CONSTRUCTION OF FULL-LENGTH PAPAYA RINGSPOT VIRUS TYPE P THAI STRAIN CASSETTES FOR *IN VIVO* TRANSCRIPTS AND PRODUCTION IN PAPAYA PLANT

GULSIRI CHAROENSILP

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Papaya ringspot virus (PRSV) is a major limiting factor in Thailand's papaya production. The synthesis of biologically functional RNA transcripts from the full-length cDNA clones *via in vitro* or *in vivo* transcription plays a key role in the further research of PRSV at the molecular level. When the molecular biology of PRSV is clearly understood, effective methods for controlling PRSV can be developed. This thesis focuses on development of an effective method that can generate infectious RNA transcripts *in vivo* from Thai isolate of PRSV, type P.

Three plasmid cassettes for *in vivo* and *in vitro* transcription of papaya ringspot virus type P were constructed. The *in vivo* expression cassettes were composed of CaMV 35S promoter or partially duplicated CaMV 35S promoter, the 5′ end combined with 3′ end of PRSV at *Sac*I site, the 127 bp of artificial poly (A) tail and the NOS terminator. The vectors were named pSA1078 (single 35S promoter) and pSA1079 (partially duplicated 35S promoter). The *in vitro* PRSV transcription cassette contains T7 promoter, the full length of papaya ringspot cDNA, the 127 bp of artificial poly(A) tail and the NOS terminator (pSA1110).

The three overlapping fragments of a 10.3-kb full-length of Thai isolate of the PRSV genomic cDNA were obtained by RT-PCR technique. All three fragments were combined by sequential cloning to obtain the full-length cDNA clone of PRSV under the T7 promoter (pSA1100 plasmid). A 9.5 kb *Sac*I fragment from pSA1100 was further cloned into both *in vivo* expression cassettes to obtain the full-length cDNA clones of PRSV under single CaMV 35S promoter (pSA1101) and partially duplicated 35S promoter (pSA1102).

The two full-length cDNA plasmids, pSA1101 and pSA1102 were used to infect papaya plants by mechanical inoculation. Plasmid pSA1110 was used as a negative control while live virus particles were used as positive control. Only 30 % of the positive control plants showed severe symptoms at three weeks post-inoculation while no symptom was so far seen in remaining plants.

These findings suggest that further research in the development of infectious RNA transcripts *in vivo* from Thai isolate of PRSV, type P is required.

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กุลศิริ เจริญศิลป์: การสร้าง cassettes ของจีโนมไวรัสใบด่างจุดวงแหวน type P สายพันธุ์ไทยสำหรับ *in vivo* transcripts และทดสอบการก่อโรคในพืช (CONSTRUCTION OF THE CASSETTES OF FULL-LENGTH PAPAYA RINGSPOT VIRUS TYPE P THAI STRAIN FOR *IN VIVO* TRANSCRIPTS AND PRODUCTION IN PAPAYA PLANT) คณะกรรมการควบคุมวิทยานิพนธ์ : Miloslav Juricek, Ph.D., สุณี เกิดบัณฑิต, Ph.D., บูรชัย สนธยานนท์, Ph.D. 167 หน้า ISBN 974-664-396-7

ไวรัสใบด่างจุดวงแหวนก่อโรคที่เป็นปัญหาสำคัญต่อการผลิตมะละกอในประเทศไทย การสร้าง RNA transcripts จาก full-length cDNA clones โดยใช้วิธี *in vitro* หรือ *in vivo* transcription นับเป็นกุญแจที่สำคัญสำหรับใช้ศึกษาไวรัสนี้ในระดับโมเลกุล เพื่อนำความรู้ไปใช้ในการควบคุมการระบาดของไวรัส ในงานวิจัยนี้ได้ทำการศึกษาและพัฒนาวิธีการสร้าง infectious RNA transcripts *in vivo* ของไวรัส PRSV type P ที่ระบาดในประเทศไทย

ได้สร้างพลาสมิดที่มี plant expression cassettes ขึ้น 3 ชนิด เพื่อใช้สำหรับ *in vivo* และ *in vitro* transcription ของ ไวรัส PRSV type P พลาสมิดสองชนิดแรกที่ใช้สำหรับ *in vivo* transcription ประกอบด้วยส่วนโปรโมเตอร์ของ CaMV 35S (แบบ single และแบบ partially duplicated), ปลายด้าน 5' และ 3' ของจีโนมของไวรัส PRSV เชื่อมต่อกันที่จุดตัดเอนไซม์ *Sac*I, ส่วนปลาย poly(A) tail ที่มีขนาด 127 เบส และส่วนควบคุม Nos terminator พลาสมิดนี้ชื่อ pSA1078 (มีส่วนโปรโมเตอร์ของ CaMV 35S แบบ single) และ pSA1079 (มีส่วนโปรโมเตอร์ของ CaMV 35S แบบ partially duplicated) พลาสมิดที่ใช้สำหรับ *in vitro* expression นั้น ประกอบด้วย T7 promoter, จีโนมของไวรัส, ส่วนปลาย poly(A) tail ที่มีขนาด 127 เบส และส่วนควบคุม Nos terminator (ให้ชื่อว่าพลาสมิด pSA1110)

ได้สร้าง genomic cDNA ของไวรัส PRSV มีความยาว 10.3 กิโลเบส โดยนำชิ้นดีเอ็นเอย่อย 3 ชิ้นที่ได้จากปฏิกิริยา RT-PCR มาเชื่อมต่อกันเป็นชิ้นเดียวในพลาสมิด pSA1100 โดยวางอยู่ต่อกับ T7 promoter ได้ทำการตัดชิ้นดีเอ็นเอขนาด 9.5 กิโลเบสจากพลาสมิด pSA1100 ด้วยเอนไซม์ SacI แล้วตัดต่อเข้ากับพลาสมิดสำหรับ *in vivo* transcription ทำให้ได้ full length cDNA clones ของไวรัส PRSV ภายใต้การควบคุมโปรโมเตอร์ CaMV35S แบบ single (ให้ชื่อว่าพลาสมิด pSA1101) และแบบpartially duplicated (ให้ชื่อว่าพลาสมิด pSA1102)

นำพลาสมิด pSA1101 และ pSA1102 มาทดสอบการก่อโรคในต้นมะละกอเปรียบเทียบกับ pSA1110 และไวรัส PRSV พบว่ามีเพียง 30% ของมะละกอที่ได้รับเชื้อ PRSV เท่านั้นที่แสดงอาการโรคขณะที่มะละกออื่นๆที่ทำการทดสอบไม่แสดงอาการ

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
^{o}C	degree Celsius
CTAB	hexadecyl tri-methyl ammonium bromide
cDNA	complementary deoxyribonucleic acid
C-terminal(us)	carboxy terminal(us)
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dATP	deoxy adenosine-5-triphosphate
dCTP	deoxy cytidine-5-triphosphate
dGTP	deoxy guanosine-5-triphosphate
dNTPs	dATP, dCTP, dGTP, dTTP
dTTP	deoxy thymidine-5-triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
g	gram(s)
HC-Pro	helper component-proteinase

LIST OF ABBREVIATIONS (CONTS.)

hr(s)	hour(s)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair(s)
kDa	kilodalton(s)
Lac Z	β-galactosidase gene
LB	Luria-Bertani media
M	molar
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mM	millimolar
nm	nanometer(s)
no.	number
N-terminal(us)	amino terminal(us)
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PRSV	papaya ringspot viruse
RNA	ribonucleic acid
RNase A	ribonuclease A
RT-PCR	reverse transcriptase-polymerase chain reaction

LIST OF ABBREVIATIONS (CONTS.)

sec	second(s)
TB	terrific broth
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit(s)
UV	ultraviolet
µg	microgram(s)
µl	microliter(s)
µM	micromolar
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4chloro-3-idocyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

1. Papaya ringspot virus (PRSV)

1.1 Characteristic and classification of PRSV

Papaya ringspot virus (PRSV) is a flexuous filamentous virus, which is approximately 780 x 12 nm in diameter (Figure 1). It consists of a single positive RNA specie (1-3) and one kind of coat protein subunit (4, 5) that induces both cylindrical inclusions (CI, pinwhelllike) and amorphous inclusions (AI) in the cytoplasm of the infected host cell (6, 7). PRSV is a member of the genus potyvirus of the family *Potyviridae* (8, 9). It is classified into two strains, which cannot be distinguished by serological method but they can be distinguished by host range (10). Papaya ringspot virus type W (PRSV-W, formerly watermelon mosaic virus 1) infects only cucurbits and causes severe losses in a wide range of economically important cucurbit crops. Papaya ringspot virus type P (PRSV-P) causes a papaya ringspot disease as well as attacks a limited experimental host range within cucurbits (11). The nucleotide sequence comparison of the 3′ terminal region of PRSV-W and PRSV-P show 98.2% identity in their NIb gene region and 97.7% identity in their coat protein gene. The sequences of these two strains are distinct from other potyvirus types (11).

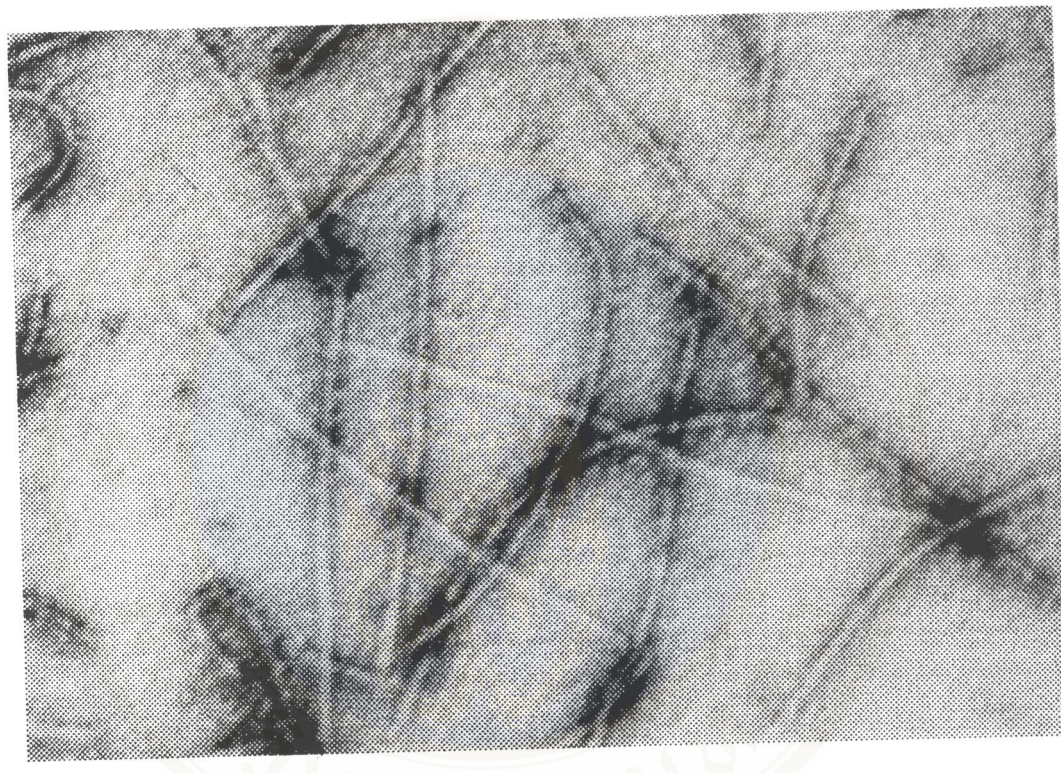


Figure 1. Papaya ringspot virus particles under the transmission electron microscope (40,000x), (taken by Central Equipment Laboratory of Electronmicroscope and Cell Biology Research Unit, Department of Anatomy, Faculty of Science, Mahidol University).

1.2 Disease caused by PRSV

The papaya ringspot disease can be found in many tropical and subtropical countries where papaya is grown and has become a major limiting factor in papaya production in many countries such as France, Germany, USA, India, Taiwan, China, and Thailand. Infected trees show symptoms within 2 to 3 weeks after virus infection. The symptoms consist of intense yellow spots on leaves, small shoestring-like new leaves (Figure 2), stunting of the plant, dark green and slightly sunken rings on the fruit, and numerous oily-looking streaks on the stem. Fruits produced after infection are usually small in size, exhibit lichenlike lesions and ringspots (Figure 3), and show uneven bumps. Trees infected at a very young age are stunted and would never produce any fruit. PRSV is transmitted by several species of aphids in a non-persistent manner. The virus can spread rapidly from one tree to other papaya trees, then to all trees in an orchard within few months (12).

2. Molecular biology of PRSV

2.1 Genome structure and organization

The complete genomic RNA sequences of PRSV type P from Hawaiian isolate (PRSV-HA) and Taiwanese isolate (PRSV-YK) were published in 1992 (13) and 1997 (14), respectively. The genomic RNA is 10326 nucleotides in length, containing the genome linked protein (VPg) at 5′end, but excluding the poly(A) tract. The genome contains one large open reading frame that starts at nucleotide 86 and ends at positions 10120, encoding a polyprotein of 3344 amino acids, which is subsequently cleaved by 3 virus-specific proteinases: P1, HC-Pro and NIa, (15) at specific points to produce smaller nine proteins. The genetic organization of PRSV RNA is proposed to be VPg

Figure 2. Symptoms of papaya ringspot disease on a papaya leaf
The infected papaya plant shows ringspot, mottling and malformation of papaya leaf.

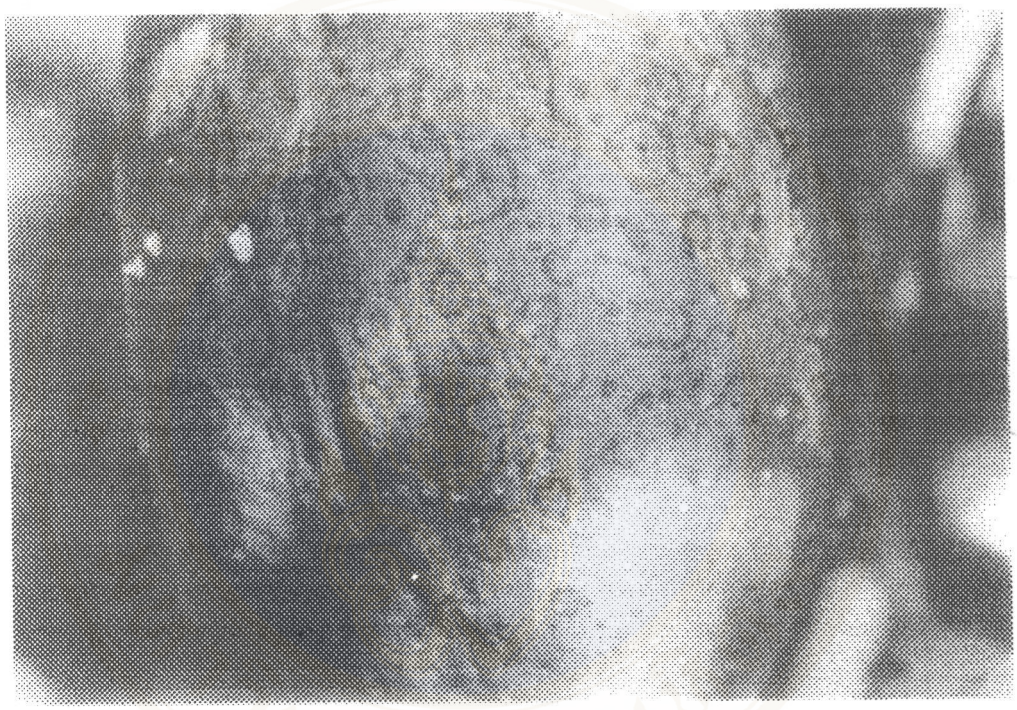


Figure 3. Symptoms of papaya ringspot disease on a papaya fruit
The infected papaya plant shows ringspot and streaking on surface of papaya fruit.

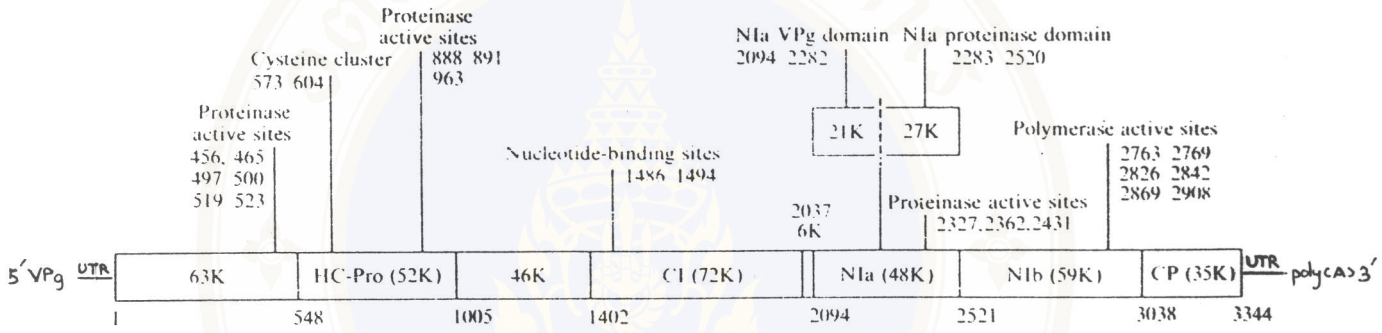


Figure 4. The genetic organization of the PRSV RNA (modified from a tentative map of PRSV polyprotein of Yeh *et al.*, 1992 (13)).

Specific motifs and their positions on the PRSV polyprotein are indicated. Solid bars indicate cleavage sites in the polyprotein. The dashed line indicates the potential internal cleavage site of the NIa protein.

– 5′leader – 63K P1 – 52K HC–Pro – 46K P3 – 72K CI – 6K – 48K NIa – 59K NIb – 35K CP – 3′non-coding region – poly(A) tract, as illustrated in figure 4. The 23 nucleotides at the 5′end (AAATAAAACATCTCAACACAACA) of PRSV Hawaii and Taiwan isolates are identical (14) and also conserved in other potyviruses (13, 16). The conservation of the 5′leader, adenine rich sequences is proposed to facilitate the melting of RNA secondary structures (17, 18), to play important role in the virus life cycle such as encapsidation, translation or replication (19). The (CAA)n motif is present in the 5′leader sequences of PRSV-HA and PRSV-YK, with 10 and 7 repeats, respectively. This kind of motif is also present in the 5′ leader sequences of two known potyviruses, tobacco etch virus (TEV) and pea seedborne mosaic potyvirus (PSbMV), and were found to enhance GUS activity to a similar level as the omega sequence of tobacco mosaic virus (TMV), in which (CAA)n motif is responsible for a translation enhancement (20-22). The 3′non-coding regions of different potyviruses have been described as heterogeneous in terms of size, sequence, and predicted secondary structures (11, 23-25). Features that seem common to all of them are the presence of AT-rich segments and the fact that each sequence can be predicted to fold into stable secondary structures (24). Among the different strains of the same potyvirus, the 3′non-coding regions are clearly more conserved (14, 19). The poly(A) tail have been found to be very heterogeneous in length (23, 26). In plum pox potyvirus (PPV), they have a very peculiar length distribution that might have some functional significance, as it is rapidly recovered upon replication in plants of PPV infectious *in vitro* transcripts (27).

2.2 Functions of PRSV gene products

The polyprotein product of PRSV can be cleaved into several functional proteins. The P1 protein of potyviruses shows high variability in molecular weight (29 KDa to 63 KDa) and sequence (10.4-18.3% identity) (13, 28, 29). The P1 proteins of the same strain but different isolates of PRSV-HA and PRSV-YK have 66.7% sequence identity (14). The C terminus of PRSV P1 protein has a structure similar to those of the other potyviruses, which is serine-type proteinase and required for polyprotein processing at its own C terminus (30-32). The active sites of P1 protein of PRSV are His_{456}, Asp_{465}, Gly-Ser-Ser_{499}-Gly, Phe_{519}-Val-Val-Arg-Gly_{523} (13) and its cleavage site is 24 amino acid downstream from a consensus sequence FVVRG (33). P1 protein of the potyvirus group functions as a trans-active accessory factor during genome amplification (34) and has RNA-binding property in a sequence-unspecific manner (35-37). It was suggested that P1 functions as a movement protein involved in cell-to-cell transport of virus in plant (19, 38, 39). However, experimental evidence for a movement function of P1 protein is still lacking.

The second protein product of PRSV is HC-Pro (helper component proteinase), the amino acid sequence of HC-Pro of PRSV shows a 45.1% to 51.8% sequence identity with HC-Pro proteins of other potyviruses. The N terminus part of potyviral HC-Pro protein affects virulence (symptom severity), genome amplification and virus accumulation (40-43). KITC and PTK amino acid motifs of HC-Pro proteins of tobacco vein mottling virus and zucchini yellow mosaic virus are required for transmissibility by aphid vectors (41, 44). The central region of HC-Pro protein of tobacco etch potyvirus affects long-distance movement of virus (45, 46), whereas the C terminus part of HC-Pro protein of bean common mosaic necrosis potyvirus is

needed for cell-to-cell movement (47) and has cysteine-type protienase activity that autocatalytically cleaves its C terminus (48). The cysteine cluster Cys-X_8-Cys-X_{13}-Cys-X_4-Cys-X_2-Cys (573-604) in the HC-Pro protein of potyviruses is considered to be similar to the zinc fingers of several nucleic acid-binding proteins (49). The proteinase active sites are Gly-Tyr-Cys-Tyr (888-891) and His_{963}. There are some reports of potyviruses HC-Pro protein which have RNA-binding property in a sequence-independent manner (37, 50).

The third protein product of PRSV is P3, lying between HC-Pro and CI in the polyprotein and showed a 25.7- 33.0% sequence identity with those of other potyviruses (13). The function of this protein remains unclear but it is proposed to be the proteolytic cofactor in the regulation of proteolytic processing of potyviral polyprotein (19). P3 protein is required for virus replication in the infected cell (51) and has been showed to interact with cytoplasmic (52) and nuclear (53) inclusions in tobacco vein mottling virus (TVMV) and pea seed-borne mosaic virus (PSbMV) infected cells, respectively. The TVMV P3 protein has been show to be present predominantly in membrane-enriched fractions of extracts of infected leaves (54).

The cylindrical inclusion protein (CI) of PRSV showed a 50.1-59.0% sequence identity with those of other potyviruses (13). The CI protein contains nucleoside triphosphatase and RNA unwinding activities and exhibits sequence similarity with several known RNA helicases (30, 55). The nucleotide binding motive GAVGSGKST, which has been identified in CI proteins of tobacco etch virus (TEV), TVMV, plum pox potyvirus (PPV), potato virus Y (PVY) and PSbMV (49, 56, 57), is also present in the CI protein of PRSV at polyprotein positions 1486 to 1494. This protein may involved in virus replication as a membrane-bound protein (13, 58). The RNA-binding

property in a sequence-unspecific manner of CI protein of PPV (59), tamarillo mosaic potyvirus (TaMV) (60) and potato A potyvirus (PVA) (37) have been studied. The functional role of CI protein in virus movement in the early infection has been suggested (61-63). There is an evidence to support that the formation of specific structures by potyviral CI protein is required and plays a direct role in the intracellular passage of viral genetic material in the form of virus particles or complexes containing viral CP and RNA in infected plants (64-66).

The 6 KDa protein product of PRSV shared a 31.6 to 35.1% sequence identity with the other potyviruses (13). The function of this protein is still unclear. It may be anchored to an endoplasmic reticulum membrane in the form of a 6K/NIa polyprotein, or a large polyprotein containing 6K/NIa and it is proposed to be play a role related to some step of the viral RNA replication process (19, 67).

The NIa protein (nuclear inclusion a) is function as a proteinase responsible for *cis* and *trans* proteolytic activity for the five cleavages in the C terminus portion of the polyprotein (68-70). The N terminus 24 KDa portion of the NIa protein of tobacco etch potyvirus has been reported to be viral VPg (71). The internal cleavage site delimiting the VPg and proteinase domains of tobacco etch potyvirus was proposed by Johansen *et al.* (1991) and confirmed by Dougherty and Parks (1991) (57, 72). This cleavage site is also found in the NIa protein of PRSV at position 2282 to 2283 (VHHE / GKS). Thus PRSV NIa can also be divided into two domains, NIa-VPg and NIa-Pro. The former shows 45.3 to 54.4% sequence identity and latter 39.4 to 46.9% sequence identity with the corresponding domains of other potyviruses (13). The catalytic triad of NIa proteinase, His, Asp and Cys (70), is conserved in all the potyviruses, including PRSV (polyprotein position 2327, 2362 and 2431) (13). The

proteolysis process at cleavage site that separates the VPg and proteinase domains of NIa protein might be temporarily or functionally connected with the RNA replication process (19, 73-75). Both NIa-VPg and NIa-Pro are found to bind RNA in a sequence independent manner (37, 76). In the potyvirus family, the potyviral VPg binds to the 5′ phosphate of the 5′terminus nucleotide of genomic RNA through the hydroxyl group of a tyrosine residue in the NIa-VPg protein (77, 78). This residue is found in a block of amino acids completely conserved in the NIa-VPg proteins of TVMV, TEV, PPV and PVY (77). Strikingly conserved of the first 20 nucleotides at 5′terminus of genomic RNA could be implicated in interaction with VPg (79). The NIa-VPg proteins of TEV and PVA are proposed to function as a long distance movement proteins (80, 81). In turnip mosaic potyvirus, the viral genome linked protein (VPg) is found to be interact with eIF(iso)4E which plays an essential role in the initiation of the translation (82). The vast majority of NIa molecules are localized in the nuclei of infected cells and these proteins contain independent nuclear localization signal (NLS) (83-85).

The nuclear inclusion b protein (NIb) of PRSV showed a 58.4 to 60.9% sequence identity with those of other potyviruses (13). The relatively high degree of identity indicates that this protein is the most conserved potyvirus protein. The consensus motifs YCDADGS, GNNSGQPSTVVDNT(S)LMV and NGDDL-X34-K of NIb are responsible for the putative RNA polymerase function of potyviruses (28, 86, 87). These motifs also present in PRSV polyprotein at positions 2763 to 2769, 2826 to 2842 and 2869 to 2908 (13). The vast majority of NIb molecules are localized in the nuclei of infected cells and these proteins contain independent nuclear localization signal (NLS) (88). The NIb proteins of TVMV and PVA are found to bind

RNA in a sequence unspecific manner (37, 89). In 1997, Xiao Hua Li *et al.* proposed the model of positive-strand RNA synthesis that the NIb polymerase might be recruited to initiation complexes through protein-protein interactions with the Pro domain of intact NIa (90). The NIa protein itself might be anchored to endoplasmic reticulum membrane in the form of a 6K / NIa polyprotein, or a large polyprotein containing 6K / NIa, as proposed previously (67, 91). How a NIa-NIb complex might be associated with viral RNA or with other replication proteins is not yet known. Initiation of RNA synthesis may be stimulated by a priming mechanism involving the VPg domain of NIa, as proposed for picornaviruses and potyviruses (piconar-like plant viruses) (74, 75, 92-94).

The coat protein (CP) protein of PRSV shares a 53.2 to 56.6% sequence identity with other potyviruses (13). A DAG amino acid motif at the N terminus of the potyvirus CP is required for aphid-transmissibility (43, 95-98). The potyvirus CP encapsidates viral RNA in a helical, filamentous particle in which both termini of CP are exposed on the virion surface (26, 99, 100) and affect long-distance movement (101-103). Mutation in the central region of potyvirus CP result in defective virus assembly and cell to cell movement (47, 64, 101, 102, 104) and reduce genome amplification (105). The potyvirus CP is found to bind RNA in a sequence independent manner (37).

3. Systemic infection

The systemic infection (Figure 5) of plants by viruses occurs via a wound created either by mechanical means or by a vector organism. There are at least three

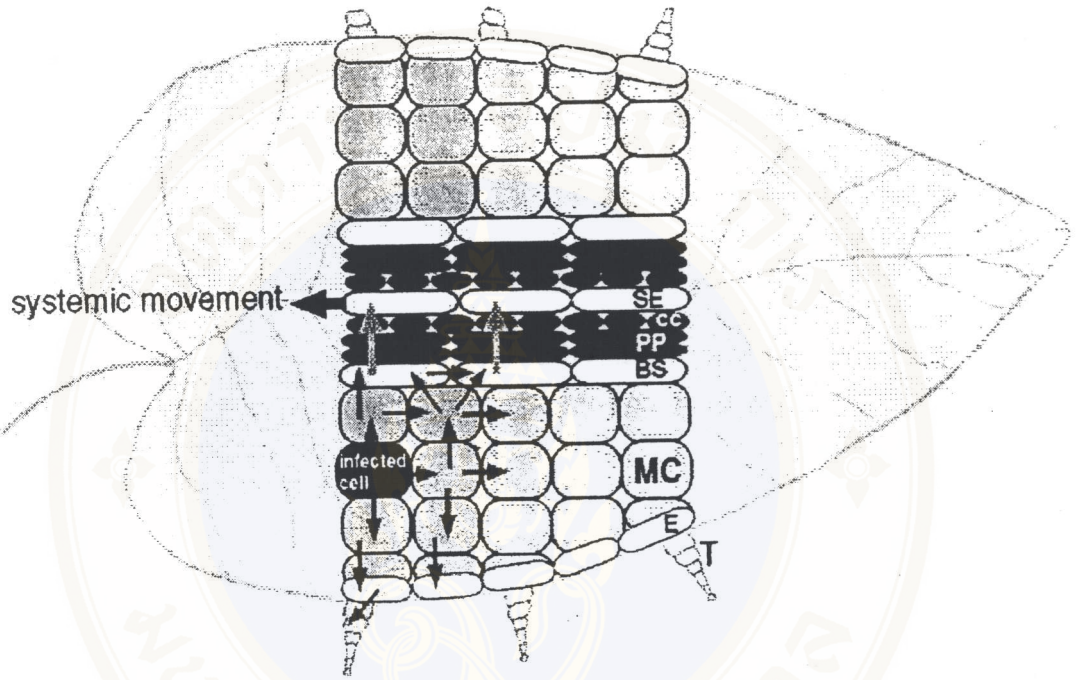


Figure 5. Cellular routes for local and systemic movement of plant viruses (122)

T, trichome; E, epidermal cells; MC, mesophyll cells; BS, bundle sheath cells; PP, phloem parenchyma cells; CC, companion cells; SE, sieve elements. Arrows indicate viral movement. Viral spread between trichome, epidermis, and mesophyll cells represents local, cell-to-cell movement. Plasmodesmata between bundle sheath and phloem parenchyma cells are thought to mediate transition from local to systemic movement, which then proceeds through the sieve elements to other plant organs.

steps that requires a series of compatible interactions between viral and host factors (106):

a) Viral gene expression and replication in initially infected cells;

b) cell to cell or short-distance movement, where the virus moves out from the initial site to adjacent cells; and

c) long-distance transport, where the virus spreads through the vascular system and moves rapidly to distant uninfected tissues and organs of the plant.

In cell to cell and long-distance movements, the infectious form of the virus, which may be virions, viral nucleic acids, viral nucleoprotein complexes, or a combination, must be able to traffic between cells and different cell types. It is generally accepted that plant viruses accomplish this by exploiting plasmodesmata (106-109). However, virus movement through plasmodesmata is an active process. Because dye-coupling studies have established that plasmodesmata have a size exclusion limit (SEL) of 800 to 1000 Da (110, 111), which is too small to allow a free passage of virions and free viral nucleic acids (112-114). It is now well established that many plant viruses encode dedicated movement proteins (MPs) that have the ability to increase size exclusion limit of plasmodesmata and mediate the passage of macromolecules between cells (109, 115-120). The function nature and evolutionary origin of MPs in different virus groups may be distinct (112, 113). Furthermore, many viral MPs possess nucleic acid binding properties, consistent with their role in the trafficking of viral nucleic acids. These viral MPs have been characterized for a number of RNA and DNA viruses. The mutation of viral MPs blocks virus spread from cell to cell but has no effect on virus replication in single cells or their ultrastructural or functional association with plasmodesmata (106, 121, 122). Many of

viruses do not require the capsid protein for cell to cell movement (115-117, 119, 123) whereas some viruses required (124-126). It is generally considered that viruses move long distance as intact virions and the CP is required for vascular movement of many plant viruses (127). Only a few viruses, such as barley stripe mosaic virus and tomato bushy stunt tombus virus, produce rapid systemic infection in the absence of the CP (128, 129). It has been suggested that virion formation is required for long-distance transport since assembly-deficient CP mutants fail to produce systemic infections (130-132). However, there are examples of assembly-competent CP mutants that fail to spread systemically (101, 102, 133), suggesting a specific function of the CP in vascular movement that is distinct from its function in cell to cell movement and virion assembly (101). This role of the CP is often host specific, which may reflect a specific interaction between the viral CP and host factors to allow efficient vascular movement (134, 135). In potyvirus group, CP is required for both cell to cell and long-distance movements (47, 101-103, 136). Many of potyviral gene products have been implicated in the process of viral movement such as NIa-VPg domain, which is covalently attached to the 5′end of genomic RNA, interacts either directly or indirectly with host components to facilitate long-distance movement (80)Cell to cell and long-distance movement also required one or more functions provided by helper component-proteinase (HC-Pro) (45-47). In 1998, Carrington proposed the model of virus movement that the CI protein interacts directly with plasmodesmata and CP-containing ribonucleoprotein complexes to facilitate potyvirus intercellular movement (64-66)

4. Infectious RNA transcript

More than 90% of all known plant viruses have RNA genomes (137). They differ in virion structure, genome organization, and replication strategy. Studies on molecular mechanisms that govern viral RNA functions shall give us understanding the molecular biology of both viral pathogens and their hosts. These features can be exploited in genetic engineering of higher organisms towards strategy to control viral infection. Recently, the advance in recombination DNA technology makes it possible to obtain infectious full-length cDNA clones of many RNA viruses which can facilitate the study of viruses when they are present only at low titers in infected cells, or whose isolation is problematic. This achievement provides precious information in the study of genetic expression and replication of RNA viruses by using of site direct mutagenesis, deletions, insertions and complementation. Moreover, these clones can also be considered as "pools" for viral genes or sequences of interest for the design of antiviral strategies and transcomplementation studies or for the development of new viral vectors (138).

4.1 *In vitro* transcription

There are several significant advantages of *in vitro* transcription.

1. Sequence can be manipulated by any means applicable to DNA sequence.
2. Resulting of RNA is homogenous in sequence, not consisting of a mixture of sequences that rapidly accumulate during viral infection.
3. Very large quantities can be easily prepared, which is important for low titer viruses.

While performing *in vitro* cDNA synthesis and cloning, several consideration must be taken in account. The choice of the RNA polymer promoter is highly important because it directly affects the yield of transcripts and the nucleotide sequence at their extremities. Several types of promoters have been used such as the *E. coli* Pm (a modified version of Pr) promoter from bacteriophage λ, and the promoters of bacteriophages SP6, T3, or T7. The full-length of RNA viruses were obtained by ligation of the overlap fragments from cloned cDNA library or from the RT-PCR products of partial viral sequence (139-145). A possibility to use PCR amplification to obtain longer full-length cDNA (more than 6 kb and up to 40 kb) was achived by a combination of *KlenTaq* and *Pfu* polymerses in the process now called long and accurate PCR (LA-PCR) (146). It is generally admitted that the presence of nonviral nucleotides at the 5′end of viral transcripts strongly reduces infectivity whereas 3′ extensions were more easily tolerated (138). The other discussed problem in generating infectious transcripts is the viral 5′cap structure. It can be introduced either post-transcription or during *in vitro* transcription. Some viruses have 5′linked protein (VPg) instead of a cap structure. When VPg is removed, some viruses show many or little loss in activity, other show a low infectivity (147-149). Unfortunately, there is no efficient method to add VPg to an RNA transcript.

In plant viruses, infectious *in vitro* RNA transcripts were first successfully prepared for brome mosaic virus (BMV) (150), and the construction of a directly infectious cDNA clone was first reported for RNA 3 of alfalfa mosaic virus (AlMV) through co-inoculation with RNA 1, 2, and 4 (151). PCR was firstly used for construction of all 3 RNA required for cucumber mosaic virus (152). In the potyvirus group, infectious *in vitro* transcripts have been synthesized from full-length cDNA

clones with bacterial phage promoters for tobacco vein mottling virus (TVMV) (153), plum pox virus (PPV) (27), zucchini yellow mosaic virus (ZYMV) (154), barley yellow dwarf virus (BYDV) (141), tobacco etch virus (TEV) (155) potato virus A (PVA) (156), tobacco vein mottling potyvirus (TVMV) (157), Hawaii strain of papaya ringspot virus (PRSV-HA) (158), and turnip mosaic virus (159).

4.2 *In vivo* transcription

An alternative way to obtain plant infection with a cloned cDNA is to construct a plant expression cassette, where the genomic viral cDNA is under the control of strong plant promoter. This expression of infectious viral RNAs through *in vivo* transcription of cDNA-containing vectors has several advantages.

1. Infectivity is less dependent on RNA degradation since it presumably occurs only within cells where the RNA are synthesized and the replication process can overcome detrimental effects resulting from degradation.
2. *In vitro* transcription is not necessary. Furthermore, costly reagents such as the cap analogues and RNA polymerases are not required.
3. It might be very convenient when studying the role and localization of proteins expressed by mutants viral RNAs unable to replicated in the cells (160).

The viral full-length cDNA clones constructed under the CaMV 35S promoter and usually contained the poly(A) tail following 3′UTR and 35S or NOS terminator or restriction site for run out of transcripts, had been reported (161, 162). Infection of the host plants was achieved by mechanical inoculation the full-length of viral cDNA plasmids. Alternatively, infection was achieved by agroinfection (infection of plants with *Agrobacterium tumefaciens* expressing a viral RNA) (163, 164) or particle

bombardment (165). The particle bombardment infection uses low amount of DNA and it is more efficient than mechanical inoculation because foreign DNA delivers directly to the host nucleus (166, 167). However, the toxicity of tungsten particles used for bombardment has been noted (168) and mechanical inoculation is more convenient than particle bombardment.

