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THESIS

INSTABILITY OF RESISTANCE TO *PAPAYA RINGSPOT VIRUS* IN R_5 TRANSGENIC PAPAYA

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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R_5 transgenic papayas line A44 were analyzed for the inheritance of transgene. The virus resistant transgene was constructed from PRSV-CP gene linked with inverted repeat of CP gene (CP-IR). This gene construct was transformed to papaya genome by *Agrobacterium*-mediated transformation. Seedlings of R_5 transgenic papaya were obtained from seeds collected from R_0 generation to R_5 generation of greenhouse grown plants. Transgenic plants containing CP-IR gene were challenge-inoculated with PRSV to screen for virus resistance. Results from PCR analysis showed that 99.5% of tested plants (378 of 380) contained CP-IR transgene. Virus resistance in transgenic papayas was on average of 51.4% as determined by ELISA and symptom expression. The selected transgenic plants of R_5 generation showed two copies number of transgene in their genome as determined by Southern blot hybridization. No PRSV coat protein was detected in all transgenic plants as analyzed by western blotting. It is concluded that CP-IR transgene inherited in the fifth generation of transgenic papayas with two copies number in its genome without an expression of PRSV-CP gene. However, resistance to PRSV was significantly decreased as the generation progressed to the fifth generation.

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Student's signature Thesis Advisor's signature

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Chutima Arlai
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LIST OF ABBREVIATIONS

bp	=	Base pair
CDP-Star	=	Disodium-4-chloro-3-(methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricycle[3.3.1.1.^{3,7}] decan}-4-yl)-1-phenyl phosphate
CTAB	=	Cethyl Trimethyl Ammonium Bromide
DIG-11-dUTP	=	Digoxigenin-11-uridine-5'-triphosphate
DNA	=	Deoxyrionucleic acid
dNTP	=	Deoxyrionucleotide triphosphate
EDTA	=	Ethylene diamine tetraacetic acid
g	=	Gram
h	=	Hour
kb	=	Kilobase
kDa	=	Kilodalton
µg	=	Microgram
µl	=	Microlitre
LB	=	Luria bertani broth
M	=	Molar
mM	=	Millimolar
mg	=	Milligram
ml	=	Millilitre
ng	=	Nanogram
nptII	=	Neomycin phosphotransferase II gene
O.D.	=	Optical density
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PRSV	=	*Papaya ringspot virus*
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcription-Polymerase chain reaction
rpm	=	Round per minute

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LIST OF ABBREVIATIONS (Continued)

SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC	=	Saline-sodium-citrate
TAE	=	Tris acetate EDTA
TEMED	=	N, N, N', N'-tetra methylethylene diamine
UV	=	Ultraviolet
V	=	Volts
siRNA	=	Small interfering RNA

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INSTABILITY OF RESISTANCE TO *PAPAYA RINGSPOT VIRUS* IN R_5 TRANSGENIC PAPAYA

INTRODUCTION

Papaya (*Carica papaya* L.) is an important economic fruit crop and grown in different geographical areas of Thailand. The fresh fruit is normally consumed as fresh dessert fruit and green fruit as salad, called Somtum. However, production of papaya was limited by papaya ringspot disease caused by *Papaya ringspot virus* (PRSV). The virus is transmitted by aphids in non-persistent manner. Infected plant shows ringspot on leaves, stem and fruits. Growers have to cut down papaya plants after virus infection leading to the severe loss in production. The disease was first discovered in 1975 at Khonkaen province in Thailand (Srisomchai, 1975). In 1982 it was observed in 10 provinces of many cultivated areas in Northeast of Thailand causing up to 100 % of destructive level (Prasartsri *et al.*, 1982). The spreading of virus is difficult to control by conventional method and there is no naturally occurring PRSV resistant papaya. Breeding programs to introduce a resistance gene from wild species into commercial cultivars have not been successful because PRSV-resistant commercial cultivars are still unavailable (Manshardt and Wenslaff, 1989).

Consequently, attempt to develop virus resistance papaya has been pointed in transformation technique which developed from the concept of pathogen-derived resistance by introducing a part of viral genome to papaya genome to enhance resistance in plant. The coat protein-mediated resistance has been used successfully to generate virus resistance plant. In 1994, a program to control PRSV by developing genetically engineered papaya that is resistance to virus was financially supported by Thai Government in collaboration with Cornell University. During that period, Kasetsart University, Kamphaengsaen Campus, International Service for the Acquisition of Agi-biotech Applications (ISAAA) and Monsanto Company, successfully developed transgenic Khaknual and Khakdam papaya with PRSV-CP via particle bombardment and *Agrobacterium*-mediated transformation. Numbers of transgenic lines were shown to be resistant to PRSV (Warin *et al*., 2003, Horaruang *et al.*, 2006). However, the

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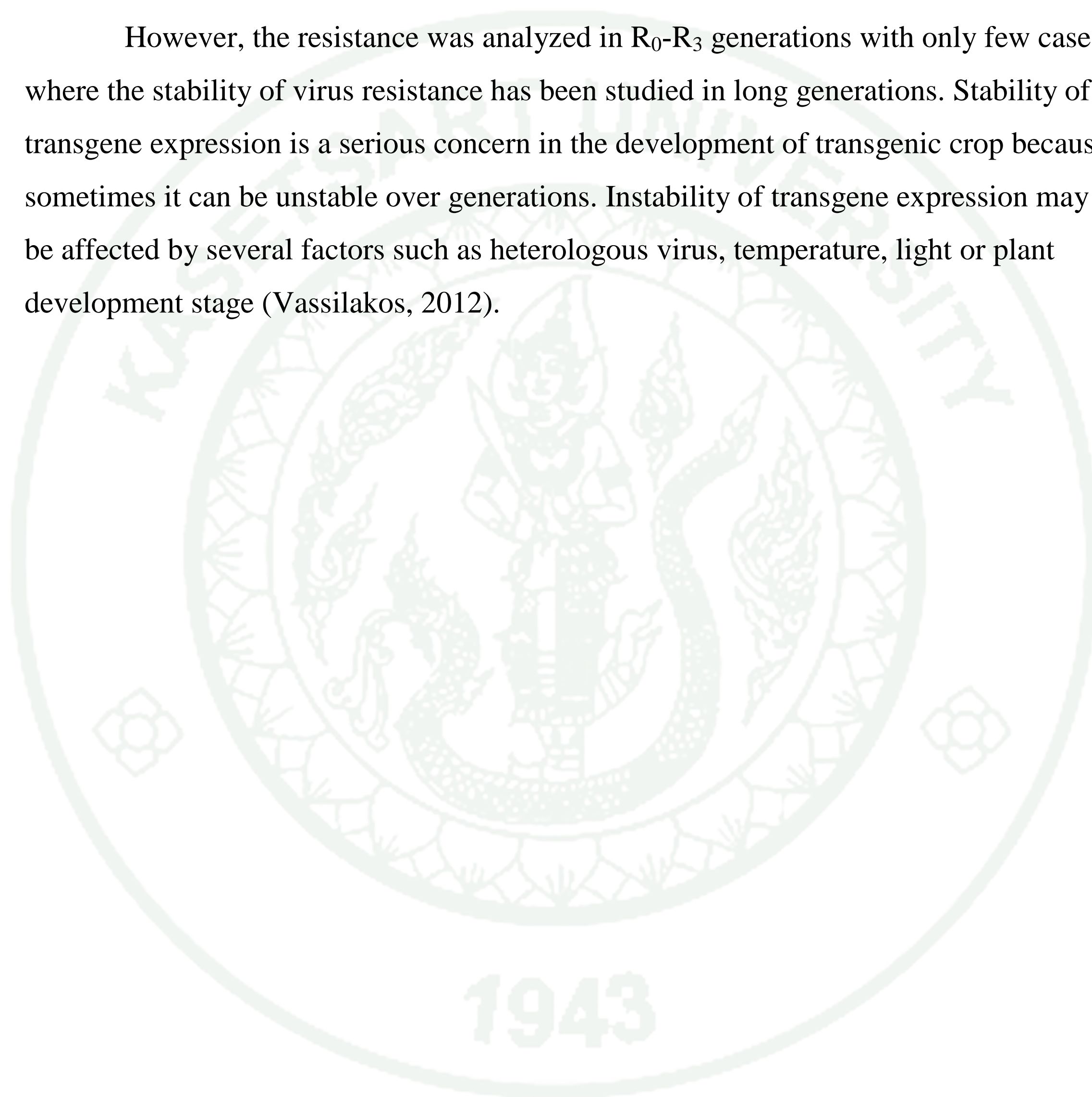
Government has placed a moratorium on field testing of GM plant since 2001 (Davidson, 2008). Only screen house testing was permitted despite the fact that information on the inheritance of transgene and its performance in the successive generation is essential for utilization for GM plant.

However, the resistance was analyzed in R_0-R_3 generations with only few cases where the stability of virus resistance has been studied in long generations. Stability of transgene expression is a serious concern in the development of transgenic crop because sometimes it can be unstable over generations. Instability of transgene expression may be affected by several factors such as heterologous virus, temperature, light or plant development stage (Vassilakos, 2012).



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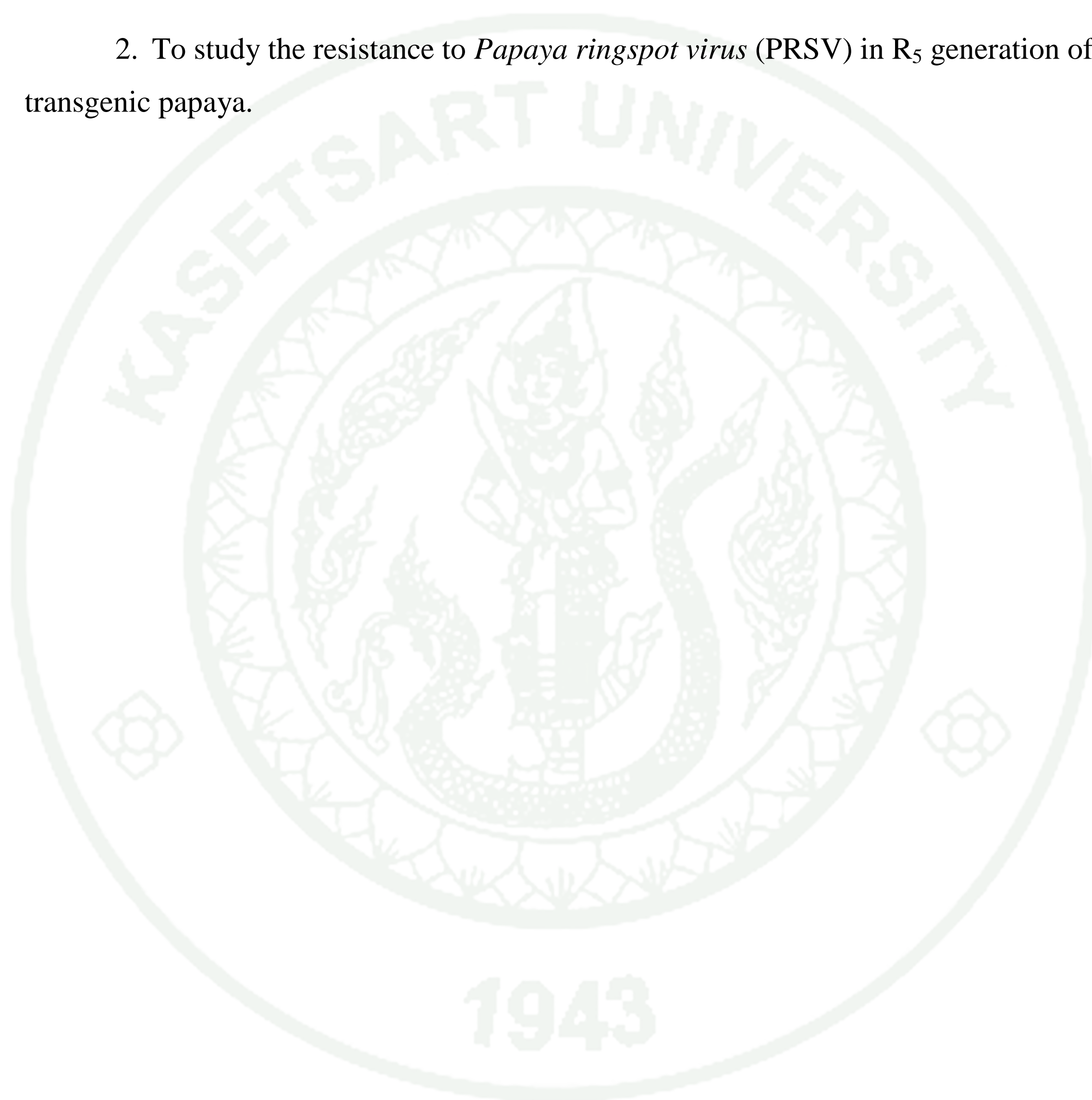
OBJECTIVES

1. To determine the stability of transgenes in R_5 generation of transgenic papaya.

2. To study the resistance to *Papaya ringspot virus* (PRSV) in R_5 generation of transgenic papaya.



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LITERATURE REVIEW

I. Papaya

Papaya (*Carica papaya* L.), a member of the *Caricaceae* family, in the genus *Carica*, is a herbaceous plant and has a single stem with a large palmately shaped leaves. Flowers are formed at the axils of the leaf petiole. The plant is polygamous, with male, female, and hermaphrodite plants (Manshardt, 1992). It is a perennial fruit crop, native of Central America and generally grown all through the tropics and subtropics. The enzyme papain obtained from immature fruit is used in the pharmaceutical and food industry (Purcifull *et al*.,1984). Papaya is diploid with 9 pairs of chromosomes. It has a small genome of 372 Mb (Arumuganathan and Earle, 1991). The fresh fruit are mostly consumed as a dessert while the green ones are commonly used as salad, called Somtum. Papaya plant is usually grown in home gardens because it is easy to grow from seed. The first mature fruits are able to harvest in nine months after sowing seeds. Papaya fruits can be produced continuously year-round. They are delicious and rich in vitamins A and C. The flesh contains about 85% water, 10-13% total sugar, and 0.6 % protein (Manshardt, 1992; Samson, 1986).

Papayas can be classified into different cultivars, depending on geographical regions, such as Kamiya (Solo type) releasing from the University of Hawaii, Sunset (Sunset Solo) originated from the University of Hawaii and Vista Solo originated from Vista, California (Samson, 1986). In Thailand, Khakdam and Khaknual cultivars are favored fruit in the markets of rural communities. They are hermaphroditic fruit with elongated shape, deep yellow-orange flesh, sweet and tasty fruit (Kasemsin, 2007).

Currently, production of papaya in Thailand has been declining due to the serious diseases especially viral disease. Papaya ringspot disease is a severe disease affecting papaya cultivation. Infected plants become stunting and severe reductions of the fruit quality and yield.

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II. *Papaya ringspot virus* (PRSV)

Papaya ringspot virus (PRSV) was first detected in Thailand in 1975 and is now widespread throughout all papaya plantations in the country and others in the region . PRSV is a member of *Potyvirus* genus in the family of *Potyviridae* which has flexuous and filamentous particle of 780 × 12 nm. The virion contains a positive-sense of single-stranded RNA of 10,326 nucleotides (Shukla *et al.*, 1994) that encapsulated by a coat protein (CP), approximately 36 kDa in size (Gonsalves and Ishii, 1980).

PRSV is transmitted by numerous species of aphids in a nonpersistent manner to a limited host range of cucurbits and papayas. PSRV also produces local lesions on *Chenopodium quinoa* and *C. amaranticolor*. A number of investigations have failed to demonstrate that PRSV is transmitted through seeds of papaya or cucurbits and it is grouped into two types: Type P (PRSV-P) infects cucurbits and papaya, whereas type W (PRSV-W) infects cucurbits but not papaya (Purcifull *et al.*, 1984).

III. Effect of PRSV on papaya production

Papaya ringspot virus (PRSV) is the most widespread and damaging virus that infects papaya. The name of the disease, papaya ringspot, is taken from the ringed spots on fruit of infected plants (Jensen, 1949).

