that value of coating buffer and healthy plant, showing PRSV susceptibility.

+ = presence of *CP* gene, - = absence of *CP* gene

Figure 18 Analysis of PCR product of amplified fragments derived from long PCR.

A: PCR product derived from IPCR together with long PCR; lane M1: 1 kb DNA ladder (Fermentas), lane M2: λ *Hin*dIII DNA ladder (Fermentas)

Lane 1-5: PCR products derived from using a pair of primers TF1 and PR1; lane 1: *Eco*RI digested A45, lane 2: *Eco*RI digested and religated A45, lane 3: undigested A45, lane 4: *Spe*I digested non-transformed papaya, lane 5: distilled water

Lane 6-10: PCR products derived from using a pair of primers CF1 and PR2; lane 6: *Spe*I digested A45, lane 7: *Spe*I digested and religated A45, lane 8: undigested A45, lane 9: *Spe*I digested non-transformed papaya, lane 10: distilled water

B: PCR blot hybridization probing with the CP gene; lane M: 1 kb DNA ladder (Fermentas), lane 1: long PCR product derived from amplification of *Eco*RI digested and religated A45 with the pair of primers TF1 and PR1, lane 2: long PCR product derived from amplification of undigested A45 with the pair of primers TF1 and PR1, lane 3: long PCR product derived from amplification of *Spe*I digested and religated A45 with the pair of primers CF1 and PR2, lane 4: undigested genomic DNA A45

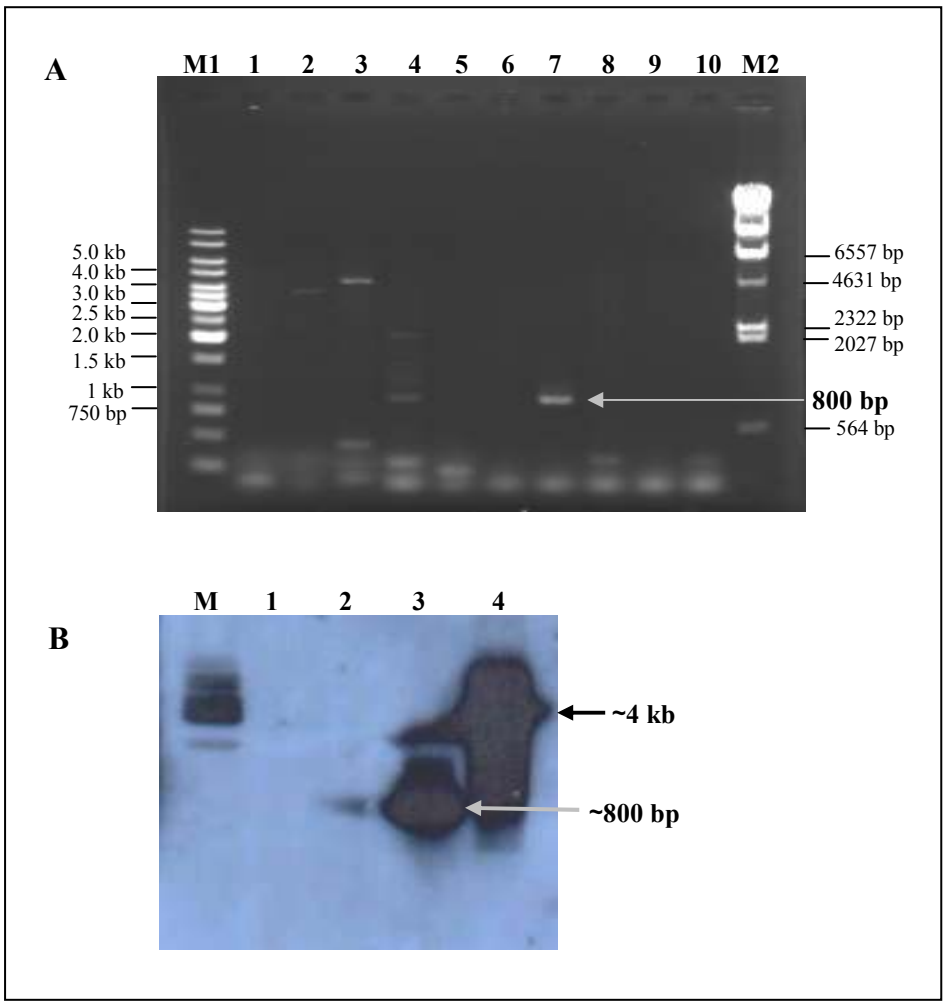

A
M1 1 2 3 4 5 6 7 8 9 10 M2
5.0 kb
4.0 kb
3.0 kb
2.5 kb
2.0 kb
1.5 kb
1 kb
750 bp
6557 bp
4631 bp
2322 bp
2027 bp
800 bp
564 bp
B
M 1 2 3 4
~4 kb
~800 bp

Figure 19 Analysis of nucleotide sequence showed the location of the elements in plant expression construct.

A: Nucleotide sequence of pMON65301; 25 bp repetitive sequences of RB was shown in red, 35S CaMV region was shown in italic, Hsp17.9 leader sequence was shown in green, CP region was shown in blue and *Spe*I site was shown in pink.