Infectious *in vivo* transcripts have been synthesized from full-length cDNA clones with CaMV 35S promoters for brome mosaic virus (BMV) (169); beet necrotic yellow vein virus (BNYVV) (170); tomato mosaic virus (ToMV) (171); pea early browning virus (PEBV) (172); cowpea mosaic virus (CPMV) (161) alfalfa mosaic virus (AIMV) (173) citrus tristeza closterovirus (CTV) (174); beet yellow closterovirus (BYV) (175). The construction of a new and convenient cloning vectors (pCass), which contains a full-length cDNA of cucumber mosaic cucumovirus (CMV) under the CaMV 35S promoter and 35S terminator, are reported (176). The *in vivo* infectious genomic cDNA in potyvirus group such as plum pox potyvirus (PPV) (177), pea seedborne mosaic virus (178), Hawaii strain of papaya ringspot virus (PRSV-HA) (158), potato virus Y (PVY) (179), clover yellow vein virus (CIYVV) (180), turnip mosaic virus (159) were successfully inoculated by mechanical inoculation. The infectious full-length cDNA clones of tomato aspermy cucumovirus (TAV) was constructed in a modified pCass vector (pCass2) as well as in a pUC19-based pCass (pCass1) resulted in the infectivity of TAV cDNAs. The cDNAs controlled under a partially duplicated CaMV 35S promoter in pCass2 is 3-fold higher than the same cDNAs driven by the single 35S promoter in pCass1. Recently the partially duplicated CaMV 35S promoter, which the activator sequence region (–343 to –90 upstream of the transcription start site +1) is tandemly repeated and inserted upstream the intact

35S promoter), had been widely used in order to increase the transcription *in vivo* such as barley mild mosaic virus (BaMMV) (162); tobacco mosaic virus (TMV) (167); lettuce mosaic virus (LMV) (181); grapevine virus A (GVA) and grapevine virus B (GVB) (182)

4.3 CaMV 35S promoter

The cauliflower mosaic virus (CaMV) 35S promoter has been shown to be the strongest plant transcription promoters (183-188). Moderate activity of the 35S promoter has also been observed in *Escherichia coli* (9), *Schizosaccharomyces pombe* (189) and *Saccharomyces cerevisiae* (190). The 35S promoter, and its engineered derivatives are very efficiently transcribed by cellular enzymes and have been extensively used to drive a high-level, near constitutive expression of many heterologous genes in transient expression assays and in several species of transgenic plants (191-198). Many details of the structure of CaMV 35S promoter have recently been determined. The sequence from –46 to +8 contains the conventional proximal element of eukaryotic promoters (TATA box) and is sufficient for the accurate initiation of transcription, albeit at low level compared with the full-length promoter (199). The majority of the promoter strength lies in the sequence of the 343 bp upstream of the transcription start (199). The studies of Fang *et al.*, 1989 on integrated constructs showed that this sequence is made up of three functional regions (Figure 6) (200). The –343 to –208 and –208 to –90 regions are responsible for about 50 and 40% of the promoter activity, respectively. The –90 to –46 region has little activity on its own, but enhances the transcriptional activity of the two upstream regions and also control tissue specificity (195). Two CCAAT box-like sequences and two TGACG

motifs (nuclear factor binding) are found in this segment. Only the TGACG motifs are essential for activation of the TATA box and for root-specific expression (201, 202). The sequence from –90 to +8 (domain A) (193) enables strong expression of a reporter gene in the roots of transgenic tobacco and much weaker expression in the aerial parts of a plant (193, 194). The other domain (B) (-343 to –90) confers expression in most cell types comprises at least five subdomains (Figure 6) that confer distinct expression patterns when fused to the A domain (193, 195). Some of the subdomains act independently of each other, so that the combination of two subdomains produces an expression pattern that is the sum of the two individual patterns; other pairs of subdomains act synergistically rather than additively (193, 200).

The –343 to –90 activator sequence was tandemly repeated and inserted upstream of the full-length CaMV 35S promoter (203). The resulting promoter directed 10-fold-higher expression of a reporter gene in transgenic tobacco plants than the native 35S promoter. Versions of the CaMV 35S promoter with the double enhancer are now widely used whenever strong constitutive expression of a transgenic is desirable.

4.4. Effect of non-viral nucleotide at the 5′end of the virus genome

Many scientists have investigated the effect of non-viral nucleotides at the extremities of viral transcripts. As a general rule, non-viral nucleotides between the promoter and 5'end of the viral genome substantially decrease or even abolish infectivity, whereas 3′nucleotide extensions are more easily tolerated. (204-206). In the infectious full-length cDNA clones of tobacco vein mottling virus (TVMV), barley yellow dwarf virus (BYDV), zucchini yellow mosaic virus (ZYMV), plum pox virus

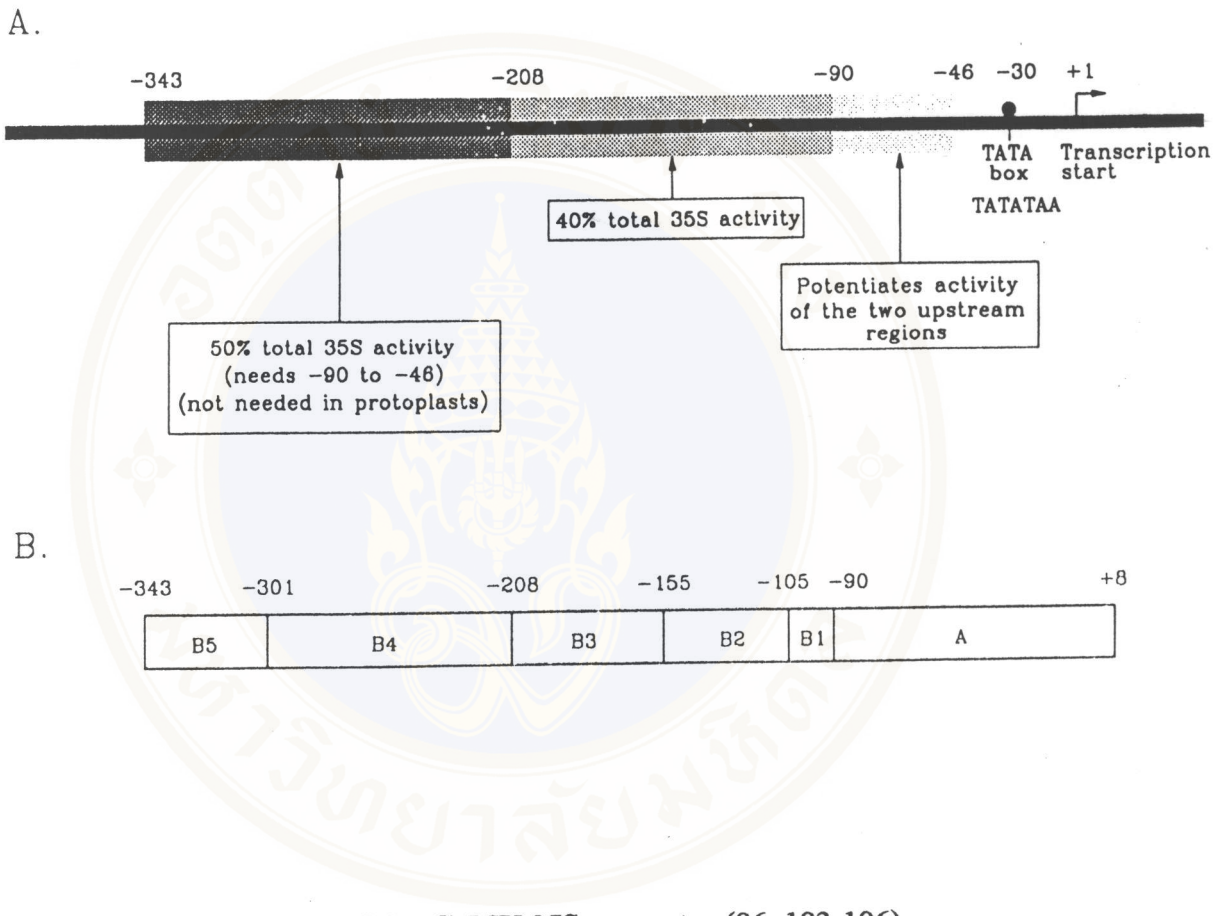


Figure 6. Domains of the CaMV 35S promoter (96, 193-196).

A: the three functional regions of the promoter, as designed by Fang *et al.* in reference 96, are showed as boxes with different densities of shading. The transcription start site is designated +1 and the locations in nucleotides of important upstream domains, including the TATA box, are indicated as negative numbers.

B: the subdomains of the promoter that confer distinct expression patterns (193-196).

(PPV), potato virus Y (PVY) and peanut stripe potyvirus (PStV), which were contained one nonviral nucleotide between promoter and viral genome, had 5%, 10%, 11%, 49%, >90% and 100% infectivity, respectively (27, 141, 153, 154, 207, 208). All of the infectious full-length cDNA clones of TVMV, ZYMV, PPV and PStV were inoculated to host plants by mechanical inoculation except infectious full-length cDNA clones of BYDV and PVY were inoculated to host plants by electroporation and particle gun bombardment, respectively. The full-length cDNA of cucumber necrosis virus (CNV) had been synthesized. This sequence had 5 non-viral nucleotides at the 5′end and was very poor infectious. After deletion of these non-viral nucleotides by site direct mutagenesis, the infectivity increased 20 times (209). The biological active full-length cDNAs of brome mosaic virus genomic RNAs 1, 2 and 3 had extra 12 non-viral nucleotides at the 5′ends of the cDNA inserts were poorly infectious (169). Infectivity is abolished when the transcripts derived from plant viruses harbor moderately long 5′additional sequences of 14 to 33 nucleotides (158, 210-212). Commandeur *et al.* (1991) have recently showed that the cDNA sequences of RNAs 3 and 4 of beet necrotic yellow vein virus (BNYVV) are biologically active when cloned downstream the CaMV 35S transcription promoter (170). The resulting *in vivo* transcripts containing up to 40 non-viral nucleotides at the 5′end are infectious *in planta*, whereas *in vitro* derived transcripts harboring such extensions are biologically inactive in the same host plant. However, the number of different extensions of 5′non-viral nucleotides tested for various viral transcripts is limited in the each case and the differences of infectivity are observed between transcripts harboring non-viral 5′ extensions similar in length but differing in their sequences (213, 214).

Different strategies have been reported for fusion of transcription promoters to the beginning of viral sequences.

Construction of universal transcription vectors in which restriction sites were inserted as close as possible to the transcription initiation site of the promoter. These were the cases of the pPM / λPm (150), pHST70 / SP6 (215), pCaP35J / 35S (216), pCass / 35S (176), pCaP35J2 / dup35S (162) and pCass2 / dup35S (217) constructs. All these vectors could theoretically direct transcription of viral cDNA sequences into RNAs devoid of vector-derived nucleotides.

Extragenous sequences between the promoter and viral cDNA had been eliminated by site-directed mutagenesis (27, 158, 177, 207).

Synthesis of second strand cDNA was primed with an oligonucleotide containing a promoter directly linked to the 5′end of the viral sequence (144, 218).

5. Aspects of infectious full-length cDNA clones of RNA viruses

The infectious transcript derived from the full-length cDNA clones by *in vitro* or *in vivo* transcription is an important tool in the study of RNA virus at the molecular level. Various aspects of virus pathogenicity such as symptom development, cell to cell and long-distance movement, seed and aphid transmission, interactions with resistance genes, replication, translation, proteolysis and disassembly have been studied by this technique (40, 219-225). Reporter genes have been inserted into full-length clones to visualize virus spreading and its accumulation following infection (155, 226, 227). In addition, viruses have been used as vectors for *in vivo* expression of foreign proteins in plants (228, 229).

CHAPTER II

OBJECTIVES

Papaya ringspot virus (PRSV) is the major limiting factor for papaya production. There are several methods that have been used to control PRSV however all of them are only partially effective. Thus, the molecular biology of PRSV should be understood for development of the effective methods by means of genetic engineering. The synthesis of biologically functional RNA transcripts from full-length cDNA clones via *in vitro* or *in vivo* transcription plays a key role in the research of PRSV at the molecular level. The infectious RNA viral transcript is usually constructed under strong bacterial promoter, which permits synthesis of genomic RNA *in vitro*. This thesis focuses on development of an alternative method that can generate infectious RNA transcripts from *in vivo* transcription of Thai PRSV isolate, type P where the viral cDNA is in the plant expression vector under the control of CaMV 35S promoter or partially duplicated 35S promoter

The aim of this thesis was:

1. Construction of the plant expression cassettes containing single CaMV 35S promoter and partially duplicated 35S promoter, poly(A) and NOS terminator suitable for cloning the full length of PRSV.

2. Construction of the full-length cDNAs of PRSV-P under the control of T7 promoter, single CaMV 35S promoter and partially duplicated 35S promoter

3. Infection of papaya plants with plasmid DNAs of full-length PRSV-P, under the control of single CaMV 35S promoter and partially duplicated 35S promoter, and observation of the symptom development

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

All chemicals used in this thesis were analytical grade.

1.2 Miscellaneous biochemical materials

Ampicillin	Sigma
Adenosine 5′-Triphosphate (ATP)	GIBCO BRL
Deoxynucleotides triphosphates (dNTPs)	Pharmacia
Diethyl pyrocarbonate (DEPC)	Sigma
*Bst*EII digested λ-DNA markers	Biolabs
*Hin*dIII digested λ-DNA markers	GIBCO BRL
Trizol™ reagent	GIBCO BRL

1.3 Enzymes

1.3.1 Modifying enzymes

Pfu DNA polymerase	Promega
BIOTOOLS DNA polymerase	BIOTOOLS
Calf intestinal alkaline phosphatase (CIAP)	GIBCO BRL
Lysozyme	Sigma

T4 DNA polymerase	Biolabs
T4 DNA ligase	GIBCO BRL
T4 polynucleotide kinase	Biolabs
RNaseA	Sigma
Superscript ™ II	GIBCO BRL

1.3.2 Restriction endonucleases

Table 1.: Information of restriction endonucleases

Enzymes	Recognition sequences	Incubation temperature(°C)	Heat inactivation at	Suppliers
*Acc*I	GT↓(A/C)(T/G)AC	37	-	Biolabs
*Bam*HI	G↓GATCC	37	65°C 15min	Promega
*Eco*RI	G↓AATTC	37	65°C 20min	Biolabs
*Eco*RV	GAT↓ATC	37	65°C 10min	GIBCO BRL
*Hin*dIII	A↓AGCTT	37	65°C 15min	Promega
*Kpn*I	GGTAC↓C	37	65°C 10min	GIBCO BRL
*Nar*I	GG↓CGCC	37	65°C 15min	Promega
*Pac*I	TTAAT↓TAA	37	65°C 20min	Biolabs
*Pst*I	CTGCA↓G	37	65°C 20min	Biolabs
*Pvu*II	CAG↓CTG	37	65°C 15min	Promega
*Sac*I	GAGCT↓C	37	65°C 10min	GIBCO BRL
*Sac*II	CCGC↓GG	37	65°C 15min	Promega
*Sph*I	G↓CATGC	37	65°C 20min	Biolabs
*Sma*I	CCC↓GGG	30	65°C 10min	GIBCO BRL
*Stu*I	AGG↓CCT	37	65°C 15min	Promega
*Xba*I	T↓CTAGA	37	65°C 10min	GIBCO BRL

Note: The cleavage site is specified by ↓.

1.4 Bacterial strains

Escherichia coli strain DH5α [ϕ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^-,m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169], originally prepared by GIBCO BRL, was employed as a host cell for recombinant DNA. This strain permits ∝-complementation with the amino terminus of β-galactosidase encoded by gene of pUC vectors.

E. coli strain JM109 [*endA*1, *recA*1, *gyrA*96, *thi*, *hsd*R17 (r_K^-, m_K^+), *rel*A1, *sup*E44, Δ(*lac-proAB*), [F', *traD*36, *proAB*+, *lacI*^{q}, lacZΔM15)] was employed as a host cell for recombinant DNA. This strain permits ∝-complementation with the amino terminus of β-galactosidase encoded by gene of pUC vectors.

1.5 Plant material

Papaya (*Carica papaya* L.) seeds were obtained from ripen fruits (Rajchaburee *cultivar*), dried and kept at 4°C until use.

1.6 Plant virus material

Isolated PRSV-P (severe-type) infected papaya plants were obtained from Virology Section, Plant Pathology Microbiology Division, Department of Agriculture, Bangkaen, Bangkok, and use as an origin of PRSV source.

1.7 Bacterial growth media

1. LB medium (Luria-Bertani medium) : 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl

2. LB agar plate : 1.5% (w/v) of bacto-agar in LB medium

3. SOB medium : 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% (w/v) NaCl, 0.01 M $MgCl_2$

4. SOC medium : 20 mM glucose in SOB medium

5. TB medium (Terrific broth medium) : 1.2% (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4

1.8 PCR primers

1.8.1 CaMV 35S primers

CaMV 35S primers were designed from nucleotide sequence of CaMV 35S promoter in pBI221. These primers were synthesized in the Institute of Molecular Biology and Genetics and were used to amplify 35S promoter and also used as sequencing primers. The sequence and location of these primers were shown in the table 2 and section 4.1 (chapter IV), respectively.

Table 2.: Information of CaMV 35S primers

No.	Name	Sequence	Size (bp)
1	35Ss	5′-TGCAGGTCCCCAGATTAGCC-3′	20
2	35Sa	5′-TTCTCTCCAAATGAAATGAACTTCC-3′	25
3	35S-seq*	5′-ATCCTTCGCAAGACCCT-3′	17

Note: T is complementary to the transcription start site.

The * represents that primer is available in our lab.

1.8.2 PRSV primers

All of the PRSV primers were available in our lab, which were designed by Dr. Miloslav Juricek and synthesized by GENSET, Singapore. They were divided into 2 sets. Set1 primers were used to amplify 5′ and 3′parts of PRSV. Set2 primers were used to amplify start, middle and end part of PRSV. Both of them were also used as sequencing primers. The sequences of set1 and set2 primers were shown in the table 3 and table 4, respectively. The location of set1 and set2 primers was shown in figure 7 and figure 8, respectively.

Table 3.: Information of set1 PRSV primers

No.	Name	Sequence	Size (bp)
4	T7GF2	5′-GTAATACGACTCACTATAGAAATAAAACA TCTCAACACAACACAA-3′	45
5	GF2	5′-AAATAAAACATCTCAACACAACACAAT-3′	27
6	GSP3	5′-CGAAGGATCCGCAAAGTTTACAGCTTCGG C-3′	30
7	1L32	5′-CGATTAGTCGAGTCCACACCACTGATAAC TGGCG-3′	34
8	PRSV5′CP	5′-CATCATCATCATATCATTCCATGGCTGTGG ATGCTGGTTTGAATG-3′	45
9	PRSV3′CP	5′-CTACTACTACTAGTCAAGCCATGGTTGCG CAGCCACNCTGTATTG-3′	45
10	Seq-IF	5′-GAGGGAGTGAGGAATGA-3′	17
11	GEN-R	5′-CGTTTTTTTTTTTTTTTTTAGCTCATTCTAA GAGGCTC-3′	38

Table 4.: Information of set2 PRSV primers

No.	Name	Sequence	Size (bp)
12	INT203	5′-CCTCATGTTGGCGAGTTCGTAGTTAGTGAA GGAG-3′	34
13	BF	5′-GCCAAAACGCACGAGATATACATGAGC-3′	27
14	BR	5′-GCGTAGGTTTTTTCCACAGCCTCACG-3′	26
15	EN	5′-GCGTCATGCGGCCGCTCTAGACTCTCATTC TAAGAGGCTCGAATAGCACG-3′	50
16	PF	5′-GCCATGACAAGTTGCACTGGTCTTAACTC-3′	29
17	XR	5′-GCACGTGTGCGGCCGCTTGTTGAATTTGGG CCTTTGCCAGC-3′	41

1.9 Vectors and recombinant plasmids

pBI221 (Figure 9) (Clonetech) : This plasmid was constructed by cloning the 3.0-kb *Hin*dIII-*Eco*RI expression cassette of pBI121 [containing the CaMV 35S promoter, β-Glucuronidase selectable marker (GUS gene), and nopaline synthase terminator] into pUC19.

pSA1074 (Figure 10) : This plasmid was constructed in our laboratory. It is derived from pUC19, and contains PRSV-P coat protein gene, 3′ untranslated region, and a poly A tail (127 bp). Ampicillin resistant gene in this plasmid is used as a selectable marker.

pUC18 (Figure 11) (254): This vector derived from a pBR322 plasmid contains ampicillin resistant gene as a selectable marker, origin of DNA replication, and a portion of lac Z gene encoding the amino-terminal fragment of β-galactosidase to display α-complementation in appropriate host.

pUC19 (Figure 12) (254): This vector is identical to pUC18 except it contains polycloning sites arranged in the opposite orientation.

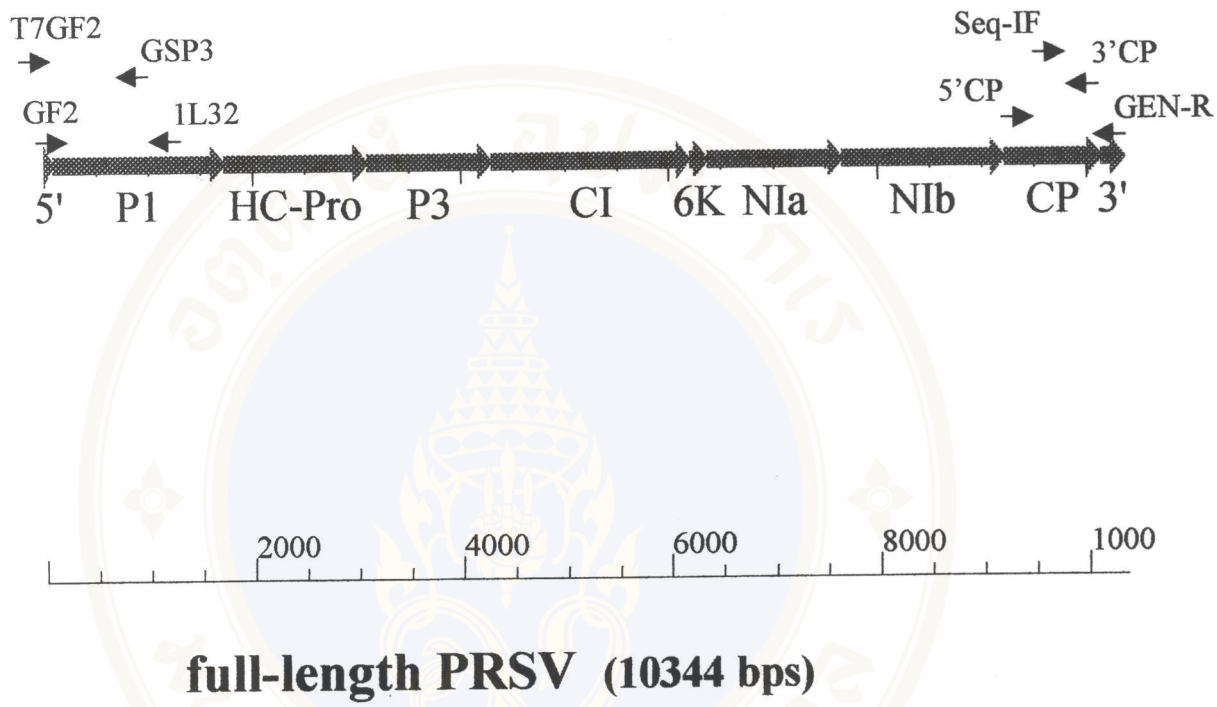


Figure 7. Locations of set1 PRSV primers

This figure showed the set1 PRSV primers (primer no.4-11) that used to amplify the 5′part and the 3′part of PRSV and also used as sequencing primers.

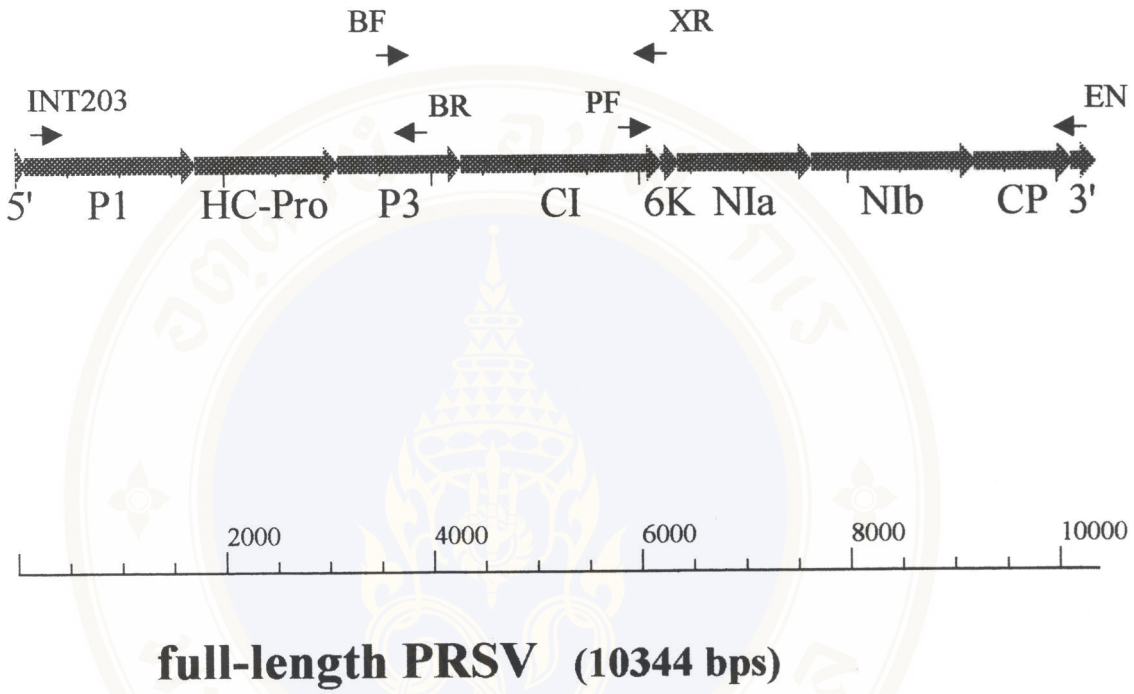


Figure 8. Locations of set2 PRSV primers

This figure showed the set2 PRSV primers (primer no.12-17) that used to amplify the start part, middle part and the end part of PRSV and also used as sequencing primers.

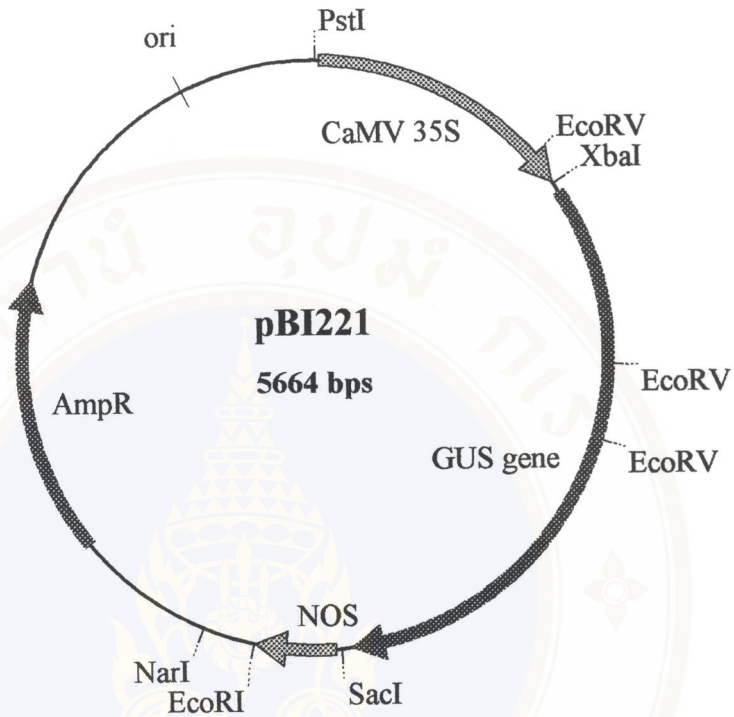


Figure 9. Physical map of pBI221 vector (Clonetech)

This plasmid is a plant expression vector, which was constructed by cloning the 3.0-kb *Hin*dIII-*Eco*RI expression cassette of pBI121 [containing the CaMV 35S promoter, β-Glucuronidase selectable marker (GUS gene), and nopaline synthase terminator (NOS)] into pUC19 vector.

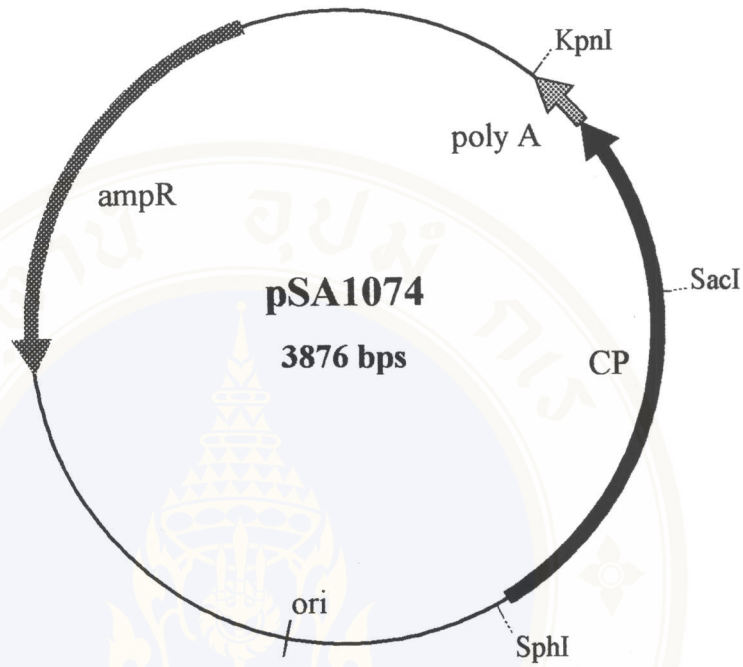


Figure 10. Physical map of pSA1074 plasmid (constructed in the Plant Molecular Virology Laboratory, Institute of Molecular Biology and Genetics, Mahidol University)

This plasmid is derived from pUC19 vector, and contains PRSV-P coat protein gene, 3′ untranslated region, and a poly A tail (127 bp). Ampicillin resistant gene in this plasmid is used as a selectable marker.

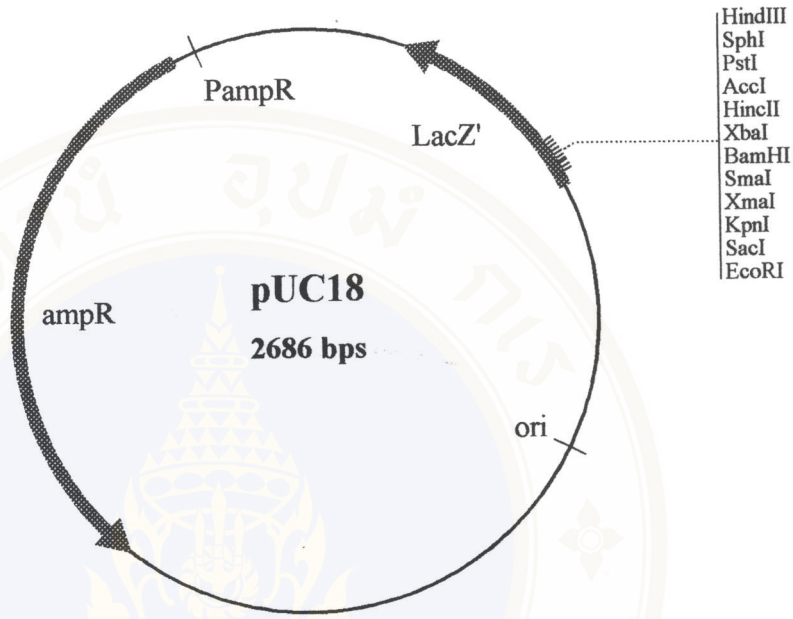


Figure 11. Physical map of pUC18 vector (254)

This vector which derived from a pBR322 plasmid contains ampicillin resistant gene as a selectable marker, origin of DNA replication, and a portion of lac Z gene encoding the amino-terminal fragment of β-galactosidase to display α-complementation in an appropriate host.

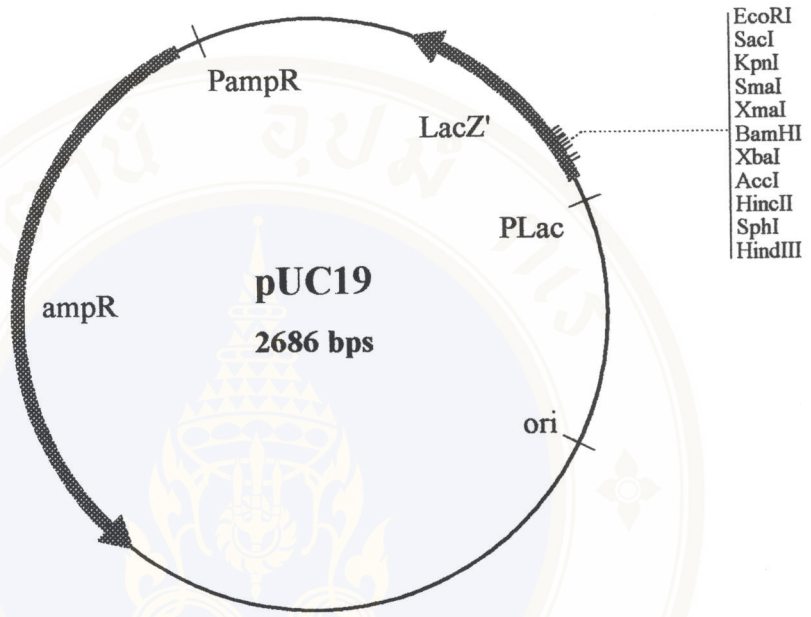


Figure 12. Physical map of pUC19 vector (254)

This vector is identical to pUC18 vector except that it contains polycloning sites arranged in the opposite orientation.

2. Methods

2.1 Polymerase chain reaction (PCR)

2.1.1 Coupling reverse transcription and polymerase chain reaction (RT-PCR)

Superscript ™ II (GIBCO BRL) was used to synthesize first strand cDNA. The reverse transcription reaction was performed using Gene Amplification System 2400 (Perkin Elmer) and nuclease-free thin-wall PCR tubes. The reaction mixture contained 10 pmol of nonspecific primer (oligo-dT) or specific primers (1L32, 3′CP), 2 µl of 100 ng purified viral RNA and sterile DEPC-treated distilled water to make a final volume of 20 µl. The reaction mixture was heated to 70°C for 10 min and quick chilled on ice. Then, 7 µl of 5x buffers (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM $MgCl_2$), 0.5 mM dNTPs and 0.01 M DTT were added to the reaction mixture and it was incubated at 42°C for 2 min. After addition of 1 µl (200 units) of Superscript™ II, the reaction mixture was incubated at 42°C for 20s and 50°C for 50 min. Finally; the reaction was inactivated at 70°C for 15 min and incubated at 4°C for 5 min. The 10-20 µl of this first strand cDNA/RNA hybrid was directly used as the template for PCR reaction.

2.1.2 DNA amplification by polymerase chain reaction (PCR)

The 50-100 ng of plasmid DNA or 1-2 µl of first strand cDNA/RNA hybrid was used as DNA template. The 50 µl of total PCR reaction was composed of DNA or cDNA/RNA hybrid template, 50 pmol of each forward and reverse primers, 200 µM of dNTPs mix, 1x PCR buffer (20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 10 mM KCl,

10 mM $(NH_4)_2SO_4$, 0.1% Triton x-100, and 100 µg/ml nuclease-free BSA), 0.5 µl (1.5 units) *Pfu* DNA polymerase and water. In the case of amplification to confirm recombinant clone, 0.5 µl BIOTOOLS DNA polymerase or 2 µl *Taq* DNA polymerase synthesized in this institute was used. The differences of the components in PCR reaction were 1x PCR buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), 10 pmol of each forward and reverse primers and 1.5 - 2.0 mM $MgCl_2$. A gene amplification system 2400 (Perkin Elmer) was used as a thermocycler for the PCR amplification according to the temperature profile in table 5.

Table 5.: Temperature profile for PCR amplification

HOLD	STEP CYCLE (35-40 cycles)			HOLD
	Denaturation	Annealing	Extension	
95°C 1 min	92°C 20 sec	45°C-55°C 30 sec	68°C or 72°C 1.3-2 min	68°C or 72°C 7 min

2.1.3 DNA amplification by touchdown polymerase chain reaction

DNA amplification by touchdown polymerase chain reaction was performed as the PCR reaction (section 2.1.2). The difference was the amplification step, which was shown in table 6.

Table 6.: Temperature profile of touchdown PCR

STEP	TIME AND TEMPERATURE		
1	1 hold at 95°C for 1 min		
2	10 cycles		
	Denaturation	Annealing	Extension
	92°C 20 sec	60°C→50°C* 30sec	72°C 1.3 min
3	30 cycles		
	Denaturation	Annealing	Extension
	92°C 20 sec	50°C 30 sec	72°C 1.3 min
4	1 hold at 72°C for 7 min		

Note: The * represents the annealing temperature in this step started at 60°C and decreased 1°C in each cycle until reached 50°C.

2.2 Agarose gel electrophoresis

The PCR product was analyzed by 1% agarose gel in 1xTBE buffer (89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH 8.0) or 1xTAE buffer (40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH 8.0) in the case of purification of DNA fragment from agarose gel. Loading dye (0.1% bromophenol blue, 40% ficoll and 5 mM EDTA) was added to the DNA samples that were loaded into the wells. The electrophoresis was performed at a constant voltage (100-volt) for 1 hour or (80 volt) for 1.3 hour in the case of DNA purification. After completion of electrophoresis, the gel was stained with 2 μg / ml ethidium bromide for 5 min and destained in distilled water for 10 min. The DNA bands were visualized under UV light and photographed by gel documentation system (BIO-RAD). In the case of DNA purification, the desired DNA band was excised from the gel and purified by GENECLEAN II Kit.

2.3 Precipitation of DNA with isopropanol or ethanol

0.1 volume of 3 M sodium acetate pH 5.2 was added into the DNA solution. After mixing the DNA solution, 1 volume of isopropanol or 2 volume of absolute ethanol were added and mixed well again. Then, the DNA solution was centrifuged at maximum speed (20,800xg) for 15 min at room temperature. The DNA pellet was washed by 70% ethanol and centrifuged again at maximum speed for 5 min at room temperature. Supernatant was removed and the DNA pellet was dissolved in sterile distilled water or TE buffer after air-drying.