Papaya plants infected with PRSV develop a range of symptoms including mosaic and chlorosis of leaf lamina, water-soaked oily streaks on the petiole and upper part of the trunk, and distortion of young leaves that resembles mite damage. Infected plants lose vigor and become stunted. When papaya plants were infected at the seedling stage or within two months after planting, they do not normally produce mature fruit. Production of fruit by infected plants at later stages resulted in severely reduction in numbers and quality (Gonsalves, 1998; Purcifull *et al.*, 1984)

The disease was first discovered in Thailand in 1975 at Khonkaen province and papayas were infected up to 100% in many cultivated areas of the province (Srisomchai,

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1975). In 1982 it was observed in 11 provinces in Northeast of Thailand causing 20-100 % of destructive level (Prasartsri *et al.*, 1982). Currently, the disease is spreading in every area of the country causing severe reduction of papaya production and farmer's incomes. In many cases farmers have to plant papaya in remote cultivation area away from infected areas to be able to produce papaya for the market.

IV. Control of papaya ringspot disease

PRSV causes a serious disease in papaya in many areas in Thailand such as Khon Kaen, Yasothorn, Suphan Buri, Nakhon Pathom and Surat Thani. In the past, several efforts were introduced to control this viral disease including rouging, chemical spray for aphid control and breeding for resistant cultivars. However, they could not solve the problems because of the highly aphid transmission rate (at 30 sec for transmission) thus making chemical control less effective. Furthermore, papaya breeding program was not so successful since no natural virus resistant germplasm is available. In 1986, attempt to control this disease by cross protection was initiated. It was based on the observation that plants systemically infected with a mild strain of PRSV could protect against the second infection by the virulent related strains (Kositratana *et al.*, 1991). This practice has been long known and used to control some virus diseases such as citrus tristeza in citrus, tobacco mosaic in tomato and zucchini yellow mosaic in zucchini (Costa and Muller, 1980; Rast, 1975; Walkey *et al.*, 1992). However, cross protection technique has not been successfully used for control of PRSV in other geographical areas. Main reasons are cross protection requires extra cultural management and care, the limitation of related PRSV mild strains and some mild strains can cause symptoms and the mutation to a severe strain by existing mild strains (Gonsalves, 1998).

V. Development of transgenic papaya with virus resistance

The concept of pathogen-derived resistance (PDR), conceived in the middle1980s (Sanford and Johnston, 1985), offers a new approach for controlling PRSV. Pathogen-derived resistance is a phenomenon whereby transgenic plants

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containing genes or sequences of a pathogen (in this case, the coat protein gene of a virus) are protected against detrimental effects of the same or related pathogens. The application of PDR for plant viruses was first demonstrated by Beachy's group (Powell-Abel e*t al*., 1986). Transgenic tobacco expressing the coat protein gene of *Tobacco mosaic virus* was protected against infection by the same virus. Subsequent reports have shown that this approach is effective in controlling many plant viruses (Lomonossoff, 1995).

In 1990, transgenic papaya was first successfully developed with a chimeric coat protein (CP) gene of PRSV from Hawaii by particle bombardment transformation method. The transgenic plant line 55-1 was resistant to a PRSV HA in the greenhouse (Fitch *et al*., 1992) and a field trial in Hawaii (Lius *et al*., 1997).The first generation (R_0) of line 55-1 was crossed with PRSV-susceptible commercial non-transgenic plant to produce SunUp and Rainbow variety for control PRSV disease in Hawaii (Manshard,1992). In Thailand, attempt was made to control the disease by using cross protection technique but it has a limited success in all geographical areas. The Department of Agriculture (DOA), Ministry of Agriculture and Co-operatives, Thailand initiated the collaboration with Cornell University to control this disease. Dr. Nonglak Sarindu and Dr. Suchirat Sakuanrungsirikul of the Department of Agriculture went to work in the laboratory at Cornell under the supervision of Dr. Dennis Gonsalves to develop transgenic papaya specifically for Thailand. By July 1997, transgenic papayas of the Khakdam, Khaknual, and Thapra varieties were transported to Thailand. They were transferred to the research station at Tha Pra, Khon-Kaen Province. Seeds from selfing and crossing among transgenic and non-transgenic lines were produced from the R_0 plants. The progeny (R1) was tested for virus resistance in the greenhouse. Selection included mechanical inoculation of PRSV isolated from five endemic areas in Thailand. The most resistant lines of each generation were subsequently evaluated under field conditions for 3 generations. Each lines of plants in R_3 generation exhibited 90%-100% resistance to PRSV (Sakuanrungsirikul *et al.*, 2005). Subsequently, there was public and private sector collaboration in the country to produce transgenic papaya. This group led by Kasetsart University, Monsanto Company and ISAAA, has successfully developed transgenic papaya Khaknaul variety with PRSV-CP via particle bombardment

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transformation. The transgenic plant lines KN1.2.3, KN13.2.3 and KN29 were resistant to PRSV in different geographical area in Thailand (Warin *et al.,*2003). Additionally, Horaruang and co-workers (2006) developed *Agrobacterium*-mediated transformation technique for gene transfer into papaya. Khakdam cultivar was transformed with coat protein (CP), RNA replicase (NIb) and CP linked to inverted repeat of CP genes from Suphan Buri (SB-PRSV) and Nakhon Pathom (NP-PRSV) isolates. Thirty four transgenic lines (R_0) exhibited high degree of resistance to PRSV under glasshouse condition.

VI. Mechanism of virus resistance in transgenic plants

A. Coat protein-mediated resistance

The molecular mechanisms that govern CP-mediated resistance (CPMR) are not fully understood. These mechanisms of CPMR may be different among viruses (Bendahmance *et al*., 2007). Several evidence support the hypothesis that CPMR against virus is a consequence of interaction between the transgenic CP and the CP of the challenging virus (Koo *et al.,* 2004). The most accepted model suggests that the transgenic CP could prevent virions from undergoing co-translational disassembly, which is an early event of infection. In this process, a virus particle has to disassemble to partially expose the 5'-end of the viral RNA so that ribosomes can displace the CP to translate the first open reading frame (ORF) (Lin *et al.*., 2007). Thus the invading virus releases its 5' terminal CP subunits, the protective transgenic CP will immediately recoat disassembling virus particles to prevent their infection (Lu *et al.,* 1998). Asurmendi *et al*. (2007) postulated that the state of aggregation of CPs is correlated with the level of CPMR. Hence, the co-translational disassembly in early step of virus infection is a key step to determine whether the virus can overcome the CPMR resistance (Lin *et al.,* 2007). The ability of CP self-assembly necessary for a virus species may not extend to other virus species. For example, transgenic plants expressing the CP assembly-deficient mutant of *Tobacco etch virus* (TEV) still conferred CPMR against TEV (Voloudakis *et al.,* 2005). However, the resistance establishment of different levels of resistance indicates that multiple mechanisms could be involved. In

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addition, a transgene can confer both protein-and RNA-mediated protection (Prins *et al.,* 2008).

B. RNA-mediated resistance

1. RNA silencing

In most case, RNA-mediated resistance is related to RNA-silencing which is probably the most important and common strategy for engineered resistance to plant virus. The mechanism of resistance in vast majority of the applications of transgenic plant strategy is based on RNA-silencing. RNA-silencing is a sequence specific RNA degradation mechanism, highly conserved between kingdom, which in plant, among other function, operates as a natural antiviral defense system (Eamens *et al.,* 2008). RNA-silencing, is activated as a response to double-stranded RNA (dsRNA). Viruses as well as inverted repeats (IR), can directly produce dsRNA, whereas highly transcribed, sense oriented, single copy transgene can also produce abberrant transcripts that serve as a substrate for producing dsRNA. In the latter case dsRNA is synthesized by cellular RNA-dependent RNA polymerases (RdRps). Subsequently, the dsRNA can be targeted by Dicer-like ribonuclease (DCL) with each of them being involed in specific sRNA pathways and generating specific size of sRNA duplexed (18-25 nt in length). The cleavage of dsRNA is facilitated by another group of dsRNA binding proteins. Then siRNAs are stabilized by 2' 0-methylation in their overhanging 3' ends and exorted to cytoplasm for post-transcriptional gene silencing (PTGS). One selected sRNA strand together with one member of the ARGONAUTE (AGO) family of proteins form the core of nuclease complex (RNA induced silencing complex, RISC) that targets and cleaves sequence-specifically homologous ssRNA (Ronemus *et al.*, 2006., Ruiz-Ferrer and Voinnet, 2009). RNA silencing impedes viral multiplication in plants by two major ways. First it degrades the dsRNA intermediates of virus replication themselves as well as the cognate mRNAs, a procedure that leads to the increase of accumulation of the respective siRNAs. Second, it generates a mobile signal that triggers the degradation of homologous mRNA in distant cells (systemic silencing) (Kalantidis *et al*., 2008). The engineering of transgenic plants to harbor

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single-stranded sense and to a less extend antisense viral sequence became a common strategy to pre-activated the silencing machinery and obtain resistance against the homologous virus from which the introduced sequence has derived (Ritzenthaler, 2005). Further exploiting this knowledge led to constructing IR transgenes from which long dsRNA precursors of siRNAs were directly generated. The utilization of such IR transgene constructs has become the method of choice for providing genetically engineered resistance to viruses. Only a single copy of IR is sufficient to provide immunity and there is no expression of viral proteins. Short genome incomplete sequence can be used with efficiencies up to 90% of all transgenic plants produced to be resistance to homologous virus were achieved (Lin *et al*., 2007, Tenllado *et al*., 2004, Ritzenthaler, 2005). However, RNA-silencing mediated resistance can be overcome by viruses whose sequence differs from that of transgene by more than 10% (Bau *et al*., 2003, Jones *et al*., 1998).

a) Factors that influence the RNA-silencing based transgenic resistance

(1) Heterologous virus

It has been established since 1998 that most known virus species carry at least one RNA silencing suppressor (Diaz-Pendon and Ding, 2008, Ding and Voinnet, 2007). The awareness of this viral counter defensive strategy against the innate antiviral defense system of plants guided several groups to investigate the effect that could invoke on transgenic resistance of plants that were immune to a virus, the infection with a different virus carrying a strong silencing suppressor.

Savenkov and Valkonen (2001) have shown that transgenic tobacco plant resistant to *Potato virus* A (PVA, *Potyvirus*) was affected by infection of *Potato virus* Y (PVY, *Potyvirus*) which was known to suppress RNA silencing through its HC-Pro protein (Diaz-Pendon and Ding, 2008 and Ding and Voinnet, 2007). However, when transgenic potato plant resistant to PVY was infected by *Potato virus* X (PVX, *Potexvirus*) which has P25 silencing suppressor, resistance to PVY remained unaffected (Missiou *et al.,* 2004). In contrast, in *N. benthamiana* plants expressing a *Grapevine*

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virus A (GVA) minireplicon and displaying high resistance to GVA, infection with *Grapevine virus B* (GVB, genus *Vitivirus*, carrying a P10 silencing suppressor) or PVY resulted in suppression of the GVA-specific defense (Brumin *et al*., 2009).

Mixed infection is a common phenomenon in field condition. The interference between the heterologous viruses on resistance in transgenic plant should be taken into consideration for an effective utilization. The reasons for the discrepancies are unclear, but could be related to the mode of action of the viral suppression proteins of the different virus tested. Viral silencing suppressors are highly diverse in sequence, structure and activity, and could target multiple points in RNA silencing pathways whereas viruses with large genomes may encode several functionally distinct proteins to achieve silencing suppression (Dıaz-Pendon and Ding, 2008, Ding and Voinnet, 2007). It is considered that suppressor proteins interfere either with siRNAs biogenesis or siRNA function without a multifunctional nature to be excluded. For instance, most studies agree that the potyviral HC-Pro probably specifically blocks accumulation of secondary siRNAs and leaves primary siRNA accumulation unimpaired, whereas P25 blocks accumulation of primary siRNAs (Dıaz-Pendon and Ding, 2008). In contrast, the 2b protein of cucumoviruses directly sequestrates siRNAs duplexes using a pair of hook-like structures that interact more promiscuously with long and short dsRNA (Dıaz-Pendon and Ding, 2008, Ding and Voinnet, 2007, Ruiz-Ferrer and Voinnet, 2009). Additionally, it binds AGO1 and blocks slicing without interfering with sRNA loading *in vitro*.

However, antiviral plant defense pathways could also be involved in the interference between the heterologous virus infection and the transgenic resistance or as yet unknown factors involved in specific virus species interactions.

(2) Temperature

It has been well known to plant virologists that temperature strongly influences plant-virus interactions. In high temperature, symptoms are frequently attenuated and virus titers in infected plants are decreased. In contrast, outbreaks of

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virus diseases are frequently associated with low temperatures (Hull, 2002). Kalantidis and co-workers (2002) examined the influence of elevated temperature on siRNAs in CMV-resistant transgenic tobacco plants. Two transgenic lines, one expressing very high and the other very low levels of siRNAs, were tested for siRNAs concentration at 25ºC and 32ºC and at two time points, 20 and 30 days post-germination. At the early time point, transgene derived siRNAs could be detected only in the first line at 25ºC and in both lines at 32ºC . However, in the first line transgene specific siRNAs were at 32ºC in a significantly higher concentration compared to that of 25ºC . The analysis of samples taken at the second time point revealed the presence of transgene derived siRNAs in both lines at 25ºC . However, at 32ºC , siRNAs were detected in both plant lines at a higher concentration.

Szittya and associates (2003) demonstrated that RNA silencing induced by viruses or transgenes is inhibited at low temperatures and enhanced with rising temperatures. They used wild type *Cymbidium ringspot virus* (CymRSV) encoding a p19 viral suppressor and a mutated one unable to express p19 (Cym19stop). In virus transfected *N. benthamiana* protoplasts, virus derived siRNAs were undetectable at 15ºC and gradually increased with temperature from 21 to 27ºC indicating that virus-induced cell autonomous silencing is temperature dependent. To study the effect of temperature on virus induced systemic RNA silencing, *N.benthamiana* plants were inoculated with CymRSV and Cym19stop and grown at different temperatures. CymRSV infected plants died within 2 weeks at 15, 21 and 24ºC whereas CymRSV symptoms were attenuated at 27ºC and associated with reduced virus level. Plants infected with the Cym19stop showed a recovery phenotype at 21 and 24ºC . At 27ºC , the mutant virus was unable to infect the plants, while at 15ºC , Cym19stop-infected plants displayed strong viral symptoms demonstrating that at low temperature, RNA silencing failed to protect the plants even when the virus lacked the silencing suppressor. In addition, using a strain of *Agrobacterium tumefaciens* carrying a green fluorescent protein (GFP) gene construct which was infiltrated sole or together with p19, to wild type *N.benthamiana* or *N.benthamiana* plants expressing GFP, it was shown that transgene-induced silencing is also temperature dependent. The stability of RNA silencing mediated transgenic virus resistance at different temperatures was

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examined using transgenic *N.benthamiana* plants expressing a CymRSV-derived RNA. After inoculation with CymRSV the plants displayed strong resistance at 24ºC whereas at 15ºC , severe symptoms were developed and CymRSV RNA accumulated to a high level demonstrating that the transgene-mediated virus resistance was broken at low temperature. The temperature effect on the development of viral diseases is closely associated to the RNA silencing antiviral pathway and consequently influences the efficiency of silencing-based transgenic resistance. However, it appears that the low temperature effect on the transgenic resistance depends on additional factors that remain to be identified.