B: Nucleotide sequence of SL2; forward primer (CF1) and reverse primer (PR2) were shown in brown, CP region was shown in underlined, 35S CaMV region was shown in italic and *Spe*I site was shown in pink.

.

A. pMON65301 (RB-*Spe*I site) sequence

25 bp repetitive sequences of RB

```
1    GTTTACCCGC CAATATATCC TGTCAAACAC TGATAGTTTA AACATGACTC TCTTAAGGTA
61   GCCAAAGCCC GGGCTTAATT AAGGCGCGCC GGCCAAGTCG GCCGCGGCCG CGTTAACTGC
121  AGGTCCGATG TGAGACTTTT CAACAAAGGG TAATATCCGG AAACCTCCTC GGATTCCATT
181  GCCCAGCTAT CTGTCACTTT ATTGTGAAGA TAGTGGAAAA GGAAGGTGGC TCCTACAAAT
241  GCCATCATTG CGATAAAGGA AAGGCCATCG TTGAAGATGC CTCTGCCGAC AGTGGTCCCA
301  AAGATGGACC CCCACCCACG AGGAGCATCG TGGAAAAAGA AGACGTTCCA ACCACGTCTT
361  CAAAGCAAGT GGATTGATGT GATGGTCCGA TGTGAGACTT TTCAACAAAG GGTAATATCC
421  GGAAACCTCC TCGGATTCCA TTGCCCAGCT ATCTGTCACT TTATTGTGAA GATAGTGGAA
481  AAGGAAGGTG GCTCCTACAA ATGCCATCAT TGCGATAAAG GAAAGGCCAT CGTTGAAGAT
541  GCCTCTGCCG ACAGTGGTCC CAAAGATGGA CCCCCACCCA CGAGGAGCAT CGTGGAAAAA
601  GAAGACGTTC CAACCACGTC TTCAAAGCAA GTGGATTGAT GTGATATCTC CACTGACGTA
661  AGGGATGACG CACAATCCCA CTATCCTTCG CAAGACCCTT CCTCTATATA AGGAAGTTCA
721  TTTCATTTGG AGAGGACACA GAAACATTCG CAAAAACAAA ATCCCAGTAT CAAAATTCTT
                  Hsp17.9 leader sequence
781  CTCTTTTTTT CATATTTCGC AAAGATTTAA AAAGATCCAT GGCGTCGAGA AGCATCGACG
                                          CP start codon
841  ATTATCAGTT TGTTGGCAGT GATGACACAC ATGTGTTTCA CCAGTCCAAA ACCGAAGCTG
901  TGGATGCTGG TCTTAATGAA AAGCTCAAAG ATAAAGAAAA ACAGAAAGGA GAAAAAGATA
961  AACAAAAAGG TAAAGAAAAT AATGAAGCTA GTGACGGAAA CGATGTGTCA ACTAGCACAA
1021 AAACTGGAGA GAGAGATAGA GATGTCAATG CCGGAACTAGT
                                    SpeI site (5'-3')
```

B. SL2 sequence

Forward primer (CF1)

```
1    GCGTCGAGAG CATCGACGAT TATCAGTTTG TTGGCAGTGA TGACACACAT GTGTTTCACC
61   AGTCCAAAAC CGAAGCTGTG GATGCTGGTC TTAATGAAAA GCTCAAAGAT AAAGAAAAAC
121  AGAAAGGAGA AAAAGATAAA CAAAAAGGTA AAGAAAATAA TGAAGCTAGT GACGGAAACG
181  ATGTGTCAAC TAGCACAAAA ACTGGAGAGA GAGATAGAGA TGTCAATGCC GGAACTAGTT
                                                        speI site (5'-3')
241  TAAACATGAC TCTCTTAAGG TAGCCAAAGC CCGGGCTTAA TTAAGGCGCG CCGGCCAAGT
301  CGGCCGCGGC CACGTTAACT GCAGGTCCGA TTGAGACTTT TCAACAAAGG GTAATATCCG
361  GAAACCTCCT CGGATTCCAT TGCCCAGCTA TCTGTCACTT TATTGTGAAG ATAGTGGAAA
421  AGGAAGGTGG CTCCTACAAA TGCCATCATT GCGATAAAGG AAAGGCCATC GTTGAAGATG
481  CCTCTGCCGA CAGTGGTCCC AAAGATGGAC CCCCACCCAC GAGGAGCATC GTGGAAAAAG
541  AAGACGTTCC AACCACGTCT TCAAAGCAAG TGGATTGATG TGATGGTCCG ATTGAGACTT
601  TTCAACAAAG GGTAATATCC GGAAACCTCC TCGGATTCCA TTGCCCAGCT ATCTGTCACT
661  TTATTGTGAA GATAGTGGAA AAGGAAGGTG GCTCCTACAA ATGCCATCAT TGCGATAAAG
721  GGAAGGCCAT CGTTGAAGAT GCCTCTGCCG ACAGTGGTCC CAAAGATGGA CCCCCACCCA
781  CGAGGAGCAT CGTGGAAAAA GAAGACGTTC CAACCACGTC TTCAAAGCAA GTGGATTGAT
841  GTGATATCTC CACTGACGTA AGGGATGACG CACAATCCCA CTATCCTTCG CAAGACCCTT
901  CC
                                                  Reverse primer (PR2)
```