2.4 Phosphorylation of 5′ end of PCR product by T4 polynucleotide kinase

The detail of procedure was according to the manufacturer recommendation. T4 polynucleotide kinase catalyzes the transfer of the γ-phosphate of ATP to a 5′ hydroxyl group of DNA. The reaction was prepared by adding PCR amplified DNA fragment, 1xT4 polynucleotide kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT), 1 mM ATP, 10 units of T4 polynucleotide kinase (Biolabs) per 300 pmole of 5′ termini and sterile distilled water to make a 50 µl final volume. After mixing the reaction thoroughly but not vigorously, it was incubated at 37°C for 1 hour. Then, the reaction was stopped by heat inactivating at 65°C for 20 min and the DNA was recovered by ethanol precipitation.

2.5 Restriction endonuclease digestion of DNA

The reaction of restriction endonuclease digestion was composed of DNA, 1x restriction endonuclease buffer, restriction endonuclease and sterile distilled water.

The restriction endonuclease buffer, the amount of restriction endonuclease used and the optimum condition for digestion was depended on the restriction endonuclease manufacturer.

2.6 Generation blunt ends by T4 DNA polymerase

The detail of procedure was according to the manufacturer recommendation. T4 DNA polymerase posses 5′→ 3′ DNA polymerase and 3′ → 5′ exonuclease activity so it can be used to generate blunt ends from sticky ends of DNA fragment by filling in the 5′ overhang bases and removal the 3′ overhang bases. The reaction was performed by adding 1xT4 DNA polymerase buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM DTT), 10 mM dNTPs, double-stranded linear DNA, T4 DNA polymerase (Biolabs), 50 µl / ml BSA and sterile distilled water to make a 30-100 µl final volume. The amount of enzyme used was 1-3 units polymerase per µg DNA. After gentle mixing, sample was incubated at 12°C for exactly 20 min and the reaction was heat inactivated at 75°C for 10 min. Finally; the DNA was precipitated from the reaction by isopropanol.

2.7 Dephosphorylation of linearized plasmid DNA by calf intestinal alkaline phosphatase (CIAP)

Plasmid DNA, digested with single restriction endonuclease for cloning, can be prevented from self-ligation by removing the 5′-phosphate group with the activity of calf intestinal alkaline phosphatase (CIAP). The reaction was composed of linearized plasmid DNA, 1 x buffer (50 mM Tris-HCl pH 8.5, 0.1 mM EDTA), CIAP (GIBCO BRL), and sterile distilled water up to the final reaction volume. The amount of

enzyme used was dependent on the pmole ends of linearized plasmid DNA (1 unit of enzyme was used for 2 pmole ends of linearized plasmid DNA) and the reaction mixture was incubated at 37°C for 30 min. The same amount of enzyme was added again and the reaction mixture was incubated at 55°C for 30 min. The dephosphorylated DNA fragment was purified by GENECLEAN II Kit.

2.8 Isolation of DNA fragment from agarose gel by GENECLEAN II Kit

GENECLEAN II Kit (BIO101) can be efficiently used to purify the DNA fragment of 0.5 - 15 kb in length. The TAE buffer was used in gel electrophoresis of DNA separation instead of TBE buffer. The desired DNA band was quickly excised from the gel under long-wave UV light and transferred to the Eppendorf tube. NaI solution was added as 3 volume per 1 volume of gel slice (e.g. 300 µl per 0.1 g of gel slice). The agarose was dissolved by incubation at 55°C for 5 min while mixing every minute by inversion. The glassmilk suspension was mixed thoroughly by vortexing and 5 µl of the suspension was added to the solution containing 5 µg or less of DNA. An additional 1 µl of glassmilk suspension was added for each of 0.5 µg of DNA above 5 µg. The mixture was placed on ice for 10 min while mixing gently by inversion to allow binding DNA to the silica matrix. The pellet of glassmilk-DNA complex was collected by 5 s centrifugation at maximum speed in a microcentrifuge. The supernatant was removed and then and the pellet centrifuged again. The rest of NaI solution was removed by pipetting. The pellet was washed 3 times with New Wash solution by using 200 µl in the first wash and 400 µl in the next 2 washes. After the third wash, all the New Wash solution was removed from pellet by centrifugation.

The DNA was eluted twice by resuspending the pellet in the equal volume of TE buffer or sterile distilled water, incubation at 55°C for 2 min and centrifugation at maximum speed in a microcentrifuge for 30 s. The DNA solution was transferred to the new Eppendorf tube.

2.9 DNA ligation

DNA ligation was carried out using T4 DNA ligase (GIBCO BRL). The linearized near length PCR products were combined together at 1:1 molar ratio or the linearized vector was combined with insert DNA at 1:3 molar ratio. The ligation mixture contained 1x ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% (w/v) polyethylene glycol-8000), T4 DNA ligase and sterile distilled water up to the final volume of 10 - 20 µl. The ligation mixture was incubated at 14 - 16°C overnight. The amount of T4 DNA ligase (1-10 units) added to the reaction is depended on the amount of total DNA in the reaction and also depended on the ligation reaction.

2.10 Preparation of competent cells by simple and efficient method (SEM) (255)

About 10 - 12 colonies (diameter of about 2-3 mm) of *E.coli* host DH5α were inoculated into 250 ml of SOB medium in a 2 liter flask. Cells were incubated at 18°C with viscous shaking until the O.D at 600 nm reached 0.6. The culture was cooled on ice for 10 min before being transferred to a 500 ml centrifuge bottle and centrifuged at 3700xg, 4°C for 10 min. The pellet was resuspended in 80 ml of ice-cold TB buffer (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$ and 250 mM KCl), incubated in an ice

bath for 10 min and centrifuged at 3,700xg, 4°C for 10 min. The cells were resuspended in 20 ml of ice-cold TB buffer. DMSO was then slowly added with stirring to give a 7% final concentration and the culture was incubated in an ice bath for another 10 min. 200 µl of resulting competent cells were aliquoted immediately into Eppendorf tubes frozen in liquid N_2 and keep at -80°C. These cells were ready for transformation. Transformation efficiency estimation was 1-3 x 10^9 cfu/µg of plasmid DNA.

2.11 Transformation of competent cells

1 - 3 µl of ligation mixture was diluted 1:5 with double distilled water, added to 200 µl of competent cells and incubated on ice for 30 min. The cells were heat-shocked at 42°C for 90 s and incubated on ice for 2 min. The transformed cells were mixed with 800 µl of LB medium or SOC media and incubated at 37°C for 60 min. The cells were then spread on LB agar plates containing 100 µg / ml ampicillin. In the case of blue-white selection, 40 µl of 20 mg / ml X-gal and 4 µl of 200 mg / ml IPTG were spread on LB agar plates containing 100 µg / ml ampicillin and incubated at 37° C overnight.

2.12 Rapid size screening

The rapid detection of recombinant plasmids procedure was developed by David Law and Neil Crickmore (230). A 1 mm diameter colony was transferred by toothpick into 20 µl of pre-warmed lysis buffer (5 mM EDTA, 10% w/v sucrose, 0.25% w/v SDS, 100 mM NaOH, 60 mM KCl, and 0.05% w/v bromophenyl blue) and

mixed by vortexing. The sample was incubated 5 min at 37°C and it was further incubated 5 min in refrigerator or in cold room. After incubation, the sample was centrifuged 1 min at 20,800xg and the supernatant was loaded onto an agarose gel. The recombinant plasmid was determined by the size of supercoil plasmid DNA and the correct clone was selected from the master plate.

2.13 Plasmid extraction

2.13.1 Cetyltrimethylammonium bromide (CTAB) plasmid DNA mini preparation

A single colony of *E.coli* was incubated in 1.5 ml LB medium containing 100 μg / ml ampicillin with shaking at 37°C overnight. The *E.coli* culture was transferred to 1.5 ml microtube and centrifuged at 20,800xg for 1 min. The pellet was resuspended in 200 μl of STET buffer (8% sucrose, 0.1% Triton X-100, 50 mM EDTA and 50 mM Tris pH 8.0). Then, 5 μl of 50 mg / ml fresh lysozyme solution was added and mixed by vortexing. After incubated at room temperature for 5 min, the mixture was heated at 100°C for 45 s and immediately centrifuged at 20,800xg for 15 min at room temperature. The cell pellet was removed by toothpick and 1/10 volume of 5% (w/v) CTAB solution was added and mixed by inversion. The mixture was centrifuged at 20,800xg for 5 min at room temperature. The pellet was dissolved in 300 μl of 1.2 M NaCl. Then, 3 μl of 5 mg / ml RNaseA were added following by incubation at 37°C for 15 min. An equal volume Chloroform was added and mixed by inversion and centrifuged at 20,800xg for 2 min at room temperature. The aqueous phase was transferred to a new Eppendorf tube and 1 volume of isopropanol was added. The mixture was centrifuged at 20,800xg for 10 min at room temperature. The

DNA pellet was rinsed in 70% ethanol, air dried and then resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or distilled water.

2.13.2 Large scale plasmid DNA preparation and purification by $CsCl_2$-EtBr ultracentrifugation

E. coli containing recombinant plasmids were activated in 5 ml of LB medium containing 100 mg / ml ampicillin and incubated at 37°C overnight. One percent activated cells was transferred to 500 ml LB medium containing 100 mg / ml ampicillin and further incubated for 6 h. Then, 170 µg / ml chloramphenicol was added in order to increase plasmid copy number. The culture was continually incubated at 37°C for 16 hrs. The bacterial cell pellet was harvested by centrifugation at 3,700xg for 10 min at 4°C and resuspended in 20 ml TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The resuspended cells were centrifuged at 3,700xg for 10 min at 4°C and the cell pellet was resuspended in 7.5 ml of 25% sucrose in 50 mM Tris pH 8.0. Then, 1 ml lysozyme solution (15 mg / ml lysozyme, 250 mM Tris pH 8.0) was added and mixed by vortexing. After incubation the mixture on ice for 5 min, 3 ml of 250 mM EDTA pH 8.0 were added into the mixture and mixed by vortexing. 12 ml of Triton solution (10% Triton X-100, 250 mM EDTA pH 8.0, 1 M Tris pH 8.0) was added into the mixture and incubated on ice for 1 - 2 hrs. Then, the mixture was centrifuged at 50,000xg for 40 min at 4°C and the supernatant was transferred to measuring cylinder in order to make up the volume to 30 ml with TE buffer. 30 g of cesium chloride (CsCl) was added to achieve the concentration of 1 g of CsCl per 1 ml of DNA solution, followed by addition of 1 ml of 5 mg / ml EtBr. After mixing, the

mixture was transferred to the quick seal tubes. The sealed tubes were centrifuged at 540,000xg for 7 h at 20°C using vTi70 rotor in Beckman XL-90 ultracentrifugation. Two bands of DNA could be visualized under UV light. The upper band consisted of chromosomal DNA and nicked circular plasmid DNA and the lower band consists of closed circular plasmid DNA. The lower band was collected with 3 ml syringe by pucturing the tube with a no. 20 hypodermic needle. The plasmid DNA was diluted with 2 volume of TE buffer and precipitated with 3 volume of isopropanol. After the plasmid DNA solution was mixed and incubated at room temperature for 10 min, it was centrifuged at 20,800xg for 30 min at 20°C and the DNA pellet was resuspended in 1 ml TE buffer. EtBr was removed from the DNA solution by adding one volume of phenol and one volume of chloroform: isoamyl alcohol (24:1). The DNA solution was centrifugation at 20,800xg for 5 min at 20°C. This procedure was repeated twice. The DNA was then precipitated with 90% ethanol and rinsed with 70% ice-cold ethanol. After air dry, the DNA was resuspended with TE buffer. The concentration of DNA was determined by UV spectrophotometer. One A_{260} unit is approximately equal to 50 μg / ml of double stranded DNA.

2.14 DNA sequencing

The DNA sequencing reactions were performed with ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and performed on ABI PRISM™ 377 DNA Sequencer (Perkin Elmer).

2.15 DNA sequence alignment

All of the sequence alignment in this thesis was performed using program Prophet 5.0 and GeneDoc.

2.16 Plant inoculations

Papaya (*Carica papaya* L.) seeds were grown in a greenhouse. At the 4 - 5 true-leaf stage (approximately 2 - 5 months), the 50 of papaya plants (10 plants per each groups) were used for inoculation. The PRSV full-length cDNA clones pSA1101 (clone 215, single 35S promoter), pSA1102 (clone 81, partially duplicated 35S promoter) and pSA1110 (T7 promoter) were prepared from CTAB plasmid extraction as mentioned in section 2.13.1 (Chapter III). Aliquots of 20 µl, containing 20 µg DNA in sterile double distilled water, were mechanically applied onto a leaf of each carborundum-dusted papaya plants with a sterilized glass spatula. Healthy plants were mock inoculated with 20 µl sterile double distilled water as a negative control and the sap inoculated with PRSV infected papaya plant that ground with phosphate buffer (0.1 M K_2HPO_4/KH_2PO_4) containing 0.5% (w/v) Na_2SO_3, pH 7.0 as a positive control. The inoculated leaves of all inoculated plants were washed with water to remove the excess abrasive and kept in a greenhouse for observation of symptom development.

2.17 RNA extraction by TRIzol™ reagent

Plant total RNA was isolated by TRIzol reagent kit (GIBCO BRL). TRIzol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate for the single-step RNA isolation. TRIzol reagent maintains the integrity of the RNA and the RNase contamination is avoided during sample homogenization. The 50 - 100 mg of

leaf tissue was homogenized with 0.5 ml of TRIzol reagent in a 1.5 ml microcentrifuge tube using power homogenizer (IKA-Labortechnik). After complete homogenization, 0.5 ml of the TRIzol reagent was added and incubated 5 min at room temperature. The 0.2 ml of chloroform was added and the sample was shaken vigorously by hand for 15 sec. The sample was incubated for 2 - 3 min at room temperature and centrifuged at 12,000xg for 5 min at 4°C. The colorless upper aqueous phase was transferred to the new Eppendorf tube and RNA was precipitated with 0.5 ml of isopropyl alcohol. After incubation for 10 min at room temperature, total RNA was recovered by centrifugation at 12,000xg for 10 min at 4°C. The supernatant was removed and the pellet was washed with 75% ethanol, air dried for 5 - 10 min and RNA pellet was dissolved in 20 µl DEPC treated water. The extracted RNA was verified by RT-PCR as mentioned in section 2.1.1 and 2.1.2.

CHAPTER IV

RESULTS

General strategy for cloning the full length of PRSV cDNA

The simplest technique to obtain the full-length clone of the PRSV genome would be RT PCR amplification and cloning the product into appropriate plasmid vector. Although we were able to obtain the full length PCR product of PRSV type P or type W using reverse transcription and LA PCR, all attempts to clone this full length PCR fragment to any plasmid available in out laboratory failed (M. Juricek, P Attasart, personal communication). The new strategy, which eventually succeeded, was based on PCR amplification and cloning of 3 overlapping PCR fragments of the PRSV genome. Moreover, this technique enabled easier modification of the final product, e.g. adding control sequences etc. For the sake of clarity, the global approach is outlined first, followed by the detailed presentation of the results. It consists of 2 parts:

1. Engineering the control sequences of the plant expression cassette and
2. Cloning the full length of PRSV into the plant expression cassette.

1. Construction of the plant expression cassettes (Overview)

CaMV 35S promoter and 5′PRSV were amplified separately by PCR using *Pfu* DNA polymerase (see Figure 13A). The purified fragments were randomly ligated by T4 ligase. The ligated fragment containing 35S promoter attached to 5′ end of PRSV was selectively reamplified from the ligation mixture using appropriate primers. This fragment was then cloned into pUC18 vectors yielding pSA1064 plasmid. In order to

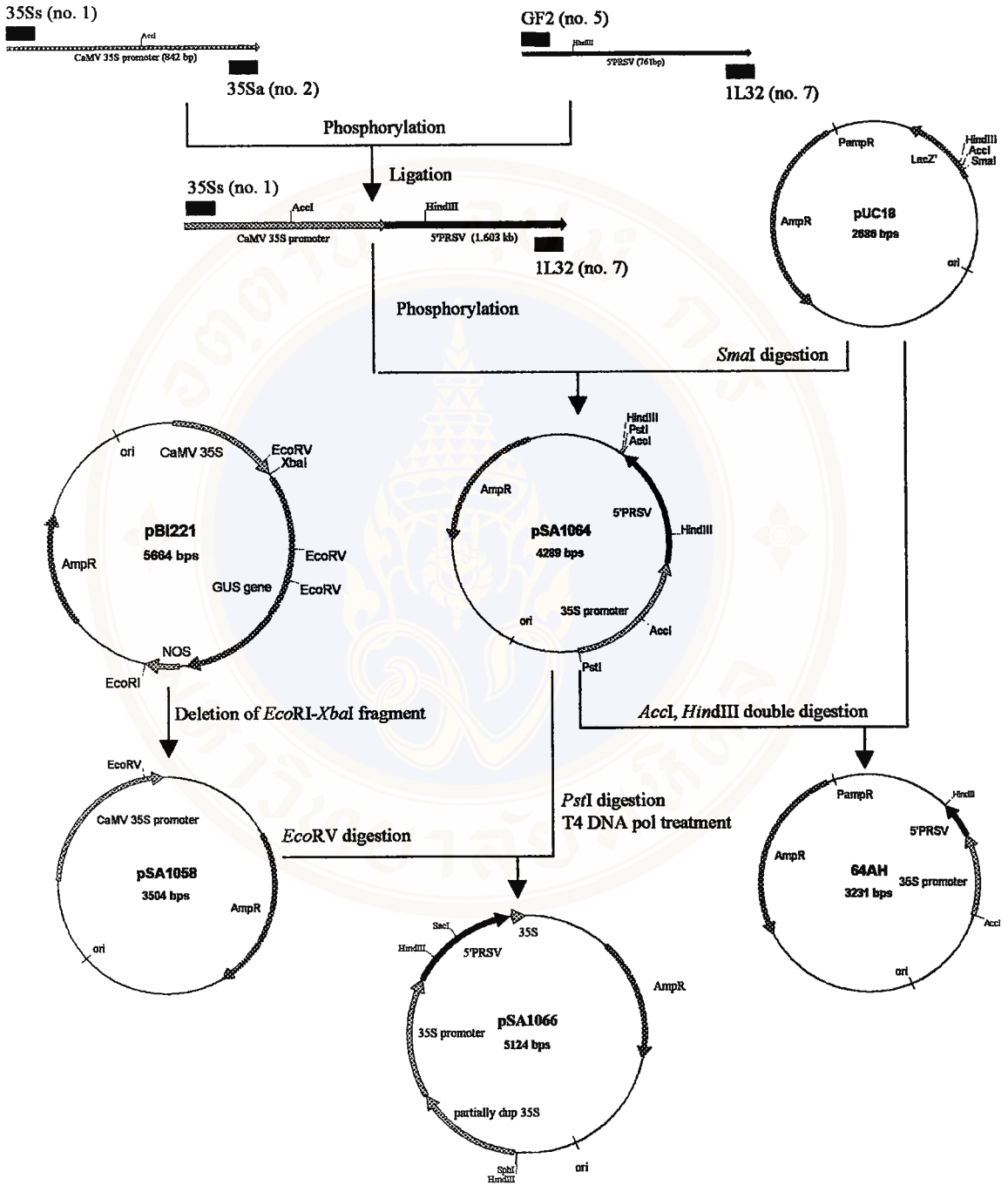


Figure 13A. Cloning strategy of plant expression cassettes

examine the ligation junction between the end of 35S promoter and beginning of PRSV sequence the *Acc*I - *Hin*dIII fragment of pSA1064 was further subcloned into pUC18 to generate plasmid 64AH. For construction of partially duplicated 35S promoter, the pSA1058 plasmid was obtained by deletion of *Eco*RI - *Xba*I fragment of pBI221 plasmid (Clonetech). Then, the *Pst*I fragment of pSA1064 plasmid was subcloned into *Eco*RV site of pSA1058 to obtain plasmid pSA1066. This plasmid is identical as pSA1064 but it contains partially duplicated 35S promoter.

The next step was adding the regulatory sequences on the 3′end of the cassette (See Figure 13B). NOS terminator fragment from *Sac*I and *Nar*I digested pBI221 plasmid was cloned into pSA1074 plasmid. pSA1074 was previously constructed in our laboratory and it contains the coat protein gene, 3′NTR and poly(A) tail of about 127 bp. Resulting plasmid pSA1077, was then used to clone of both 35S+5′PRSV and partially duplicated 35S +5′PRSV from pSA1064 and pSA1066 plasmids, respectively, to obtain plant expression cassettes that are composed of a single or partially duplicated promoter, 5′PRSV, 3′PRSV, poly(A) tail and NOS terminator. These plasmids were names as pSA1078 or pSA1079, respectively. Both plasmids have a unique *Sac*I site, which was later used for cloning the remaining part of PRSV genome.

1.1. Amplification of target DNA fragments by polymerase chain reaction

The PCR conditions that were employed for amplification of 35S promoter, 5′ PRSV and combined 35S+5′PRSV were optimized for specific condition to obtain high yield of PCR products in each PCR reaction.

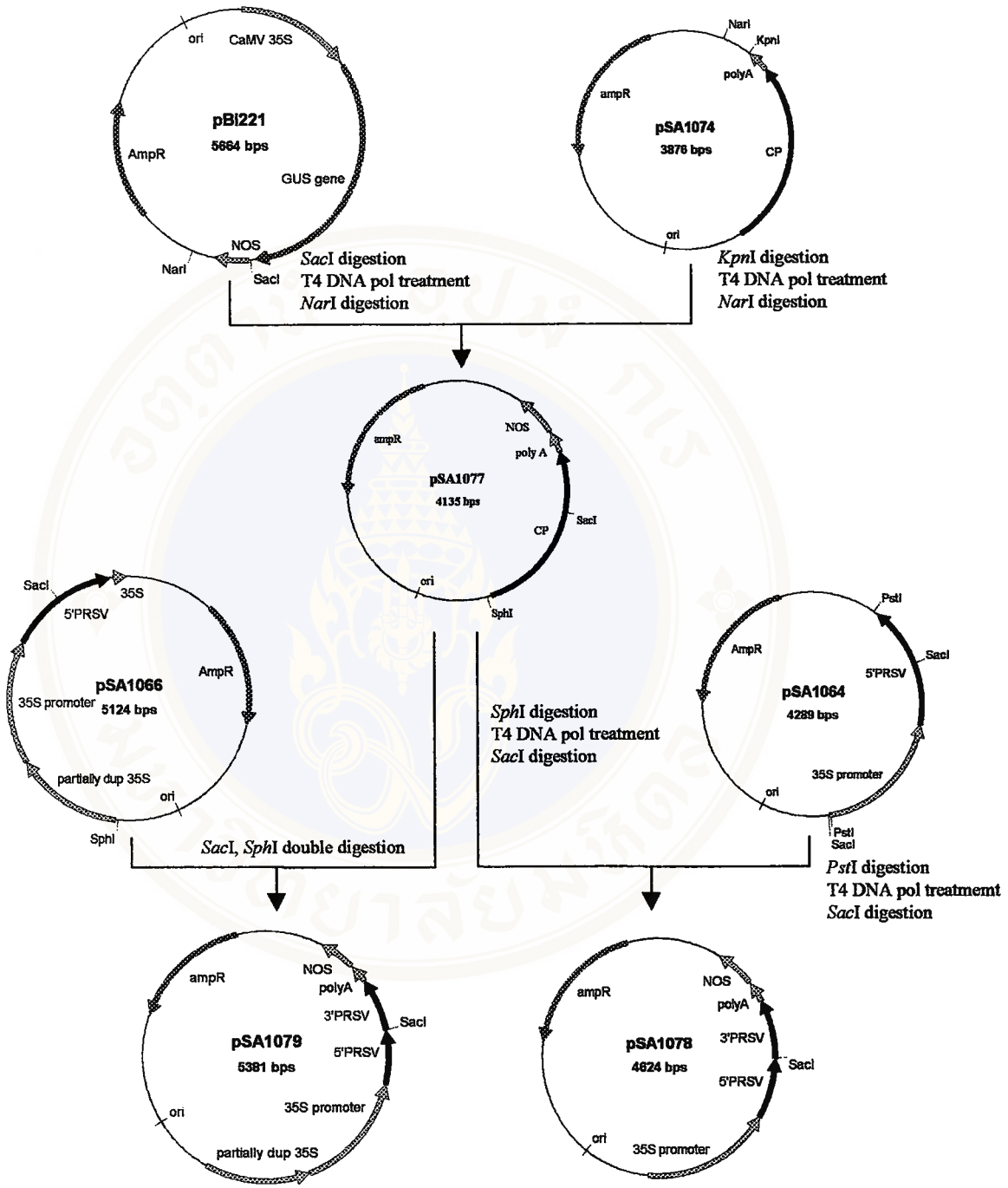


Figure 13B. Cloning strategy of plant expression cassettes

1.1.1 Amplification of CaMV 35S promoter

The CaMV 35S promoter, which is 842 bp in length, was amplified from pBI221 vector (Figure 9) by touchdown PCR using cloned *Pfu* DNA polymerase and the two primers 35Ss and 35Sa. The touchdown PCR condition was mentioned in section 2.1.3 (Chapter III). The PCR products show the major band corresponding to the size of the CaMV 35S promoter (842 bp) and a very faint band of non-specific product on 1% of agarose gel electrophoresis when stained with EtBr (Figure 14).

1.1.2 Amplification of 5′PRSV

The 5′PRSV was amplified by RT-PCR using Superscript II reverse transcriptase in the step of cDNA synthesis and by cloned *Pfu* DNA polymerase in the step of PCR amplification and with two specific primers GF2 and 1L32. The RT-PCR condition was mentioned in section 2.1.1 (Chapter III). The PCR products showed the major band corresponding to the size of 5′PRSV (761 bp) and a very faint band of non-specific product on 1% agarose gel electrophoresis when stained with EtBr (Figure 14).

1.1.3 Amplification of combined 35S+5′PRSV

The combined 35S+5′PRSV was amplified by PCR from ligation mixture of 35S+5′PRSV using cloned *Pfu* DNA polymerase and the two primers 35Ss and 1L32. The PCR condition was mentioned in section 2.1.2 (Chapter III), the PCR products showed the single band corresponding to the size of 35S+5′PRSV (1.6 kb) on 1% agarose gel electrophoresis when stained with EtBr (Figure 14).

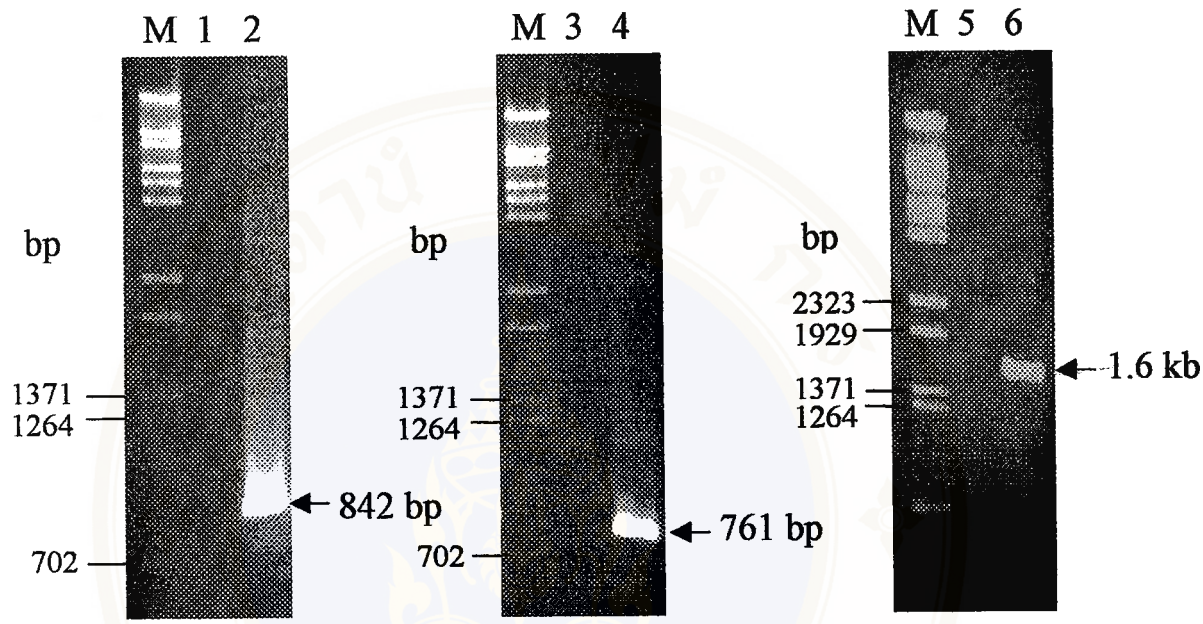


Figure 14. PCR amplification of CaMV 35S promoter, 5′PRSV and 35S+5′PRSV

The 2 - 5 µl from 50 µl of PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Bst*EII digested markers

Lane 1, 3, 5: Negative control; amplification without template

Lane 2: PCR products of CaMV 35S promoter amplified by using pBI221 vector as DNA template and 35Ss, 35Sa primers

Lane 4: RT-PCR products of 5′PRSV amplified by using 1 µl cDNA / RNA template and GF2, 1L32 primers

Lane 6: PCR products of combined 35S+5′PRSV amplified by using 1µl from 5 times dilution of ligation mixture (PCR products of 35S and 5′PRSV) as DNA template and 35Ss, 1L32 primers

1.2. Construction of plasmid pSA1064

According to the construction strategy mentioned in section 1, the combined 35S+5'PRSV PCR product (1.6 kb) was first phosphorylated with T4 DNA polynucleotide kinase. The pUC18 vectors were digested with *Sma*I and dephosphorylated with calf thymus intestinal alkaline phosphatase. Then, the digested plasmid and 35S+5'PRSV PCR product were purified, from agarose gel as mentioned in section 2.8 (Chapter III), and used for the blunt ends ligation reaction to generate the recombinant plasmids as showed in figure 15.

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a blue-white selective LB agar plate. After incubation at 37°C overnight, the white colonies were picked and cultured in LB-Amp medium. Plasmid from each clones was extracted following the method described in section 2.13.1 (Chapter III). Then the recombinant plasmids were digested with *Hin*dIII where the correct orientation of the insert in the recombinant plasmid generates two bands of 0.6 and 3.6 kb and the other pattern is the incorrect orientation as shown in Figure 16. The correct orientation clones were further confirmed by PCR amplification. By using 35S primer, 1L32 primer and BIOTOOL DNA polymerase, the PCR products of four clones showed the major band correspond to the size of 35S+5'PRSV (1.6 kb) and some of non-specific bands as showed in figure 16. The sequences of recombinant plasmids (clone no. 1 and 3) were confirmed using universal forward or reverse sequencing primers, 35Sa primer, 35S-seq primer and GSP3 primer. The resulting sequence is shown in section 4.1- 4.4 (Chapter IV). The recombinant plasmid clone no. 1 was named pSA1064.

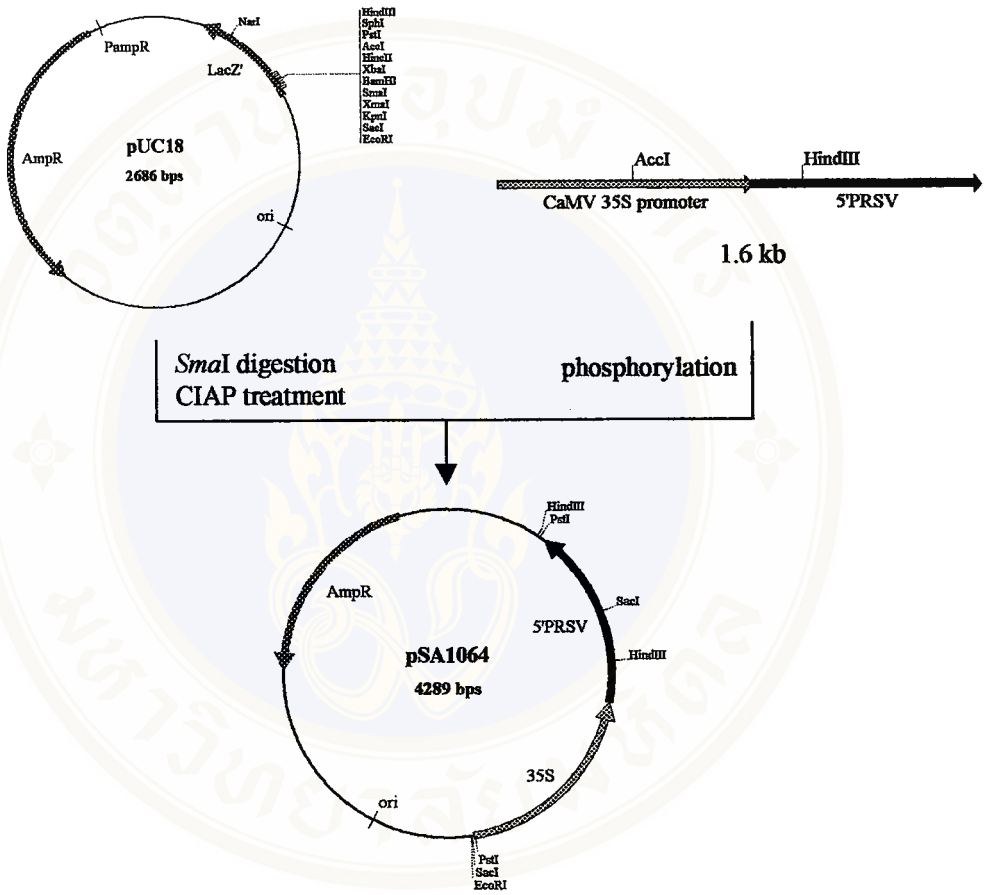


Figure 15. Construction of plasmid pSA1064

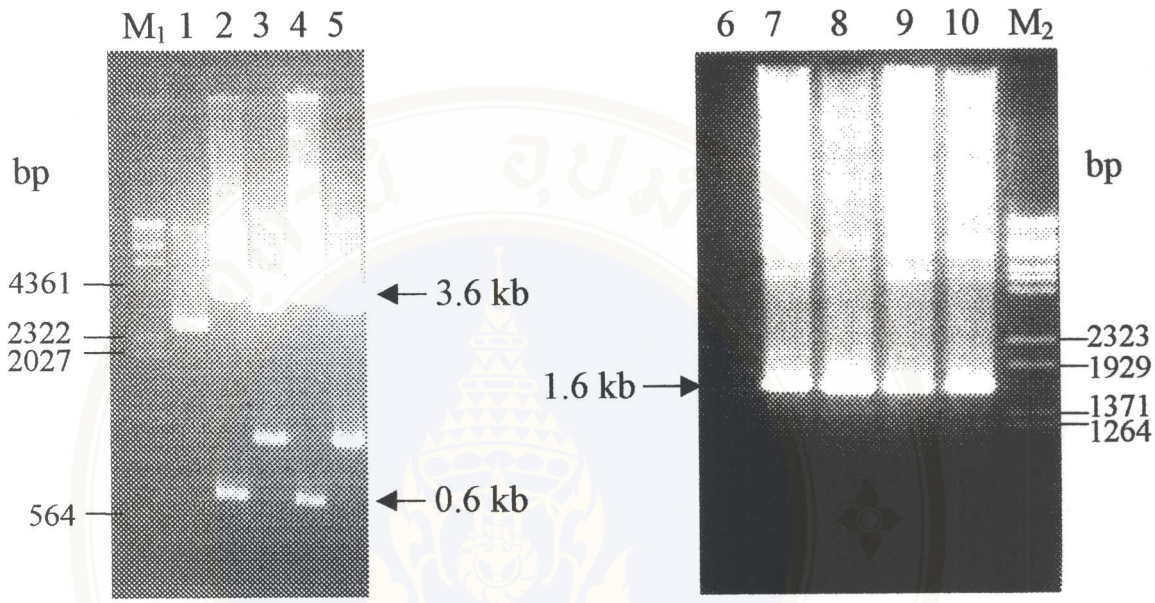


Figure 16. Restriction analysis and PCR amplification of pSA1064 plasmid

The 4 recombinant plasmids digested with *Hin*dIII and 10 µl from 50 µl of 35S+5′ PRSV PCR products of each recombinant plasmids were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M_1: Lambda DNA / *Hin*dIII digested markers
Lane M_2: Lambda DNA / *Bst*EII digested markers
Lane 1: pUC18 digested with *Hin*dIII
Lane 2-5: Clone no.1, 2, 3, 4 digested with *Hin*dIII, respectively
Lane 6: Negative control; amplification without template
Lane 7-10: PCR products of 35S+5′PRSV amplified by using recombinant plasmid DNA clone no. 1, 2, 3, 4 as DNA template, respectively and 35Ss, 1L32 primers

1.3. Subcloning of plasmid pSA1064 for sequencing the junction between 35S promoter and 5′ PRSV

Both pSA1064 and pUC18 were double digested with *Acc*I and *Hin*dIII. The 0.5 kb fragment from pSA1064 and 2.6 kb fragment of pUC18 vector were purified and used in a sticky end ligation reaction to generate the recombinant plasmids (Figure 17). The ligation products were transformed into the *E.coli* host DH5α and the culture was spreaded on a blue-white selective LB agar plate. After incubation at 37°C overnight, the white colonies were picked and cultured in LB-Amp medium. Plasmid from each clones was extracted following the method described in section 2.13.1 (Chapter III). Then the recombinant plasmids were double digested with *Acc*I and *Hin*dIII yielding two bands of 0.5 and 2.6 kb. The sequence of recombinant plasmid clone no. 4 (64AH) was confirmed using universal forward and reverse sequence primers. The resulting sequence is shown in section 4.1-4.4 (Chapter IV).