(3) Light

Studies on the effect of light on transgenic resistance to viruses are not available, however light has been implicated as one of the factors that affect RNA silencing initiation and maintenance in several studies. Although in most of them light effect on silencing was not clearly isolated from that of temperature (Nethra *et al*., 2006; Vaucheret *et al*., 1997). Recently, Kotakis *et al*. (2010) investigated solely the role of light intensity in physiological ranges on RNA silencing. They used *N. benthamiana* transgenic lines engineered to express GFP, which exhibited spontaneously silencing at different frequencies and of different spreading intensities as a model. The result showed that high light intensity increased the frequency of plants displaying both short range and systemic silencing. In contrast, plants grown under low light conditions, showed lower silencing frequencies. In addition, increased light intensity positively affected siRNA levels corresponding to the GFP transgene (sense) transcript. In a different set of experiments, *N. benthamiana* plants incorporating an IR structure derived from the NIb gene of *Plum pox virus* (PPV) were used. It was shown that levels of all distinguishable siRNA classes corresponding to the IR transcript were also positively affected by high light intensity. However, the effect of light intensity on virus resistance should be taken under consideration when transgenic virus resistance is going to be applied in the field.

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(4) Plant development stage

A few studies with plants carrying sense transgenes and displaying RNA-silencing mediated resistance have suggested an influence of plant developmental stage on the degree of the expressed resistance. Tenllado and Diaz-Ruiz (1999) reported that a higher percentage of transgenic *N. benthamiana* plants, transformed with the 54K read-through domain of the replicase gene of *Pepper mild mottle virus* (PMMoV), displayed complete virus resistance at maturity than at an earlier stage of development. Tennant *et al.*(2001) demonstrated that transgenic papaya plants were susceptible to *Papaya ringspot virus* (PRSV) at a younger stage but resistant when inoculated at an older stage. Moreover, Kalantidis and associates (2002) showed that siRNA accumulation in transgenic tobacco, incorporating an IR construct carrying CMV sequences, was higher at later developmental stages. No significant differences in the siRNA concentration were observed between leaves of different age from a single plant or from the seven-leaf stage. Vassilakos *et al*. (2008) transformed *N. tabacum* plants with the 57-kDa read-through domain of the replicase gene of TRV. Transgenic plants were highly resistant to homologous (to the transgene sequence) TRV isolates and moderately resistant to the genetically distinct TRV-GR. Very young transgenic plants with detectable levels of transgene transcript were resistant only systemically to homologous isolates and were susceptible to TRV-GR. Conversely, older plants (at a five-leaf stage) containing a low steady state level of transcripts were immune to homologous isolates and displayed moderate resistance against TRV-GR.

VII. Stability of transgene and virus resistance in transgenic plants

For commercial production of transgenic cultivars, transgene must be inherited to several generations and the level of resistance must be maintained. Xiangdong *et al.* (2007) analyzed virus resistance and fruit quality in T_4 generation of transgenic papaya. All transgenic papaya plants with the mutated replicase (RP) gene of PRSV showed high resistance to PRSV in the field. The RP transgene can be stability inherited to the progenies. The growth characteristics of transgenic papaya were much better than those of non-transgenic papaya in the field. Most of the transgenic papaya plants (about

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91.8%) did not show any symptom of PRSV but exhibited more productive with bigger and better fruit quality. In Thailand, a number of resistant transgenic papaya lines, in particular Khakdam and Khaknual, were developed. Twenty-five lines of Khaknual and two of Khakdam in the R_2 generation that showed various levels of resistance to virus when inoculated in the greenhouse were subsequently tested in a field that was severely infected with the virus. The transgenic lines showed excellent resistance under field conditions. The excellent lines were then carried on to the third generation. Among these, one excellent line of Khaknual (R_3 319-1KN-181) and one of Khakdam (R_3 300KD-9) have been identified. The progeny from these lines exhibit 90% to 100% resistance to PRSV (Sakuanrungsirikul *et al.,* 2005).

Hu *et al.* (2011) demonstrated that transgenic tobacco plants expressed partial TMV movement gene and partial CMV replicase gene in the form of an intermolecular intron-hairpin RNA exhibited complete resistance to TMV or CMV infection and the transgenic plants could be inherited and kept stable for the fourth generation.

However, some instances of unstable pathogen-derived virus resistance have been observed, although the cause of instability was not determined. For example, Retuta *et al.* (2012) evaluated three selected transgenic papaya lines for inheritance of resistance to PRSV in R_1 and R_2 generation in field condition. The result showed that the percentage of resistance progenies to PRSV in R_1 generation decreased in the R_2 generation, indicating that the inherited resistance of the three transgenic line was unstable. Furthermore, in Malaysia, it has been observed that the resistance is also unstable as the number of resistant progenies (119) in the R_0 (original transgenic plants) regeneration decreases to 13 in the R_1 generation and no resistant progenies were observed in R_2 generation (Habibuddin *et al.*, 2007). In addition, transgenic tobacco (*Nicotiana benthamiana*) containing a translatable *Pea early browning virus* (PEBV) 54K sequence from the 201K replicase gene has been analyzed. The resistance observed in R_2 and R_3 generations became less consistent in R_4 and R_5 generation (Van den Boogaart *et al*., 2001). Moreover, Liu *et al.*(2003) produced transgenic doubled-haploid tobacco with the *Tomato spotted wilt virus* (TSWV) nucleocapsid gene from the Hawaiian L isolate. The result showed that some lines of transgenic plants in R_1

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generation were completely resistant to TSWV but in R_2 generation about a quarter of the plants exhibited lower levels of resistance. However, some lines of transgenic plants exhibited high resistant to TSWV through to the R_9 generation.

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MATERIALS AND METHODS

I. Transgenic plants

R_5 generation of transgenic papayas, Khakdam cultivar (line A44) used in this study were obtained from *Agrobacterium*-mediated transformation with chimeric constructs of PRSV derived genes. Plant expression vectors were obtained by cloning of CP of PRSV isolate from Suphan Buri (PRSV-SB) into a binary vector for *Agrobacterium*-mediated papaya transformation. The vector contains PRSV- CP genes linked with 250 bp inverted repeat sequence of the CP gene, neomycin phosphotransferase II (*nptII*) gene that used for selection of transformants and 35S CaMV promoter for regulation of gene expression, respectively (Kasemsin, 2007).

II. Molecular analysis of transformed papaya lines

A. Genomic DNA extraction from papaya

Genomic DNA was extracted from young fresh leaves of papaya using modified protocol from CTAB method (Rogers *et al*., 1996). One gram of leaves was ground in liquid nitrogen then transferred the powder into 1.5 ml micro-centrifuge tube and added 700 µl of CTAB buffer. The mixture was incubated at 65°C for 30 min and followed by extraction with an equal volume of chloroform : isoamyl alcohol (24 : 1) and then mixed by inverting tube about 200 times. Supernatant was harvested by centrifugation at 12,000 rpm for 10 min. DNA pellet was precipitated with an equal volume of cold isopropanol, centrifugation at 12,000 rpm for 5 min, rinsed with cold 70% ethanol and centrifugation at 12,000 rpm for 2 min. Ethanol residues were removed as much as possible by a vacuum for overnight. Dry pellet was resuspended in distilled water containing 20 µg/ml of RNase and kept in -20°C until use.

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B. PCR analysis of transgenic papaya

Genomic DNA from papaya was used as a template for PCR reaction. PCR mixture was composed of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTPs, 10 pmol of SC501 primer (5'- AAAGTGGTATGAGGGAGTGAGGAA-3') and SC104 primer (5'-ATTGCGCATACCTAGGAGAGAGTG-3'), 1 unit of *Taq* DNA polymerase (Invitrogen), genomic DNA 1 µl and followed by adding distilled water to adjust the total volume in 20 µl. Amplification involved a 5 min denaturation step at 94°C and 30 cycles consisting of 45 sec denaturation at 94 °C, 45 sec primer annealing at 58°C and 45 sec primer extension at 72°C followed by a final 5 min extension step at 72°C. PCR product was separated on 1% (w/v) agarose gels in 0.5X tris borate EDTA buffer (TBE).

C. Preparation of PCR DIG-labeling DNA probe

The DIG-labelling DNA probes were used for *in vitro* hybridization with complementary DNA in order to confirm the integration of PRSV genes in transformed papaya genome. They were synthesized by PCR using DIG-11-dUTP labelling mixture (Roche). PCR mixture was composed of 1X PCR buffer (200 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl2, 1X DIG-PCR labeling mixture, 20 pmol of each primer (Table 4), 2 unit of Taq DNA polymerase (Invitrogen), 50 ng of plasmid DNA harboring PRSV gene that used as a template for PCR reaction and followed by adding distilled water to adjust the total volume in 50 µl. The PCR reaction was done as described above (2.2). The DIG-labelled DNA product was investigated by comparing with non-labelled DNA product on 0.8% agarose gel. The DNA probes were denatured by boiling for 10 min and immediately chilled on ice for 10 min before hybridized with complementary DNA.

D. Southern blot hybridization of R_5 transgenic papaya DNA

Genomic DNA was digested with *Eco*RI which does not have a restriction site inside the PRSV-derived transgenes. Reaction for digestion was composed of 1X

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buffer (Roche), 100 units of restriction enzyme, 20 µg of genomic DNA and followed by adding distilled water to adjust the total volume in 200 µl. The reaction was incubated at 37°C for 16-18 h. A five microlitre of digestion was separated on 0.8% agarose gel in 1X TBE buffer. Smeared DNA bands were observed to confirm complete digestion. Digested DNA was purified by phenol chloroform extraction to remove the buffer, precipitated with 1.5 volumes of isopropanol and 0.1 M sodium acetate, pH 5.4 and incubated at -20°C for 3 h. DNA pellet was harvested by centrifugation at 12,000 rpm for 15 min and rinsed with 70% ethanol. It as then was resuspended in distilled water and kept at -20°C until use.

Southern blot hybridization as described by Southern (1975) was used for determination of number of inserted DNA in papaya genome. The digested DNA was separated on 0.8% agarose gel in 1X TBE buffer at 50 V for 90 min. Then, the gel was depurinated in 0.25 N HCl for 10 min, rinsed with distilled water and denatured in alkaline transfer buffer (0.4 M NaOH and 1M NaCl) for 10 min. The DNA from gel was transferred to the positive charge nylon membrane (Roche) by capillary transfer in alkaline transfer buffer at room temperature for 16-18 h. The digested DNA was fixed on the membrane by soaking in neutralization buffer (Tris-HCl pH 7.2 and 1M NaCl) for 15 min. The membrane was incubated in hybridization buffer [5X SSC (0.75 M NaCl, 0.075 M sodium citrate), 0.1% N-lauroylsarcosine, 1% blocking reagent (Roche)] at 65°C for 1 h. Then, the pre-hybridization buffer was removed, added a new hybridization buffer containing 2 µg of denatured DIG-labelled DNA probe and incubated at 65°C for 16-18 h. After hybridization, the expected bands of hybridized DNA were detected by chemiluminescent procedure. The membrane was soaked twice in low stringency buffer (2X SSC, 0.1% SDS) at room temperature for 5 min and then soaked twice in high stringency buffer (0.1X SSC, 0.1% SDS) at 65°C for 15 min. The membrane was soaked in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20) at room temperature for 2 min and then incubated in blocking buffer [1% blocking reagent (Roche), 100 mM maleic acid, 150 mM NaCl, pH 7.5] with shaking at room temperature for 30 min. The membrane was incubated in blocking buffer containing anti-digoxiginin-AP at room temperature for 30 min. Ratio of anti-digoxiginin-AP to blocking buffer was 1/5,000. The membrane was washed twice in

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washing buffer at room temperature for 15 min and then soaked briefly in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl). After that, the membrane was incubated briefly in CDP-Star substrate (Roche) that was diluted to 1/200 in detection buffer. The hybridized DNA bands were visualized by exposing membrane on X-ray film (KODAK) for at least 1 hr. After exposure, the X-ray film was soaked in developing solution for 3 min, rinsed with water, soaked in fixing solution for 3 min and washed with water.

III. Evaluation of PRSV resistance under greenhouse condition

A. Mechanical inoculation of PRSV

Plantlets from transgenic papaya lines (R_5 generation) with 5-6 leaves stage (45 days after germination) were evaluated for PRSV resistance. Transgenic papaya lines were inoculated with PRSV. Virus infected young fresh leaves were ground in a cold mortar and pestle containing 0.1 M phosphate buffer (NaH_2PO_4,Na_2HPO_4, pH 7.0). The ratio of leaf to buffer was 1:5. The homogenate was mixed with 0.5% w/v of Celite (diatomaceous earth) in order to assist the viral infection into papaya cells. Transgenic papayas were inoculated by gently rubbing onto 2 leaves of papaya seedling. Non-transformed papayas were used as positive control. After inoculation, inoculated leaves were rinsed off with water to remove Celite and prevent shading. The inoculated plants were kept in greenhouse for symptom observation.

B. Detection of viral protein by ELISA (Enzyme-linked immunosorbent assay)

After 30 days of inoculation, the inoculated plants were evaluated for symptom development. They were also identified for virus infection by detection of viral protein with polyclonal antibody against PRSV. One gram of inoculated leave was ground in small plastic bag containing 1 ml of coating buffer (0.05 M carbonate buffer, pH 9.6). A 50 µl of homogenate was loaded into 96-well microplate and incubated at 4°C for 16-18 h. The microplate was washed with 1x PBST 3 times, each time for 5

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min, to remove excess residues. Coated microplate was incubated with polyclonal antibody against the CP of PRSV diluted 1/1,000 at 37°C for 1 h 30 min. The microplate was washed as above to remove uninteracted proteins and then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase diluted 1/10,000 for 1 h 30 min. The microplate was washed as above to remove the goat anti-rabbit IgG residues. The interaction between antigens and antibodies was visualized by adding PNPP (para-nitrophenyl phosphate) substrate of alkaline phosphatase, incubated at 37°C for 1 h. and measured by ELISA microplate reader at absorbent 405 nm ($O.D._{405}$).

IV. Analysis of Mechanism of PRSV resistance in R_5 transgenic papaya

A. Analysis of transgene expression in the PRSV-resistant transgenic papaya

1. Protein extraction

Total protein was extracted from fresh leaves of papaya using modified protocol from Macintosh *et al*. (1992). Approximately 1 g of fresh leaves was ground in liquid nitrogen, added protein extraction buffer (0.05 M Tris-HCl, 0.06 M sodium sulphite, pH 8.5) 500 µl and transferred the homogenate into a 1.5 ml microcentrifuge tube. The homogenate was centrifuged at 10,000 rpm for 5 min. The supernatant was transferred into a new microcentrifuge tube that used as total protein for further analysis and kept at -20°C until use.

2. Western blot analysis

Western blot analysis used for detection of CP translated protein using polyclonal antibody against the CP of PRSV, kindly provide by Dr. Srimek Chowpongpang, Kasetsart University. Fifteen microlitre of total protein was separated on SDS-PAGE (5% staking and 12% separating polyacrylamide gels) (Sambrook *et al*., 1989). Protein samples were mixed with 1X loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% bromophenol blue, 8% glycerol, 2.5% 2x mercapto ethanol), boiled for

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10 min and cooled at room temperature for 10 min. Then the protein samples were loaded into the lanes of staking gel and run at 50 V for 30 min in 1X running buffer and then at 100 V for 120 min. After electrophoresis, protein from gel was transferred to the nitrocellulose membrane (Amersham) in cold transfer buffer at 40 V for 100 min. Then, the membrane was soaked in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) with shaking at room temperature for 5 min and incubated in 4% blocking buffer (5% skim milk in TBS buffer) with shaking for 1 h. The membrane was incubated in TBS buffer containing diluted 1/5,000 polyclonal antibody to the CP of PRSV at room temperature with shaking for 1 h. Then, the membrane was washed in TBST buffer 3 times each time for 5 min and incubated in TBS buffer containing diluted 1/10,000 goat anti-rabbit IgG conjugated with alkaline phosphatase (ZYMED) at room temperature with shaking for 1 h. The membrane was washed as described above. The expected band of the CP was visualized by adding NBT/BCIP substrate (ZYMED) for alkaline phosphatase.