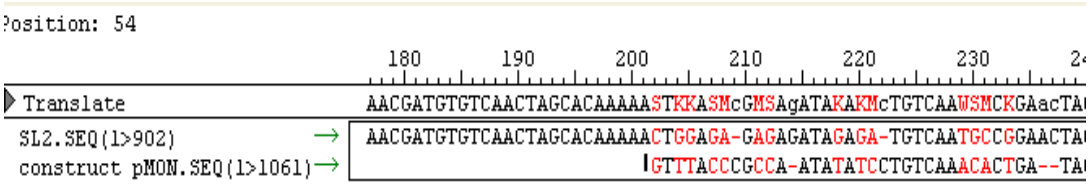


Figure 20 Sequence analysis of SL2 and pMON65301 using SeqMan program. The positions of mismatch base pairing were shown in red.

DISCUSSION

Gene transformation techniques using *Agrobacterium tumefaciens* and particle bombardment have been used to introduce foreign DNA into genome of both monocotyledonous and dicotyledonous plants (Christou, 1995; Horaruang *et al*., 2006; Kertbundit *et al*., 2007; Yang *et al*., 1996). In this study, somatic embryos derived from immature zygotic embryos of papaya seeds were used as a target tissue for *Agrobacterium*-mediated transformation. Forty one of R_0 putative transgenic papaya lines from transformation events were determined for the presence of *CP* or *NIb* transgenes in their genomes. From PCR and Southern hybridization analysis, it was showed that the expected bands of either *CP* or *NIb* transgenes were detected in 38 transgenic papaya lines, but not in these lines: A4, A7 and A10 (Table 6). This result suggested that *Agrobacterium*-mediated transformation is the efficient gene transfer method for papaya transformation and subsequently obtained transgenic papaya with PRSV resistance (Chen *et al*., 2001; Davis *et al*., 2004). The insertion number of PRSV transgene in papaya genome was ranged from 1 to 3 copies, especially two copies of transgene were mostly found in the resistant lines. Those lines (A26, A29, A30, A31, A33, A35, A36, A39, A40, A43 A44) exhibited the equivalent fragment size of transgene (Table 6 and Figure 11A). Therefore, these lines might originally derive from the same clone in transformation event and these plants were assumed as identical lines. However, different size of insertion fragments derived from other lines that exhibited the equivalent of transgene copy number were also found (Table 6). It can be concluded from this result that these plants were originated from the different clone in transformation event and were assumed as individual lines.

In the previous study, transgenic papayas were successfully generated by introduction of the *CP* gene of PRSV, Chiang Mai (PRSV-CM) isolate, into somatic embryos of Thai papaya, Khaek Nual cultivar, via particle bombardment (Chowpongpang *et al*., 2002). These transformed papaya lines in R_0 generation were exhibited various insertion events, varied from 2 to 10 copy number of transgene,

based on the result of Southern blot hybridization. This result suggested that transformed papaya lines derived from particle bombardment occurred a chance of multiple copies of transgene in papaya genome.

PRSV-SB and PRSV-NP were isolated from severely damaged the papaya cultivated areas, Suphan Buri (SB) and Nakhon Pathom (NP) provinces, respectively. Thirty eight lines from R_0 generation carrying *CP*, *CP-IR* or *NIb* transgenes were challenged with PRSV-SB or PRSV-NP isolates. Symptom development appeared within one week after inoculation of non-transformed control plants but was delayed for 2 to 3 weeks in the transgenic susceptible lines. In this work, we evaluated the PRSV resistance in transformed papaya lines at only young stage with 5-6 fully expanded leaves in order to confirm that the confidence of PRSV resistance was really obtained from the mechanism of the transgenes but not obtained from the plant's natural defense system. However, the developmental stage of plant influenced the degree of PRSV resistance. Inoculated plants were susceptible when inoculated at young stage (~6 weeks after germination) otherwise the plants from the same germination batch were resistant when inoculated at older stage (~16 weeks after germination) (Tennant *et al*., 2001). Moreover, *CP* gene dosage and CP sequence identity of the challenge isolate were also affected the degree of PRSV resistance (Tennant *et al*., 2001).

All of the resistant lines carrying *CP*, *CP-IR* and *NIb* transgenes which all transgenes were contained the coding regions from start codon, ATG to stop codon, TGA. In addition, *CP-IR* transgene was composed of *CP* gene and ~250 nucleotides of the inverted repeat of *CP* gene that were inserted at downstream position of the stop codon, TGA. Interestingly, all of transformed papaya lines, R_0 generation, derived from *CP-IR* transgene exhibited highly resistant to the challenged isolate. Moreover, transgenic papaya progenies, R_1, containing *CP* (A44) or *CP-IR* (A6) transgenes were also conferred PRSV resistance (Table 8). This result suggested that the translatable *CP* gene or modified *CP* gene (*CP-IR*) were the promising PRSV resistance in transgenic papaya. Moreover, the modified *CP* gene in the following

forms; antisense orientation (AS-CP), sense orientation with a frame-shift mutation (FS-CP) and sense orientation mutated with three-in-frame stop codons (SC-CP) were evident of PRSV resistance in transgenic papaya obtained from other groups (Davis and Ying, 2004).