1.4 Construction of plasmid pSA1058

This procedure was used to remove the GUS reporter gene from pBI221, which was then used for further engineering. pBI221 (Clonetech) was double digested with *Eco*RI and *Xba*I and blunted with T4 DNA polymerase. The 3.5 kb fragment was purified and self ligated (Figure 18). The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, five colonies were picked and cultured in selective LB medium. Plasmid from each clone was extracted. To confirm the correct plasmid

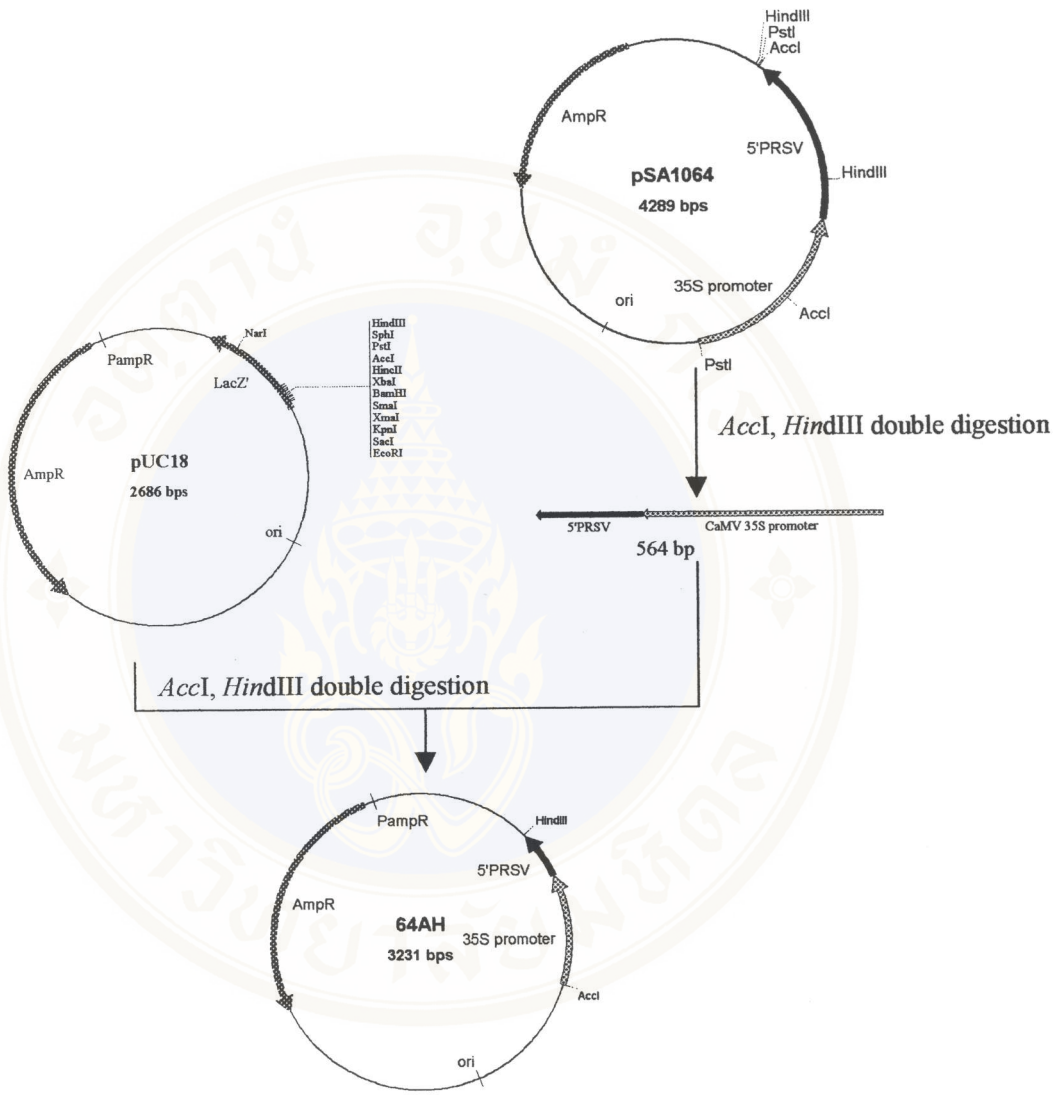


Figure 17. Construction of plasmid 64AH

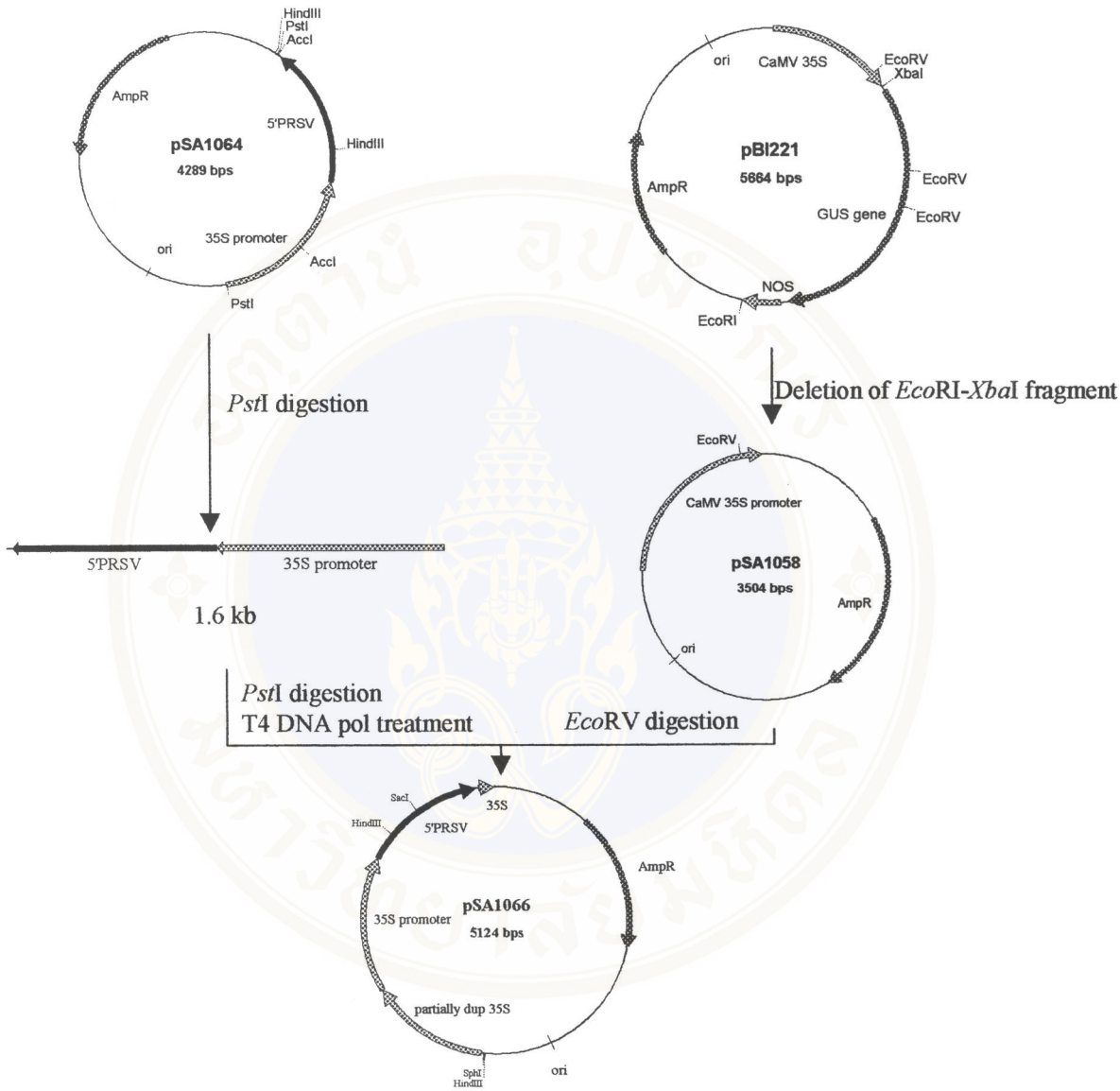


Figure 18. Construction of plasmids pSA1058 and pSA1066

construction, plasmid DNA was linearized with *Eco*RV generating 3.5 kb band and also digested with *Pvu*II giving 3 bands of 0.09 kb, 1.1 kb and 2.3 kb as showed in figure 19.

1.5 Generation of partially duplicated 35S promoter of plasmid pSA1066

The pSA1058 plasmid was digested with *Eco*RV and dephosphorylated with calf thymus intestinal alkaline phosphatase. The pSA1064 plasmid was digested with *Pst*I and blunted with T4 DNA polymerase. The 1.6 kb fragment of digested plasmid containing 35S+5′PRSV was purified and cloned into *Eco*RV site of pSA1058 (Figure 18).

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in LB-Amp medium. Plasmid from each clone was extracted following the method described in section 2.13.1 (Chapter III). Then, the recombinant plasmids were digested with *Hin*dIII to determine the correct orientation. The corrected orientation of recombinant plasmid gives two bands of 1.7 and 3.3 kb where the incorrected orientation gives other pattern as shown in figure 20. The clone was further confirmed by PCR amplification using DNA from clone no. 6 with 35Ss primer, 1L32 primer and BIOTOOL DNA polymerase. The PCR condition was mentioned in section 2.1.2 (Chapter III). The PCR revealed two major bands correspond to the size of 35S+5′PRSV (1.6 kb), the size of partially duplicated 35S+5′ PRSV (2.3 kb) and some of non-specific bands as shown in figure 20. The recombinant plasmid clone no. 6 was named pSA1066.

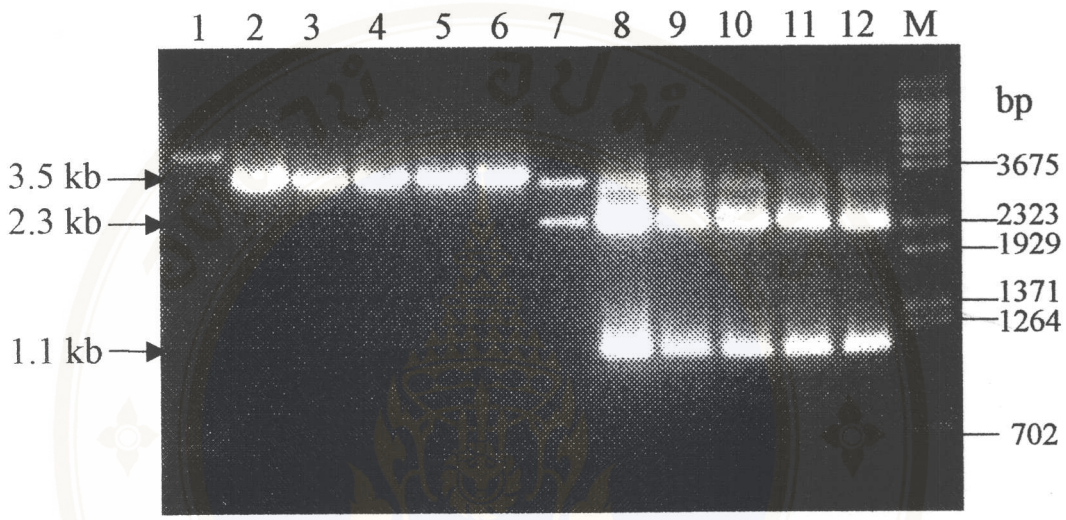


Figure 19. Restriction analysis of pSA1058 plasmid

The 5 recombinant plasmids digested with *Eco*RV or *Pvu*II were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Bst*EII digested markers
Lane 1: pBI221 digested with *Eco*RV
Lane 2-6: clone no.1, 2, 3, 4, 5 digested with *Eco*RV, respectively
Lane 7: pBI221 digested with *Pvu*II
Lane 8-12: clone no.1, 2, 3, 4, 5 digested with *Pvu*II, respectively

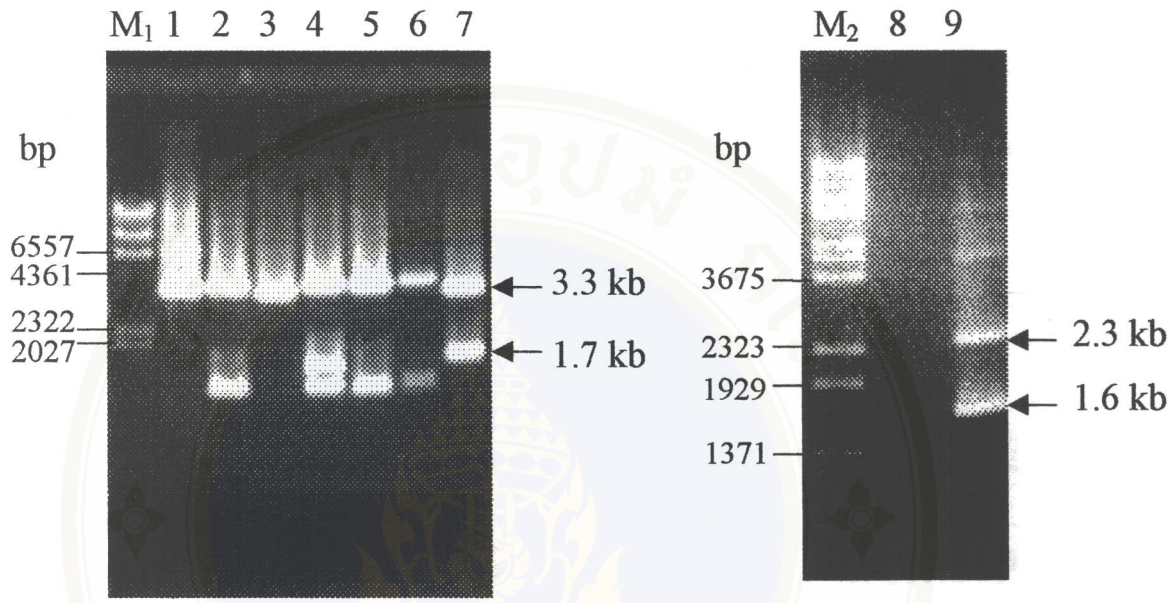


Figure 20. Restriction analysis and PCR amplification of pSA1066 plasmid

The 6 recombinant plasmids digested with *Hin*dIII and 10 µl from 50 µl of partially duplicated 35S+5′PRSV PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M_1: Lambda DNA / *Hin*dIII digested markers
Lane M_2: Lambda DNA / *Bst*EII digested markers
Lane 1: pSA1058 digested with *Hin*dII
Lane 2-7: clone no.1, 2, 3, 4, 5, 6 digested with *Hin*dIII, respectively
Lane 8: Negative control; amplification without template
Lane 9: PCR products of 35S+5′PRSV and partially duplicated 35S+5′ PRSV amplified by using recombinant plasmid DNA clone no. 6 as DNA template and 35Ss, 1L32 primers

1.6 Construction of plasmid pSA1077

The pSA1074 plasmid was digested with *Kpn*I, blunted with T4 DNA polymerase and then digested with *Nar*I. NOS terminator from the pBI221 vector (Clonetech) was obtained by *Sac*I digestion, blunting with T4 DNA polymerase then by digesting with *Nar*I. The 0.4 kb NOS fragment as well as digested plasmid pSA1074 were purified and ligated together (Figure 21). The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Pvu*II. The correct orientation of the insert gives two bands of 1.7 kb and 2.3 kb where the other orientation gives the other pattern as shown in figure 22. The sequences of recombinant plasmids clone no. 5 and 10 were confirmed using universal forward and reverse sequence primers. The resulting sequence is shown in section 4.7-4.10 (Chapter IV). The recombinant plasmid clone no. 5 was named pSA1077.

1.7 Subcloning of single promoter fragment into plasmid pSA1077

According to the construction strategy mentioned in section 1 (Chapter IV), the pSA1077 plasmid was digested with *Sph*I, blunted with T4 DNA polymerase and digested with *Sac*I. The 3.4 kb fragment was purified. The pSA1064 plasmid were digested with *Pst*I, blunted with T4 DNA polymerase and digested with *Sac*I. After purification, the 1.2 kb fragment containing 35S promoter + 5′ end PRSV was ligated with 3.4 kb fragment of plasmid pSA1077 (Figure 23).

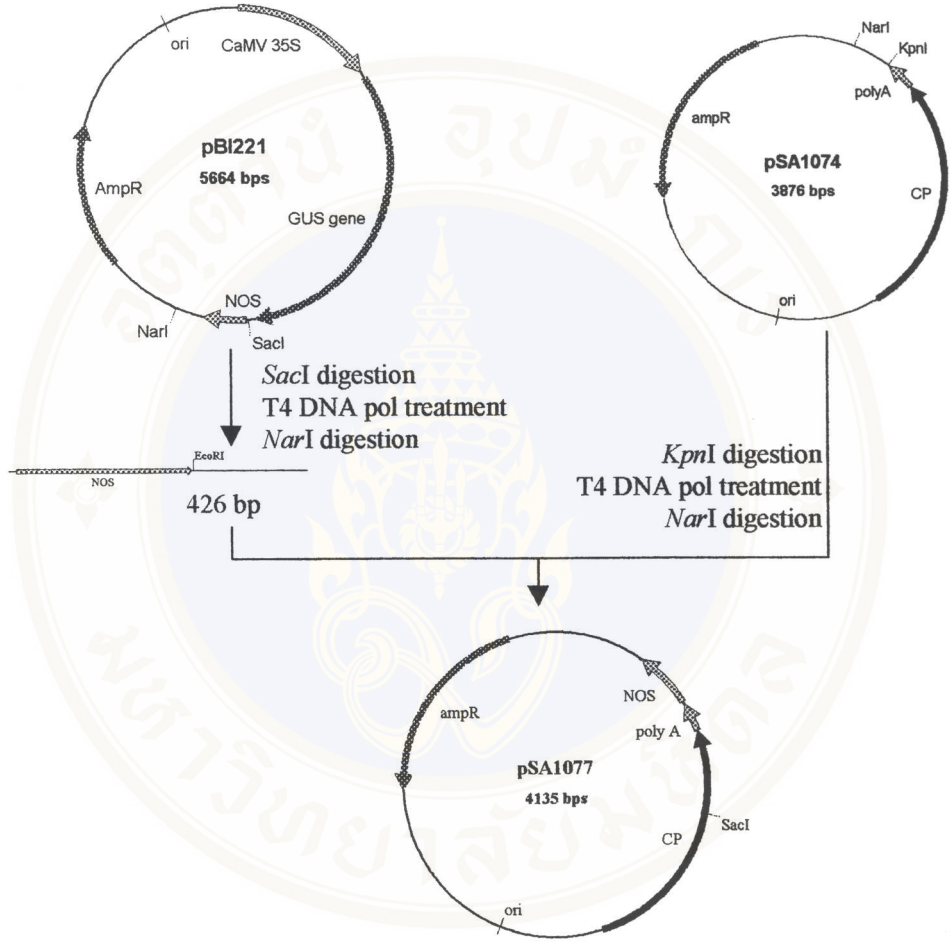


Figure 21. Construction of plasmid pSA1077

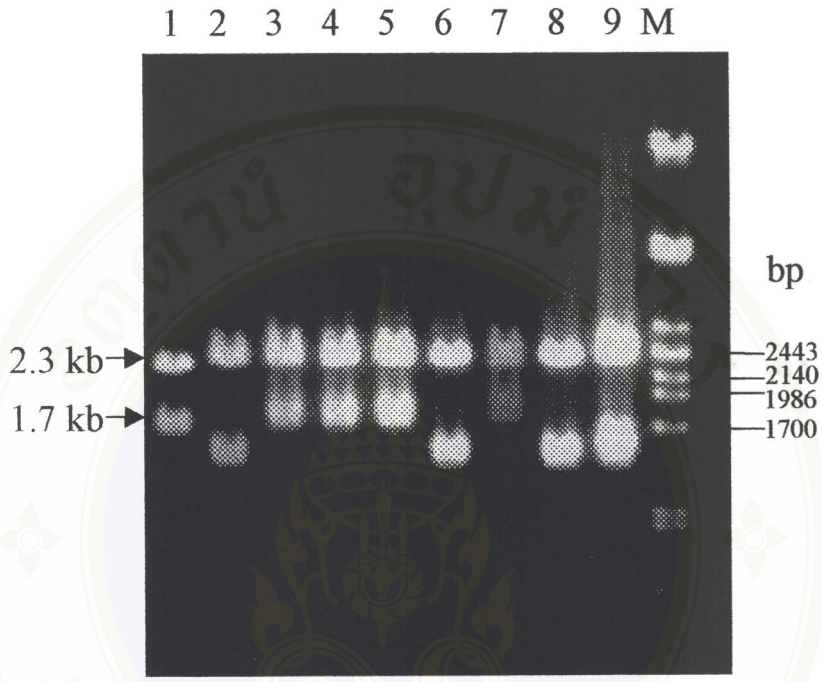


Figure 22. Restriction analysis of pSA1077 plasmid

The 9 recombinant plasmids digested with *Pvu*II were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Pst*I digested markers

Lane 1-9: clone no.5, 6, 10, 11, 14-18 digested with *Pvu*II, respectively

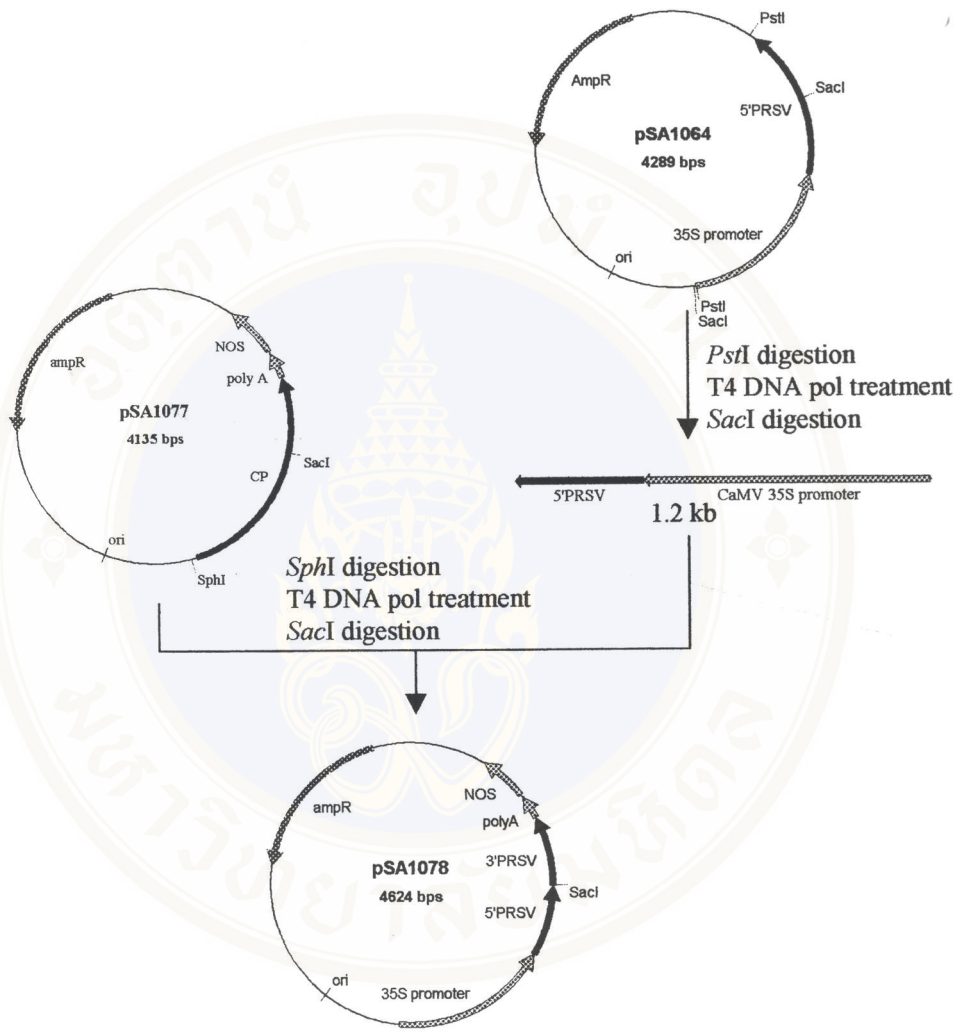


Figure 23. Construction of plasmid pSA1078

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were double digested with *Eco*RV and *Sac*I, which generated two bands at 0.4 kb and 4.2 kb if the orientation is correct (Figure 24). The correct clone was further confirmed by PCR amplification using DNA from clone no. 1, 35Ss primer, GEN-R primer and BIOTOOL DNA polymerase. The PCR products showed the major band corresponding to the size of about 1.6 kb (Figure 24). The sequences of recombinant plasmid clone no. 1 were confirmed using universal reverse sequence primer and GEN-R primer. The resulting sequence is showed in section 4.1 and 4.7 (Chapter IV). The recombinant plasmid clone no. 1 (pSA1078) contains the single 35S promoter plant expression cassette used for cloning the full length of PRSV into the single *Sac*I site.

1.8 Subcloning of partially duplicated promoter fragment into plasmid pSA1077

According to the construction strategy mentioned in section 1 (Chapter IV), the pSA1066 plasmid and pSA1077 plasmid were double digested with *Sph*I and *Sac*I. The 1.9 kb fragment of pSA1066 plasmid and 3.4 kb fragment of pSA1077 plasmid were purified and used in a sticky end ligation reaction to generate the recombinant plasmid pSA1079 (Figure 25)

The ligation product was transformed into the *E.coli* host DH5α and the

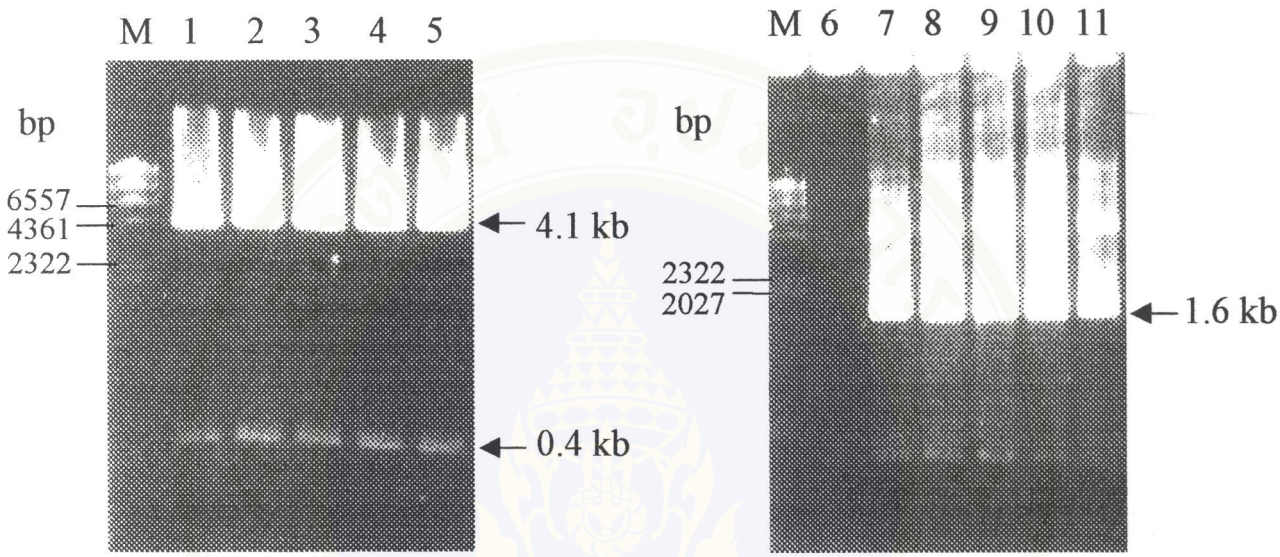


Figure 24. Restriction analysis and PCR amplification of pSA1078 plasmid

The 5 recombinant plasmids, double digested with *Eco*RV and *Sac*I and 10 µl from 50 µl of PCR products of each recombinant plasmid, were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers
Lane 1-5: clone no.1-5 double digested with *Eco*RV and *Sac*I, respectively
Lane 6: Negative control; amplification without template
Lane 7-11: PCR products of clone no.1-5, respectively amplified by using 35Ss and GenR primers

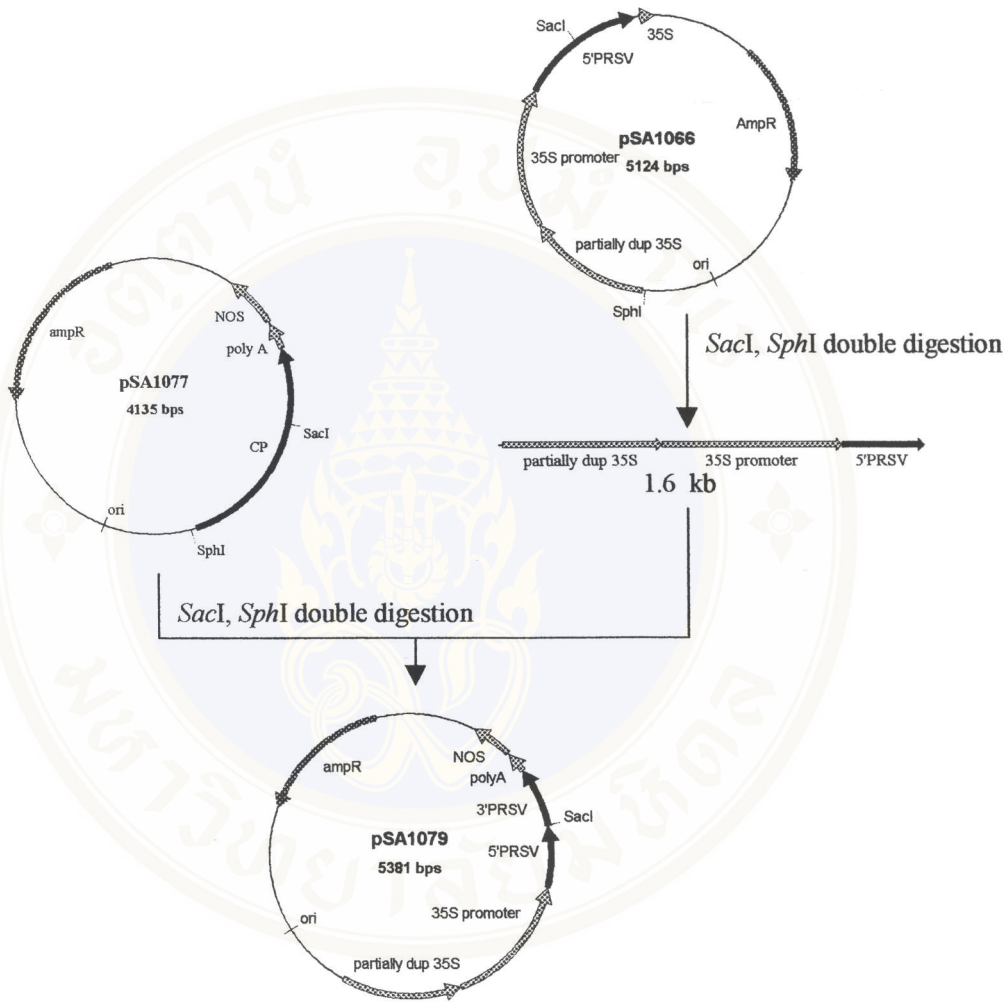


Figure 25. Construction of plasmid pSA1079

culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were double digested with *Eco*RV and *Sac*I. The plasmids with correct orientation of the insert showed bands of 0.4 and 4.9 kb (Figure 26). The plasmid was further verified by PCR amplification using DNA from clone no. 1 with 35Ss primer, GEN-R primer and BIOTOOL DNA polymerase. The PCR amplification resulted in the two major bands corresponding to the size of about 1.6 kb and the size of about 2.3 kb (Figure 26). The sequences of recombinant plasmid clone no. 1 were confirmed using reverse universal sequence primers and GEN-R primers. The resulting sequence is shown in section 4.1 and 4.7 (Chapter IV). This recombinant plasmid clone no. 1 was named pSA1079 (partially duplicated 35S promoter plant expression cassette). This plasmid was used for cloning the full length of PRSV into the single *Sac*I site.

2. Construction of full-length PRSV type P (Overview)

Obtaining of the full length PRSV genomic cDNA was achieved by cloning three overlapping sequences of the genomic PRSV cDNA which were previously amplified in our laboratory (M. Juricek, unpublished results). These sequence fragments are referred as "start", which is 3.5 kb long 5′end DNA sequence, "end", which is 4.3 kb long 3′end DNA sequence and the "middle" part, 2.7 kb long DNA

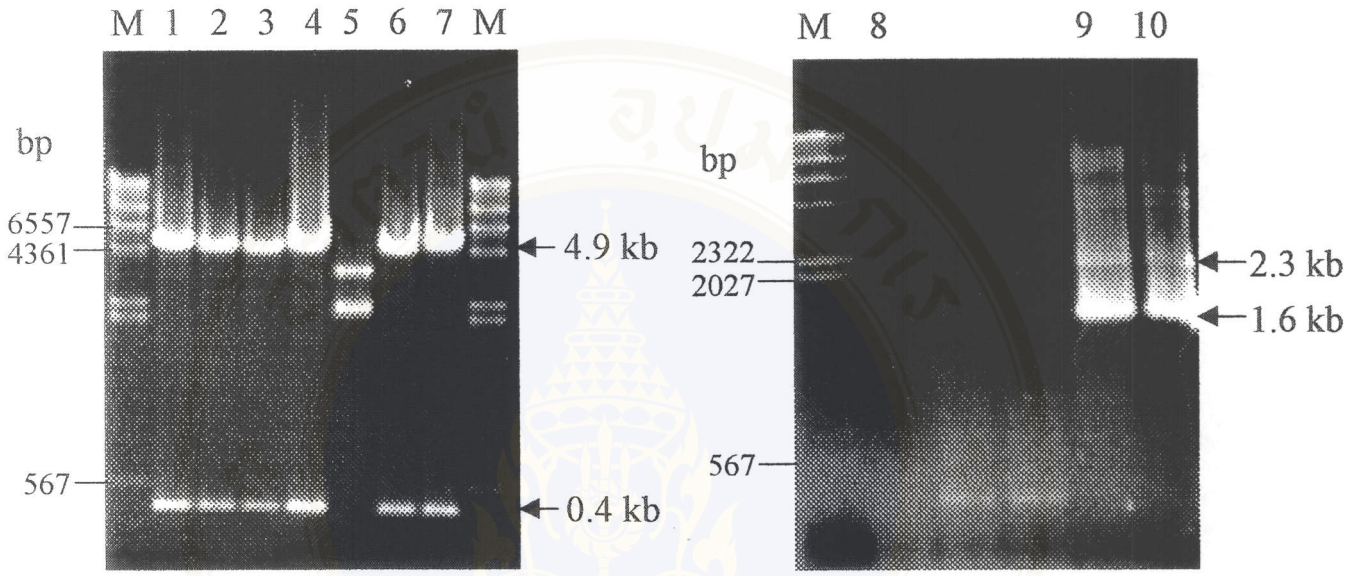


Figure 26. Restriction analysis and PCR amplification of pSA1079 plasmid

The 7 recombinant plasmids double digested with *Eco*RV and *Sac*I and 10 µl from 50 µl of PCR products of each recombinant plasmid were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers
Lane 1-7: clone no.1-7 double digested with *Eco*RV and *Sac*I, respectively
Lane 8: Negative control; amplification without template
Lane 9-10: PCR products of clone no.1-2 amplified by using 35Ss and GenR primers

sequence overlapping the middle and the start sequences.

The pSA1099 plasmid containing the "start" PRSV sequence under the T7 promoter was constructed first, by cloning of RT-PCR amplification of first 781 bp of PRSV into *Sma*I site of pSA1065 plasmid followed by cloning of the "start" part of PRSV into *Bam*HI-*Pac*I site (Figure 27A).

Plasmid pSA1091 was constructed by ligating the "middle" PRSV sequence into single *Pvu*II site of pSA1080, while plasmid pSA1092 was constructed by ligating the "end" PRSV sequence into the same vector. After digesting both pSA1091 and pSA1092 with *Pvu*II and ligating together, the plasmid pSA1098 was obtained, which contains both "middle" and "end" parts connected (Figure 27B).

By digesting the plasmid pSA1098 with *Stu*I and *Xba*I, this sequence was cut out and ligated into pSA1099. Resulting plasmid pSA1100 has the full-length PRSV sequence under the control of T7 promoter (Figure 27C).

The pSA1100 plasmid was partially digested with *Sac*I to obtain the 9.5 kb fragment, which was further cloned into *Sac*I site of both pSA1078 plasmid (single 35S promoter plant expression cassette) and pSA1079 plasmid (partially duplicated 35S promoter plant expression cassette). Resulting plasmids pSA1101 and pSA1102 have the full-length of PRSV under the control of single and partially duplicated 35S promoter (Figure 27C).