B. Study of CP sequence in transgenic papaya and PRSV

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

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

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2. Isolation of PRSV-CP gene from infected papaya leaves

a) Extraction of total RNA

Total RNA was extracted from PRSV-infected fresh leaves. One gram of leaves was ground in liquid nitrogen then transfer the powder into 1.5 ml micro-centrifuge tube and added 650 µl of TLES buffer (100 mM Tris HCl, 100 mM LiCl, 100 mM EDTA and 1% SDS) with 2 % Na_2SO_3 then followed with an equal volume of phenol: chloroform: isoamyl (25:24:1). The mixture was mixed by vortex for 2 min. Supernatant was harvested by centrifugation at 12,000 rpm, 4°C for 10 min. DNA pellet was precipitated by adding 450 µl of 4M LiCl then incubated at 4°C for 16 h and centrifugation at 12,000 rpm, 4°C for 10 min. DNA pellet was resuspended with RNase-free water and incubated at 37°C for 30 min. The mixture was precipitated with 250 µl of absolute ethanol and 10 µl of 2.5 M CH_3COONa, pH 6.0 and incubated at -20°C for 30 min. DNA pellet was precipitated by centrifugation at 12,000 rpm, 4°C for 10 min and rinsed with 70% ethanol followed by centrifugation at 12,000 rpm, 4°C for 10 min. DNA pellet was dried and resuspended with RNase-free water and kept at -20°C until use.

b) Preparation of CP-cDNA of PRSV

Total RNA from PRSV-infected fresh leaves was used as template for complementary DNA (cDNA) synthesis. The cDNA was synthesized by Revert Aid™ First strand cDNA Synthesis kit followed by manual of production then amplified by PCR technique as described above (B).

3. Ligation of PCR product into plasmid cloning vector

DNA template was used for ligation with cloning vector (pGEM®-T Easy vector). Reaction of ligation was composed of 1X Rapid Ligation Buffer (Promega), 50 ng of pGEM®-T Easy vector , 2 µl of PCR product (molar ratio of PCR product:vector is 3:1), 3 Weiss unit of T_4 DNA ligase (Promega) and followed by adding distilled

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water to adjust the total volume in 10 µl. The mixture was incubated at 4°C for 16-18 h. Then, the mixture was transformed into competent cells by heat-shock transformation.

4. Heat-shock transformation of the ligation mixture into competent cells

The ligation mixture from step 3 was mixed with the competent cells on ice for 30 min, incubated at 42°C for 1 min and immediately placed on ice for 5 min. The competent cells were grown in 1 ml of LB liquid medium at 37°C with shaking for 1 h. Cell pellet was harvested by centrifugation at 8,000 rpm for 1 min, resuspended in 100 µl of LB liquid medium, spread on solid LB medium containing 100 mg/ml of ampicillin and incubated at 37°C for 16-18 h. The colonies were picked and cultured in LB liquid medium for isolation of plasmid DNA.

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

Bacterial colonies were grown in LB liquid medium containing 100 mg/ml of ampicillin at 37°C with shaking for 16-18 h. Two millilitre of bacterial culture was centrifuged at 12,000 rpm for 1 min. Plasmid DNAs were extracted from bacterial transformants by innuPREP Plasmid Mini Kit (Analytik Jena) as described in the procedure.

6. Sequencing and sequence analysis

The sequences of transgene and PRSV-CP gene were analyzed using DNASTAR™ program (Lasergene) and compared to sequences in GenBank database. DNASTAR™ contained the following program subunits; EditSeq, SeqMan, MegAlign, PrimerSelect, GeneQuest, MapDraw and Protein.

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RESULTS AND DISCUSSION

Results

I. Analysis of CP-IR transgene by PCR technique

Three hundred and eighty seedlings of R_5 transgenic papaya from 13 fruits of line A44 were used to detect transgene by PCR with PRSV-CP specific primer. With specific primers for PRSV-CP gene and PCR technique, bands corresponding to 526 bp in size were amplified from R_5 transgenic and PRSV infected papaya. There was no such band observed in non-transgenic one (Figure. 1). The CP-transgene was detected in all 13 fruits of R_5 transgenic papayas except in line A44R5-T1F36 (73 out of 74) and A44R5-T25F3 (24 out of 25) contained CP gene (Table 1). Results indicated that the inserted transgene was successfully transferred to R_5 generation in 99.5% (378 of 380) plants tested.

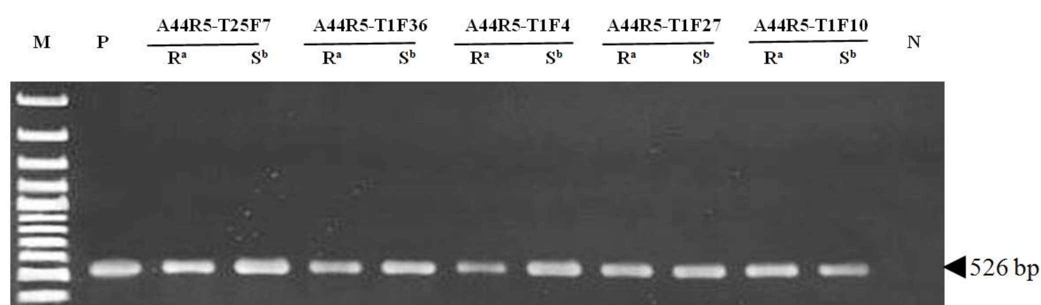

Figure 1 Agarose gel electrophoresis of PCR products from transgenic papaya plants. PCR products amplified from genomic DNA of R_5 seedlings. M: DNA molecular marker (100 bp plus DNA ladder), P: Positive control (R_4 transgenic papaya contained transgene), N: Non-transgenic papaya, R^a: PRSV-resistance transgenic plant and S^b: PRSV-susceptible transgenic plant.

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Table 1 Stability of CP-IR transgene in R_5 transgenic papaya

Fruits	#Tested plants	Transgene		
		+T	-T	%T
A44R5-T1F4	70	70	0	100
A44R5-T1F7	7	7	0	100
A44R5-T1F10	16	16	0	100
A44R5-T1F14	21	21	0	100
A44R5-T1F15	24	24	0	100
A44R5-T1F27	5	5	0	100
A44R5-T1F36	74	73	1	98.7
A44R5-T25F3	25	24	1	96
A44R5-T25F7	74	74	0	100
A44R5-T25F16	7	7	0	100
A44R5-T27F2	13	13	0	100
A44R5-T27F3	42	42	0	100
A44R5-T27F11	2	2	0	100
Total	380	378	2	99.5

+T = Number of R_5 transgenic papaya containing CP-IR transgene

-T = Number of R_5 transgenic papaya no present CP-IR transgene

%T = Percentage of CP-IR transgene inheritance in the fifth generation of transgenic papaya

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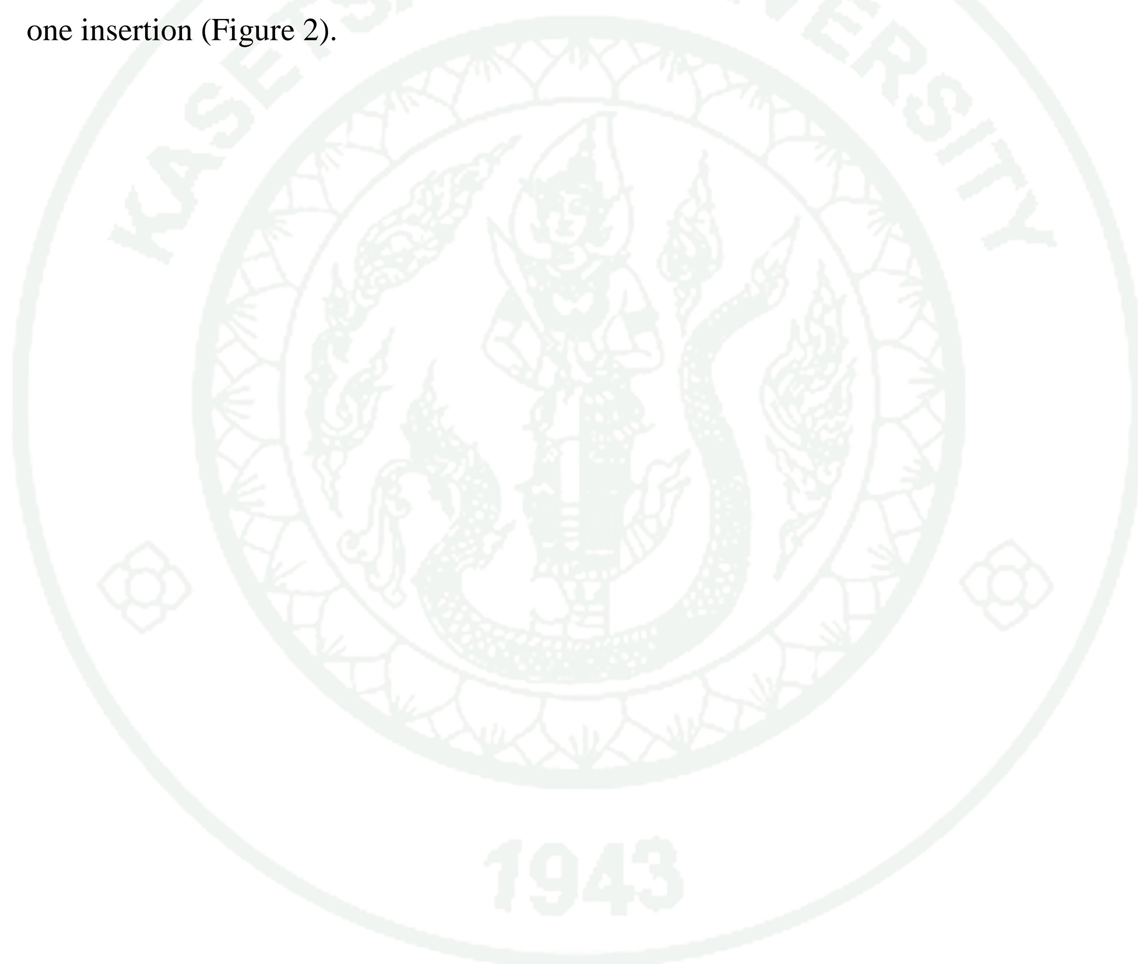
II. Analysis of copy number inserted in R_5 transgenic papaya genome by Southern hybridization

Numbers of insertion transgenes in papaya genome were determined by Southern hybridization. Five selected PRSV-resistant and susceptible transgenic plants were used for analysis of copy number inserted in papaya genome. Genomic DNA was completely digested with *Eco*RI and the small size DNA bands were generated. Result showed that the insertion number of transgene was 2 copies as indicated by one band for one insertion (Figure 2).



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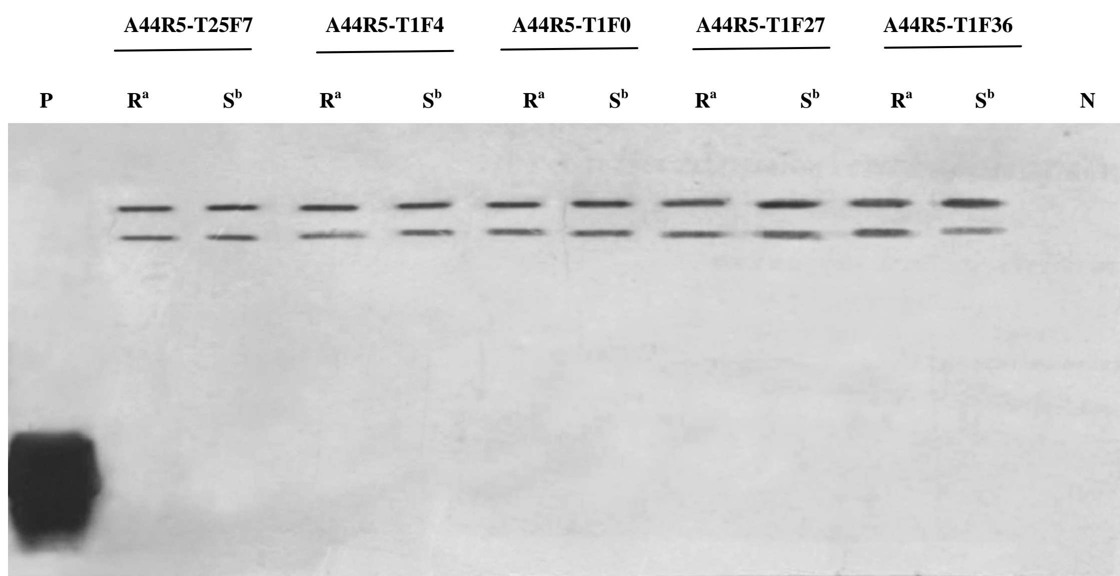

Figure 2 Southern blot analysis of genomic DNAs from non-transgenic and transgenic papaya digested with *Eco*RI and probed for CP-PRSV gene label with Digoxigenin, as determined by chemiluminescence with CDP star^{TM} substrate. All transgenic lines revealed two copies of transgene. P: PCR product of PRSV-CP gene, N: Non-transgenic papaya, R^a: PRSV-resistant transgenic plant and S^b: PRSV-susceptible transgenic plant.

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III. Evaluation of PRSV resistance

R_5 transgenic plantlets from 13 fruits were challenge-inoculated with PRSV under greenhouse condition (Figure 3A). Symptom was observed after 14 days of every inoculation and resistance to PRSV was confirmed by detection of PRSV coat protein using ELISA technique. Result showed that susceptible transgenic papaya and non-transgenic plant developed light chlorotic spot and systemic mosaic on new shoot after 14 days of the first inoculation (Figure 3B and 3C). Incontrast, the PRSV-resistance transgenic plants did not show any PRSV symptoms as well as non-transgenic uninoculated plants (Figure 3D). Thirty days after inoculation, there were transgenic plants (51.4%) that showed resistance to PRSV inoculation without symptom (Figure 4C) as compared to non-transgenic, uninfected planted plant (Figure 4A). However, 48.6% of transgenic plants developed disease symptom consisting of leaf distortion, systemic mosaic, and dwarfing (Figure 4D). They were classified as susceptible plants as compared to non-transgenic, infected plant (Figure 4B). A_{405} values of ELISA reading for the susceptible plants were greater than 0.3 (about 2 times of the A_{405} values of the healthy plants), while the A_{405} values of the transgenic resistant plant were less than 0.3 nearly to that values of coating buffer and healthy plants which were used as negative control (Table 2). Resistance to PRSV infection in R_5 transgenic papaya was reduced to nearly 50% in most of tested plants except in line A44-T27F2 where its resistance was the lowest at 23.1% (Table 2). An average resistance in R_5 transgenic papaya seedlings was 51.4%.

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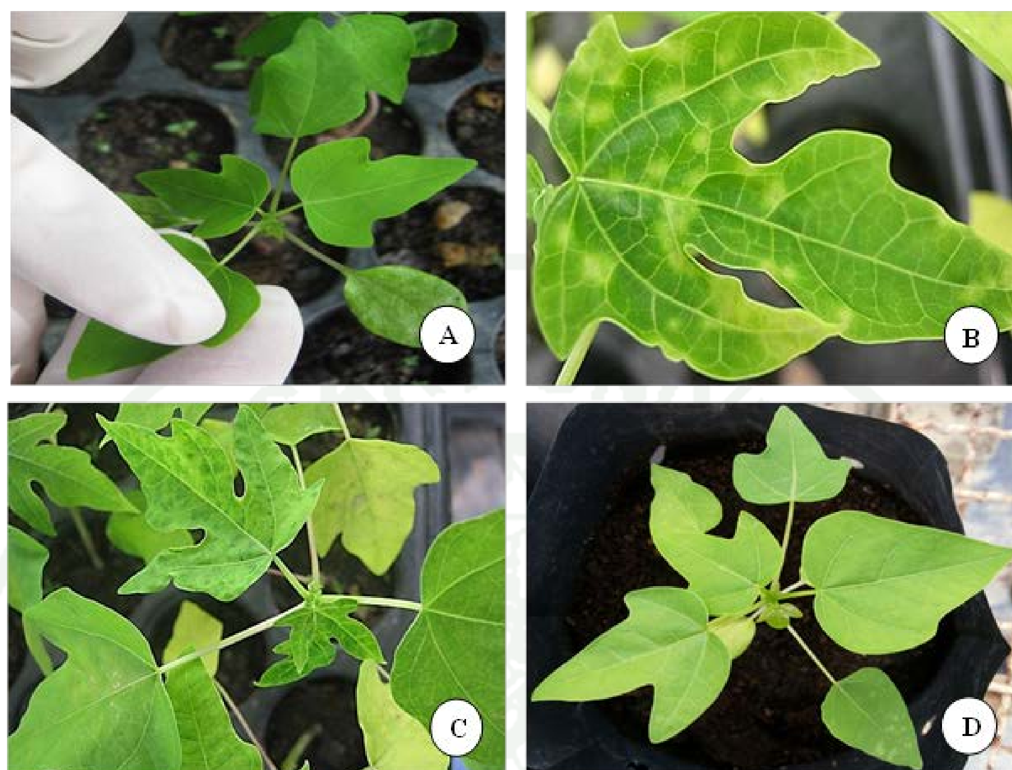

Figure 3 Mechanical inoculation and symptom expression of PRSV on papaya. Forty-five days-old seedlings of R_5 transgenic papaya were inoculated with PRSV-infected papaya leaf sap (A). The seedlings showed light chlorotic spot on young leaf (B) and systemic mosaic on shoot (C) at 14 days after inoculation. Non-transgenic uninoculated as negative control (D).