To consider the characterization of viral resistance, nucleotide sequence comparison of the *CP* gene of PRSV isolates from different geographical areas was done (Flasinski *et al*., 2002; Tennant *et al*., 2001; Tripathi *et al*., 2004). Sequence alignment revealed that the nucleotide identity between Southeast Asian (SE) isolates, Malaysia (M), Thailand (TSb), the Philippines (Ph) and Vietnam (Vn) ranged from 89.7 to 91.7 (Flasinski *et al*., 2002). This result was used to describe the degree of resistance that depended on the homology of CP gene of PRSV isolate and transgene (Tennant *et al*., 2001). However, previous result observed by Tripathi and colleagues (2004) concluded that the PRSV resistance was not correlated with higher degree of sequence divergence from the transgene that investigated in the transgenic resistant line carrying the *CP* gene (Tripathi *et al*., 2004). From our result, the resistant lines carrying the *CP* or *CP-IR* transgenes of PRSV-SB were resistant to SB isolate and other isolates originally from different geographical areas in Thailand, Nakhon Pathom, Sakon Nakhon, Yasothon and Surat Thani (data not shown). Moreover, the resistant lines carrying the *NIb* transgene of PRSV-SB or PRSV-NP were also resistant to those SB and NP isolates. This result was similar to a result observed by Chen and colleagues (2001) which concluded that the viral replicase (*NIb*) gene was conferred PRSV resistance in transgenic papaya (Chen *et al*., 2001).

The *CP* transgene expression was investigated in the resistant lines of R_0 generation at transcriptional and translational levels. CP transcript was found in both uninoculated and inoculated resistant lines (Figure 14 and 15). The expression of the CP transcript showed high level in both the resistant lines carrying *CP* and *CP-IR* transgenes, A18, A19, A28 and A37, whereas the resistant lines, A20, A23, A25 and A35 showed lower level of CP transcript (Figure 14). In addition, the resistant lines with low level of CP transcript showed less expression during PRSV inoculation

(Figure 15). The resistant lines with high level of CP transcript were detected the protein 32 kDa insize of coat protein (CP) of PRSV by interaction with polyclonal antiserum against the CP of PRSV, Thai isolate. Western blot and ELISA analyses revealed that the coat protein was the least expression in those resistant lines (Figure 15). However, CP expression was found at both transcription and translation in the susceptible line (Figure 14B and 16). From our result, the resistant lines with high level of CP transcript exhibited high degree of PRSV resistance, while other resistant lines with lower CP transcript also exhibited high degree of PRSV resistance. Previous report revealed that the expression level of the transgene is negatively correlated with the degree of PRSV resistance. Low level of transgene expression was found in highly resistant and immune lines while high level of transgene expression was found in resistant lines and susceptible line (Bau *et al*., 2003). To describe this observation, the study of transgene expression in the resistant lines with purposed mechanism of PRSV resistance was carried out (Bau *et al*., 2003; Ruanjan *et al*., 2007). The resistant lines with the lowest transgene expression were investigated the mechanism of resistance by detection for the presence of small interfering (siRNA) in papaya's cell. The presence of siRNA is now considered the most important characteristic of post-transcriptional gene silencing (PTGS), the potential mechanism of PRSV resistance in transgenic papaya (Ruanjan *et al*., 2007). In this work, the resistant lines exhibited the *CP* transgene expression in only transcriptional level. Therefore, the possible mechanism of PRSV resistance might be involved in the process of PTGS.

The resistant lines carrying *NIb* transgene with high degree of PRSV resistance were also investigated transgene expression at transcriptional level. The result revealed high level of NIb transcript in the resistant lines, A11, A16 and A17 (Figure 17), while the lowest expression of NIb transcript was found in the resistant lines, A12 and A14 (Figure 17). In this study, the translational NIb expression was not analyzed because the antiserum to the NIb of PRSV, Thai isolate, is not available. However, the possible mechanism of resistance was still unclear (Chen *et al*., 2001).

The release of commercialized transgenic crops requires the comprehensive analysis of transferred DNA (T-DNA) in plant's genome. In order to obtain the finding, plant's DNA sequences that flanked with the sequences of T-DNA were investigated (Cottage *et al*., 2001; Forsbach *et al*., 2003; Kim *et al*., 2003). T-DNA flanking sequences have been reported in many transgenic plants, such as *Arabidopsis thaliana* (Forsbach *et al*., 2003), *Oryza sativa* (Kim *et al*., 2003) and transgenic potato (Cottage *et al*., 2001). Study of T-DNA flanking sequence was an important factor to describe the characterization of T-DNA integration in the plant's genome which was done by the proteins encoding in *Agrobacterium*-Ti plasmid (Kim *et al*., 2003). For transgenic papaya, T-DNA flanking sequence has not been reported, this is the first investigation with the purpose of the location of T-DNA integration in papaya genome. In this work, we selected the resistant line (A45) that carried one copy *CP* transgene for amplification of T-DNA flanking sequence using inverse PCR (IPCR) together with long PCR. For IPCR strategy, genomic DNA was digested with sticky-end restriction enzymes that cut inside the T-DNA once and completely digested inside the plant's DNA. Then, these fragments were religated in the form of circular fragments that served as a template for PCR amplification. This method was successful amplification of T-DNA flanking sequence in transgenic rice (*Oryza sativa*) (Kim *et al*., 2003). From our result, T-DNA flanking sequence was not successfully obtained and might be due to the following problems in the experiment, (i) there were a few of restriction enzymes that cut inside the T-DNA once and completely digested in the plant's DNA, (ii) there were many fragments, linear and circular forms obtained after religation which caused many unexpected fragments. In addition, another technique that we have applied to separate the target DNA fragment by using the magnetic beads conjugated with streptavidin. The magnetic beads could bind the DNA fragment that hybridized with biotin labelling probe. Genomic DNA was digested with restriction enzyme to give many small fragments, then allowed hybridization with biotin labelling CP probe and separated this complex by adding magnetic beads conjugated with streptavidin. After magnetization, the magnetic beads were removed, then the DNA fragments were separated from the biotin labelling CP probe and used as a template for PCR amplification. From this