The last constructed clone was pSA1110 plasmid, which was full-length of PRSV under T7 promoter and contained poly A and NOS terminator. This clone was constructed by cloning of 9.8 kb *Kpn*I - *Sac*II fragment of pSA1100 plasmid into *Sph*I - *Sac*II site of pSA1077 plasmid, which contained coat protein gene, 3'UTR, poly(A)

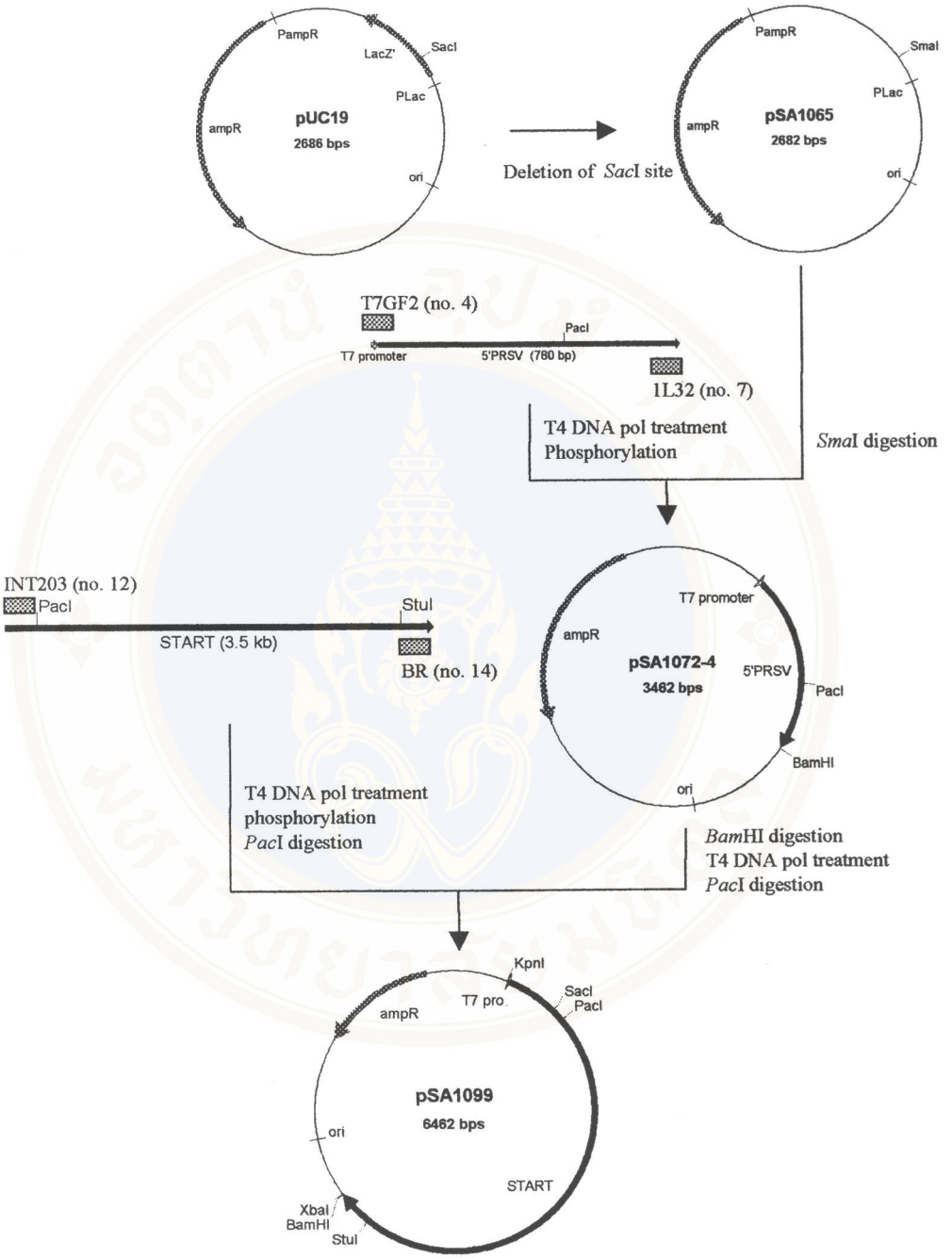


Figure 27A. Cloning strategy of full-length PRSV

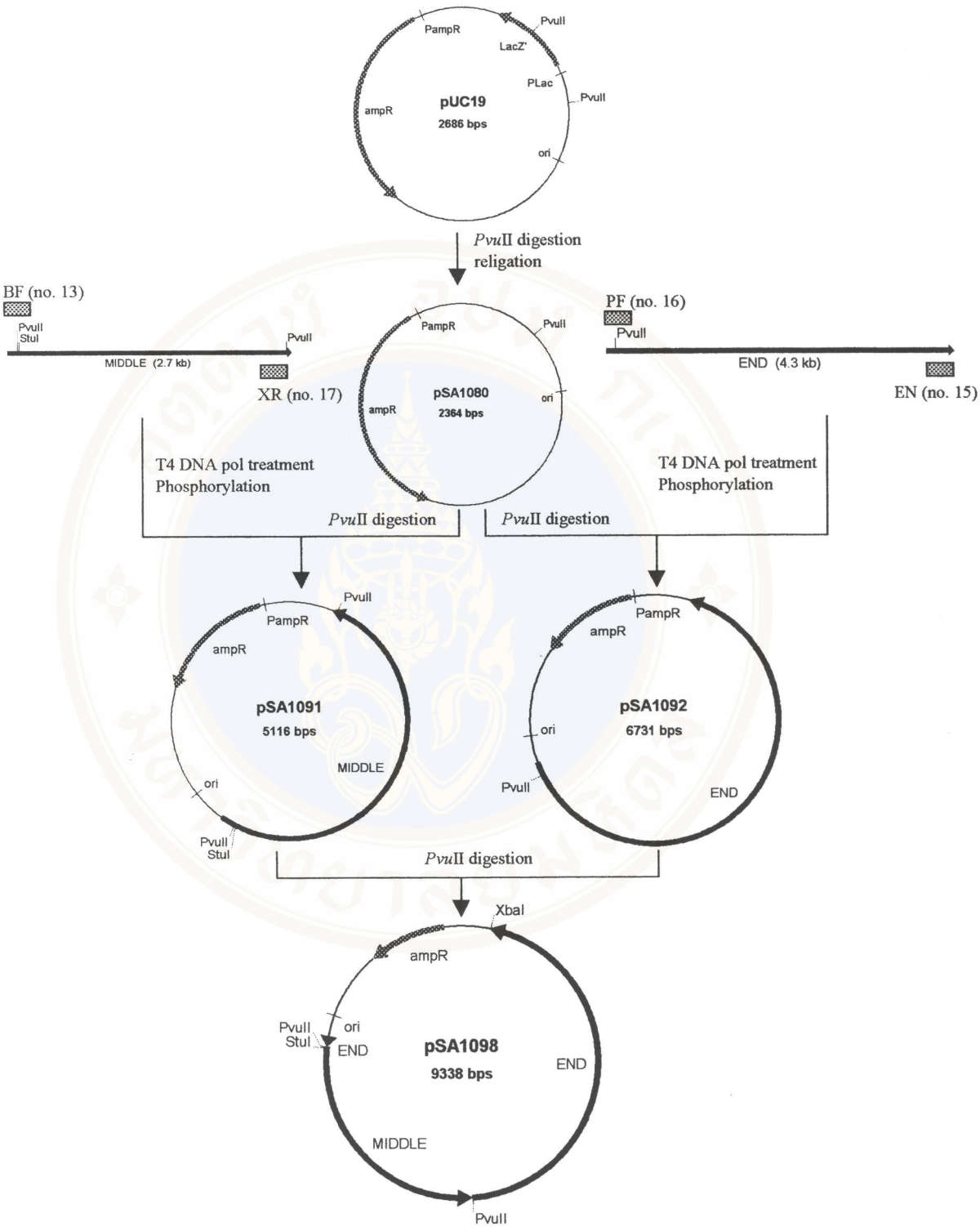


Figure 27B. Cloning strategy of full-length PRSV

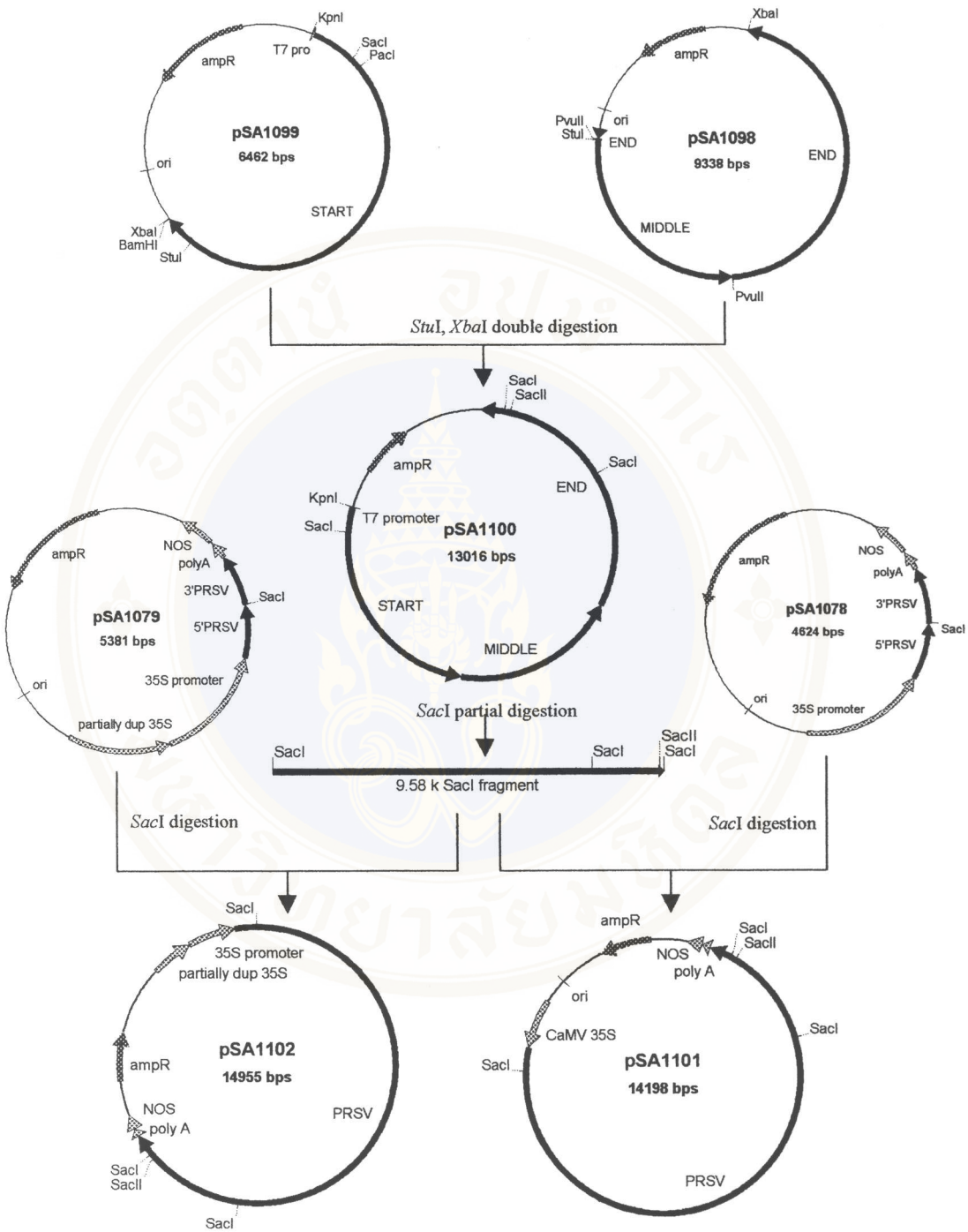


Figure 27C. Cloning strategy of full-length PRSV

tail and NOS terminator (Figure 27D).

2.1 Amplification of T7 promoter + 5′PRSV

Since the "start" PRSV sequence begins at nucleotide 201, the beginning of the PRSV genome was added first. The 5′PRSV was amplified by RT-PCR using Superscript II reverse transcriptase in the step of cDNA synthesis, *Pfu* DNA polymerase in the step of PCR amplification and the two primers T7GF-2 and 1L32. The RT-PCR condition was mentioned in section 2.1.1 (Chapter III), the PCR product showed the major band corresponding to the size of T7-5′PRSV (781 bp) and a very faint band of non-specific product on 1% agarose gel electrophoresis when stained with EtBr (Figure 28).

2.2 Construction of plasmid pSA1065

The only available restriction enzyme site for cloning the final 9.5 kb PRSV fragment into the plasmids pSA1078 and pSA1079 was *Sac*I. This site had to be removed first from pUC19. The pUC19 vector was digested with *Sac*I and blunted with T4 DNA polymerase. After self-ligation, the product was transformed into the *E.coli* host DH5α and the culture was spreaded on a blue-white selective LB agar plate. After incubation at 37°C overnight, the white colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Sac*I. The correct recombinant plasmid, not digested with *Sac*I, was named pSA1065 (Figure 29).

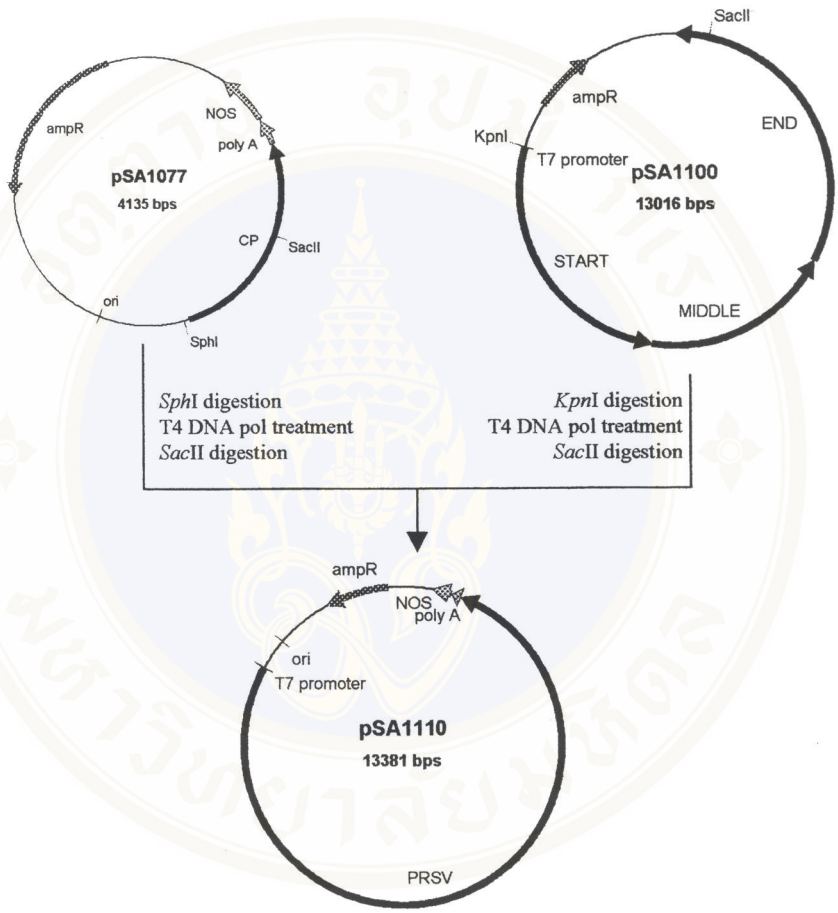


Figure 27D. Cloning strategy of full-length PRSV

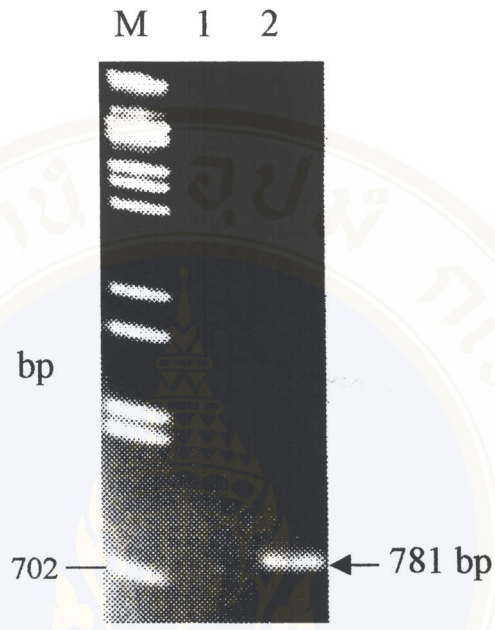


Figure 28. PCR amplification of T7 promoter+5′PRSV

The 5 µl from 50 µl of RT-PCR product was analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Bst*EII digested markers

Lane 1: Negative control; amplification without template

Lane 2: RT-PCR products of T7 promoter+5′PRSV amplified by using 1µl of cDNA/RNA as template and T7GF2, 1L32 primers

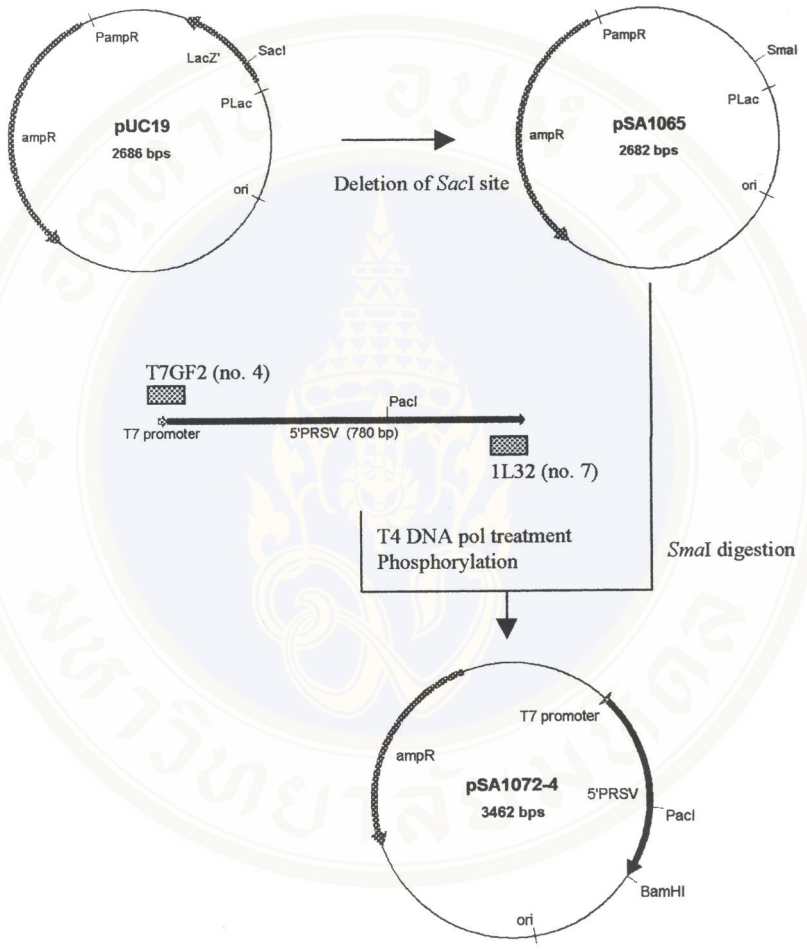


Figure 29. Construction of plasmids pSA1065 and pSA1072-4

2.3 Construction of plasmid pSA1072-4

Plasmid pSA1065 was digested with *Sma*I and dephosphorylated with calf thymus intestinal alkali phosphatase. The T7+5′PRSV PCR product (781 bp) was phosphorylated with T4 DNA polynucleotide kinase. Both digested plasmids and T7+5′ PRSV PCR products were purified and used in a blunt end ligation reaction (Figure 29). The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Hin*dIII. The corrected orientation of recombinant plasmid generates two bands at 0.6 and 2.8 kb where the incorrect recombinant plasmid generates other pattern (Figure 30). The correct clone was further confirmed by PCR amplification by using T7GF-2 primer, 1L32 primer and BIOTOOL DNA polymerase. The PCR product shows the major band correspond to the size of T7+5′PRSV (781 bp) (Figure 30).

2.4 Construction of plasmid pSA1080

The *Pvu*II site is important for the above-mentioned cloning strategy. There are two *Pvu*II sites in pUC19 fanking the multiple cloning sites, plasmid pSA1080 is essentially pUC19 with removed cloning site and one *Pvu*II site. The pUC19 plasmid was digested with *Pvu*II and the 2.3 kb fragment was purified and self ligated (Figure 31). The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a blue-white selective LB agar plate. After incubation at 37°C

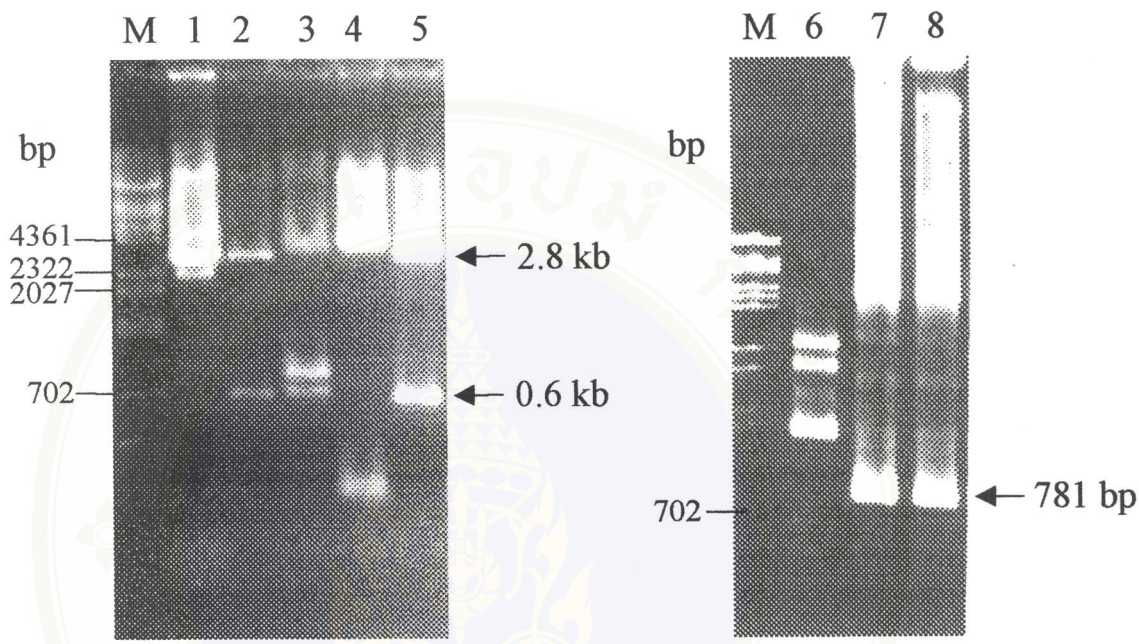


Figure 30. Restriction analysis and PCR amplification of pSA1072-4 plasmid

The 4 recombinant plasmids digested with *Hin*dIII and 10 µl from 50 µl of PCR products of each recombinant plasmid were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Bst*EII digested markers
Lane 1: pSA1065 plasmid digested with *Hin*dIII (vector)
Lane 2-5: clone no.1-4 digested with *Hin*dIII, respectively
Lane 6 Negative control; amplification without template
Lane 7-8: PCR products of clone no.1 and 4, amplified by using T7GF2 and 1L32 primers

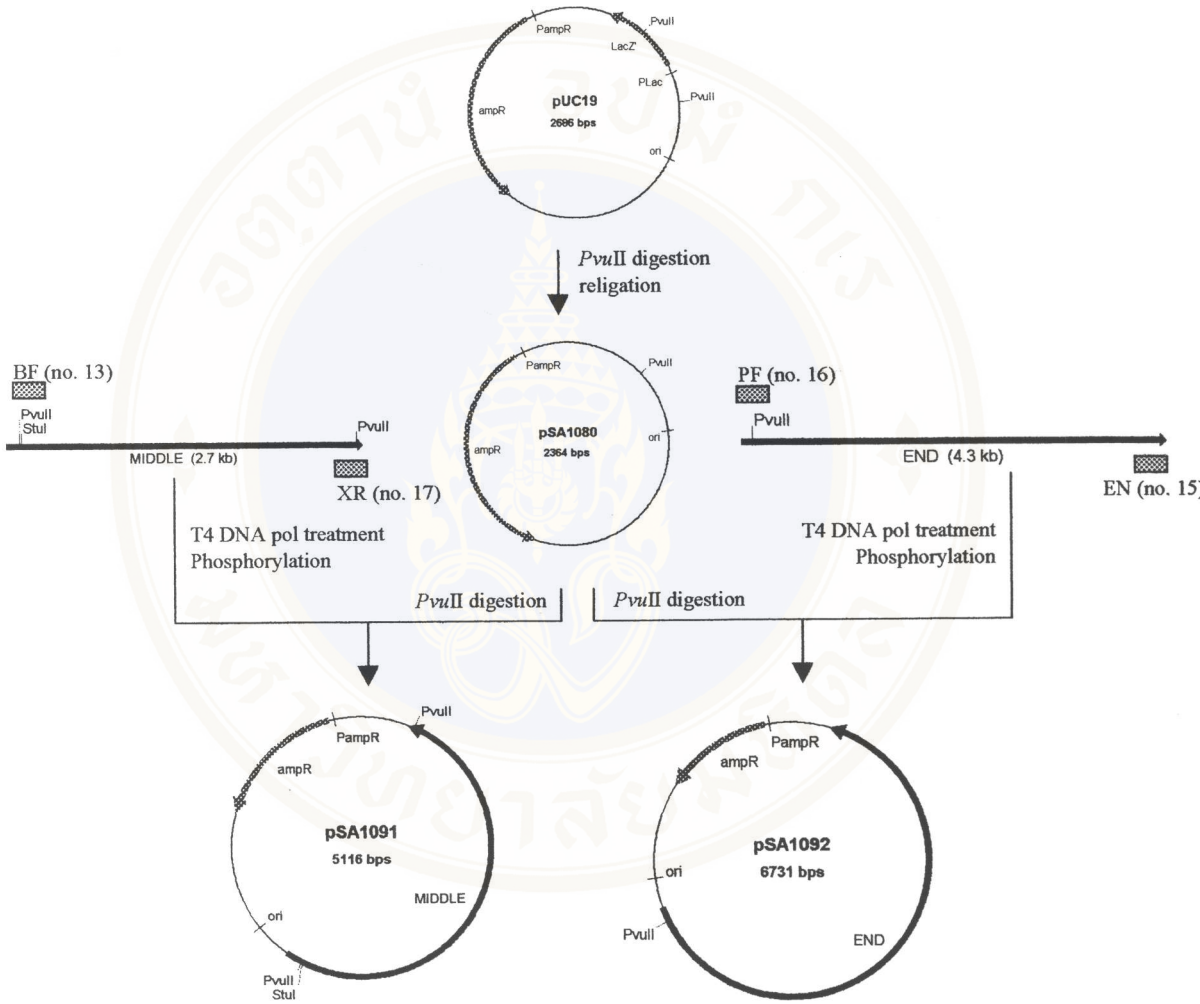


Figure 31. Construction of plasmids pSA1080, pSA1091 and pSA1092

overnight, the white colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Pvu*II giving one band of 2.3 kb.

2.5 Construction of plasmid pSA1099

The "start"RT PCR product (3.5 kb) (Figure 32) was previously obtained by M. Juricek using Expand Taq DNA polymerase kit, Int203 primer and BR primer. The fragment was blunted with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase. Then, it was digested with *Pac*I and purified by GENECLEAN II KIT. The pSA1072-4 was digested with *Bam*HI and blunted with T4 DNA polymerase. After digestion with *Pac*I and purification, they were ligated with the purified "start" fragment (Figure 33).

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked to do the master plate for rapid size screening as mentioned in section 2.12 (Chapter III). The colonies that gave the correct pattern were picked form master plate and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Stu*I. The correct orientation of the insert gave the single band of 6.4 kb. The correct clone was further confirmed by PCR amplification using T7GF-2 primer, 1L32 primer and BIOTOOL DNA polymerase. The PCR condition was mentioned in section 2.1.2 (Chapter III). The PCR products showed the major band corresponding to the size of T7+5'PRSV

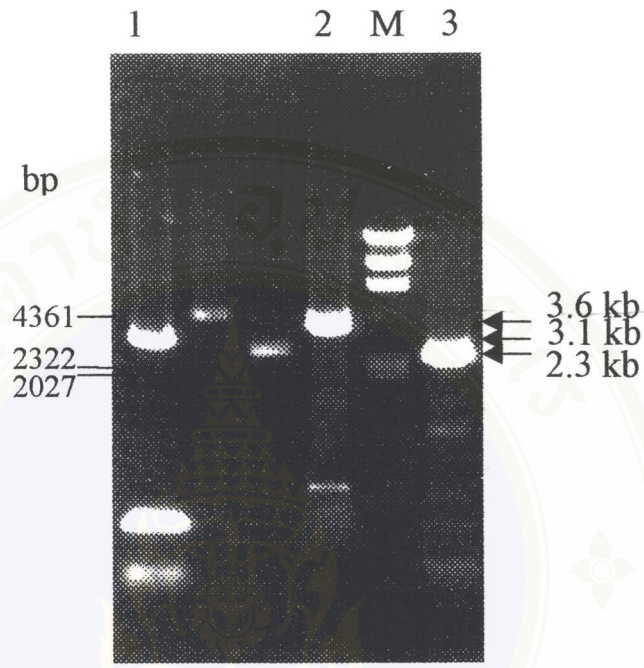


Figure 32. Restriction analyses of PCR amplification of start, middle and end part of PRSV-P Thai isolate

The 3 µl from 60 µl of RT-PCR products was analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers

Lane 1: RT-PCR product of start part amplified by using INT203 and BR primers and digested with *Bam*HI

Lane 2: RT-PCR product of end part amplified by using PF and EN primers and digested with *Pvu*II

Lane 3: RT-PCR product of middle part amplified by using BF and XR primers and digested with *Pvu*II

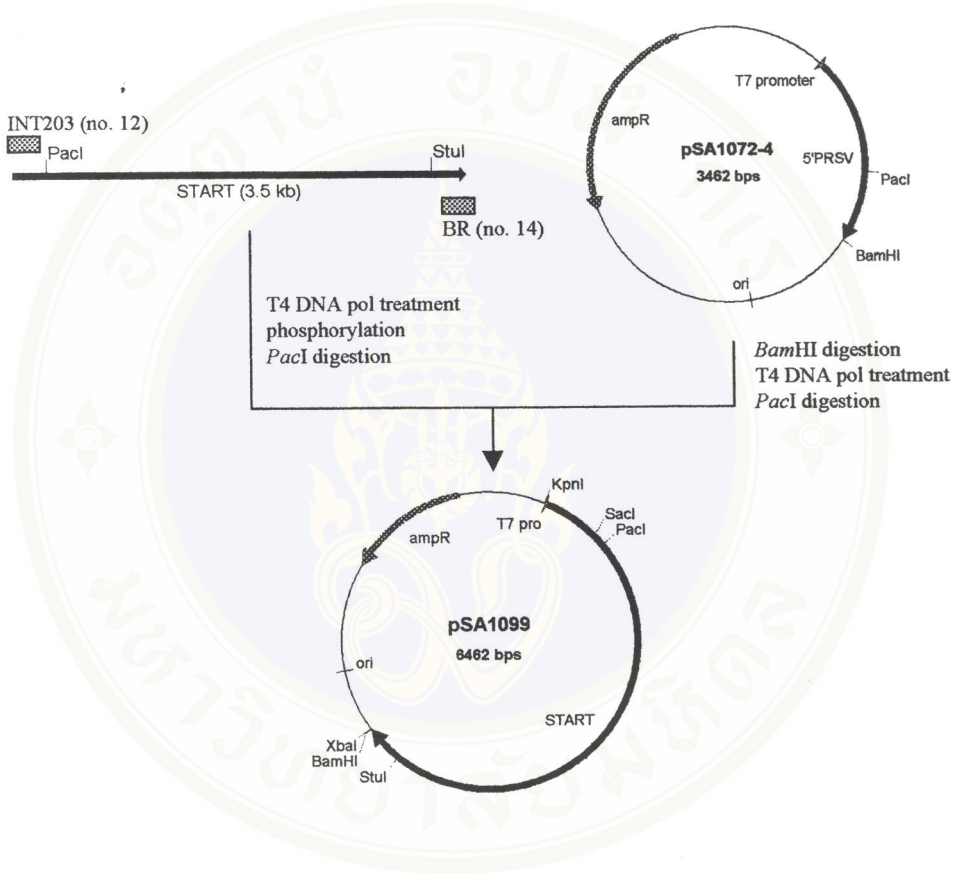


Figure 33. Construction of plasmid pSA1099

(781 bp) and some of non-specific bands (Figure 34). The sequences of recombinant plasmid clone no. 35 were confirmed using primers T7GF-2 and BR. The resulting sequence was shown in section 4.3 and 4.5 (Chapter IV). The recombinant plasmid clone no. 35 was named pSA1099.

2.6 Construction of plasmid pSA1091

The RT-PCR product of "middle" part of PRSV (2.5 kb) (Figure 32) was previously amplified by M. Juricek using Expand Taq DNA polymerase, BF primer and XR primer. It was blunted with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase and purified. The plasmid pSA1080 was digested with *Pvu*II and dephosphorylated with calf intestinal alkaline phosphatase. After purification, they were ligated with the purified middle part of PRSV (Figure 31).

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked to do the master plate for rapid size screening as mention in section 2.12 (Chapter III). The colonies that gave the corrected pattern were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Nar*I that supposed to be one band at 5.1 kb. The correct clone was further confirmed by PCR amplification using BF primers, XR primers and BIOTOOL DNA polymerase. The PCR products showed the major band corresponding to the size of 2.5 kb and some of non-specific bands (Figure 34). The sequences of recombinant plasmids clone no. 30 and 33 were confirmed using BF and XR primers. The resulting sequence is shown in section 4.5 and 4.6 (Chapter IV).

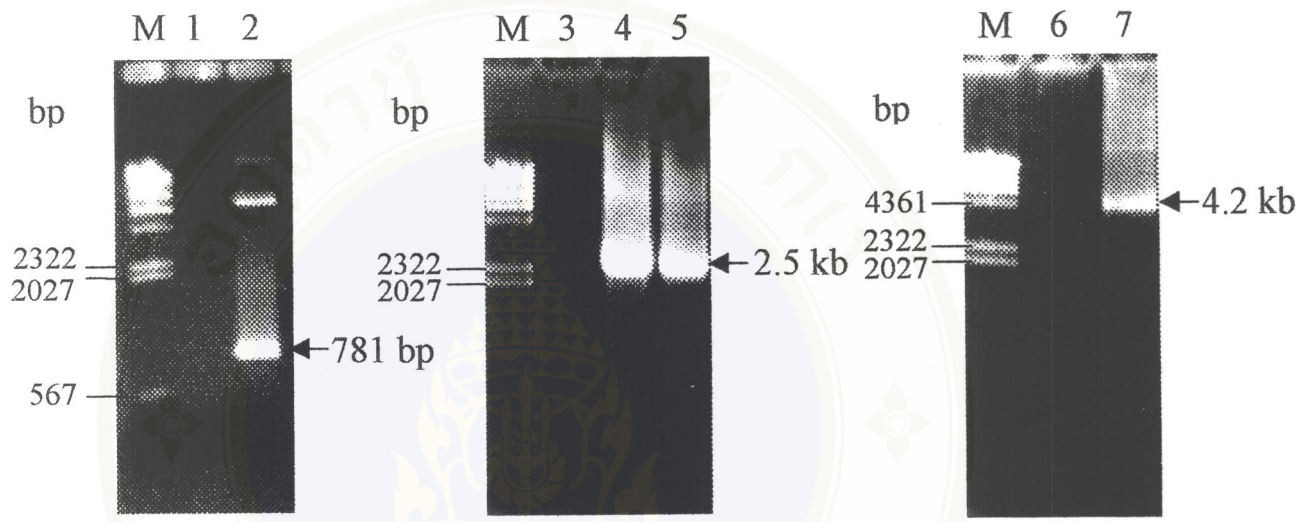


Figure 34. PCR amplification of pSA1099 (start part), pSA1091 (middle part) and pSA1092 (end part) plasmids

The 10 µl from 50 µl of PCR products was analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M:	Lambda DNA / *Hin*dIII digested markers
Lane 1, 3, 6:	Negative control; amplification without template
Lane 2:	PCR product of pSA1099 (start part) amplified by using T7GF2 and 1L32 primers
Lane 4-5	PCR products of pSA1091 (middle part) clone no. 30 and 33, respectively amplified by using BF and XR primers
Lane 7	PCR product of pSA1092 (end part) amplified by using PF and XR primers

The recombinant plasmid clone no. 30 was named pSA1091.

2.7 Construction of plasmid pSA1092

The RT-PCR products of "end" part of PRSV (4.2 kb) (Figure 32) were previously amplified by M. Juricek using Expand Taq DNA polymerase, PF primer and EN primer. The fragment was blunt ended with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase and purified. The plasmid pSA1080 was digested with *Pvu*II and dephosphorylated with calf intestinal alkaline phosphatase. After purification, it was ligated with the purified "end" part of PRSV (Figure 31).

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked to do the master plate for rapid size screening. The colonies that gave the corrected pattern were picked and cultured in a selective LB medium. Plasmid from each clones was extracted. Then, the recombinant plasmids were digested with *Pvu*II giving one band at 6.7 kb in case of the correct orientation. The correct clone was further confirmed by PCR amplification using PF primer, EN primer and BIOTOOL DNA polymerase. The PCR products showed the major band correspond to the size of 4.2 kb and some of non-specific bands (Figure 34). The sequences of recombinant plasmids clone no. 4 and 16 were confirmed using PF and EN primers. The resulting sequence is shown in section 4.6-4.7 (Chapter IV). The recombinant plasmid clone no. 4 was named pSA1092.

2.8 Construction of plasmid pSA1098: joining the "middle" and "end" parts

The plasmid pSA1092 was digested with *Pvu*II and dephosphorylated with calf intestinal alkaline phosphatase. The pSA1091 plasmid was digested with the same restriction enzyme that generating 2.5 kb fragment. After purification, it was ligated with purified fragment of pSA1092 plasmid (Figure 35).

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked to do the master plate for rapid size screening. The colonies that gave the corrected pattern were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Stu*I that supposed to be one band of 9.3 kb. The sequences of recombinant plasmid clone no. 35 were confirmed using PF primer and XR primer to determine the orientation of the insert. The resulting sequence is shown in section 4.6 (Chapter IV). The recombinant plasmid clone no. 35 is correct orientation and had a named as pSA1098.