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Figure 4 The resistance and susceptibility to PRSV in R_5 transgenic papaya at 30 days after inoculation. An example of 51.4% R_5 transgenic papaya showed resistance to PRSV infection without any symptom (C) as well as non-transgenic uninfected (A). An example of 48.6% R_5 transgenic papaya showed susceptibility to PRSV infection with symptoms consisted of leaf distortion, systemic mosaic on leaf and dwarf (D) as well as non-transgenic infected (B).

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Table 2 Evaluation of PRSV-resistance in R_5 transgenic papaya by ELISA

Fruits	#Tested plants	PRSV resistance		
		R	S	%R
A44R5-T1F4	70	37	33	52.9
A44R5-T1F7	7	3	4	42.9
A44R5-T1F10	16	8	8	50.0
A44R5-T1F14	21	12	9	57.1
A44R5-T1F15	24	15	9	62.5
A44R5-T1F27	5	3	2	60.0
A44R5-T1F36	74	44	30	59.5
A44R5-T25F3	25	13	12	52.0
A44R5-T25F7	74	33	41	44.6
A44R5-T25F16	7	4	3	57.1
A44R5-T27F2	13	3	10	23.1
A44R5-T27F3	42	24	18	57.1
A44R5-T27F11	2	1	1	50.0
Total	380	200	180	51.4
Average of A_{405} reading of PRSV infected non-transgenic papaya = 0.125				
Average of A_{405} reading of non-infected papaya (Healthy) = 0.854				

R = Number of PRSV-resistant of R_5 transgenic papaya

S = Number of PRSV-susceptible of R_5 transgenic papaya

%R = Percentage of PRSV resistance of R_5 transgenic papaya

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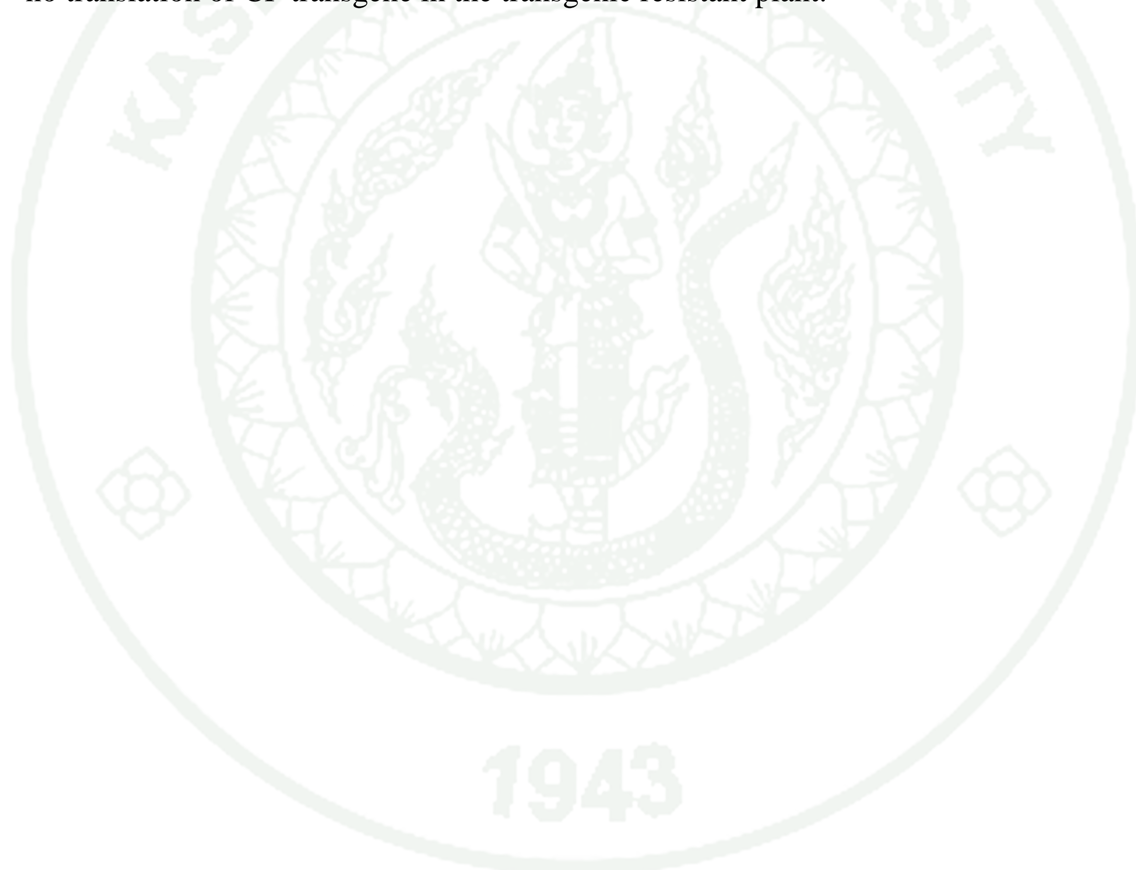
IV. Transgene expression in PRSV-resistant R_5 transgenic papaya

Transgene expression was focused on the accumulation of PRSV coat protein in resistant transgenic papayas. Five selected transgenic resistant lines namely, A44R5T25F7, A44R5-T1F36, A44R5- T1F4, A44R5-T1F27 and A44R5-T1F10 were determined for the expression of PRSV coat protein by western blot analysis with polyclonal antibody against the CP of PRSV. The expected band of the CP, approximately 32 kDa in size, was observed in PRSV infected non-transgenic papaya but not in the transgenic resistant plants (Figure 5). This result suggested that there was no translation of CP transgene in the transgenic resistant plant.



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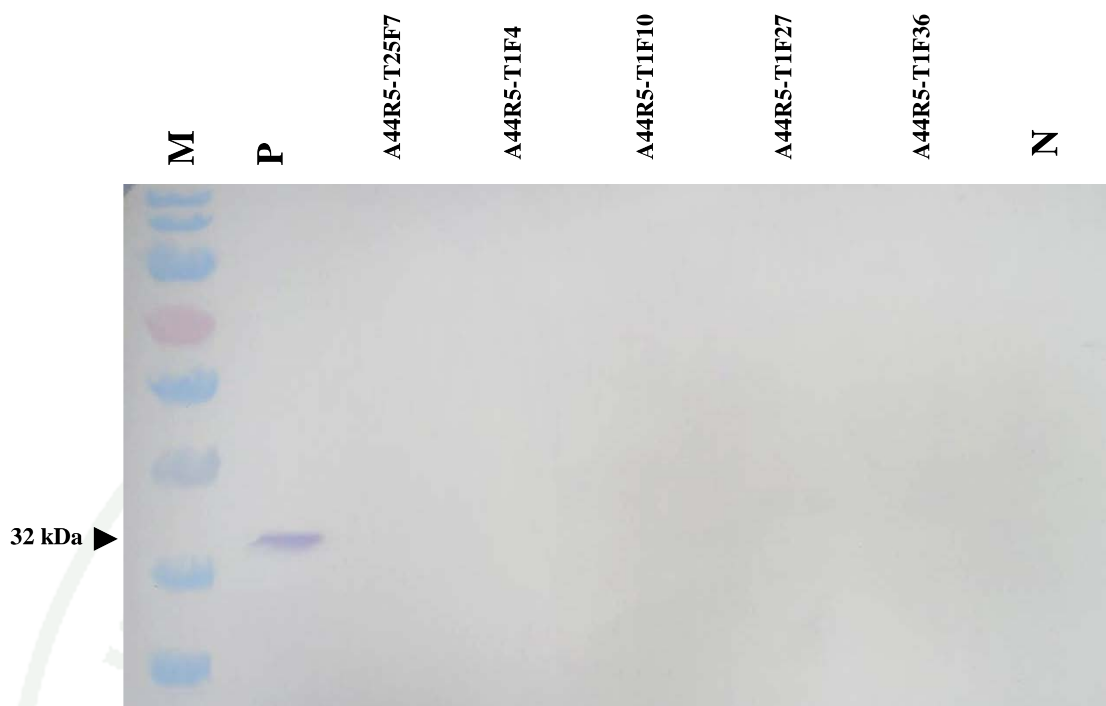

Figure 5 Western blot analysis of transgene expression in the PRSV-resistance of R_5 transgenic papaya. Protein band was detected by PRSV-CP polyclonal antibody. PRSV-resistant R_5 transgenic papaya showed no PRSV-CP transgene expression while in an infected non-transgenic papaya as a positive control showed PRSV-CP protein of 32 kDa in size (P). Non-transgenic uninoculated as a negative control did not show any PRSV-CP protein (N). Lane M as a protein ladder (Fermentus).

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V. Cloning of PRSV-CP gene from transgenic papaya and PRSV

By using DNASTAR™ program (Lasergene) and SC501 and SC104 primer, four clones of PRSV-CP transgene from transgenic papayas were obtained and subjected to nucleotide sequencing. The CPS1 and CPS2 nucleotide sequence were transgenes from susceptible transgenic lines and CPR1 and CPR2 were from resistant lines, respectively.

VI. Comparison of CP sequence in transgenic papaya and PRSV

A. Comparison of nucleotide and amino acid sequence of transgene in PRSV resistant and susceptible transgenic papayas

From multiple alignments there was no significantly difference in nucleotide sequence of 526 bp of inserted transgene in all tested lines(Figure 6). PRSV resistant and susceptible lines showed 99.6 to 100% nucleotide identities (Table 3). Multiple alignments of amino acid sequence derived from inserted transgene also showed no significantly difference among PRSV resistant and susceptible lines (Figure 7).

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CPS1      AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGAT 60
CPS2      AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGAT 60
CPR1      AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGAT 60
CPR2      AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGGGAT 60
          ******************************************************** ***

CPS1      GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCCGGTGT 120
CPS2      GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCTGGTGT 120
CPR1      GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCTGGTGT 120
CPR2      GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCTGGTGT 120
          ****************************************************** *****

CPS1      CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
CPS2      CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
CPR1      CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
CPR2      CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
          ************************************************************

CPS1      TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
CPS2      TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
CPR1      TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
CPR2      TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
          ************************************************************

CPS1      CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
CPS2      CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
CPR1      CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
CPR2      CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
          ************************************************************

CPS1      TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
CPS2      TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
CPR1      TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
CPR2      TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
          ************************************************************

CPS1      TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
CPS2      TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
CPR1      TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
CPR2      TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
          ************************************************************

CPS1      AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
CPS2      AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
CPR1      AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
CPR2      AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
          ************************************************************

CPS1      GGAAGATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
CPS2      GGAAGATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
CPR1      GGAAGATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
CPR2      GGAAGGTGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
          ***** ****************************************
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Figure 6 Multiple alignment of the 526 bp PRSV-CP transgene nucleotide sequences from transgenic papayas. CPS1 and CPS2 are from PRSV-susceptible lines 1 and 2, respectively. CPR1 and CPR2 are from PRSV-resistant transgenic lines 1 and 2, respectively. The difference in positions nucleotide sequence are highlighted.

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```
CPS1            KWYEGVRNDYGLNDNEMQVMLNGLMVWCVENGTSPDISGVWVMMDGETQVDYPIKPLIEH 60
CPS2            KWYEGVRNDYGLNDNEMQVMLNGLMVWCVENGTSPDISGVWVMMDGETQVDYPIKPLIEH 60
CPR1            KWYEGVRNDYGLNDNEMQVMLNGLMVWCVENGTSPDISGVWVMMDGETQVDYPIKPLIEH 60
CPR2            KWYEGVRNDYGLNDNEMQGMLNGLMVWCVENGTSPDISGVWVMMDGETQVDYPIKPLIEH 60
                ****************** *****************************************

CPS1            ATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLARYAFDFYEVNSKTPD 120
CPS2            ATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLARYAFDFYEVNSKTPD 120
CPR1            ATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLARYAFDFYEVNSKTPD 120
CPR2            ATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLARYAFDFYEVNSKTPD 120
                ************************************************************