technique, the preliminary result was not successful due to poor binding of DNA fragment and the probe. For further investigation, optimization of procedure in this method is required, especially the concentration of the DNA fragment and the probe should be designed and tested for more specific or strong binding. In the previous report, *ACO1* 5' flanking region was successfully determined in *C. papaya* using Adaptor-Ligation-Mediated PCR (Chuaboonmee, 2005). This method was described by Cottage *et al*. (2003) and successfully used for isolation of T-DNA flanking sequence in transgenic potato and transgenic tobacco. For Adaptor-Ligation-Mediated PCR strategy, blunt end yielding restriction fragments were ligated with adaptor and used as a template for PCR amplification using a pair of primers that were specific to the adaptor and the known sequence. This method will be applied for identification of T-DNA flanking sequence in the resistant line for further study.

CONCLUSION

A total thirty-eight transgenic lines of R_0 generation derived from independent transformation event consisted of 13 lines represented *CP* transgene, 20 lines represented *CP-IR* transgene and 5 lines represented *NIb* transgene.

Thirty-four (R_0) transgenic lines were completely resistant to PRSV-SB or PRSV-NP under glasshouse condition. These resistant lines exhibited transgene copy number, ranging from 1 to 3 copies, especially 2 copies of transgene were mostly found in the resistant lines. Moreover, R1 transgenic lines, 13-46#A44 and 39-231#A6 containing *CP* gene were also completely resistant to PRSV-SB that revealed 74% and 83% of transgene segregation, respectively.

High level of CP transcript was found in the resistant lines, A18, A19, A28 and A37, while the resistant lines, A20, A23, A25 and A35 were lower transcriped CP. Moreover, high and low levels of NIb transcript were found in the resistant lines, A11, A16, A17, A12 and A14. However, the coat protein of PRSV, 32 kDa in size, was not found in the highly expressed resistant lines and the accumulation of CP transcript level did not differ during PRSV infection.

The T-DNA sequence of pMON65301 construct, especially the position from enhanced 35S CaMV promoter through the *CP* gene, was completely integrated into the papaya's genome.

LITERATURE CITED

Adams, M. J., J. F. Antoniw and C. M. Fauquet. 2005. Molecular Criteria for genus and species discrimination within the family *Potyviridae*. **Arch. Virol.** 150: 459-479.

Arumuganathan, K. and E. D. Earle. 1991. Nuclear DNA content of some important plant species. **Plant Mol. Biol. Rep.** 9: 208-218.

Ballester, D. G., A. D. Montaigu, A. Galvan and E. Fernandez. 2005. Restriction enzyme site-directed amplification PCR: A tool to identify region flanking a marker DNA. **Anal. Biochem.** 340: 330-335.

Bateson, M. F., E. L. Rosemarie, P. Revill, W. Chaleeprom, C. V. Ha, A. J. Gibbs and J. L. Dale. 2002. On the evaluation and molecular epidemiology of the potyvirus *Papaya ringspot virus*. **J. Gen. Virol.** 83: 2575-2585.

Bau, H-J, Y-H Cheng, T-A Yu, J-S Yang and S-D Yeh. 2003. Broad-Spectrum Resistance to Different Geographic Strains of *Papaya ringspot virus* in Coat Protein Gene Transgenic Papaya. **Virology**. 93: 112-120.

Binns, A. N. 2002. The T-DNA of *Agrobacterium tumefaciens*: 25 years and counting. **Trends Plant Sci.** 7: 231-233.

Birch, R. G. 1997. Plant transformation: problem and strategies for practical application. **Annu. Rev. Plant Physiol. Plant Mol. Biol.** 48: 297-326.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. **Anal. Biochem.** 72: 248-254.

Brunaud, V., S. Balzergue, B. Dubreucq, S. Aubourg, F. Samson, S. Chauvin, N. Bechtold, C. Cruaud, R. DeRose, G. Pelletier, L. Lepiniec, M. Caboche and A. Lecharny. 2002. T-DNA integration into *Arabidopsis* genome depends on sequences of pre-insertion sites. **EMBO Rep.** 3: 1152-1157.

Bustamante, P. I. and R. Hull. 1998. Plant virus gene expression strategies. **Electron. J. Biotechnol.** 1: 65-80.