2.9 Construction of plasmid pSA1100: generation of full-length of PRSV under T7 promoter

Both pSA1099 and pSA1098 plasmids were double digested with *Stu*I and *Xba*I generating 6.4 kb or 6.8 kb fragments. After purification, these were ligated together (Figure 36). The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in a selective LB medium. Plasmid

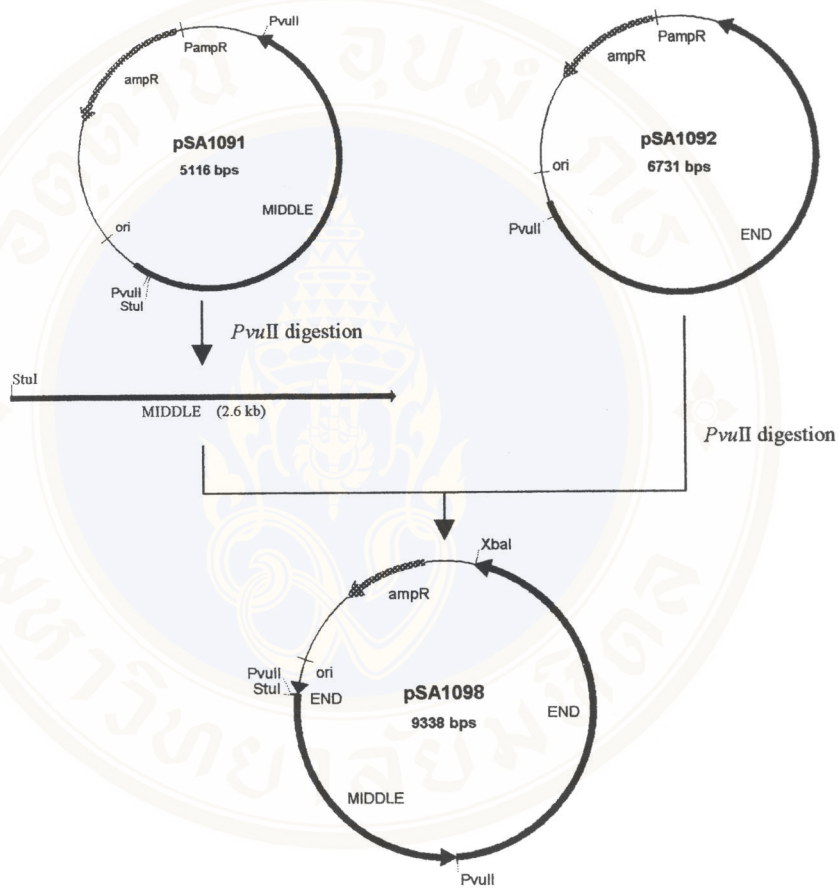


Figure 35. Construction of plasmid pSA1098

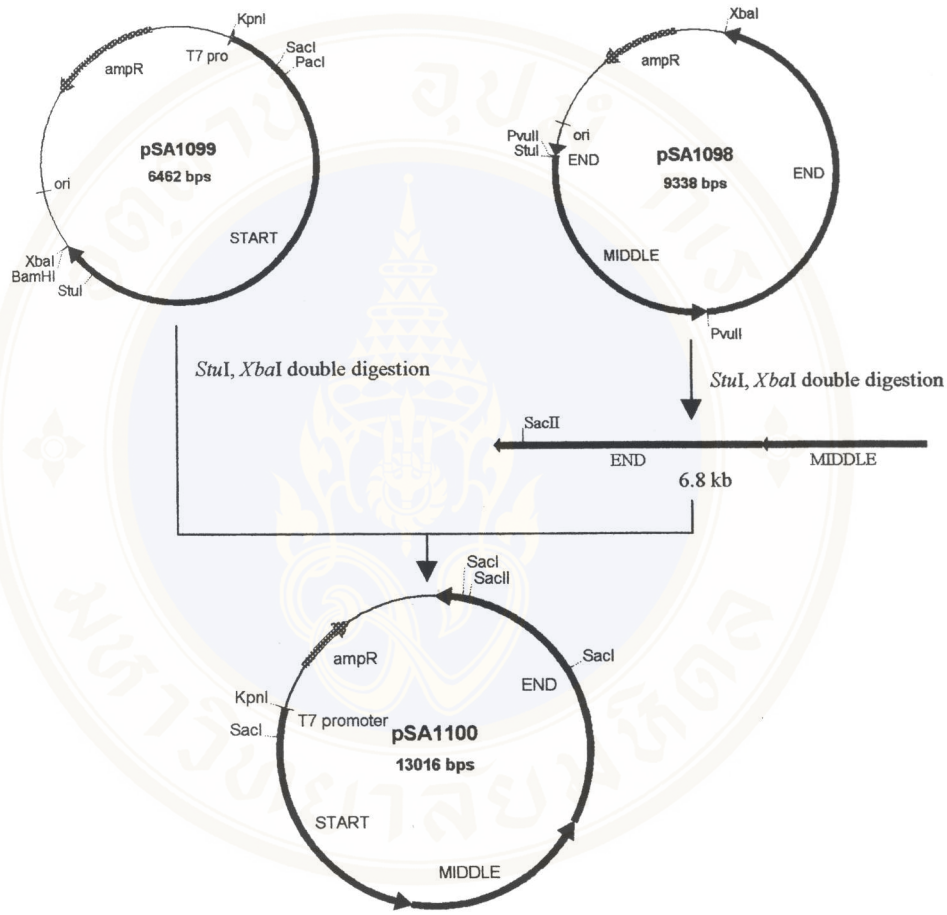


Figure 36. Construction of plasmid pSA1100

from each clone was extracted. Then, the recombinant plasmids were linearized with *Xba*I and digested with *Sac*I. The correct orientation gives one band at 13.8 kb with *Xba*I and three bands at 7.8, 3.8 and 1.7 kb with *Sac*I, respectively (Figure 37). The full-length PRSV sequence was further confirmed by PCR amplification of 5′part and the 3′part of the genome by using T7GF-2 primer and 1L32 primer or 5′CP primer and GEN-R primer using BIOTOOL DNA polymerase. The PCR products of 5′end showed the major expected band corresponding to the size of 781 bp (Figure 38) and the PCR products of 3′end showed the major expected band of 1.2 kb (Figure 38). The sequences of recombinant plasmid clone no. 17 (named pSA1100) were confirmed using universal forward and reverse sequencing primers, BR primer and also 1L32 primer. The resulting sequence is shown in section 4.1, 4.2, 4.5 and 4.7 (Chapter IV).

2.10 Construction of plasmids pSA1101 and pSA1102; generation of full-length of PRSV under single 35S promoter and partially duplicated 35S promoter

The pSA1100 plasmid was partially digested with *Sac*I (1μg DNA/ 0.5 unit enzyme for 1.30 hours) that generated 6 fragments. (Figure 39). The 9.5 kb fragment was excised from the gel and purified (Figure 40). The plasmids pSA1078 and pSA1079 were digested with *Sac*I, dephosphorylated with calf intestinal alkaline phosphatase and purified by GENECLEAN II KIT. The 9.5 kb fragments of pSA1100 plasmid were ligated with purified pSA1078 and pSA1079 plasmid fragments, respectively (Figure 40).

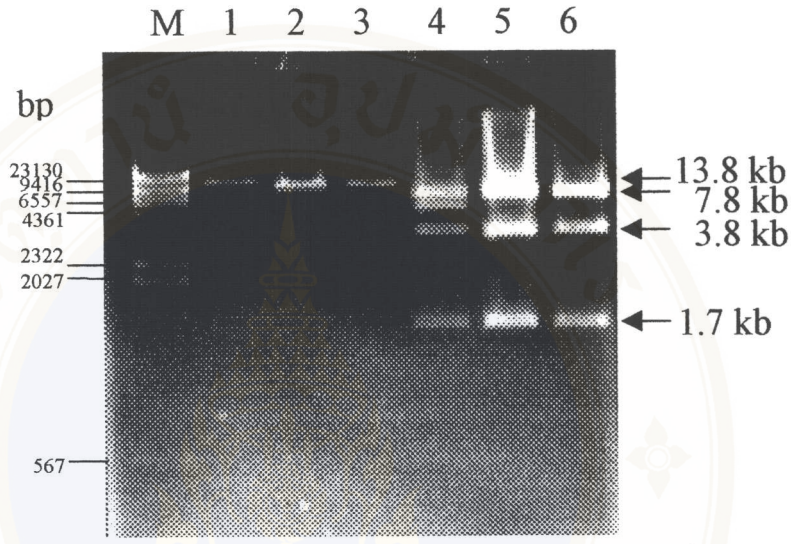


Figure 37. Restriction analysis of pSA1100 plasmid (full-length PRSV-P under T7 promoter)

The 3 recombinant plasmids digested with *Xba*I and *Sac*I, respectively each recombinant plasmid were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers
Lane 1-3: clone no. 14, 17 and 19 digested with *Xba*I, respectively
Lane 4-6: clone no. 14, 17 and 19 digested with *Sac*I, respectively

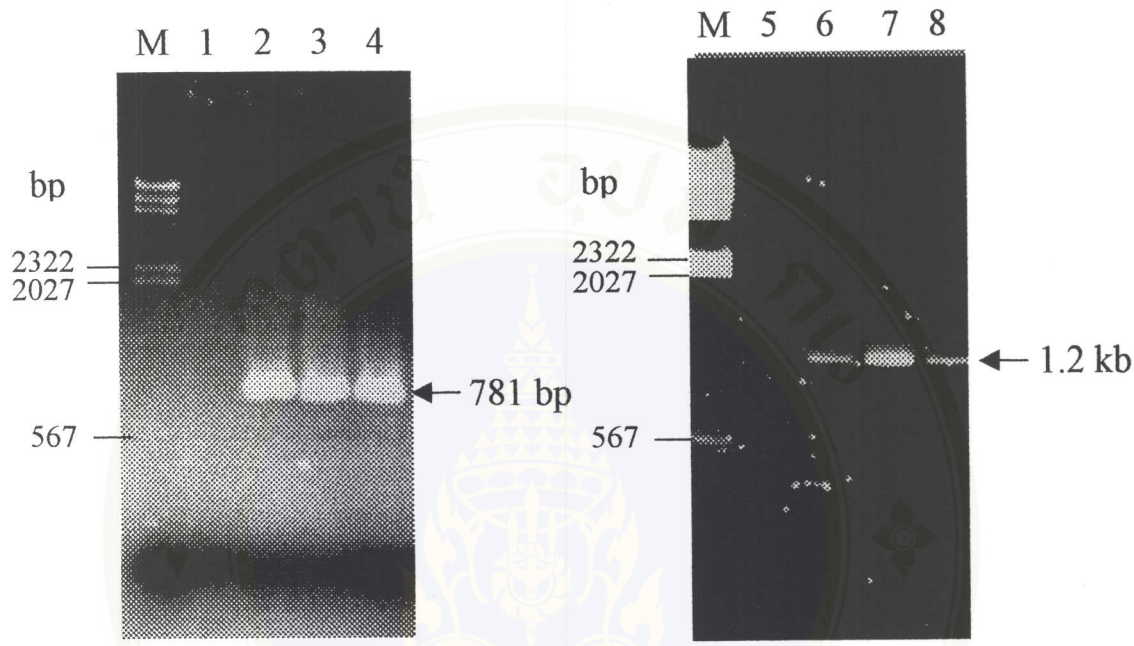


Figure 38. PCR amplification of pSA1100 plasmid (full-length PRSV-P under T7 promoter)

The 10 µl from 50 µl of PCR products of 3 recombinant plasmids were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers

Lane 1, 5: Negative control; amplification without template

Lane 2-4: 5′part PCR products of clone no. 14, 17 and 19, respectively amplified by using T7GF2 and 1L32 primers

Lane 6-8: 3′part PCR products of clone no. 14, 17 and 19, respectively amplified by using 5′CP and GEN-R primers

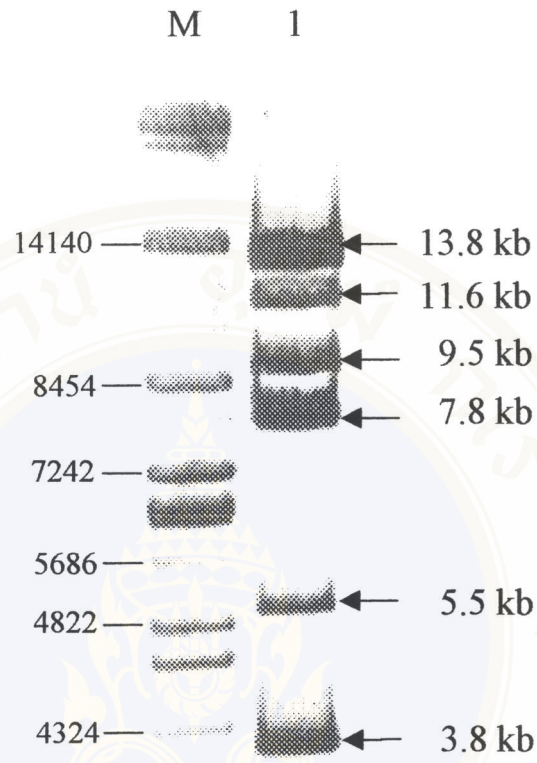


Figure 39. Partially digestion of plasmid pSA1100

Lane M: Lambda DNA double digested with *Bst*EII and *Hin*dIII markers (400 ng)

Lane 1: Plasmid pSA1100 (1µg) partially digested with 0.5 U. *Sac*I at 37°C for 1 hr.

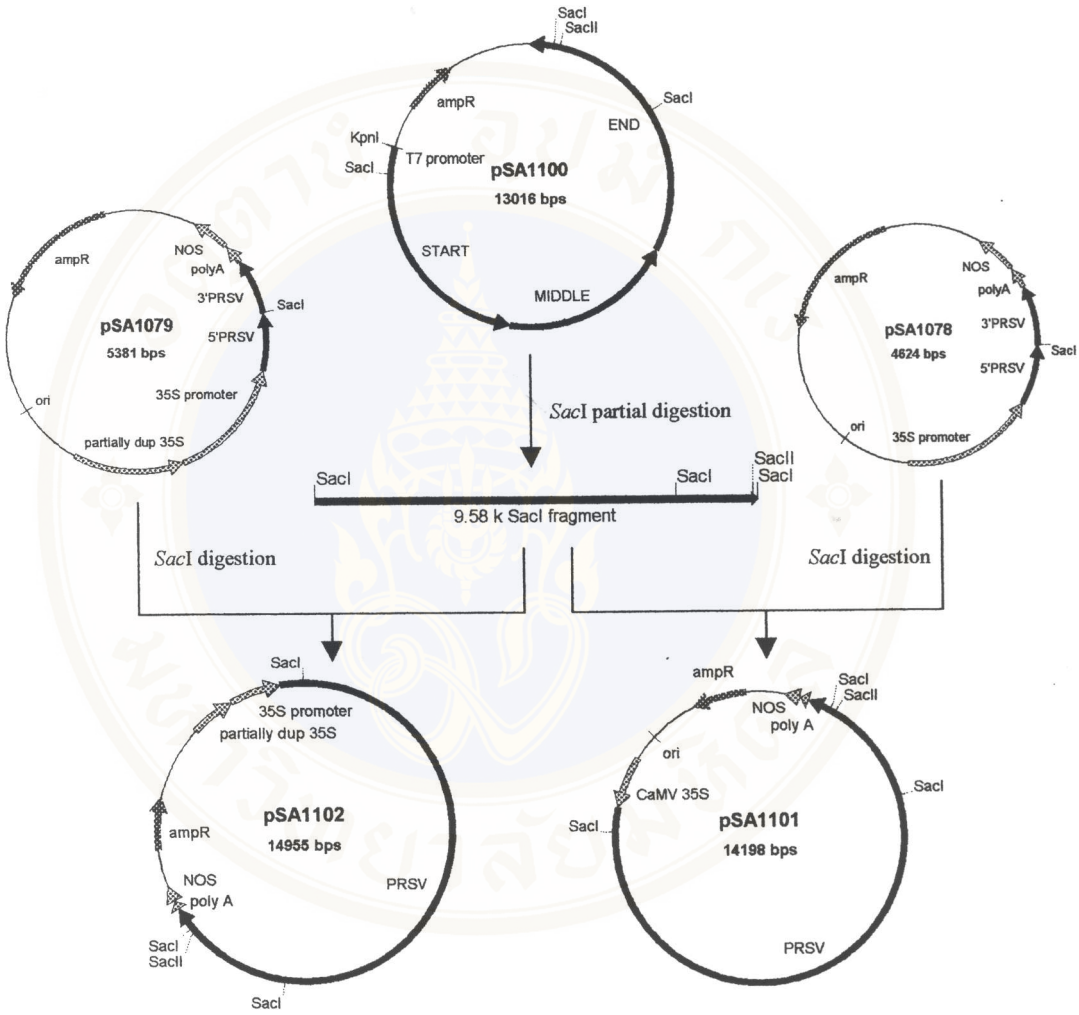


Figure 40. Construction of plasmids pSA1101 and pSA1102

Both ligation products were transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked to do the master plate for rapid size screening. The colonies that gave the corrected pattern were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. Then, the recombinant plasmids were digested with *Sac*I yielding three bands for the correct orientation of approximately 9.5 kb, 4.3 kb and 1.7 kb for single 35S promoter clones, three bands approximately at 9.5 kb, 5.1 kb and 1.7 kb for partially duplicated 35S promoter clones and there were some of incomplete cut bands (Figure 41).

Further confirmation was done by PCR amplification. Both single 35S promoter clone (no. 215) and partially duplicated 35S promoter clone (no. 81) were confirmed by amplification of the promoter part, 5′part (checking the orientation) and 3′part (also checking the orientation) using 35Ss, 35Sa, GEN-F, 1L32, 5′CP, 3′CP primers and BIOTOOL DNA polymerase. The PCR products had the expected sizes of 842 bp (single promoter) (Figure 42) or 0.8 and 1.6 kb (partially duplicated promoter) (Figure 42), 761 bp (5′PRSV) (Figure 43) and 1.0 kb (coat protein gene) (Figure 43). The sequences of recombinant plasmids clone no. 81 (pSA1102) containing full-length of PRSV sequence under the control of partially duplicated 35S promoter and clone no. 215 (pSA1101) containing the full-length PRSV sequence under the control of single 35S promoter were confirmed using universal reverse sequencing primer, 35S-seq primer, GSP3 primer and Seq-IF primer. The resulting sequences are shown in section 4.1, 4.2, 4.3, 4.7 and 4.10 (Chapter IV).

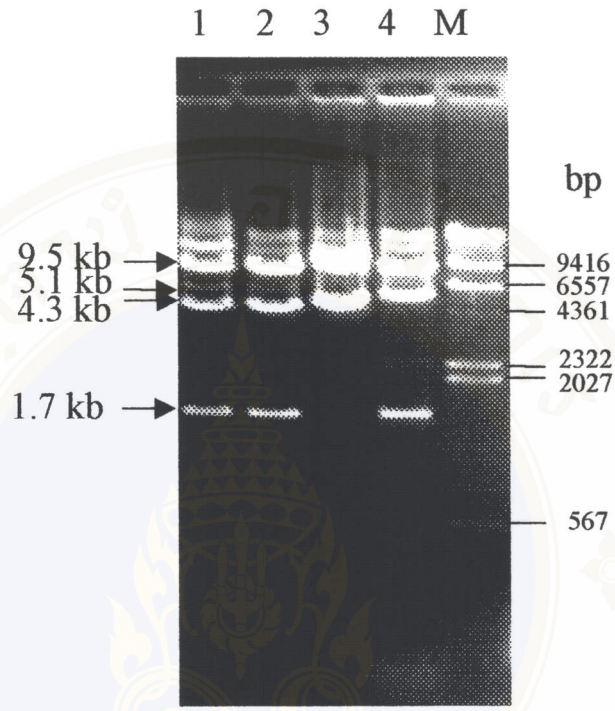


Figure 41. Restriction analysis of pSA1101 and pSA1102 plasmids (full-length PRSV-P under single and partially duplicated CaMV 35S promoter, respectively)

The 4 recombinant plasmids digested with *Sac*I, respectively each recombinant plasmid were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers

Lane 1-3: clone no. 194, 215 and 216 (single 35S promoter) digested with *Sac*I, respectively

Lane 4: clone no. 81 (partially duplicated 35S promoter) digested with *Sac*I

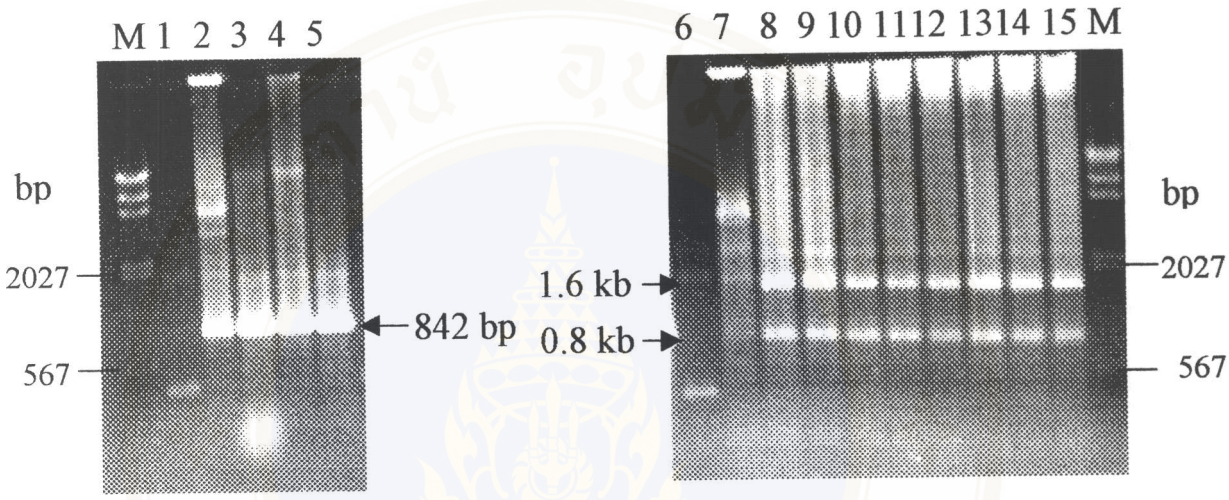


Figure 42. PCR amplification of CaMV 35S promoter of pSA1101 and 1102 plasmids (full-length PRSV-P under single and partially duplicated CaMV 35S promoter, respectively)

The 10 µl from 50 µl of PCR products of 11 recombinant plasmids were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers

Lane 1, 6: Negative control; amplification without template

Lane 2, 7: PCR products of pSA1064 and pSA1066 plasmids (positive control) amplified by using 35Ss and 35Sa primers, respectively

Lane 3-5: PCR products (35S promoter) of clone no. 4, 194 and 215 (single 35S promoter) amplified by using 35Ss and 35Sa primers, respectively

Lane 8-15 PCR products (35S promoter) of clone no. 81, 100, 145, 149, 151, 161, 162 and 184 (partially duplicated 35S promoter) amplified by using 35Ss and 35Sa primers, respectively

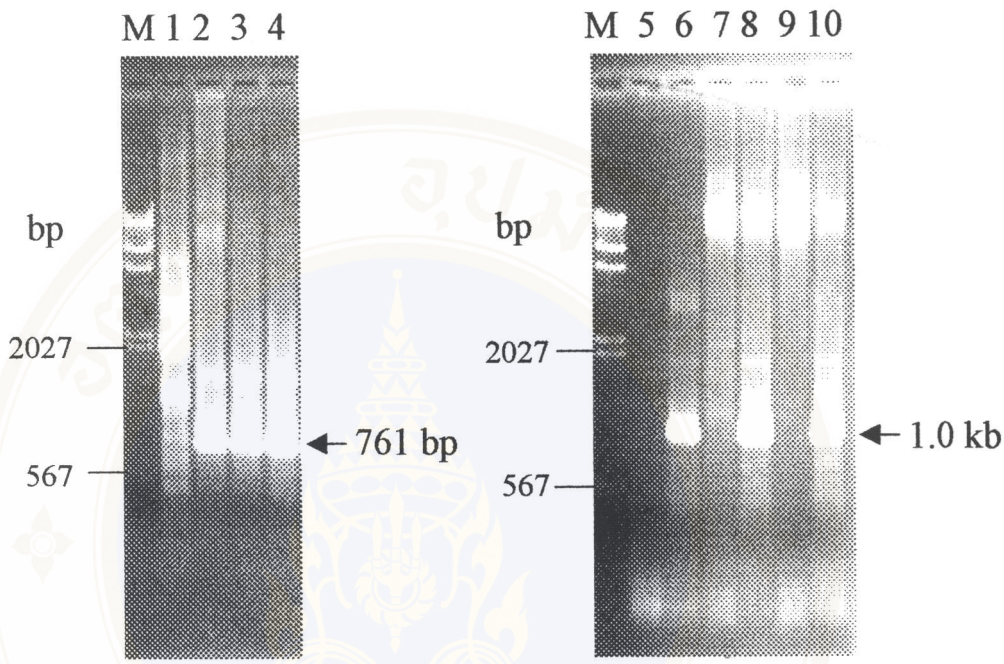


Figure 43. PCR amplification of 5′ and 3′part of PRSV of pSA1101 and 1102 plasmids (full-length PRSV-P under single and partially duplicated CaMV 35S promoter, respectively)

The 10 µl from 50 µl of PCR products of 4 recombinant plasmids were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M: Lambda DNA / *Hin*dIII digested markers

Lane 1, 5: Negative control; amplification without template

Lane 2, 6: 5′part and 3′part PCR products of pSA1100 and pSA1077 plasmids (positive control) amplified by using GF-2, 1L32 primers and 5′CP and 3′CP primers, respectively

Lane 3-4: 5′part PCR products of clone no. 215 (single 35S promoter) and 81 (partially duplicated 35S promoter) amplified by using GF-2 and 1L32 primers, respectively

Lane7-10 3′part PCR products of clone no. 194, 215, 216 (single 35S promoter) and 81 (partially duplicated 35S promoter) amplified by using 5′CP and 3′CP primers, respectively

2.11 Construction of plasmid pSA1110; generation of full-length of PRSV under the control of T7 promoter that contained poly A and NOS terminator

The pSA1077 plasmid containing the coat protein gene, 3′UTR, poly A tail and NOS terminator was digested with *Sph*I, blunted with T4 DNA polymerase and digested with *Sac*II, which generated the 3.4 kb fragment. After purification, this 3.4 kb fragment was ligated with 9.8 kb fragment of pSA1100 plasmid that was digested with *Kpn*I, blunted with T4 DNA polymerase, digested again with *Sac*II and purified (Figure 44).

The ligation product was transformed into the *E.coli* host DH5α and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked and cultured in a selective LB medium. Plasmid from each clone was extracted. The recombinant plasmids were digested with *Xba*I. The recombinant plasmid with the correct orientation of the insert cannot be cut by this enzyme. Then, the sequences of recombinant plasmids clone no. 16 and 25 were confirmed using universal forward, reverse sequencing primers and Seq-IF primer. The resulting sequence is shown in section 4.1, 4.2, 4.7 and 4.10 (Chapter IV). The recombinant plasmid clone no. 16 was named pSA1110.

3. Plasmid preparation and estimation

All of the three full-length of PRSV plasmids, pSA1101 (single 35S promoter), pSA1102 (partially duplicated 35S promoter) and pSA1110 (T7 promoter), were transformed into the *E.coli* host DH5α, JM109 and the culture was spreaded on a selective LB agar plate. After incubation at 37°C overnight, the colonies were picked

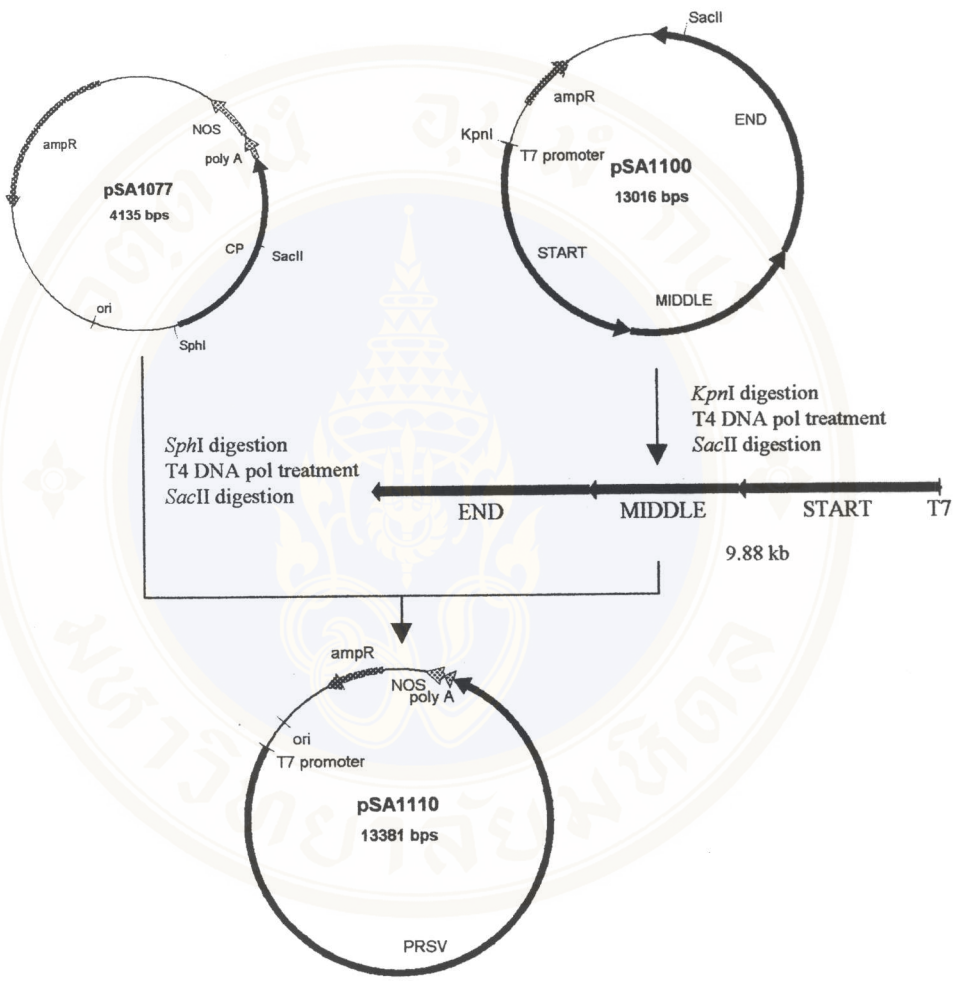


Figure 44. Construction of plasmid pSA1110

and cultured at 30°C and 37°C in both selective LB and TB medium. Plasmid from each clone was extracted following the method described in section 2.13.1 (Chapter III). The comparison of the yield of plasmid DNA among the different hosts, different temperature and different culture medium was determined. The yield of plasmid DNA was shown in the table 7. Once the best condition (JM109 host, 30°C or 37°C, a selective TB medium) was obtained, the plasmids DNA were estimated the concentration by comparison with 100 and 200 ng of standard DNA marker (Lambda DNA digested with *Hin*dIII) as shown in figure 45.

Table 7.: Comparison of the yield of plasmid DNA in different condition.

Plasmid DNA	*E.coli* host*		LB-Amp medium+		TB-Amp medium+	
	DH5α	JM109	30°C	37°C	30°C	37°C
pSA1101	Small	Large	3	0.5-1.0	3	3
pSA1102	Small	Large	3	0.2-0.5	3	3
pSA1110	Small	Large	3	0.2-0.5	3	3

NOTE: * represents the size of colony obtained from *E.coli* host

+ represents the yield of plasmid DNA (μg / ml culture)

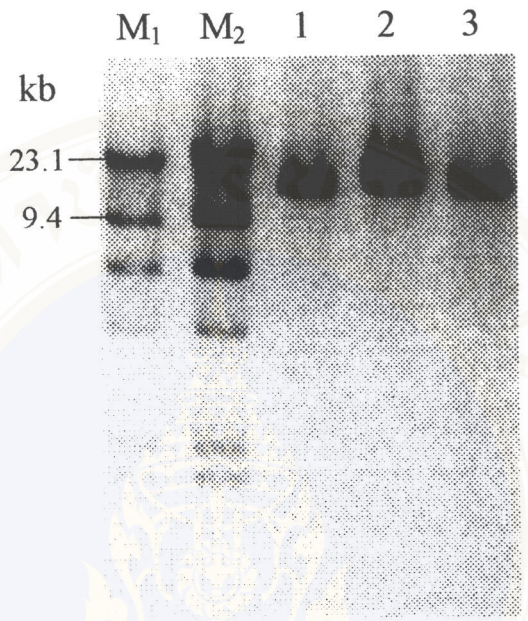


Figure 45. Estimation the concentration of pSA1101, pSA1102 and pSA1110 plasmids (full-length PRSV-P under single 35S promoter, partially duplicated 35S promoter and T7 promoter, respectively)

Lane M_1: Lambda DNA / *Hin*dIII digested markers (100 ng)
Lane M_2: Lambda DNA / *Hin*dIII digested markers (200 ng)
Lane 1: pSA1110 (full-length under T7) digested with *Sac*II (diluted 1:5)
Lane 2: pSA1101 (full-length under 35S) digested with *Sac*II (diluted 1:5)
Lane 3: pSA1102 (full-length under partially duplicated 35S) digested with *Sac*II (diluted 1:5)

The concentration of pSA1101 plasmid (full-length under 35S) was 2μg/μl. The concentration of pSA1102 (full-length under partially duplicated 35S) and pSA1110 (full-length under T7) plasmids were 1μg/μl.

4. Sequence analysis

4.1 Nucleotide sequence of CaMV 35S promoter of plasmid pSA1064

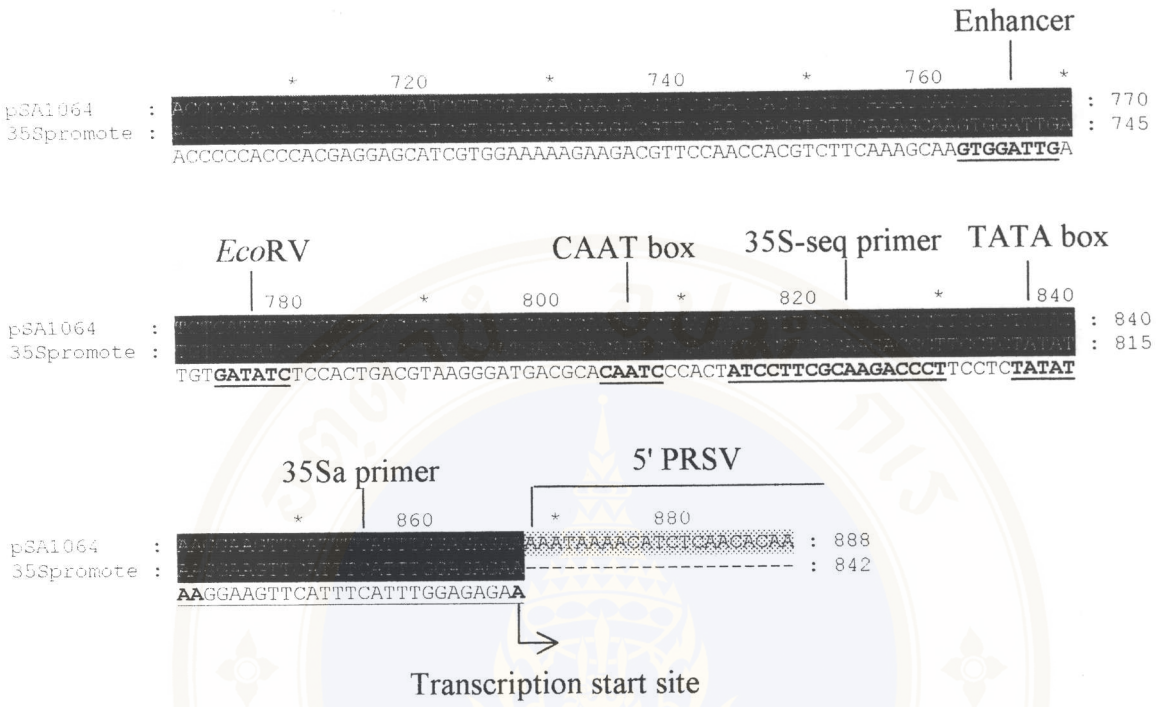


Figure 46. Nucleotide sequence comparison between CaMV 35S promoter of plasmid pSA1064 (intact 35S promoter) and pBI221 vector (Clonetech)

There was no mutation found in 35S promoter of plasmid pSA1064 when compared with its original from pBI221 vector (Colnetech). The underlined sequences represent the locations of 35Ss, 35Sa, 35S-Seq primers and the regions of enhancer, *Eco*RV site, CAAT box, TATA box, and transcription start site.

The partially duplicated 35S promoter of plasmid pSA1066 (approximately 1.6 kb) started at the first base of 35Ss primer until *Eco*RV site (751 bp) and following with intact 35S promoter (started at the first base of 35Ss primer until transcription start site, 842 bp) (Figure 46). The partially duplicated 35S promoter of pSA1066 plasmid was constructed, by subcloning of intact 35S promoter+5′ PRSV of pSA1064 into *Eco*RV site of pSA1058 (pBI221 that deleted of *Xba*I - *Eco*RI fragment, Figure 18), and confirmed by restriction analysis and also PCR amplification (Figure 20). No sequencing result of 35S promoter of plasmid pSA1066 was available.

4.2 Nucleotide sequences between the junction of 35S promoter + 5′PRSV and T7 promoter + 5′PRSV

```
                *         20         *         40         *         60         *
35S+5' : TCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAG :  70

              80         *        100         *        120         *        140
35S+5' : GCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGG : 140

                *        160         *        180         *        200         *
35S+5' : AAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGA : 210

             220         *        240         *        260         *        280
35S+5' : TGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGA : 280

                Transcription start site
                *        300         *        320         *        340         *
35S+5' : AAAATAAAACATCTCAACACAACACAATTCAAAGCATTCAAACATACTCAAGCAAACTTTATCTTCATAA : 350

             360         *        380         *        400         *        420
35S+5' : TTCACAATTCGCAATCATGTCTTCATTGTACCAACTGCAACCGATAGCACTGAAGGACCGTCTCTTGGCT : 420

                *        440         *
35S+5' : CACGAGAGAGGGAAAGGCTGGATTGAGCACAAGCTTG : 457
```

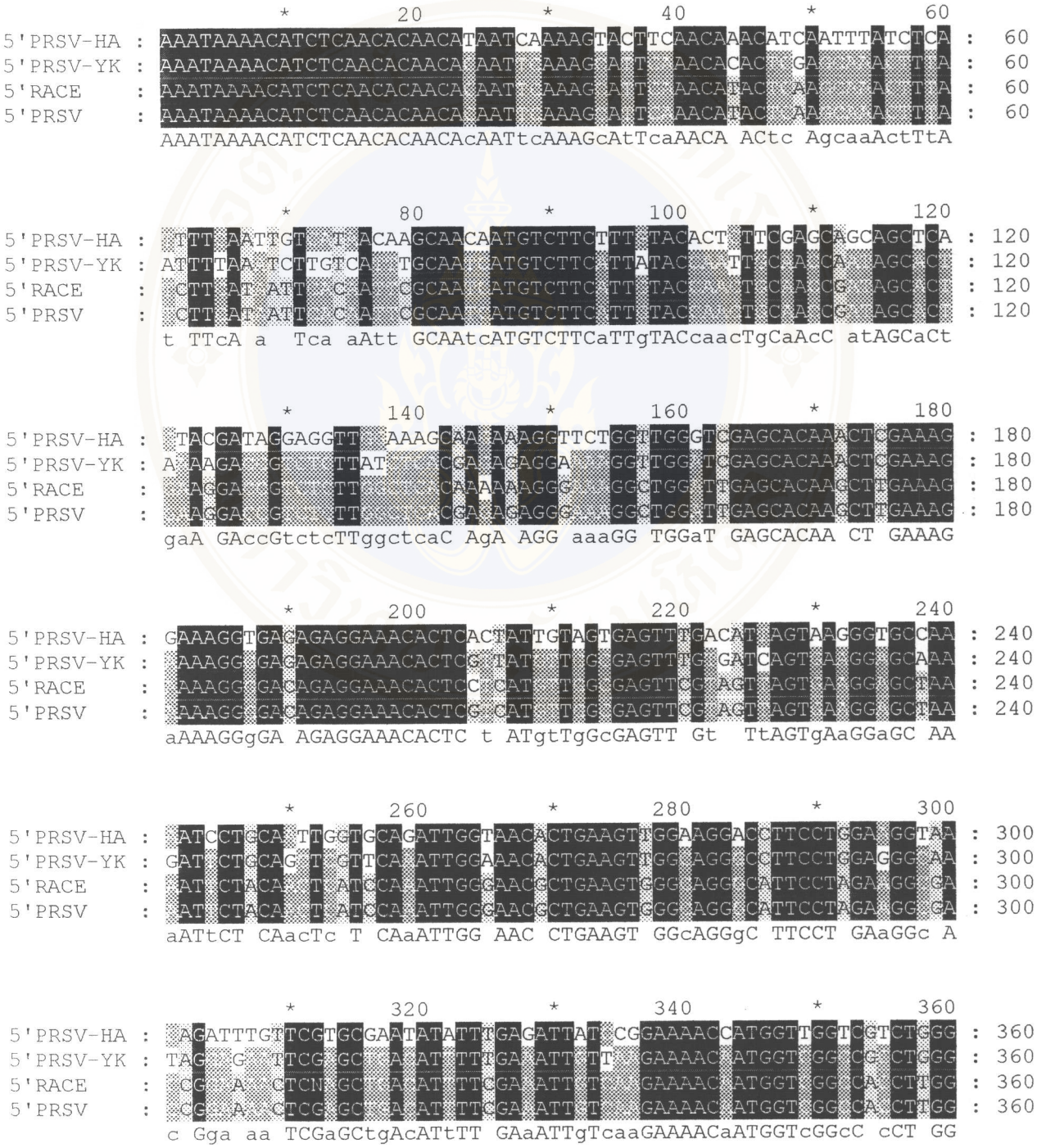
Figure 47. Nucleotide sequence of the junction between CaMV 35S promoter and 5′PRSV of Thai isolate of plasmid 64AH

This figure showed the nucleotide sequence of the junction between CaMV 35S promoter and 5′ PRSV Thai isolate of plasmid 64AH (subcloning of *Acc*I – *Hin*dIII fragment of plasmid pSA1064 for sequence the junction). The underlined sequence represented a 5′ PRSV of Thai isolate and the transcription start site was indicated. A mistake in designing of 35Sa primer caused a non-viral nucleotide (A) on the 5′ end of PRSV when plasmid was transcribed.