CPS1            RAREAHMQMKAAALRNTSRRMFGMDGSVSNKEENTERHTVEDVNRDMHSLLGMRN 175
CPS2            RAREAHMQMKAAALRNTSRRMFGMDGSVSNKEENTERHTVEDVNRDMHSLLGMRN 175
CPR1            RAREAHMQMKAAALRNTSRRMFGMDGSVSNKEENTERHTVEDVNRDMHSLLGMRN 175
CPR2            RAREAHMQMKAAALRNTSRRMFGMDGSVSNKEENTERHTVEGVNRDMHSLLGMRN 175
                ***************************************** *************
```

Figure 7 Multiple alignment of the 175 amino acid residues derived from PRSV-CP transgene in transgenic papayas. CPS1 and CPS2 are from PRSV-susceptible lines 1 and 2, respectively. CPR1 and CPR2 are from PRSV-resistant transgenic lines 1 and 2, respectively. The difference in positions of aminoacid sequence are highlighted.

Table 3 Comparison of percent identities between transgene nucleotide sequences of PRSV in resistant and susceptible transgenic papayas

	CPR1	**CPR2**	**CPS1**	**CPS2**
CPR1				
CPR2	99.8			
CPS1	99.9	99.7		
CPS2	100.0	99.8	99.9	

CPR1, CPR2 = PRSV resistant plants

CPS1, CPS2 = PRSV susceptible plants

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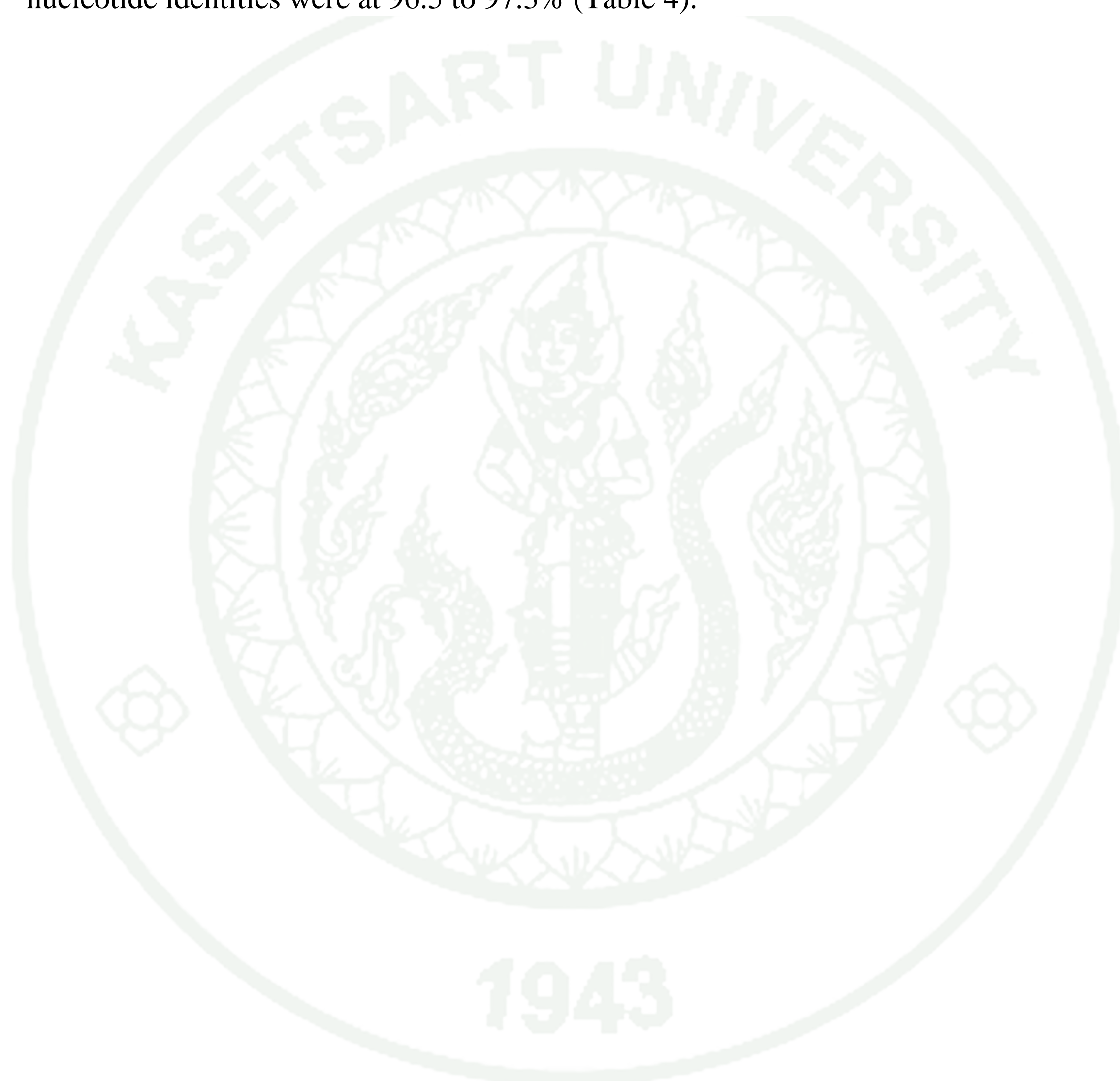
B. Comparison of nucleotide sequences of the PRSV-CP gene with PRSV-CP transgene in transgenic papaya

Multiple alignments of nucleotide sequences of PRSV-CP transgenes in resistant (CPR1 and CPR2) and susceptible (CPS1 and CPS2) transgenic lines indicated that they were no obvious difference from those of PRSV (Figure 8). Their nucleotide identities were at 96.5 to 97.3% (Table 4).



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```
CPR2          AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGGGAT 60
CPS2          AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGAT 60
CPR1          AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGAT 60
CPS1          AAAGTGGTATGAGGGAGTGAGGAATGATTACGGTCTTAATGATAACGAAATGCAAGTGAT 60
G2-1          AAAGTGGTATGAGGGAGTGAGGAATGATTATGGTCTTAATGACAAAGAAATGCAAGTGAT 60
              ****************************** *********** ** ********** ***

CPR2          GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCTGGTGT 120
CPS2          GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCTGGTGT 120
CPR1          GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCTGGTGT 120
CPS1          GTTAAATGGTTTGATGGTTTGGTGCGTCGAAAATGGAACATCCCCAGACATATCCGGTGT 120
G2-1          GTTAAATGGTTTGATGGTTTGGTGCATCGAAAATGGAACATCCCCGGACATATCTGGTGT 120
              ************************* ******************* ******** *****

CPR2          CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
CPS2          CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
CPR1          CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
CPS1          CTGGGTGATGATGGATGGGGAAACTCAAGTCGATTATCCCATCAAGCCTTTGATCGAACA 180
G2-1          CTGGGTGATGATGGATGGGGAATCCCAAGTCGATTATCCAATCAAACCTTTAATCGAACA 180
              ********************** * ************** ***** ***** ********

CPR2          TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
CPS2          TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
CPR1          TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
CPS1          TGCAACTCCTTCGTTCAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
G2-1          TGCAACTCCTTCGTTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAGGCATACAT 240
              *************** ********************************************

CPR2          CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
CPS2          CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
CPR1          CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
CPS1          CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
G2-1          CGCAAAGAGGAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGGAATTTGAC 300
              ************************************************************

CPR2          TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
CPS2          TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
CPR1          TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
CPS1          TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAGACACCTGA 360
G2-1          TGACATTAGTCTCGCTAGATATGCTTTCGACTTCTATGAGGTGAACTCAAAAACACCTGA 360
              *************************************************** ********

CPR2          TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
CPS2          TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
CPR1          TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
CPS1          TAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTAGTCGCAG 420
G2-1          CAGGGCTCGTGAAGCTCATATGCAGATGAAGGCTGCAGCGCTGCGCAACACTGGTCGCGG 420
               *************************************************** ***** *

CPR2          AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
CPS2          AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
CPR1          AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
CPS1          AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
G2-1          AATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACACAGT 480
              ************************************************************

CPR2          GGAAGGTGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
CPS2          GGAAGATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
CPR1          GGAAGATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
CPS1          GGAAGATGTTAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
G2-1          GGAAGATGTCAACAGAGACATGCACTCTCTCCTAGGTATGCGCAAT 526
              ***** *** ************************************
```

Figure 8 Multiple alignment of the transgene nucleotide sequences in R_5 transgenic papaya genome and a part of PRSV-CP nucleotide sequences. The highlights represent the different position of nucleotide sequences. CPS1 and CPS2 are transgene nucleotide sequences from PRSV-susceptible transgenic papaya

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clone number 1 and 2, respectively. CPR1 and CPR2 are transgene nucleotide sequences from PRSV-resistant transgenic papaya clone number 1 and 2, respectively. G2-1 is nucleotide sequences of CP gene of PRSV that used for screening the resistance for R_5 transgenic papaya.

Table 4 Comparison of percent identities between PRSV-CP transgene nucleotide sequences in R_5 transgenic papayas (CPR1-2 and CPS1-2) and 526 bp nucleotide sequences of CP gene of PRSV (G2-1).

	CPR1	CPR2	CPS1	CPS2	G2-1
CPR1					
CPR2	99.6				
CPS1	99.8	99.4			
CPS2	100.0	99.6	99.8		
G2-1	96.9	96.5	96.7	96.9	

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C. Comparison of nucleotide sequences of the CP gene of PRSV collected from Kamphaeng Saen, Nakorn Pathom with other PRSV isolates in Thailand

Nucleotide sequences of CP gene of sixteen PRSV isolates from Thailand were used to multiple align with G2-1, PRSV isolates that used for screen virus resistance for R_5 transgenic papaya (Table 5). The phylogenetic tree (Figure 9) showed a branching pattern of relation between the 16 isolates of PRSV coat protein nucleotide sequences in other areas of Thailand and PRSV coat protein nucleotide sequences collected at Kamphaeng Saen, Nakorn Pathom (G2-1). Based on the branching pattern of the tree, the 18 isolates of PRSV aligned could be divided into two clusters. PRSV isolates from Kamphaeng Saen formed their own phylogenetic branch within the same cluster of PRSV isolates SMK, KJR, PKT, CM1, CM2, KK1, CP, SBR, CB1, CB2, and BK with the similar ranges of identities 93.7 to 97.8% (Table 6).

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Table 5 Sequences accession numbers of coat protein of PRSV isolates from Thailand in NCBI database

PRSV isolate	Source	Accession number
BK	Bangkok	AY010712.1
CB1	Chonburi	AY010715.1
CB2	Chonburi	AY010716.1
CM1	Chiangmai	AY010719.1
CM2	Chiangmai	AY010720.1
CP	Chumphon	AY010713.1
KJR	Kanchanaburi	DQ085859.1
KK1	Khonkaen	AY010714.1
KK2	Khonkaen	DQ085860.1
KPS	Kamphaeng Saen	AF374862.1
NPT	Nakorn Pathom	DQ085861.1
PKT	Phuket	DQ085862.1
RB	Ratchaburi	AY010721.1
ROI	Roi-et	DQ085863.1
SBR	Saraburi	DQ085865.1
SMK	Samutsakorn	DQ085864.1

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Table 6 Percent identities between coat protein gene of PRSV nucleotide sequences in NCBI database and those of PRSV isolates (G2-1) used for screening resistance for R_5 transgenic papaya.

	BK	**CB1**	**CB2**	**CM1**	**CM2**	**CP**	**G2-1**	**KJR**	**KK1**	**KK2**	**KPS**	**NPT**	**PKT**	**RB**	**ROI**	**SBR**	**SMK**
BK																	
CB1	98.2																
CB2	98.3	99.9															
CM1	96.8	97.3	97.4														
CM2	97.4	97.7	97.8	98.2													
CP	97.3	97.4	97.6	97.1	97.4												
G2-1	97.3	97.4	97.6	96.2	96.8	96.7											
KJR	95.5	95.8	95.9	96.3	96.7	95.8	94.9										
KK1	97.4	97.6	97.7	97.2	97.6	99.9	96.8	95.9									
KK2	96.0	96.4	96.5	96.3	96.4	96.3	95.3	94.7	96.4								
KPS	97.2	97.3	97.4	96.7	97.3	96.9	96.2	95.5	97.1	96.7							
NPT	94.6	95.0	95.1	94.6	95.1	94.7	93.7	93.8	94.9	94.9	97.3						
PKT	96.2	96.5	96.7	96.4	96.8	96.2	95.7	95.6	96.3	95.1	96.3	94.0					
RB	97.7	97.8	97.9	97.1	97.8	97.4	96.8	95.8	97.6	96.4	97.8	95.3	96.3				
ROI	96.2	96.3	96.4	95.7	96.3	95.9	95.4	94.6	96.1	95.7	98.1	95.5	95.5	97.2			
SBR	96.8	97.2	97.3	96.2	96.9	96.3	96.3	94.8	96.4	95.8	96.3	94.2	95.2	96.8	95.5		
SMK	96.9	97.2	97.3	97.8	98.1	97.4	96.3	97.0	97.6	96.2	96.9	96.2	96.8	97.2	95.9	96.3	

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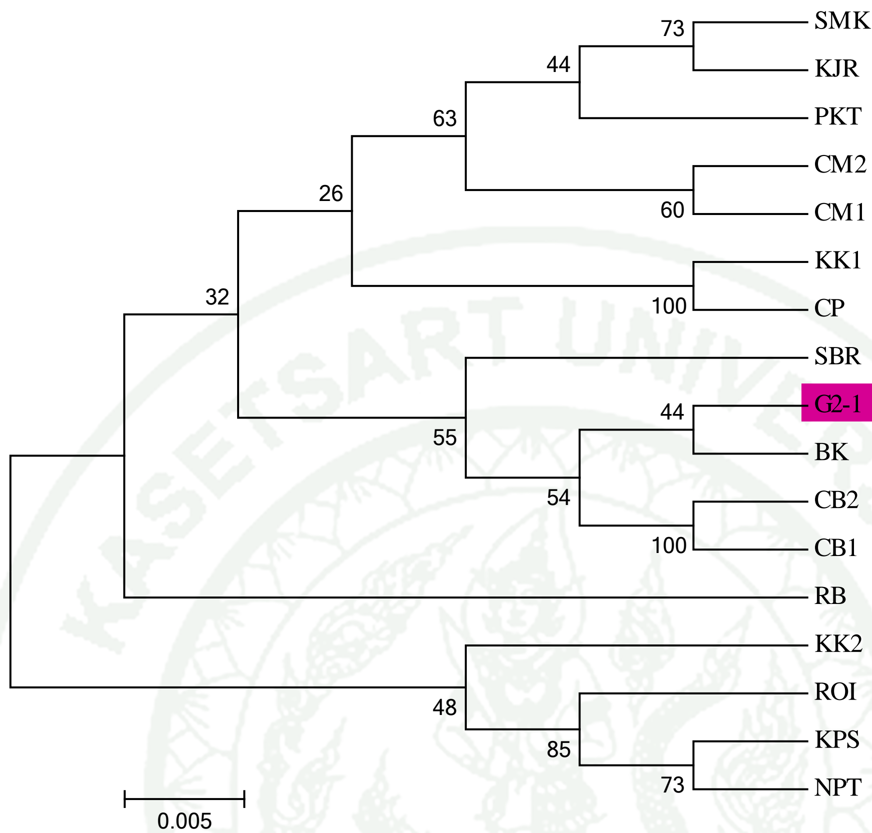

Figure 9 Phylogenetic trees constructed from the multiple alignments of the nucleotide sequences of PRSV-CP of 16 PRSV isolates from NCBI database and PRSV isolates (G2-1) used for screening the resistance for R_5 transgenic papaya. Detailed description of PRSV isolates is listed in Table 5.

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Discussion

This study clearly demonstrated the stability of transgene inheritance, from generation to generation via PCR technique. Xiangdong *et al*. (2007) reported similar result on the stability of transgene in T_4 generation of transgenic papaya in China. Therefore, the stability of transgene should not be an issue on biosafety and risk assessment for commercialization. However, these results point to an unforeseen problem, namely the sharp reduction in resistance to PRSV to about a half (51.4%) by propagation to the R_5 generation. There was no difference among resistant and susceptible lines in term of transgene inheritance and copy number of inserted gene. Lui *et al.* (2003) have also shown that there was no correlation between copy number of inserted gene and level of virus resistance.

The mechanism of resistance to PRSV in transgenic papaya has been previously proposed as a post-transcriptional gene silencing (PTGS) (Ruanjan *et al*., 2007). We have demonstrated in this study that there was no PRSV coat protein expressed in transgenic papaya (Figure 5). ELISA tests also confirmed an absence of PRSV protein in transgenic resistant plant ($O.D._{405} = 0.125$) while the ELISA readings in PRSV infected non-transgenic susceptible plant were at 0.854 (Appendix table B1). Detailed molecular analysis on transgene and viral genome that may affect transgene silencing is needed to elucidate the remaining question.

Kasemsin (2007) challenge inoculated forty-six plants of R_1 generation transgenic papaya line A44 with PRSV-SB and found that thirty-four plants (73.9%) were resistance but 12 plants (26.1%) were systemic infected after 14 days of the first inoculation. Transgene segregation was found at 74% (Kasemsin, 2007). Although segregation of transgene was unstable in the first generation, virus resistance was also shown to vary from 54% (30 of total 56) and 79% (26 of total 33) in R_3 and R_4 generation of transgenic papaya, respectively (data not show). For R_5 generation, 200 of the total 380 plants were resistant to PRSV (51.4% virus resistance) with 99.5% of

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transgene segregation. It is concluded that inheritance of transgene in R_5 generation has no direct correlation with PRSV resistance.

Southern blot analysis for determination of copy number of transgene in R_5 transgenic papaya showed two copies of CP-IR transgene in both PRSV resistance and susceptible transgenic papayas similar to R_0 generation of line A44 transgenic plants that contain CP-IR transgene (Kasemsin, 2007). Our result indicated that virus resistance or susceptible characters do not correlate with copy number of transgene. It can only show that CP-IR transgene was integrated into papaya genome and can be inherited into the fifth generation. Demeke *et al.*(1999) also observed the inheritance of transgene in T_4 and T_5 transgenic wheat lines with no evidence of transgene rearrangement.

In an absence of PRSV coat protein, the mechanism of resistance is therefore not applicable for protein-mediated resistance. To proof that resistance is derived from RNA-mediated, small RNAs should be detected. In our studies, we were not able to detect small RNAs due to limited facility.

Comparison of nucleotide sequences of transgene in PRSV resistance with those of PRSV susceptible transgenic papaya genome showed no significantly difference indicating no new rearrangement of the transgene and the virus resistance was not correlated to the nucleotide sequences of transgene in transgenic papaya genome. However, the length of transgene used in this study was too small to suggest any other event that might take place in transgenic plants.