Bytebier, B., F. Deboeck, H. De Greve, M. Van Montagu and J. P. Hernalsteens. 1987. T-DNA organization in tumor cultures and transgenic plants of monocotyledon *Asparagus officianalis*. **Proc. Natl. Acad. Sci. USA.** 84: 5345-5349.

Charoensilp, G., P. Attasart, M. Juricek, S. Panyim and S. Kertbundit. 2003. Sequencing and Characterization of Thai *Papaya ringspot virus* isolate Type P (PRSVthP). **Sci. Asia.** 29: 89-94.

Chen, G., C. M. Ye, J. C. Huang, M. Yu and B. J. Li. 2001. Cloning of the *Papaya ringspot virus* (PRSV) replicase gene and generating of PRSV-resistant papayas through the introduction of the PRSV replicase gene. **Plant Cell Rep.** 20: 272-277.

Chiang, C. H., J. J. Wang, F. J. Jan, S. D. Yeh and D. Gonsalves. 2001. Comparative reaction of recombinant *Papaya ringspot viruses* with chimeric coat protein (CP) genes and wild-type viruses on CP-transgenic papaya. **J. Gen. Virol.** 82: 2827-2836.

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

Chuaboonmee, S. 2005. **Identification and characterization of a tissue-specific 1-aminocyclopropane-1-carboxylate (ACC) oxidase promoter in *Carica papaya***. M.S. Thesis, Kasetsart University.

Chowpongpang, S., N. Warin, A. Bhunchoth, R. Rodaree, K. Romyanon, S. Attathom, W. Kositratana, S. Wasee and T. Burns. 2002. **Screening of transgenic Thai varieties of papaya for resistance to papaya ringspot virus**. BIOTEC Research Project. 80p.

Cottage, A., A. Yang, H. Maunders, R. C. de Lacy and N. A. Ramsay. 2001. Identification of DNA Sequences Flanking T-DNA Insertions by PCR-Walking. **Plant Mol. Biol. Reptr.** 19: 321-327.

Dasgupta, I., V. G. Malathi and S. K. Mukherjee. 2003. Genetic engineering for virus resistance. **Curr. Sci.** 84: 341-354.

Davis, M. J. and Z. Ying. 2004. Development of Papaya Breeding Lines with Transgenic Resistance to *Papaya ringspot virus*. **Plant Dis.** 88: 352-358.

Dolja, V. V., R. Haldeman, N. L. Robertson, W. G. Dougherty and J. C. Carrington. 1994. Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. **EMBO J.** 13: 1482-1491.

Flasinski, S., V. M. Aquino, R. A. Hautea, W. K. Kaniewski, N. D. Lam, C. A. Ong, V. Pillai and K. Romyanon. 2002. Value of Engineered Virus Resistance in Crop Plants and Technology Cooperation with Developing Countries. CAB Internation 2002. **Economic and Social Issues in Agricultural Biotechnology**. 251-268.

Ferreira, S. A. and K. Y. Pitz. 2001. Virus Coat Protein Transgenic Papaya Provides Practical Control of *Papaya ringspot virus* in Hawaii. **Plant Dis.** 86: 101-105.

Forsbach, A., D. Schubert, B. Lechtenberg, M. Gils and R. Schmidt. 2003. A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. **Plant Mol. Biol.** 52: 161-176.

Foyet, M. 1972. 'L' extraction de la papaine', **Fruits**. 27: 303-306.

Gonsalves, D. 1998. Control of *Papaya ringspot virus* in Papaya : A Case Study. **Annu. Rev. Phytopathol.** 36: 415-437.

Gonsalves, D. and M. Ishii. 1980. Purification and serology of *papaya ringspot virus*. **Phytopathology**. 70: 1028-1032.

Graaff, E., A. Dulk-Ras and P. J. J. Hooykaas. 1996. Deviating T-DNA transfer from *Agrobacterium tumefaciens* to plants. **Plant Mol. Biol.** 31: 677-681.

Hong, Y., K. Levay, J. F. Murphy, P. G. Klein, J. G. Shaw and A. G. Hunt. 1995. A potyvirus polymerase interacts with the viral coat protein and VPg in the yeast cells. **Virology**. 214: 159-166.

Horarung, W., K. Romyanon, M. Boonthum, S. Flasinski and S. Attathom. 2006. Factors affecting *Agrobacterium*-mediated transformation of somatic embryo papaya explant. **Agric. Sci. J.** 3: 231-239.

Jain, R. K., J. Sharma, A. S. Sivakumar, P. K. Sharma, A. S. Byadgi, A. K. Verma and A. Varma. 2004. Variability in the coat protein gene of *Papaya ringspot virus* isolates from multiple locations in India. **Arch. Virol.** 149: 2435-2442.

Jones, A. L., I. E. Johansen, S. J. Bean, I. Bach and A. J. Maule. 1998. Specificity of resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (NIb) gene. **J. Gen. Virol.** 79: 3129-3137.

Kertbundit, S, N. Pongtanon, P. Ruanjan, D. Chantasingh, A. Tanwanchai, S. Panyim and M. Juricer. 2007. Resistance of transgenic papaya plants to *Papaya ringspot virus*. **Biol. Plant.** 2: 333-339.