Figure 48. Nucleotide sequence of the junction between T7 promoter and 5′PRSV of Thai isolate of plasmid pSA1100

This figure showed the nucleotide sequence of the junction between T7 promoter and 5′ PRSV of Thai isolate of plasmid pSA1100 (full-length cDNA of Thai isolate PRSV under T7 promoter without poly(A) tail and NOS terminator). The italic sequence represented a T7 promoter and the underlined sequence represented a 5′ PRSV of Thai isolate. The transcription start site was also indicated. There was a non-viral nucleotide (G) on the 5′ end of PRSV when plasmid was transcribed.

4.3 Nucleotide sequence comparison between 5′PRSV of Hawaiian isolate (HA), Taiwanese isolate (YK), Thai isolate 5′RACE clone and Thai isolate pSA1100 plasmid



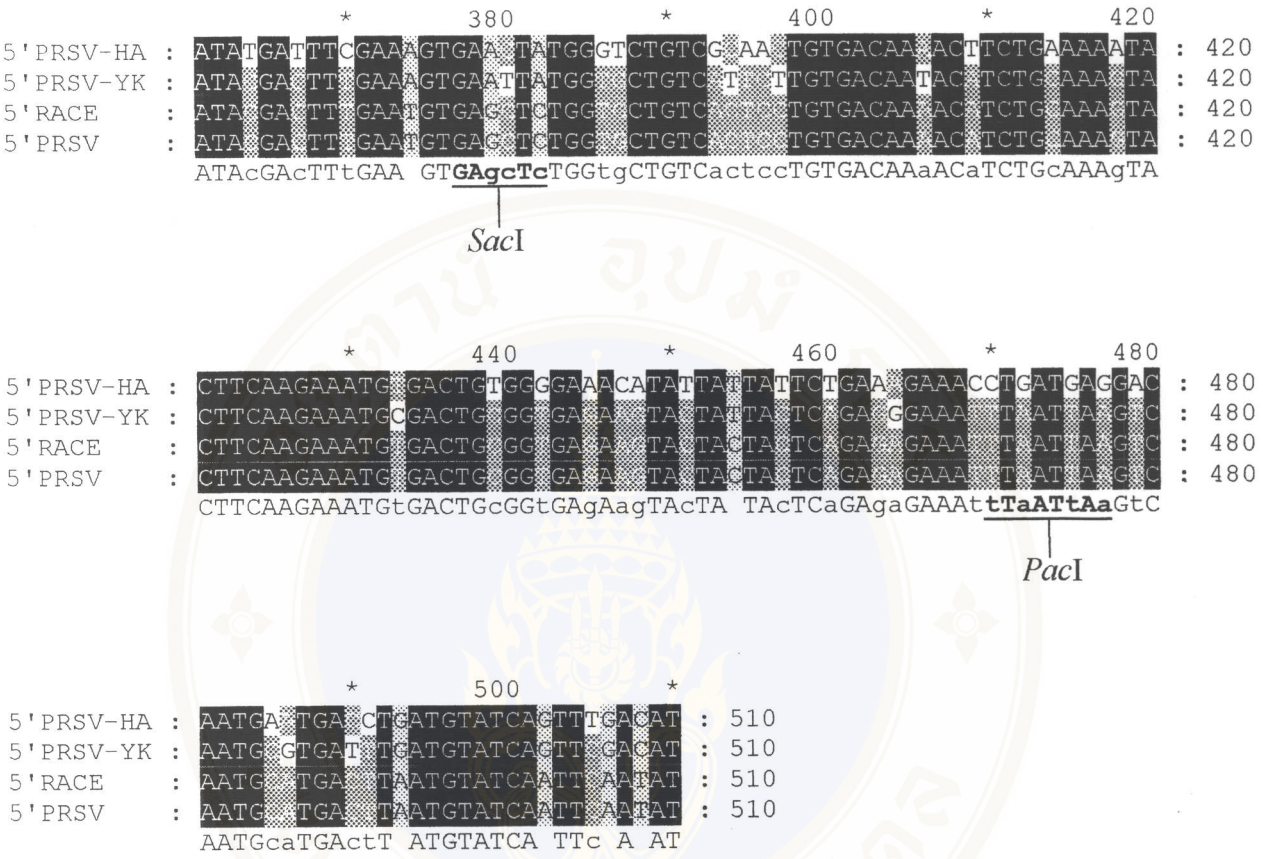


Figure 49. Nucleotide sequence comparison of 5′PRSV

The nucleotide sequence comparison of 5′PRSV (510 nucleotides) between Thai isolate of pSA1100 plasmid (T7 full-length without poly(A) and NOS), Thai isolate 5′RACE clone obtained previously, Taiwanese isolate, and Hawaiian isolate showed the sequence homology of 99%, 86% and 69%, respectively. In the same strain of Thai isolate 5′RACE clone and the clone in the plasmid pSA1100, there were 4 nucleotides mutation at the position 143, 145, 147 from A to G and at the position 204 from C to G. The restriction enzymes that were used to subclone the 5′PRSV part were *Sac*I (GAGCTC) and *Pac*I (TTAATTAA), which no mutation found in these junction. Both two restriction enzymes *Sac*I and *Pac*I were located at position 377 and 470 as indicated in the figure.

4.4 Amino acid sequence comparison between 5′PRSV encoding P1 gene of Hawaiian isolate (HA), Taiwanese isolate (YK), Thai isolate 5′RACE clone and Thai isolate pSA1100 plasmid

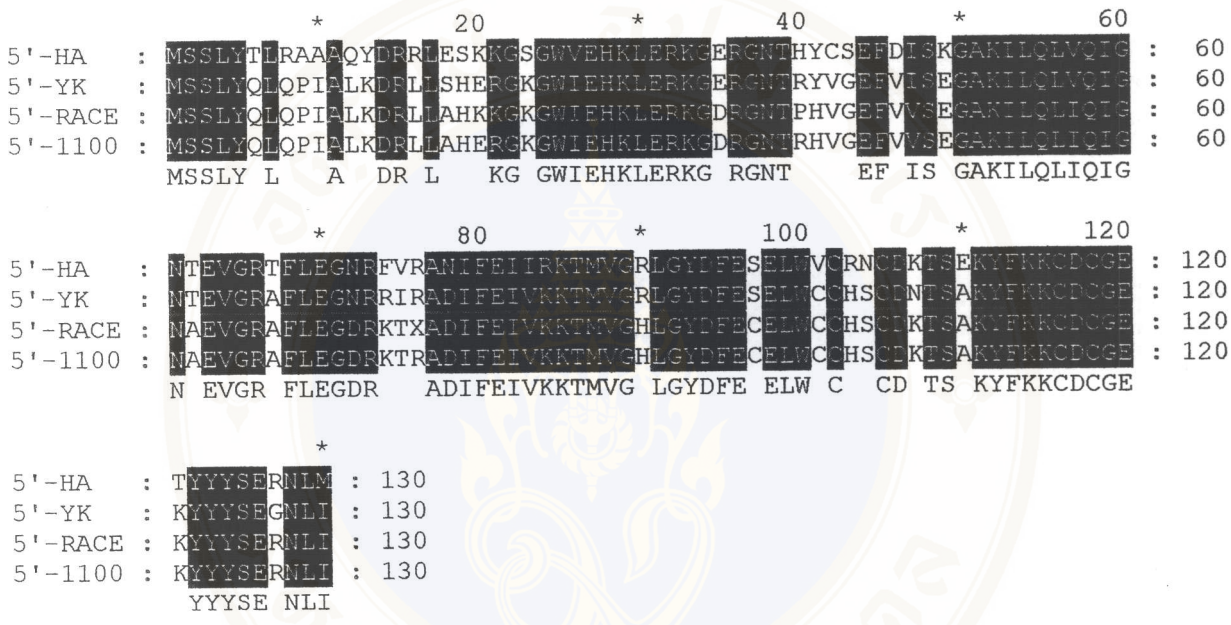
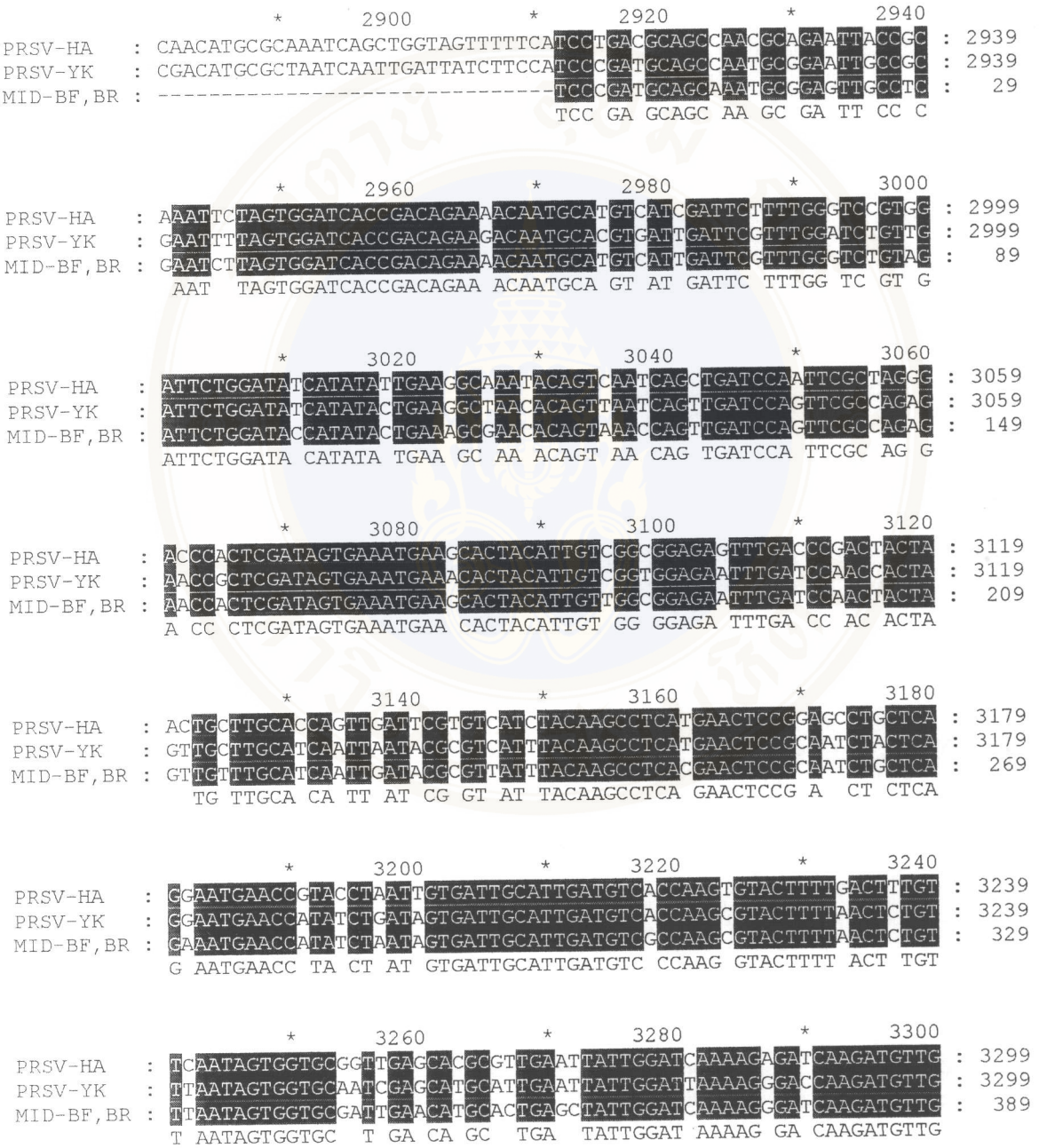
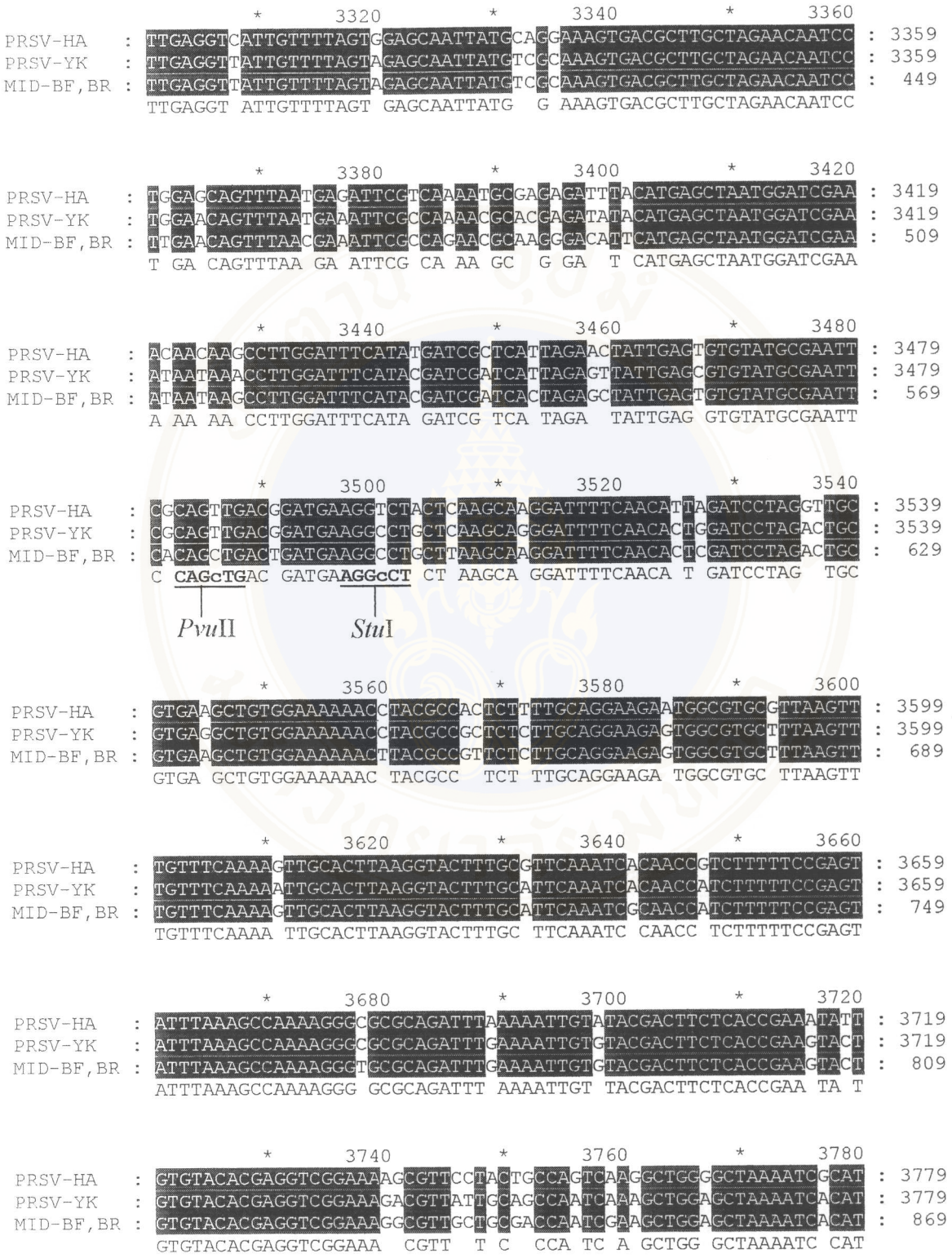


Figure 50. Amino acid sequence comparison of 5′PRSV encoding P1 gene

The amino acid sequence comparison of 5′PRSV encoding P1 gene (130 amino acids) between Thai isolate of pSA1100 plasmid, Thai isolate 5′RACE clone, Taiwanese isolate, and Hawaiian isolate showed the amino acid homology of about 96%, 90% and 70%, respectively. In the same strain of Thai isolate 5′RACE clone and the clone in the plasmid pSA1100, there were 3 amino acids change at the position 20 from lysine (K) to glutamate (E), at the position 21 from lysine (K) to arginine (R) and at the position 40 from proline (P) to arginine (R).

4.5 Nucleotide sequence comparison between middle part of PRSV from Hawaiian isolate (HA), Taiwanese isolate (YK) and Thai isolate





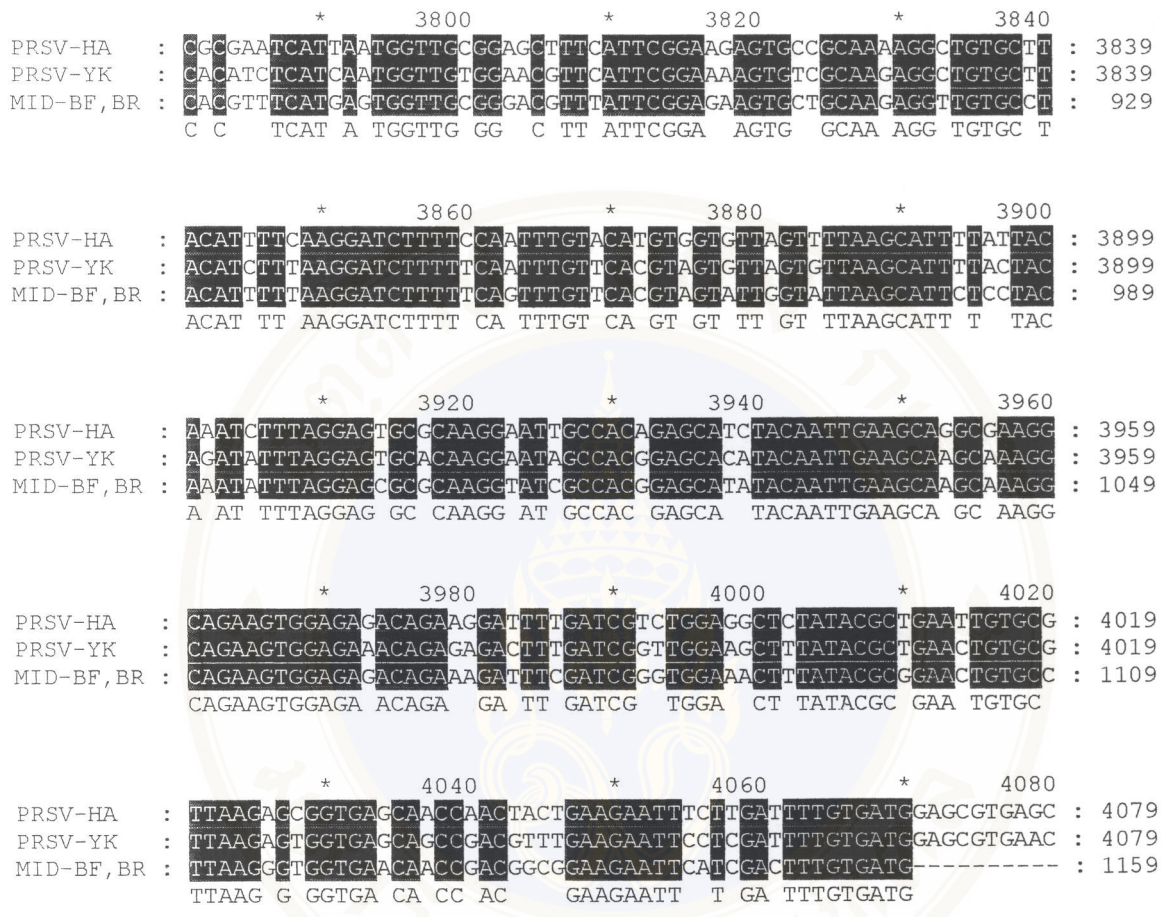


Figure 51. Nucleotide sequence comparison of middle part of PRSV

The nucleotide sequence comparison of middle part of PRSV position 2912-4071 (1,159 nucleotides) between Hawaiian (PRSV-HA), Taiwanese (PRSV-YK) isolates and Thai isolate (MID-BF, BR came from the sequences of plasmid pSA1091 that contained middle part and pSA1100 that contained T7 full-length of PRSV Thai isolate without poly(A) tail and NOS) showed the sequence homology of about 81% and 89%, respectively. The restriction enzymes that were used to subclone the middle part of PRSV were *Pvu*II (CAGCTG) and *Stu*I (AGGCCT) that located on the position 3482 and 3496 as indicated in the figure. No mutation were found in the junction that was used to assemble the full-length of PRSV.