Although the biosafety of GM plants has been a hot topic and has caused a growing public concern around the world since their first emergence in the 1980s, it is still not known whether GM plants and their products have effects on the environment and food (Fitch *et al*., 1992). However, transgenic papaya has not caused any biosafety problems since CP transgenic papaya was commercialized in Hawaii since 1998. Our results from western blot hybridization confirmed no viral protein

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expression in R_5 transgenic papayas. It could clearly eliminate the public concern on health risk based on consuming foreign protein in transgenic food products, especially transgenic papaya.

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CONCLUSION

This study has demonstrated that the transgene (PRSV-CP) in transgenic papaya inherited in R_5 generation at 99.5%. The inheritance of the transgene did not correlate with the resistance to PRSV infection. The percentages of virus resistance of R_5 generation were 51.4%. The PRSV-resistance transgenic papaya did not express viral protein in their cellular. Preliminary analysis of transgene nucleotide sequence showed no significantly difference in resistant and susceptible lines. The PRSV isolates used in the screening for resistance shared high percentages of nucleotide identities at 93.7-97.8% with other including SMK, KJR, PKT, CM1, CM2, KK1, CP, SBR, CB1, CB2 and BK, It is suggested that the performance of an advance generation of transgenic papaya for PRSV resistance may follow the same pattern. To improve the resistance in subsequent generation of papaya is therefore essential for sustainable application of transgenic PRSV resistant papaya.

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LITERATURE CITED

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

Asurmendi, S., R. H. Berg, T. J. Smitha, M. Bendahmance and R. N. Beachy. 2007. Aggregation of TMV CP plays a role in CP functions and in coat protein-mediated resistance. **Virolog**y 366: 98-106.

Bau, H. J., Y. H. Cheng, T. A. Yu, J. S. Yang and S. D. Yeh. 2003. Broad-spectrum resistance to difference geographic strains of *Papaya ringspot virus* in coat protein gene transgenic papaya. **Phytopathology** 93: 112-120.

Bendahmance, M., I. Chen, S. Asurmendi, A. A. Bazzini, J. Szecsi and R. N. Beachy. 2007. Coat protein-mediateed resistance to TMV infection of *Nicotianatabacum* involves multiple modes of interference by coat protein. **Virology** 366: 107-116.

Brumin, M., S. Stukalov, S. Haviv, M. Muruganantham, Y. Moskovitz, O. Batuman, A. Fenigstein and M. Mawassi. 2009. Post-transcriptional gene silencing and virus resistance in *Nicotianabenthamiana* expressing a *Grapevine virus* Aminireplicon. **Transgenic Research** 18: 331-345.

Costa, A. S. and G. W. Muller. 1980. Tristeza control by cross protection: a U. S. - Brazil cooperative success. **Plant Dis**. 64: 538–41.

Davidson, S. N. 2008. Forbidden Fruit: Transgenic Papaya in Thailand. **Plant Physiology** 147: 487-493.

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Demeke, T., P. Hucl, M. Båga, K. Caswell, N. Leung and R. N. Chibbar. 1999. Transgene inheritance and silencing in hexaploid spring wheat. **Theor Appl Genet**. 99: 947-953.

Diaz-Pendon, J. A. and S-W- Ding. 2008. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. **Annual Review of Phytopathology** 46: 303-326.

Ding, S. W. and O. Voinnet. 2007. Antiviral immunity directed by small RNAs. **Cell** 130: 413-426.

Eamens, A., M-B Wang, N. A. Smith and P. M. Waterhouse. 2008. RNA Silencing in Plants: Yesterday, Today, and Tomorrow. **Plant Physiology** 147: 456-468.

Fitch, M. M. M., R. M.. Manshardt, D. Gonsalves, L. Jerry, J. L. Slightom,and J. C. Sanford. 1992. Virus resistant papaya plants derived from tissues bombarded with coat protein gene of papaya ringspot virus. **Biotechnology** 10: 1466–1472.

Gonsalves, D. 1998. Control of *Papaya ringspot virus* in papaya: A case study. **Phytopathol** 36: 415-437.

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

Habibuddin, H., V. K. Abubakar, C. S. Tan, C. A. Ong, V. Pillai and A. N. Suhana. 2007. Evaluation of the transgenic Eksotika papaya lines for resistance against the *Papaya Ringspot Virus* in Malaysia. Unpublished country progress report presented at Papaya Biotechnology Network of Sutheast Asia Technical and Coordination Meeting held in Makati City and Los Banos, Laguna Phillipines, June 14-15, 2007.

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Horaruang, W., K. Romyanon, M. Boomthum, S. Flansinki and S. Attathom. 2006. Effect of pre-culture condition on *Agrobacterium*-mediated transformation in papaya. **Agricultural Science Journal** 37(3): 231-239.

Hu, Q., Y. Niu, K. Zhang, T. Liu and X. Zhou. 2011. Virus-derived transgenes expressing hairpin RNA give immunity to *Tobacco mosaic virus* and *Cucumber mosaic virus.* **Virology Journal** 8(41): 11pp.

Hull, R. 2002. **Matthews' Plant Virology**. Academic Press. San Diego, USA.

Jensen, D. D. 1949. Papaya virus diseases with special reference to papaya ringspot. **Phytopathology** 39:191–211.

Jones, A. L., I. E. Johansen, S. J. Bean, I. Bach and A. J. Manle. 1998. Spacificity of resistance to pea seed-borne mosaic potyvirus in transgenic peas expressing the viral replicase (NIb) gene. **Journal of General Virology** 79: 3129-3127.

Kasemsin, P. 2007. **Screening for *Papaya ringspot virus* (PRSV) resistance and analysis of transgenes in transgenic papayas**. M. S. Thesis, Kasetsart University.

Kalantidis, K., H. T. Sohumacher, T. Alexiadis and J. M. Helm. 2008. RNA silencing movement in plants. **Biology of the cell** 100: 13-26.

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

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Koo, J. C., S. Asurmendi, J. Biek, T. Woodford-Thomas and R. N. Beachy. 2004. Ecdysone agonist-inducible expression of a coat protein gene from *tobacco mosaic virus* confer viral resistance in transgenic Arabidopsis. **Plant J.** 37(3): 439-448.

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

Kotakis, C., N. Vrettos, D. Kotsis, M. Tsagris, K. Kotzabasis and K. Kalantidis. 2010. Light intensity affects RNA silencing of transgene in *Nicotiana benthamiana* plants. **BMC Plant Biology** 10(220): 11pp.

Lin, S. S., R. Henriques, H. W. Wu, Q. W. Niu, S. D. Yeh and N. H. Chua. 2007. Strategies and mechanisms of plant virus resistance. **Plant Biotechnol Rep** 1: 125–134.

Liu, Y. S., J. S. Levin, J. S. Murray, E. A. Wernsman and A. K. Weissinger. 2003. A multi-generation analysis of the stability of transgenic virus resistance in doubled-haploid tobacco lines. **Molecular Breeding** 12: 145-156.

Lius, S., R. M. Manshardt, M. M. Fitch, J. L. Slightom, J. C. Sanford and D. Gosalves. 1997. Pathogen-derived resistance provide papaya with effective protection against *papaya ringspot virus*. **Molecular Breeding** 3: 161-168.

Lomonossoff, G. P. 1995. Pathogen-derived resistance to plant viruses. **Annu. Rev. Phytopathol** 33: 323–43.

Lu, B., G. Stubbs and J. N. Culver. 1998. Coat protein interactions involved in tobacco mosaic tobamovirus cross-protection. **Virology** 248: 188-198.

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Lui, Y. S., J. S. Levin, J. S. Murray, E. A. Wernsman and A. K. Weissinger. 2003. A multi-generation analysis of the stability of transgenic virus resistance in doubled-haploid tobacco lines. **Molecular Breeding** 12: 145-156.

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

Manshardt, R. M. 1992. Papaya. 489–511 pp. *In* **Hammerschlag F.A. and Litz R.E. (eds), Biotechnology of Perennial Fruit Crops**. CAB International, Wallingford.

_________ and T. F. Wenslaff. 1989. Interspecific hybridization of papaya with other *Carica* species. **J Am Soc Hortic Sci** 114: 689–694.

Missiou, A., K. Kalantdis, A. Boutla, S. Tzortzakaki, M. Tabler and M. Tsagris. 2004. Generation of transgenic potato plants highly resistance to *Potato virus* Y (PVY) through RNA silencing. **Molecular Breeding** 14: 185-197.

Nethra, P., K. N. Nataraja, N. Rama and M. Udayakumar. 2006. Standardization of environmental conditions for induction and retention of posttranscriptional gene silencing using *tobacco rattle virus* vector. **Current Science** 90: 431-435.

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

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Prasartsri, V., A. Phungkeartphaibool and K. Chomphoonuchprapha. 1982. Preliminary studies of *papaya ringspot virus* in the Northeast in Thailand. **Annual Rep**. 15p.

Prins, M., M. Laimer, E. Noris, J. Schubert, M. Wassesegger and M. Tepfer. 2008. Strategies for antiviral resistance in transgenic plants. **Molecular plant pathology** 9(1): 73-83.

Purcifull, D. E., J. Edwardson, E. Hiebert and D. Gonsalves. 1984. *Papaya ringspot virus.* **Descriptions of Plant Viruses** 292: 8 pp.

Rast, A. T. B. 1975. Variability of tobacco mosaic virus in relation to control of tomato mosaic in glasshouse tomato crops by resistance breeding and cross protection. **Agric. Res. Rep.** 834: 1–76.

Retuta, A. M. O., P. M. Magdalita, E. T. Aspuria and R. R. C. Espino. 2012. Evaluation of selected transgenic papaya (*Carica papaya* L.) lines for inheritance of resistance to *papaya ringspot virus* and horticultural traits. **Plant Biotechnology** 29: 339–349.

Ritzenthaler, C. 2005. Resistance to plant viruses: old issue news answer?. **Current Opinion in Biotechnology** 16: 118-122.

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

Ronemus, M. W. Vaughn and R. A. Martienssen. 2006. Micro RNA-Targeted and Small Interfering RNA-Dependent RNA polymerase in *Arabidopsis*. **Plant cell** 18: 1559-1574.

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Ruanjan, P., S. Kertbundit and M. Juříček. 2007. Post-transcriptional gene silencing is involved in resistance of transgenic papayas to *papaya ringspot virus*. **Biologia plantarum** 51 (3): 517-520**.**

Ruiz-Ferrer, V. and O. Voinnet. 2009. Roles of Plant Smaal RNAs in Biotic Stress Responses. **Annual Review of Plant Biology** 60: 485-510.

Sakuanrungsirikul, S., N. Sarindu, V. Prasartsee, S. Chaikiatiyos, R. Siriyan, M. Sriwatanakul, P. Lekananon, C. Kitprasert, P. Boonsong, P. Kosiyachinda, G. Fermin and D. Gonsalves. 2005. Update on the development of virus-resistant transgenic papaya for people in rural communities of Thailand. **Food Nutr Bull** 26: 422-426.

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

Samson, J. A. 1986. **Tropical fruits**. 2nd ed. Longman Inc., New York, USA.

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

Savenkov, E. I. and J. P. T. Valkonen. 2001. Coat protein gene-mediated resistance to *Potato virus* A in transgenic plants is suppressed following infection with another potyvirus. **Journal of Gerneral Virology** 82: 2275-2278.

Srisomchai, T. 1975. A report of *papaya ringspot virus* in Thailand. **Annual Rep**. 5p.

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Shukla, D. D., C. W. Ward and A. A. Brunt. 1994. **The Potyviridae**. Wallingford, UK: CAB Int.

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

Szittya, G., D. Silhavy, A. Molnar, Z. Havelda, A. Lovas, L. Lakatos, Z. Banfalvi and J. Burgyan. 2003. Low temperature inhibits RNA silencing-mediated defense by the control of siRNA generatio. **EMBO Journal** 22: 633-640.

Tenllado, F. and J. R. Diaz-Ruiz. 1999. Complete resistance to pepper mild mottle tobamovirus mediated by viral replicase sequence partially depends on transgene homozygosity and is based on a gene silencing mechanism. **Transgenic Research** 8: 83-93.

Tenllado, F., C. Llave and J. R. Diaz-Roiz. 2004. RNA interference as a new technological tool for control of virus disease in plants. **Virus Research** 102: 85-96.

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

Van den Boogaart, T., F. Wen, J. W. Davies and G. P. Lomonossoff. 2001. Replicase-derived resistance against *pea early browning virus* in *Nicotiana benthamiana* is an unstable resistance based upon posttranscriptional gene silencing. **MPMI** 14(2): 196-203.

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Vassilakos, N. 2012. Stability of transgenic resistance against plant virus, 219-236 pp. *In* Y. O. Çiftçi, eds. **Transgenic Plants Advances and Limitations**. InTech.

Vassilakos, N., F. Bem, A. Tzima, H. Barker, B. Reavy, E. Karanastasi and D. J. Robinson. 2008. Resistance of transgenic tobacco plants incorporating the putative 57-kDa polymerase read-through gene of *Tobacco rattle virus* against rub-inoculated and nematode-transmitted virus. **Transgenic Research** 17: 929-41.

Vaucheret, H., L. Nussaume, J. C. Palauqui, I. Quillere and T. Elmayan. 1997. A transcriptionally active state is required for post-transcriptional silencing (cosuppression) of nitrate reductase host genes and transgenes. **Plant Cell** 9: 1495-1504.

Voloudakis, A. E., M. E. Aleman-Verdaquer, H. S. Padgett and R. N. Beachy. 2005. Characterization of resistance in transgenic *Nicotiana benthamiana*en coding N-terminal deletion and assembly mutant of the Tobacco etch potyvirus oat protein. **Arch Viral** 150: 2567-2582.

Walkey, D. G. A., H. Lecoq, R. Collier and S. Dobson. 1992. Studies on the control of zucchini yellow mosaic virus in courgettes by mild strain protection. **Plant Pathol.** 41:762–71.

Warin, N., S. Choapongpang, A. Bhuchoth, K. Romyanon, R. Rodaree, W. Kositratana and S. Attathom. 2003. New papaya cultivars for *papaya ringspot virus* resisitance, 539-546 p. *In* **The Proceedings of 41st Kasetsart University Annual Conference**. Bangkok.

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Xiangdong, W., L. Congyu, L. Zhijing and Y. Changming. 2007. Analysis on virus resistance and fruit qulity for T_4 generation of transgenic papaya. **Front.Biol.China** 2(3): 284-290.