Kim, S. R., J. Lee, S. H. Jun, S. Park, H. G. Kang, S. Kwon and G. An. 2003. Transgene structures in T-DNA-inserted rice plants. **Plant Mol. Biol.** 52: 761-773.

Kositratana, W., N. Thaveechai, S. Attathom, R. Hongprayoon and O. Chatchawankanphanich. 1991. Control of papaya ringspot disease by cross protection. **Kasetsart J. (Natural Science)**. 25: 33-39.

Liu, Y. G. and N. Huang. 1998. Efficient Amplification of Insert End Sequences from Bacterial Artificial Chromosome Clones by Thermal Asymmetric Interlaced PCR. **Plant Mol. Biol. Reptr.** 16: 175-181.

Liu, Y. G., N. Mitsukawa, T. Oosumi and R. F. Whitter. 1995. Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. **Plant J.** 8: 457-463.

Lohiya, N. K., B. Manivannan and S. Garg. 2006. Toxicological investigations on the methanol sub-fraction of the seeds of *Carica papaya* as a male contraceptive in albino rats. **Reprod. Toxicol.** 22: 461-468.

Macintosh, S., D. J. Robinson and B. D. Harrison. 1992. Detection of three whitefly transmitted geminiviruses occurring in Europe by tests with heterologous monoclonal antibobies. **Ann. Appl. Biol.** 121: 297-303.

Manshardt, R. M. 1999. **"UH Rainbow" papaya**. University of Hawaii Collage of tropical Agriculture and Human Resources, New Plants for Hawaii-Honolulu: University of Hawaii Collage of tropical Agriculture and Human Resources. 2p.

Michiels, A., M. Tucker, W. V. D. Ende and A. V. Laere. 2003. Chromosome Walking of Flanking Regions From Short Known Sequences in G-C Rich Plant Genomic DNA. **Plant Mol. Biol. Reptr.** 21: 295-302.

Ponce, M. R., V. Quesada and J. L. Micol. 1998. Rapid discrimination of sequences flanking and within T-DNA insertions in the *Arabidopsis* genome. **Plant J.** 14: 497-501.

Powell-Abel, P., R. S. Nelson, B. De, N. Hoffmann, S. G. Roger. 1986. Delay of disease development in transgenic plants that express tobacco mosaic virus coat protein. **Science**. 232: 738-743.

Reavy, B. and M. A. Mayo. 1994. Genetic Engineering of Virus Resistance, pp. 183-214. *In* A. M. R. Gatehouse, V. A. Hilder and D. Boulter, eds. **Biotechnology in Agriculture No. 7 Plant Genetic Manipulation for Crop Protection**. Redwood Books, UK.

Rogers, H. J., N. A. Burns and H. Parkes. 1996. Comparison of small-scale method for the rapid extraction of plant DNA suitable for PCR analysis. **Plant Mol. Biol. Reptr.** 14: 170-184.

Ruanjan, P., S. Kertbundit and M. Juricer. 2007. Post-transcriptional gene silencing is involved in resistance of transgenic papayas to papaya ringspot virus. **Biol. Plant.** 51: 517-520.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. **Molecular Cloning: A Laboratory Manual**. 2nded. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Sanford, J. C. and S. A. Johnston. 1985. The concept of parasite-derived resistance-deriving resistance genes from the parasite's own genome. **J. Theor. Biol.** 113: 395-405.

Samson, J. A. 1986. **Tropical fruits**. 2nded. Longman Inc., New York.

Sha, Y., S. Li, Z. Pei, L. Luo, Y. Tian and C. He. 2004. Generation and flanking sequence analysis of a rice T-DNA tagged population. **Theor. Appl. Genet.** 108: 306-314.

Simon-Mateo, C., J. J. Lopez-Moya, H. S. Guo, E. Gonzalez and J. A. Garcia. 2003. Suppressor activity of potyviral and cucumoviral infections in potyvirus-induced transgene silencing. **J. Gen. Virol.** 84: 2877–2883.

Southern, E. M. 1975. Detection of Specific Sequences among DNA Fragments Separated by Gel Electrophoresis. **J. Mol. Biol.** 98: 503-517.

Tennant, P., G. Fermin, M. M. Fitch, R. M. Manshardt, J. L. Slightom and D. Gonsalves. 2001. *Papaya ringspot virus* resistance of transgenic Rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. **Eur. J. Plant Pathol.** 107: 645–653.

Tripathi, S., H. J. Bau, L. F. Chen and S. D. Yeh. 2004. The ability of Papaya ringspot virus strains overcoming the transgenic resistance of papaya conferred by the coat protein gene is not correlated with higher degrees of sequence divergence from the transgene. **Eur. J. Plant Pathol.** 110: 871–882.

Tzfira, T., J. Li, B. Lacroix and V. Citovsky. 2004. *Agrobacterium* T-DNA integration : molecules and models. **Trends Genet.** 20: 375-383.

Urcuqui-Inchima, S., A. L. Haenni and F. Bernardi. 2001. Potyvirus proteins: a wealth of functions. **Virus Res.** 74: 157-175.

Verwoerd, T. C., B. M. Dekker and A. Hoekema. 1989. A small-scale procedure for the rapid isolation of plant RNAs. **Nucleic Acids Res.** 17: 2362.