4.6 Nucleotide sequence comparison between end part of PRSV from Hawaiian isolate (HA), Taiwanese isolate (YK) and Thai isolate

```
                        *       5480            *       5500            *       5520
PRSV-HA     : CGTATGGCCTGCCTGTTATGACCCACAATGTAGGGTTAAGCTTGCTCAAAAACTGCACTG : 5519
PRSV-YK     : CGTATGGCTTGCCTGTGATGACTCATAATGTGGGATTAAGTTTACTCAAAAACTGCACTG : 5519
END-PF,XR   : ----------------------------------------TTGCTCAAAAACTGCACCG :   19
                                                       TT CTCAAAAACTGCAC G

                        *       5540            *       5560            *       5580
PRSV-HA     : TGAGACAAGCACGCACAATGCAACAGTATGAACTAAGCCCGTTCTTTACACAAAATTTAG : 5579
PRSV-YK     : TAAGGCAAGCACGCACAATGCAACAGTACGAATTGAGTCCGTTCTTCACACAAAATTTGG : 5579
END-PF,XR   : TGAGGCAAGCACGCACAATGCACCAGTATGAGCTGAGCCCTTTTTTTACACAAAATTTGG :   79
              T AG CAAGCACGCACAATGCA CAGTA GA  T AG CC TT TT ACACAAAATTT G

                        *       5600            *       5620            *       5640
PRSV-HA     : TGAACTTTGATGGGACAGTGCACCCCAAGATTGATGTCTTGTTACGTCCCTATAAGCTGA : 5639
PRSV-YK     : TCAATTTTGATGGCACAGTACACCCCAAGATCGATGTGCTATTACGTCCTTACAAGTTGA : 5639
END-PF,XR   : TCAACTTTGATGGCACAGTGCACCCGAAGATTGATGTATTACTACGTCCCTACAAGTTGA :  139
              T AA TTTGATGG ACAGT CACCC AAGAT GATGT  T  TACGTCC TA AAG TGA

                        *       5660            *       5680            *       5700
PRSV-HA     : GAGATTGTGAAGTCAGATTAAGTGAAGCAGCAATACCGCATGGGGTACAGTCTATTTGGT : 5699
PRSV-YK     : GGGATTGTGAGATCAGATTAAGTGAAGCAGCGATACCGCATGGAGTGCAATCTATTTGGA : 5699
END-PF,XR   : GGGATTGTGAGATCAGATTGAGCGAGGCAGCAATACCGCACGGAGTGCAATCTATTTGGA :  199
              G GATTGTGA  TCAGATT AG GA GCAGC ATACCGCA GG GT CA TCTATTTGG

                        *       5720            *       5740            *       5760
PRSV-HA     : TGTCTGCTCGGGATTATGAAGCAGTTGGAGGCCGTCTTTGCCTGGAGGGTGATGTCCGAA : 5759
PRSV-YK     : TGTCTGCTAGGGAGTATGAAGCAGTTGGTGGTCGTCTTTGCCTGGAAAGTGATGTTCGAA : 5759
END-PF,XR   : TGTCTGCTAGGGAGTATGAAGCGGTTGGTGGTCGCCTTTGCCTGGAAGGTGATGTTCGAA :  259
              TGTCTGCT GGGA TATGAAGC GTTGG GG CG CTTTGCCTGGA  GTGATGT CGAA

                        *       5780            *       5800            *       5820
PRSV-HA     : TACCATTCCTCATTAAGGATGTTCCTGAGCGATTATATAAGAATTGTGGGACATCGTGC : 5819
PRSV-YK     : TACCGTTCCTCATTAAGGATGTGCCTGAGCGATTATATAGGGAATTGTGGGATATTGTTC : 5819
END-PF,XR   : TACCGTTCCTCATTAAGGATGTGCCTGAGCGGTTGTATAAGGAATTGTGGGACATTGTTC :  319
              TACC TTCCTCATTAAGGATGT CCTGAGCG TT TATA  GAATTGTGGGA AT GT C

                        *       5840            *       5860            *       5880
PRSV-HA     : AGACCTACAAGCGTGACTTTACATTTGGGCGAATTAATTCTGTGTCTGCTGGAAAAATTG : 5879
PRSV-YK     : AAACCTATAAGCGTGATTTCACATTCGGGCGAATTAATTCTGTATCTGCTGGAAAAATTG : 5879
END-PF,XR   : AAACATACAAGCGTGACTACACATTTGGACGAATTAATTCTGTATCTGCTGGGAAAATTG :  379
              A AC TA AAGCGTGA T  ACATT GG CGAATTAATTCTGT TCTGCTGG AAAATTG
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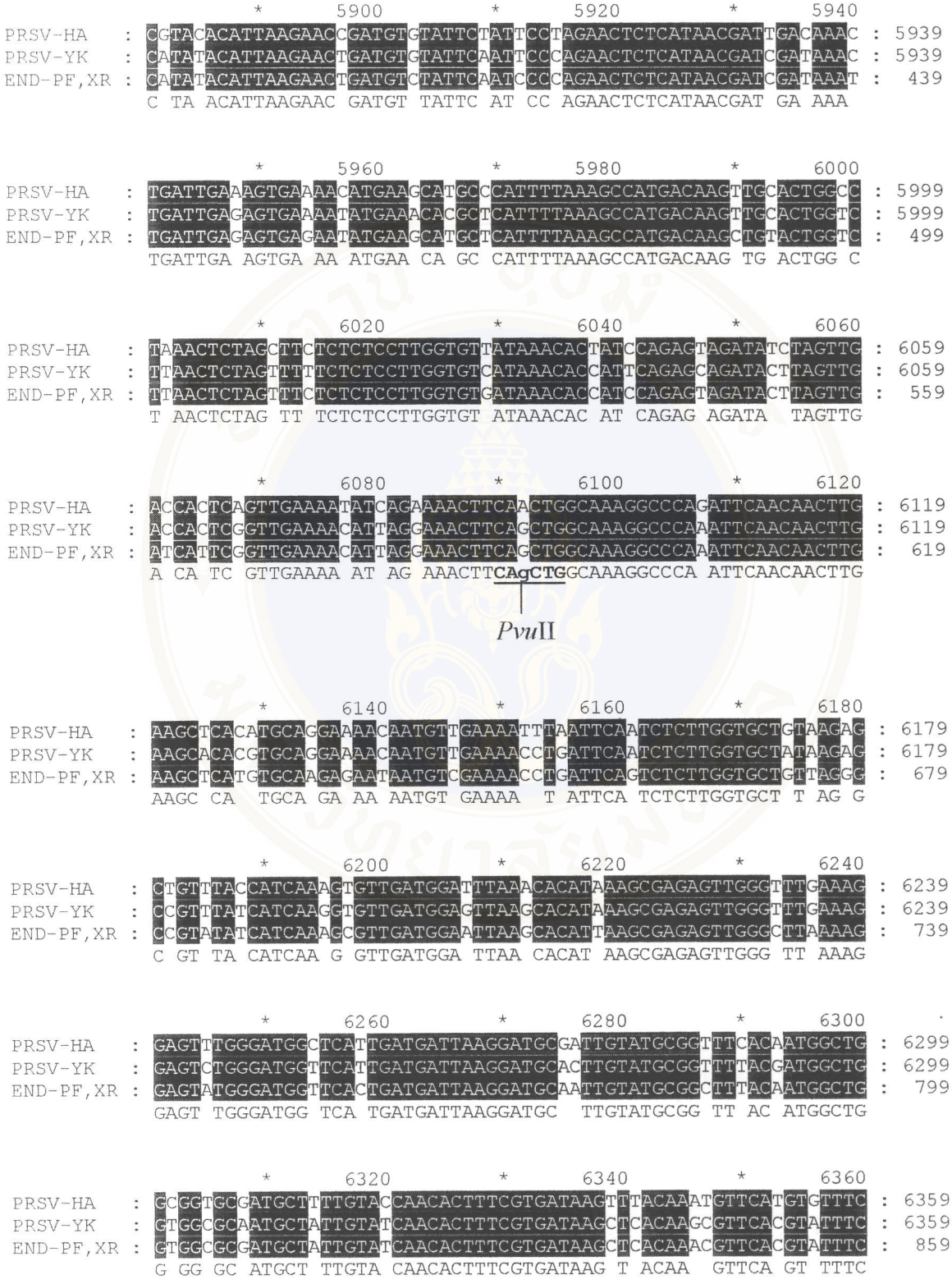


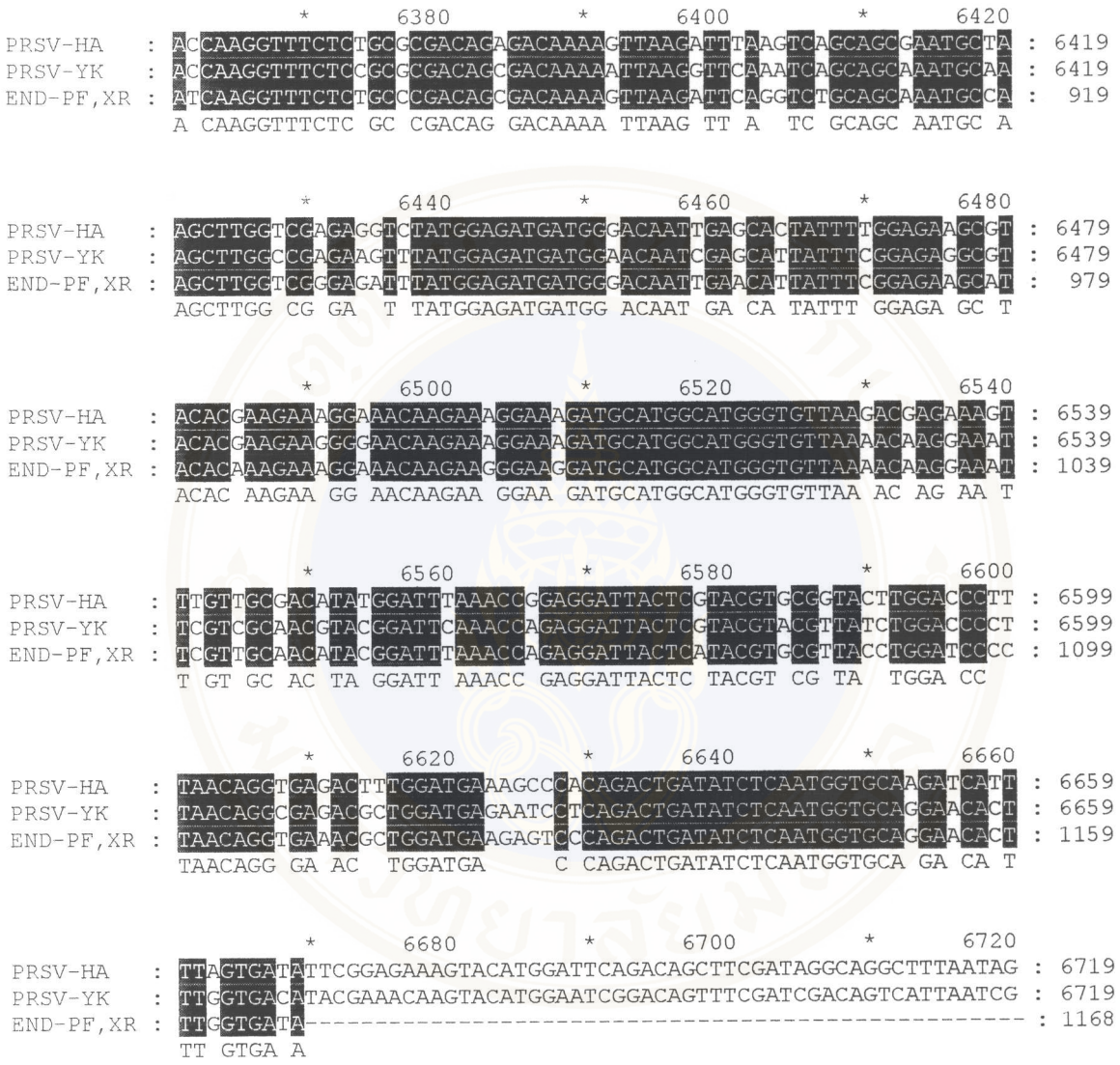
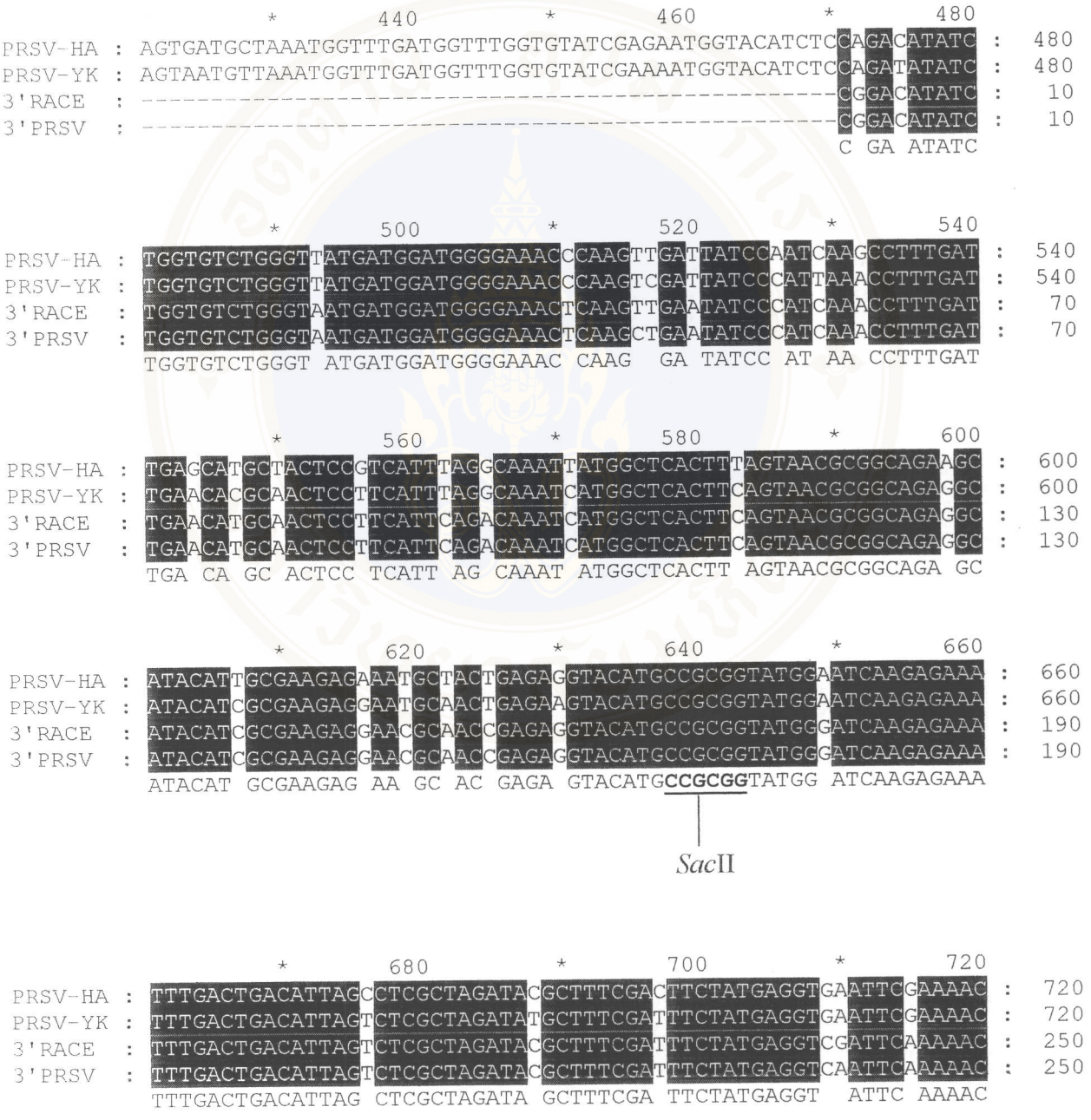
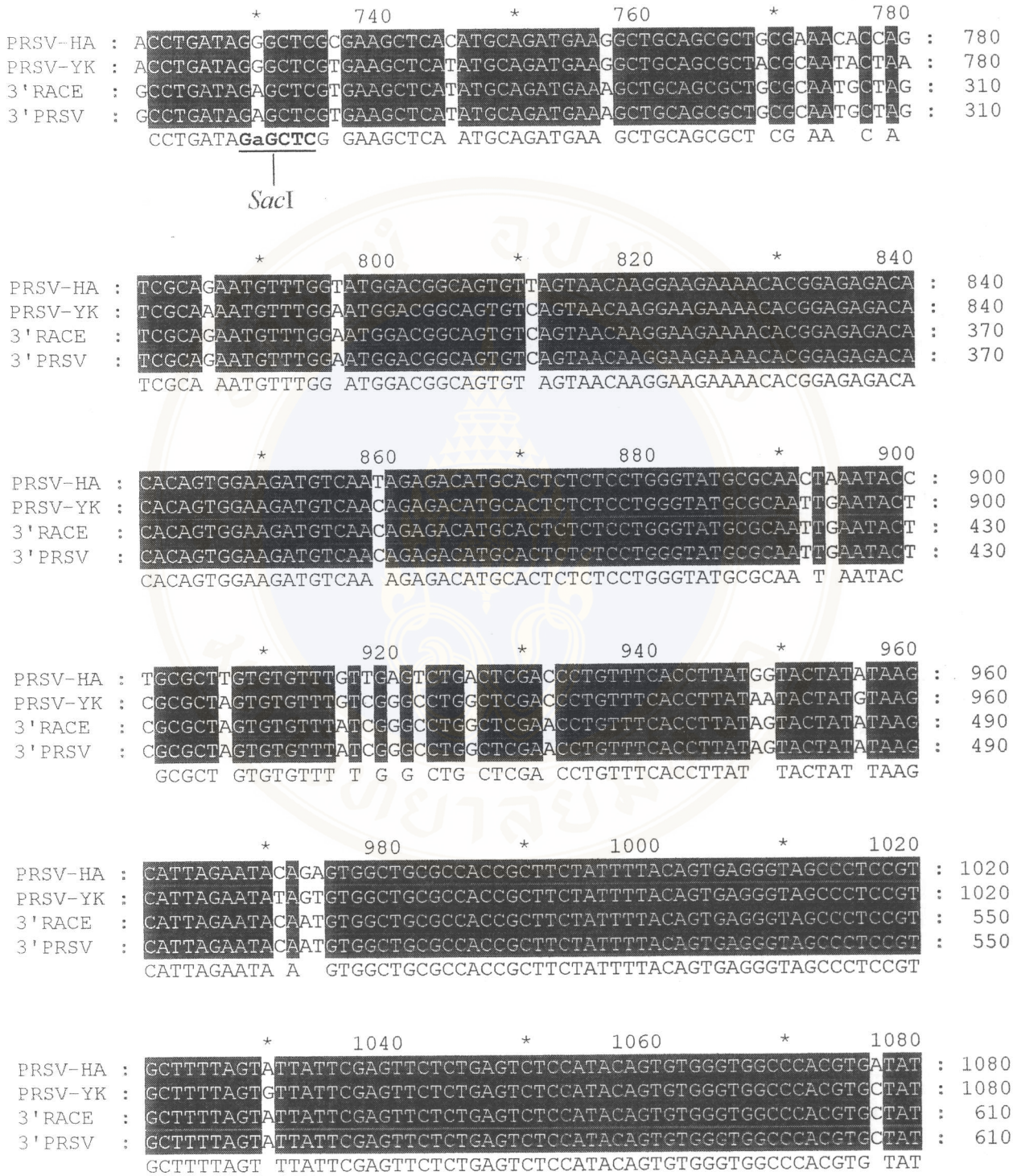


Figure 52. Nucleotide sequence comparison of end part of PRSV

The nucleotide sequence comparison of end part of PRSV position 5501-6668 (1,168 nucleotides) between Hawaiian (PRSV-HA), Taiwanese (PRSV-YK) isolates and Thai isolate (END-PF, XR came from the sequences of plasmid pSA1092 that contained end part and pSA1098 that contained middle-end part of PRSV Thai isolate) showed the sequence homology of 80% and 87%, respectively. The restriction enzyme used to subclone the end part of PRSV was *Pvu*II (CAGCTG) that is located on the position 6089 as indicated in the figure. No mutation were found in the junction.

4.7 Nucleotide sequence comparison between 3′PRSV from Hawaiian isolate (HA), Taiwanese isolate (YK), Thai isolate 3′RACE clone and Thai isolate full-length clone (3′PRSV)





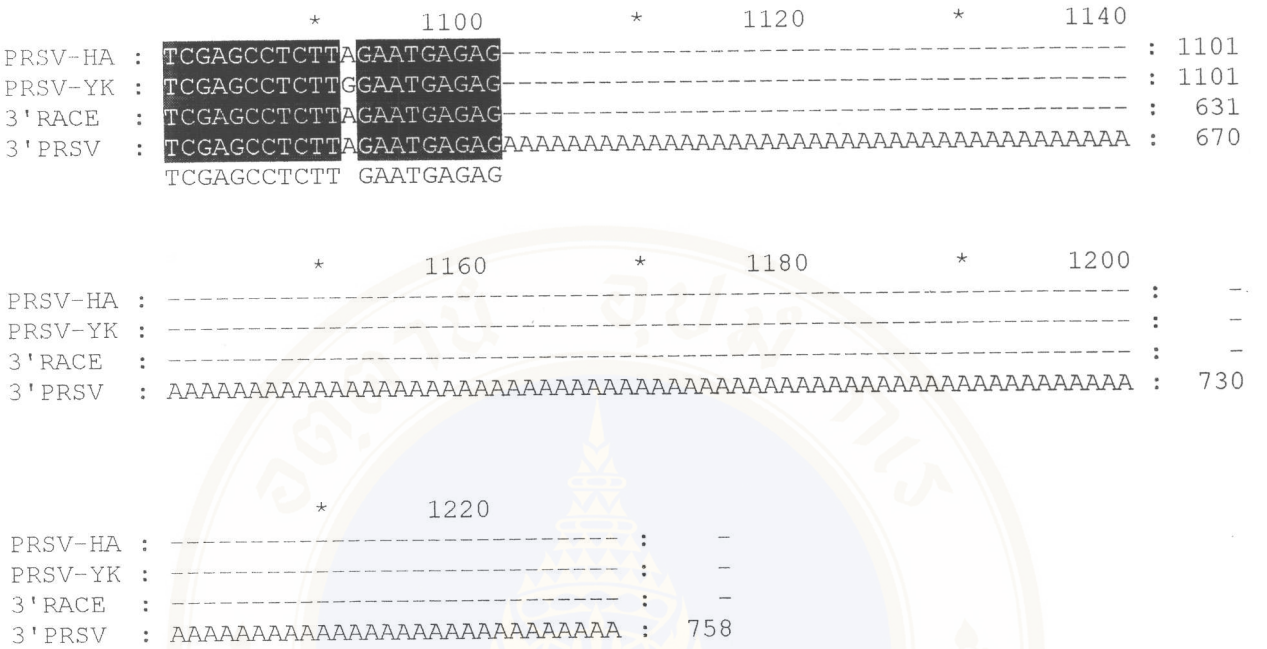


Figure 53. Nucleotide sequence comparison of 3′ PRSV

The nucleotide sequence comparison of 3′PRSV (631 nucleotides) between Thai isolate of full-length clone (the sequence came from plasmid pSA1110, pSA1101 and pSA1102 under T7, single 35S and partially duplicated 35S promoter, respectively), Thai isolate 3′RACE clone, Taiwanese isolate, and Hawaiian isolate showed the sequence homology of about 98%, 92% and 90%, respectively. In the same strain of Thai isolate 3′RACE clone and Thai isolate full-length clone, there were 2 nucleotides mutation in the position 516 from T to C and in the position 710 from G to A. The restriction enzymes, *Sac*I (GAGCTC) and *Sac*II (CCGCGG) were used to subclone 3′PRSV part. Both two restriction enzymes were located at position 729 and 538 as indicated in the figure. No mutation were found in this junction and no non-viral nucleotide was inserted between the junction of 3′PRSV and poly(A) tail.

4.8 Amino acid sequence comparison between 3′PRSV encoding coat protein gene of Hawaiian isolate (HA), Taiwanese isolate (YK), Thai isolate 3′RACE clone and Thai isolate full-length clone (3′PRSV-TH)

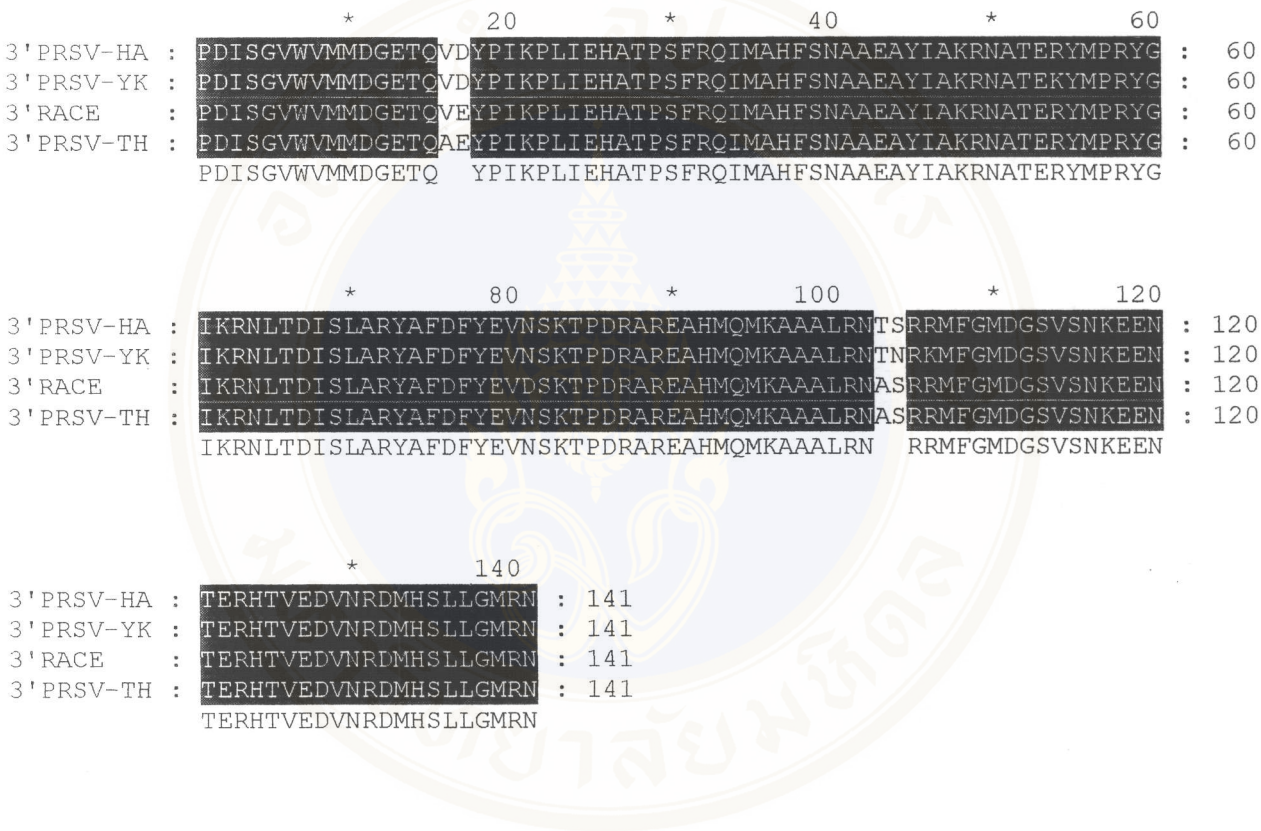


Figure 54. Amino acid sequence comparison of 3′ PRSV encoding coat protein gene

The amino acid sequence comparison of 3′PRSV encoding coat protein gene (141 amino acids) between Thai isolate of full-length clone (the sequence came from plasmid pSA1110, pSA1101 and pSA1102 under T7, single 35S and partially duplicated 35S promoter, respectively), Thai isolate 3′RACE clone, Taiwanese isolate, and Hawaiian isolate showed the sequence homology of about 97%, 97% and 97%, respectively. In the same strain of Thai isolate 3′RACE clone and full-length clone, there was 1 amino acids change in the position 16 from valine (V) to alanine (A).

4.9 Nucleotide sequence between the junction of poly(A) and NOS terminator

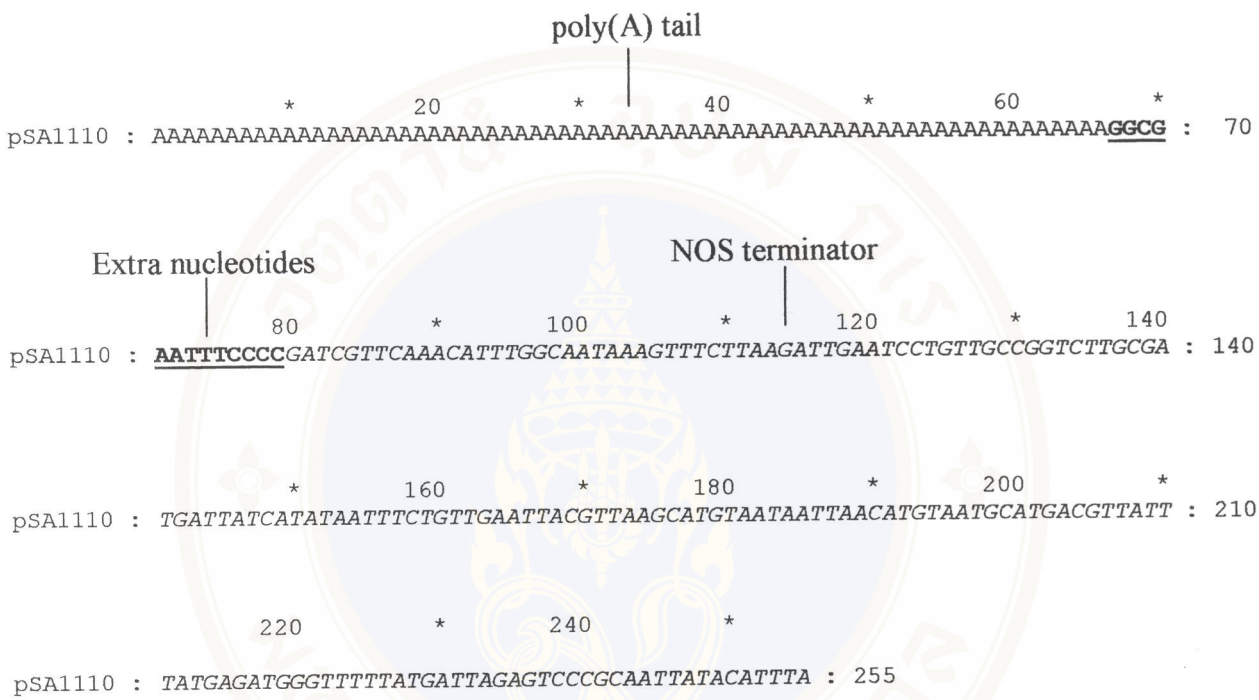


Figure 55. Nucleotide sequence of the junction between poly(A) tail and NOS terminator

This figure showed the nucleotide sequence of the junction between poly(A) tail and NOS terminator of plasmid pSA1110 (T7 full-length cDNA of PRSV Thai isolate). The underlined sequence represented of 13 extra nucleotide between the junction of poly(A) tail and NOS terminator. The italic letters represented a NOS terminator.

4.10 Nucleotide sequence of NOS terminator (253 bp) of plasmid pSA1110

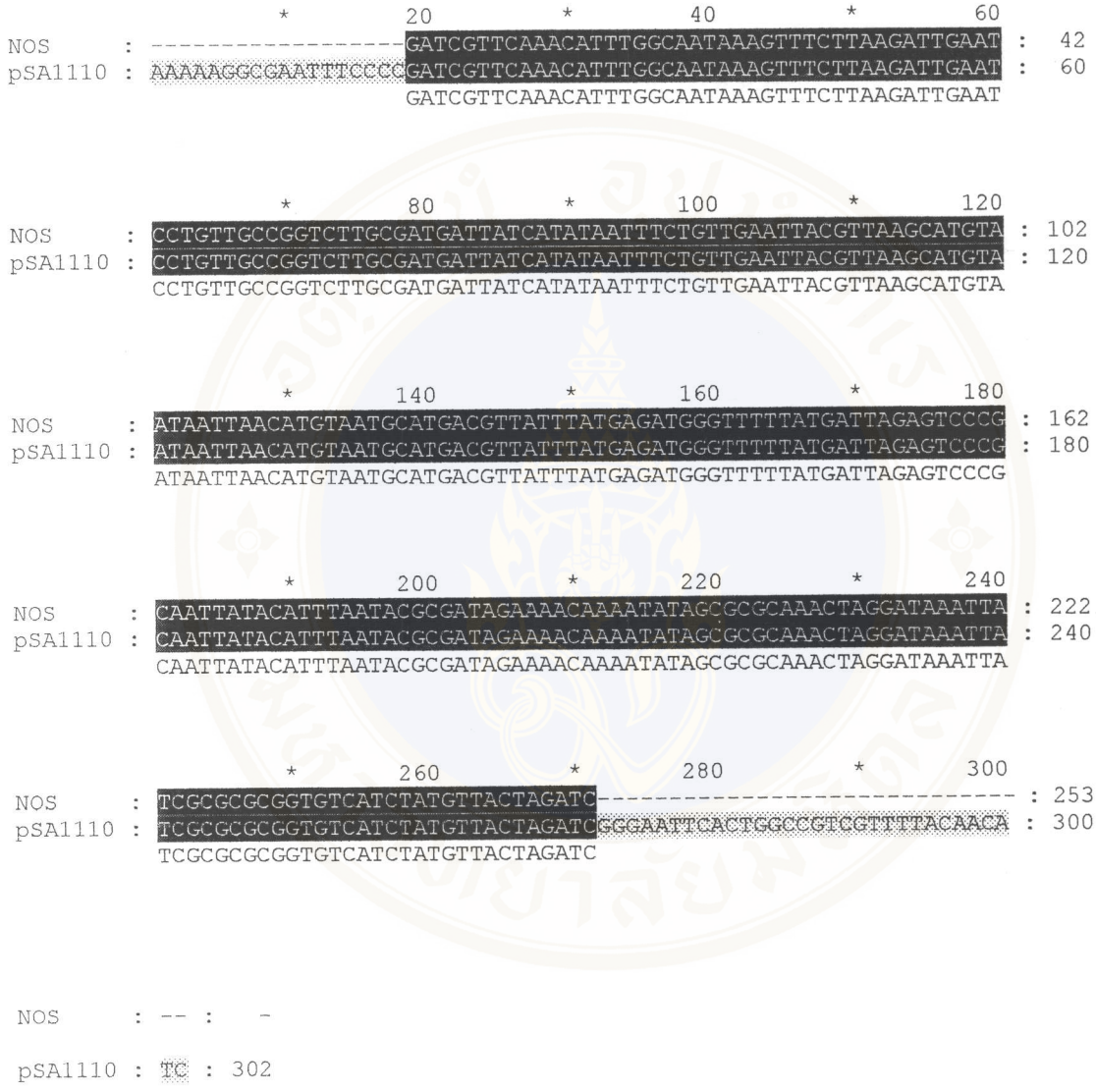


Figure 56. Nucleotide sequence comparison between NOS terminator of plasmid pSA1110 and pBI221 vector (Clonetech)

There was no mutation found in NOS terminator of plasmid pSA1110 when compared with its original from pBI221 vector (Clonetech).

All of the sequences alignment was shown in table 8.

Table 8.: Comparison of partial sequences of full-length PRSV type P cDNA Thai isolate with 5′ and 3′ RACE sequences of Thai isolate PRSV, PRSVs of Hawaiian isolate (PRSV-HA) and Taiwanese isolate (PRSV-YK)

Full-length cDNA clone of Thai isolate		5′RACE 3′RACE	PRSV-YK	PRSV-HA
Position	Number of residues			
5′	478 nt	99%	86%	69%
	130 aa	96%	90%	70%
2912 – 4071	1159 nt	-	89%	81%
5501 – 6668	1168 nt	-	87%	80%
3′	631 nt	98%	92%	90%
	141 aa	97%	97%	97%

5. Plant inoculation

For the first test of the infectivity of the PRSV genomic cDNA clones, five groups of ten papaya plants were used. The plants were approximately 4 months old. Each group of the youngest leaf of papaya plant was inoculated as mentioned in section 2.16 (Chapter III) with virus particles in the sap (positive control), pSA1110 plasmid (full-length PRSV under T7 promoter serving as negative control), pSA1101 plasmid (single 35S-full-length of PRSV, clone no. 215), pSA1102 plasmid (partially duplicated 35S-full-length PRSV, clone no. 81) and sterile

double distilled water. Three of full-length plasmids were inoculated in concentration of 20 μg / μl of plasmid DNA per each papaya plant. Only three papaya plants of the positive control group showed the severe symptom development in three weeks post-inoculation. No symptom was observed in other remaining groups. RT PCR product from amplification with coat protein PCR primers was used to verify this observation 2 months post-inoculation. Again, only the plant showed symptom from positive control group showed clear band of the amplified PRSV coat protein (Figure 57). The same mixture and condition for RT-PCR amplification was used in RT-PCR amplification of the extracted RNA from each group of papaya plant inoculation. Further experiments are now in progress.

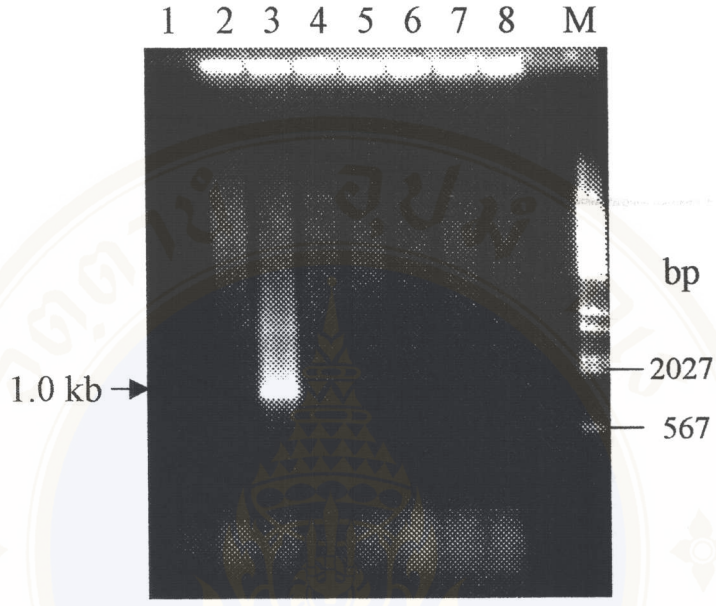


Figure 57. Verification of plants inoculation by RT-PCR

The 10 µl from 50 µl of RT-PCR products of 7 inoculated plants amplified by using 5′CP and 3′CP primers were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining.

Lane M:	Lambda DNA / *Bst*EII digested markers
Lane 1:	Negative control; amplification without template
Lane 2-3:	RT-PCR products of plant inoculated with sterile DDW (negative control) and sap solution of infected leaf (positive control), respectively
Lane 4:	RT-PCR products of plant inoculated with pSA1101 plasmid
Lane 5-7:	RT-PCR products of 3 plants inoculated with pSA1102 plasmid
Lane 8:	RT-PCR products of plant inoculated with pSA1110 plasmid

CHAPTER V

DISCUSSION

1. Cloning strategy

Although we were able to obtain the full length of PRSV cDNA by RT PCR, attempt to clone it in all available plasmids in our laboratory was not successful. Restriction sequence analysis of the amplified PRSV sequence allowed us to design an alternative method based on subsequent cloning of 3 parts of the full-length sequence into pUC19 vector engineered as plant expression cassette.

The cloning strategy took an advantage of the presence of two *Sac*I restriction enzyme sites near 5′ and 3′ end of the PRSV genome. Essentially, the 9.5 kb *Sac*I fragment can be cut out of the genome, the remaining 5′ and 3′ end sequences can be engineered to form a plant expression cassette and the *Sac*I fragment can be cloned back yielding the full length PRSV clone under the control of desired promoters. Unfortunately, it proved experimentally impossible to clone the *Sac*I fragment generated from the RT-PCR amplification to this cassette. This could be attributed to the low yield or the RT-PCR of the 10.3 kb fragment. Finally, subsequent cloning of three overlapping parts proved more successful as well as less expensive.

Engineering of the plant expression cassette took advantage of the well-known regulatory sequences from commercial source (Clontech) and from our previously constructed vectors. The 5′ end of the cassette has either CaMV 35S or partially duplicated CaMV 35S promoter, which are well documented. The 3′ end has both poly(A) tail over 100 bp long as well as NOS terminator sequence.

There are, however, contradictory reports on the use of these sequences for infectious transcripts. In some viruses such as barley yellow dwarf virus (BYDV), brome mosaic virus (BMV), cowpea mosaic virus (CPMV), and cucumber mosaic virus (CMV), their full-length viral cDNAs under the control of T7 promoter or CaMV 35S promoter did not have a poly(A) tail following their 3′UTR, yet they were infectious (141, 161, 169, 176). In the case of poliovirus, Sarnow (1989) found that transcripts terminating with 12 adenine nucleotides were 10% infectious as virion RNA whereas long homopolymeric adenine sequences (100 adenine residues) increased RNA infectivity into 100% as same as virion RNA. From previous studies, it is known that adenine sequence shorter than 20 nucleotides reduce the infectivity of virion RNA 20 fold (247). The poly(A) tail is also important for the tobacco vein mottling virus (TVMV) and peanut stripe potyvirus (PStV), which belong to potyvirus group. The transcripts derived from the full-length cDNA clone of TVMV with very short (12-nt) poly(A) tail is not infectious (153) as well as capped transcripts derived from full-length cDNA clone of PStV with 17 adenosine residues at 3′end of viral sequence (208). The NOS termination is also important for preventing the synthesis of transcripts longer than the genome. In the construction of full-length cDNA of zucchini yellow mosaic virus (ZYMV) without the NOS terminator was not infectious by mechanical inoculation (165). Instead of inserting a transcription terminator, the cDNA clones were linearized downstream of the viral sequence prior to inoculation (161, 162). We have therefore included poly(A) tail longer than 100 nucleotides as well as NOS terminator.

All attempts to clone the full-length PRSV PCR fragments to any plasmid available in out laboratory failed. So, the new strategy base on restriction mapping of

full-length PRSV PCR fragment, which eventually succeeded, were cloning of 3 overlapping PCR fragments of the PRSV genome. These sequence fragments are referred as "start", which is 3.5 kb long 5′end DNA sequence, "end", which is 4.3 kb long 3′end DNA sequence and the "middle" part, 2.7 kb long DNA sequence overlapping the middle and the start sequences. The three overlapping PCR fragments were combined together with appropriate unique restriction enzymes, which are *Pvu*II for joining middle and end part, *Stu*I for joining middle-end fragments with start part, *Pac*I for joining start part with the T7 promoter + 5′PRSV, to obtain the full-length PRSV cDNA clone. Then, the full-length clone of PRSV was partially digested with *Sac*I in order to obtain 9.5 kb fragment. This fragment was inserted into *Sac*I site of both plant expression cassettes (under control of single and partially duplicated 35S promoter). In this time, the subcloning of large fragment from the full-length cDNA clones was easier than cloning of large PCR products due to the low amount of PCR products and some of them lost in the purification step. The full-length cDNA of PRSV under the T7 promoter and with poly(A) tail followed 3′UTR and NOS terminator was obtained (pSA1110 plasmid) for serving as negative control in plant inoculation experiment. All of the plasmid constructions were confirmed by restriction analysis and also PCR amplification. There are some of PCR product bands in the negative control lane (amplified without template) in figures 30, 42, and 43 (Chapter IV) that caused from a *Taq* DNA polymerase, which synthesized in this institute without purification, was used. This less expensive polymerase enzyme could be use if the expected size of PCR product was not the same size as the PCR product in the

negative control lane. The junctions that used to assembly the full-length clones were ensured by sequencing as mentioned in section 4 (Chapter IV).

2. Influence of non-viral nucleotide at the 5′ end of the genome

As mentioned earlier, the presence of an extra non-viral nucleotide at 5′ end of the genome can greatly reduce infectivity of the transcript (discussed later). The 761 bp long 5′end fragment was directly ligated with amplified 842 bp CaMV 35S sequence. Theoretically, the random ligation gives up to 25 % probability of having the correct product in the ligation mixture. Using 5′ 35S primer and 3′ primer of 761 bp sequence 1.6 kb PCR product was obtained and cloned into *Sma*I site of pUC18 yielding pSA1064 plasmid. The nucleotide sequence of the 35S promoter in pSA1064 plasmid was confirmed by sequencing and compared with the sequence of 35S promoter from pBI221 plasmid (Clonetech). As expected for the *Pfu* polymerase used for amplification, there was no mutation found in 35S promoter sequence.

In this thesis, the transcription start site of the CaMV 35S promoter was designed to correspond to the first base of 5′PRSV. This was achieved by direct ligation of PCR product of 35S promoter with PCR product of 5′PRSV and re-amplification of this ligation mixture using 35Ss primer and 1L32 primer. Unfortunately, by a mistake in the design of 35Sa primer, one non-viral nucleotide (A) was introduced in the 5′ PRSV sequence as mentioned in section 4.2 (Chapter IV). It is difficult to deduce a role for this one extra nucleotide. It is usually assumed that the presence of these extra nucleotides could influence the proper initiation of (+) RNA synthesis from the 3′end of the (-) strand. It seems unlikely that this nucleotide will interfere with the viral gene translation *in vivo* because it does not possess an initiation codon and the *in vitro*

translation products are similar to those of wild-type RNA (144, 154, 204, 211, 245, 246). In the case of the T7 promoter, there is intentionally introduced an extra G in the primer design, which is required for the proper transcription by this pomoter. Thus, an addition of this extra shall not hamper the infectivity, although it may lower it.

3. Partial characterization of Thai isolate PRSV-P sequence

The nucleotide and amino acid sequences of 5′PRSV were analyzed and compared with the sequences of Thai isolate PRSV 5′RACE clone, which has been obtained previously in our laboratory using RACE PCR technique (M. Juricek, unpublished results), 5′ part of Hawaiian isolate and 5′part of Taiwanese isolate. The nucleotide and amino acid sequence alignments between Thai isolate 5′PRSV of pSA1064 plasmid and 5′RACE clone showed 4 nucleotides mutation in position 143, 145 and 147 from A to G, the position 204 from C to G. Three amino acids were changed in the position 20 from lysine (K) to glutamate (E), position 21 from lysine to arginine (R) and position 40 from proline (P) to arginine. These mutations were probably not caused by the PCR amplification. First, the amplified fragment is rather small (761 bp) and, second, the higher fidelity *Pfu* DNA polymerase was used (232). All mutations were found in the P1 protein generated from N terminus of the polyprotein. This protein showed a wide variation in molecular weight from 29 - 63 kDa and represented the most variable protein sharing less than 20% identity with those of other potyviruses and it is known to be the most variable among all PRSV proteins (13). Most probably, these mutations occurred spontaneously during virus propagation. For RNA the mutation rate per nucleotide site and per round of copying

is in the range of 10^{-3} to 10^{-5} for 10 kb genome ensure that each progeny RNA or DNA molecule includes an average of 0.1-10 mutations (233-237). Due to the absence or the low efficiency of proof reading-repair activities that are associated with RNA replicase and transcriptase (238, 239), the mismatch repair mechanisms are unlikely to operate on replicating RNA (240) and cannot operate on single-stranded RNA progeny genomes. The molecular recombination is also very active in plant and animal positive strand RNA viruses (241) (242, 243). Different isolates of virus have been used for RNA preparation for 5′ RACE PCR and for the cloning of the full length PRSV presented in this thesis. Similar variations have been documented also for the coat protein sequence of PRSV, which is more conserved than P1 protein (244).

4. Instability of full-length PRSV cDNA clones

All the full-length cDNA clones of PRSV (pSA1101, pSA1102 and pSA1110) proved to be difficult to maintain as a stock culture and the plasmid yield after isolation was very low. The colonies of *E. coli* host strain DH5α carrying these plasmids were small. Although the *E. coli* host strain JM109 carrying these plasmids formed larger colonies, the plasmid yield was still rather low. Moreover, the restriction enzyme analysis of this plasmid DNA revealed, that the band pattern is usually different, not matching the expected restriction enzyme map. This is an indication of an instability of the viral clones in those plasmids. Therefore, before inoculation of these plasmids on papaya plant, restriction enzyme analysis with *Sac*I was performed to ensure that the viral cDNA is intact.

The instability of full-length viral cDNA clones in bacteria due to toxicity of some virus sequences has been already reported (179, 208). So far, there are several

explanations for this phenomenon. Expression of virus proteins in *E.coli* has been shown to have toxic effects on the host cells (54, 248). It is not known how virus proteins are expressed from full-length clones, since the vector sequences do not contain promoters expected to transcribe the virus RNA in bacterial cells. Fakhfakh *et al.* (1996) suggested that virus RNA is transcribed from cryptic promoters and protein synthesis initiated at cryptic ribosomal binding sites present in the virus cDNA sequences (207). In CaMV 35S promoter, also found that it is active in *E.coli* although transcription is not initiated at the start site, which has been mapped in plants (199, 249). The toxic effects from undesired protein expression can be relieved by cloning in *E.coli* strains that reduce the plasmid copy number or using low copy number cloning vectors such as pMC18 and pBR322 (250-253). Introduction of a plant intron into the full-length cDNA was shown to stabilize the full-length cDNA constructs which were otherwise unstable or difficult to multiply in *E.coli* (178, 181).

The yield of all three plasmids carrying the full length of PRSV in a large scale preparation was as low as 0.2-1 μg/ml culture (Table 7). It was found, that there is an effect of prolonged storage time on both yield and instability of the clones. When bacterial cultures were inoculated immediately with colonies from agar plates immediately after transformation, the restriction enzyme pattern corresponded with the expected plasmid map. If inoculum was taken from plates, which had been stored at 4° C, the plasmid yield was very low (0.1-0.2 μg / ml culture) and re-arranged restriction enzyme pattern was observed when compared with the expected restriction enzyme map. These observations are very similar to Forns et al. (1997) who found, that *E. coli* can alter the cloned virus sequence by preferential selection of defective genomes. This negative selection may be eliminated or reduced by modifying the vector, insert

or changing host or growth condition. To eliminate this possible negative selection, all three plasmids with the full length PRSV clone were then grown in LB-Amp broth at 30°C or in TB-Amp broth at 30°C or 37°C. The yield of plasmid DNA was increased from 0.2-1 μg / ml culture to 3 μg / ml culture and the *Sac*I digestion pattern of these plasmids were confirmed again before inoculation on papaya plant.

5. Infectivity

Due to the space limitation the greenhouse, we could use only 10 papaya plants per plasmid for the infectivity testing. Another 10 plants were used as a positive and negative controls.

The youngest leaf in all papaya plants was mechanically inoculated with an aliquots of 20 μl containing 20 μg DNA in sterile double distilled water of each full-length plasmid pSA1101 (clone no. 215, single 35S-full-length), pSA1102 (clone no. 81, partially duplicated 35S-full-length), and pSA1110 (T7-full-length). No symptom development was observed and no RT-PCR product of viral coat protein was detected. This might be accounted to the age of the plants. Due to the technical problems, the papaya plants were overgrown and actually not suitable for mechanical inoculation. Therefore, even in a positive control experiment, only 3 papaya plants showed severe symptom development from out of 10 (sap inoculation with phosphated buffer). Usually, we obtain 90 – 100 % infection for the positive control.

There are several other parameters that influence the infectivity of viral transcripts such as hot weather in the greenhouse (more than 35°C during the day), and the low number of papaya plants inoculation (10 plants per each groups).

The infectivity of the viral transcripts is generally lower than the virus particles. Usually, depending on the type of the virus, it can range from 5 to 50 % when compared to the same virus particles. Since the positive control gave only 30 % infectivity, the infection with the virus transcript under these conditions was unlikely to appear.

The concentration of each three full-length plasmid DNA (20 μg / μl in sterile double distilled water) that was used for plant inoculation should be enough for viral transcripts due to the same experiment was successfully done in the infectious full-length cDNA clone of PRSV type P, Hawaiian strain (50% infection) (158).

The pH of sterile double distilled water that used to dissolve the full-length plasmid was low (5.7). It might be influence the stability of plasmid DNA.

In further experiment the plant inoculation will be done again with the young plants, with increased the number of plant inoculated to about 50 per transcript and phosphate buffer (pH 8.0) will be use instead of sterile double distilled water. The other possibility, if in the next experiment the papaya plants do not show the infection via symptom development or RT-PCR detection, the particle bombardment can be used. In a number of infectious virus cDNAs, this was the only successful technique due to the very low infectivity of the viral RNA transcript. (165, 167, 177, 179, 207).

CHAPTER VI

CONCLUSION

1. Two plasmids of plant expression cassettes for *in vivo* expression were constructed. Transcription of PRSV genome was designed to be driven either by a single or a partially duplicated CaMV 35S promoter. An artificial 127 bp poly(A) tail as well as a NOS terminator were added.

2. Full-length cDNA clones of a Thai isolate of PRSV, type P, under the control of CaMV 35S promoter (pSA1101), partially duplicated 35S promoter (pSA1102) and T7 promoter (pSA1110) with an artificial 127 bp poly(A) tail as well as a NOS terminator were constructed by sequential cloning of three overlapping PCR fragments of the full-length genome.

3. Partial nucleotide sequence of the full-length PRSV cDNA clone was determined. The comparison between of the full-length PRSV cDNA of Thai isolate with PRSVs of Hawaiian isolate (PRSV-HA) and Taiwanese isolate (PRSV-YK) showed a sequence identity of 70-92% on nucleotide level and 70-97% identity on amino acid level. From this comparison, the Thai isolate was more closely-related to the Taiwanese strain than to the Hawaiian strain.

4. Both full-length PRSV cDNA clones (pSA1101 and pSA1102 plasmids) have been used to infect papaya plants. No symptom was observed yet.

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