Wang, J., L. Li, Z. Y. Shi, X. S. Wan, L. S. An and J. L. Zhang. 2005. T-DNA Integration Category and Mechanism in Rice Genome. **J. Integr. Plant Biol.** 47: 350-361.

Wang, C. H. and S. D. Yeh. 1997. Divergence and Conservation of the genomic RNAs of Taiwan and Hawaii strains of *papaya ringspot potyvirus*. **Arch. Virol.** 142: 271-285.

Webb, K. J. and P. Morris. 1994. Methodologies of Plant Transformation, pp. 7-18. *In* A. M. R. Gatehouse, V. A. Hilder and D. Boulter, eds. **Biotechnology in Agriculture No. 7 Plant Genetic Manipulation for Crop Protection**. Redwood Books, UK.

Yang, J. S., T. A. Yu, Y. H. Cheng and S. D. Yeh. 1996. Transgenic papaya plants from *Agrobacterium*-mediated transformation of petioles in vitro propagated multishoots. **Plant Cell Rep.** 15: 459-464.

Zhai, W., C. Chen, X. Zhu, X. Chen, D. Zhang, X. Li and L. Zhu. 2004. Analysis of T-DNA-*Xa21* loci and bacterial blight resistance effects of the transgene *Xa21* in transgenic rice. **Theor. AppI. Genet.** 109: 534-542.

APPENDIX

Appendix Table 1 Composition of bacterium culture medium

Compound	LB medium	2X-YT medium	SOB medium
Bacto-tryptone	10 g/l	16 g/l	20 g/l
Bacto-yeast extract	5 g/l	10 g/l	5 g/l
NaCl	10 g/l	5 g/l	-
agar	15 g/l	15 g/l	-
1 M KCl	-	-	1 ml/l
1 M $MgCl_2$	-	-	10 ml/l
1 M $MgSO_4$	-	-	10 ml/l

Source: Sambrook *et al*., (1989)

1. Material and Reagent for estimation of total protein concentration by Bradford method

1.1 Preparation of Comassie Brilliant Blue solution

Comassie Brilliant Blue G250 0.02 g

95% Ethanol 10 ml

Comassie Brilliant Blue was dissolved in 95% Ethanol first.

Phospholic acid (85% w/v) 20 ml

Distilled water 170 ml

The solution was mix and filtered through Whatman filter paper No. 1 and stored in the dark until use.

Appendix Table 2 Preparation of BSA standard curve for measuring the protein concentrations

Protein standard (μg/tube)	μl of stock BSA solution (0.1 mg/ml)	μl of distilled water
0	0	200.00
1.25	12.50	187.50
2.50	25.00	175.00
5.00	50.00	150.00
7.50	75.00	125.00
10.00	100.00	100.00

2. Material and Reagent for separation of total protein by SDS-PAGE

2.1 Preparation of 5X running buffer: 1000 ml

Tris-base	15.1	g
Glycine	94	g
10% (w/v) SDS	50	ml
Distilled water	1000	ml

2.2 Preparation of transfer buffer: 1000 ml

Tris-base	2.42	g
Glycine	11.26	g
Methanol	100	g
Distilled water	1000	ml

Appendix Table 3 Solutions for preparing 12% resolving gels for Tris-glycine SDS-polyacrylamide gel electrophoresis

Solution components	Component volumes (ml) per gel mold volume of					
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml
H_2O	1.6	3.3	4.9	6.6	8.2	9.9
30% Acrylamide/Bis solution 19:1	2.0	4.0	6.0	8.0	10.0	12.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3
10% ammonium persulphate	0.05	0.1	0.15	0.2	0.25	0.3
TEMED	0.002	0.004	0.006	0.008	0.01	0.012

Source: Sambrook *et al*., (1989)

Appendix Table 4 Solutions for preparing 5% stacking gels for Tris-glycine SDS-polyacrylamide gel electrophoresis

Solution components	Component volumes (ml) per gel mold volume of					
	1 ml	2 ml	3 ml	4 ml	5ml	6 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1
30% Acrylamide/Bis solution 19:1	0.17	0.33	0.5	0.67	0.83	1.0
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06
10% ammonium persulphate	0.01	0.02	0.03	0.04	0.05	0.06
TEMED	0.001	0.002	0.003	0.004	0.005	0.006

Source: Sambrook *et al*., (1989)

3. Material and Reagent for ELISA

3.1 Coating buffer (0.05 M Carbonate buffer, pH 9.6): 1000 ml

Na_2CO_3	0.795	g
$NaHCO_3$	1.465	g

These compounds were dissolved in distilled water, autoclaved at 121°C for 15 min and stored at 4°C for maximum 14 days.

3.2 5X PBST: 1000 ml

NaCl	146.1	g
Na_2HPO_4	4.6	g
KH_2PO_4	1.0	g
Tween 20	2.5	g

These compounds were dissolved in distilled water, Tween 20 was added after sterilization.

3.3 Diethanolamine buffer, pH 9.8: 1000 ml

Diethanolamine	97	ml
distilled water	800	ml

The solution was adjusted pH to 9.8 with NaOH and stored at 4°C.