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APPENDICES

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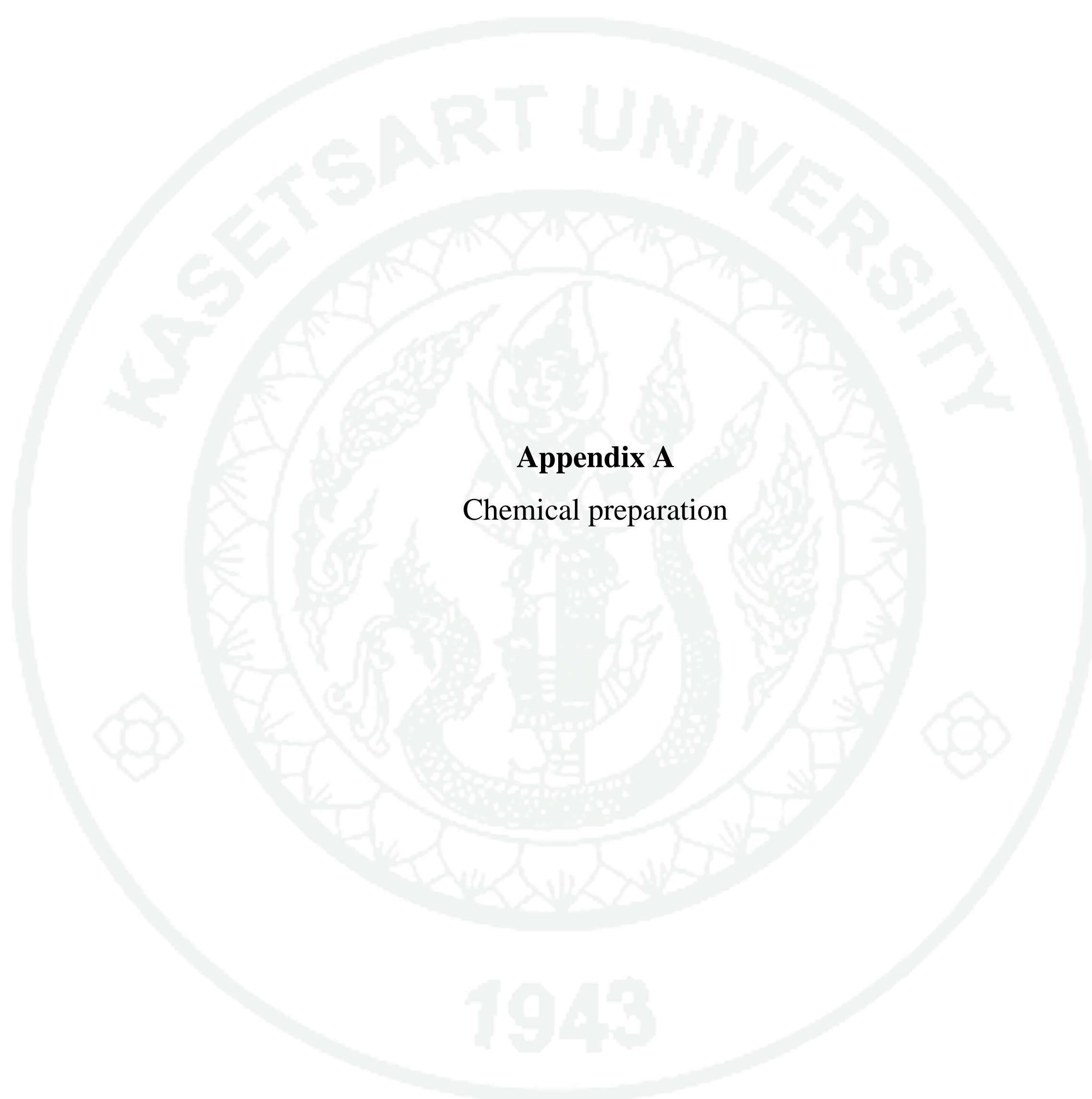

Appendix A

Chemical preparation

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Appendix Table A1 Composition of bacterium culture medium

Compound	LB medium	2X-YT medium	SOB medium
Bacto-tryptone	10 g/l	16 g/l	20 g/l
Bacto-tryptone	5 g/l	10 g/l	5 g/l
NaCl	10 g/l	5 g/l	-
Agar	15 g/l	15 g/l	-
1 M KCl	-	-	1 ml/l
1 M MgCl2	-	-	10 ml/l
1 M MgSO4	-	-	10 ml/l

Source: Sambrook *et a*l., (1989)

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

A. Preparation of 5X running buffer: 1000 ml

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

B. **Preparation of transfer buffer: 1000 ml**

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

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Appendix Table A2 Solutions for preparing 12% resolving gels for Tris-glycine SDS polyacrylamide gel electrophoresis

Solution components	Component volumes (ml) per gel mold volume of					
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml
H_2O	1.6	3.3	4.9	6.6	8.2	9.9
30% Acrylamide/Bis solution 19:1	2.0	4.0	6.0	8.0	10.0	12.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5
10% SDS	0.05	0.1	0.15	0.20	0.25	0.3
10% ammonium persulphate	0.05	0.1	0.15	0.20	0.25	0.3
TEMED	0.002	0.004	0.006	0.008	0.010	0.012

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

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Appendix Table A3 Solutions for preparing 5% stacking gels for Tris-glycine SDS polyacrylamide gel electrophoresis

Solution components	Component volumes (ml) per gel mold volume of					
	1 ml	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**
H_2O	0.68	1.4	2.1	2.7	3.4	4.1
30% Acrylamide/Bis solution 19:1	0.17	0.33	0.5	0.67	0.83	1.0
1.5 M Tris (pH 8.8)	0.13	0.25	0.38	0.5	0.63	0.75
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06
10% ammonium persulphate	0.01	0.02	0.03	0.04	0.05	0.06
TEMED	0.001	0.002	0.003	0.004	0.005	0.006

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

II. Material and Reagent for ELISA

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

Na_2CO_3 0.795 g

$NaHCO_3$ 1.465 g

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

B. 5X PBST: 1000 ml

NaCl 146.1 g

Na_2HPO_4 4.6 g

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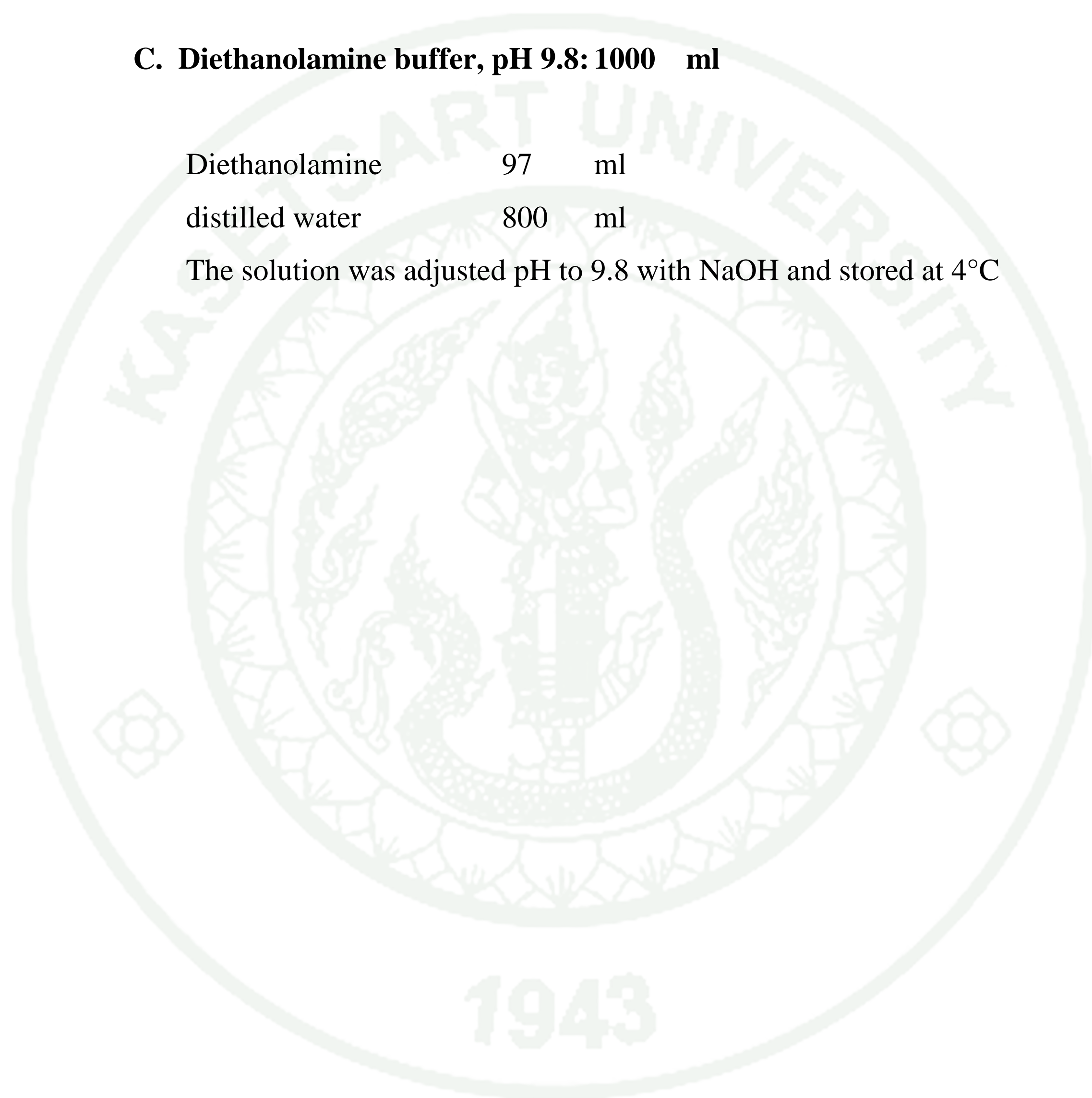
KH_2PO_4	1.0	g
Tween 20	2.5	g

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

C. Diethanolamine buffer, pH 9.8: 1000 ml

Diethanolamine	97	ml
distilled water	800	ml

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



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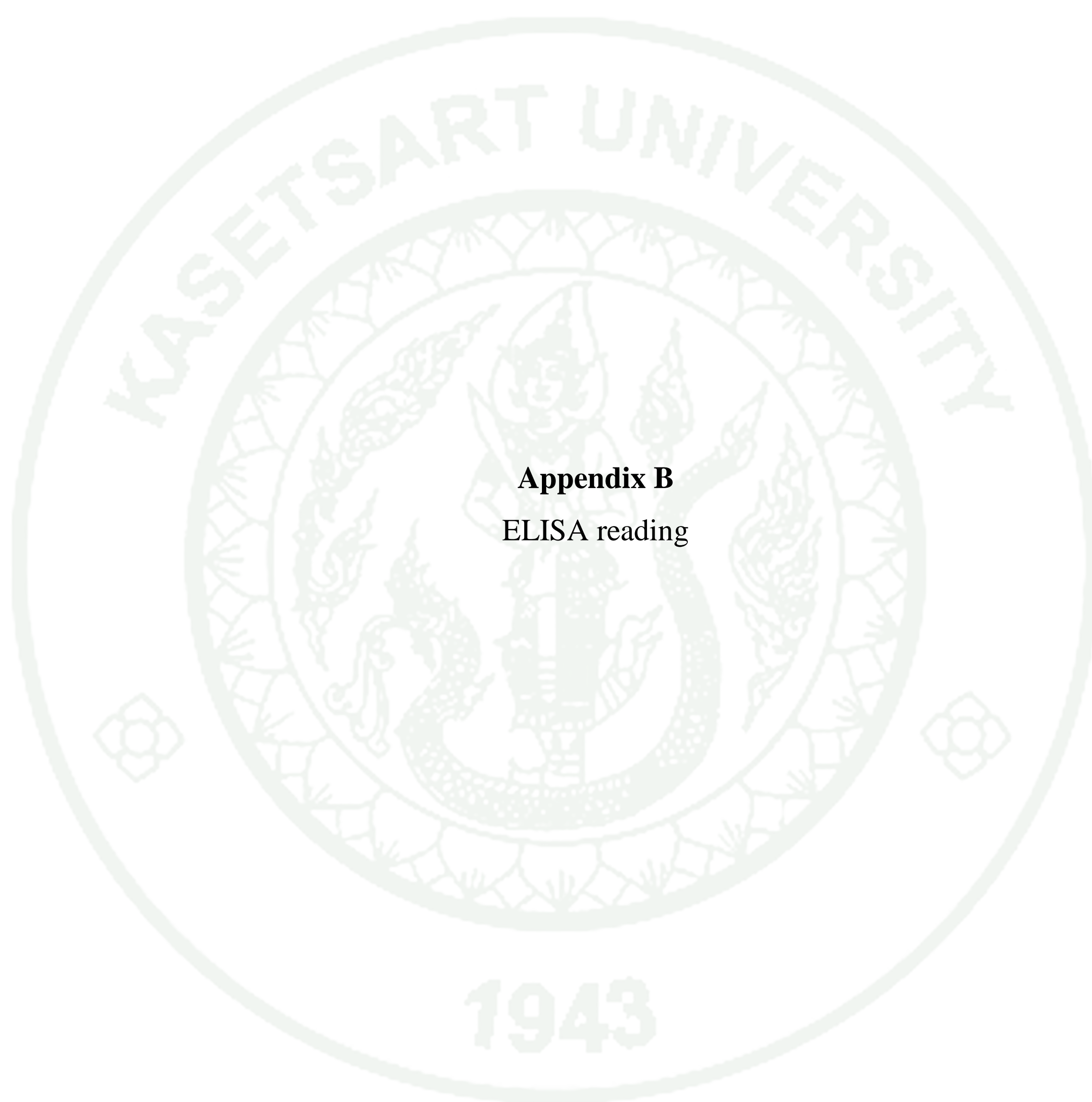

Appendix B

ELISA reading

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Appendix Table B1 ELISA reading (O.D.$_{405}$) of R_5 transgenic papaya after 14 days of the last inoculation

#tested plant	A_{405} value 0f A44R5-												
	T1F4	**T1F7**	**T1F10**	**T1F14**	**T1F15**	**T1F27**	**T1F36**	**T25F3**	**T25F7**	**T25F16**	**T27F1**	**T27F3**	**T27F11**
1	0.118	0.669	0.128	0.412	0.143	0.113	0.133	0.132	0.868	1.055	1.357	0.6	0.112
2	0.119	0.935	0.143	0.122	0.567	0.112	1.163	0.129	0.886	0.124	0.936	0.139	0.325
3	1.011	0.139	1.133	1.385	0.139	0.595	0.137	1.239	1.255	0.547	0.261	0.27	-
4	1.055	0.129	0.583	0.387	0.726	0.221	0.132	0.135	0.146	0.133	0.355	0.525	-
5	0.119	0.787	0.125	0.130	0.14	1.089	0.431	0.135	0.126	0.146	0.78	0.362	-
6	0.186	0.694	1.484	1.154	0.13	-	0.13	0.265	1.100	0.576	0.435	0.419	-
7	0.145	0.128	0.128	0.152	0.915	-	0.13	0.732	1.130	0.113	0.825	1.284	-
8	0.642	-	0.14	0.135	0.134	-	0.129	0.129	0.372	-	0.098	0.125	-
9	0.967	-	0.719	0.461	0.137	-	0.962	0.631	0.133	-	0.677	0.125	-
10	0.369	-	0.646	0.131	0.123	-	0.903	0.835	0.71	-	1.009	0.572	-
11	0.128	-	0.13	1.083	1.372	-	0.293	0.121	0.132	-	0.937	0.122	-
12	1.055	-	1.154	0.124	0.128	-	0.131	0.138	0.915	-	0.736	0.721	-

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Appendix Table B1 (Continued)

#tested plant	A_{405} value 0f A44R5-												
	T1F4	**T1F7**	**T1F10**	**T1F14**	**T1F15**	**T1F27**	**T1F36**	**T25F3**	**T25F7**	**T25F16**	**T27F1**	**T27F3**	**T27F11**
13	0.536	-	0.618	0.128	2.727	-	0.346	0.124	0.124	-	0.124	0.279	-
14	0.759	-	1.021	0.141	0.124	-	0.134	0.358	0.123	-	-	0.122	-
15	0.198	-	0.133	1.442	1.368	-	0.136	0.273	0.121	-	-	0.12	-
16	0.12	-	0.127	1.086	0.387	-	0.134	0.12	0.124	-	-	0.628	-
17	0.371	-	-	0.136	0.77	-	0.131	0.309	0.12	-	-	0.124	-
18	0.406	-	-	0.119	0.125	-	0.502	0.259	1.279	-	-	0.136	-
19	0.12	-	-	0.12	0.163	-	0.135	0.121	0.135	-	-	0.764	-
20	1.055	-	-	0.121	0.142	-	0.134	0.332	0.129	-	-	0.126	-
21	0.126	-	-	0.615	0.127	-	0.135	0.344	1.055	-	-	0.129	-
22	0.373	-	-	-	0.132	-	0.148	0.127	0.132	-	-	1.116	-
23	0.665	-	-	-	0.13	-	0.128	0.121	0.124	-	-	0.131	-
24	0.472	-	-	-	0.198	-	0.13	0.121	0.536	-	-	0.123	-
25	0.123	-	-	-	-	-	0.132	0.504	0.122	-	-	0.402	-

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Appendix Table B1 (Continued)

#tested plant	A_{405} value 0f A44R5-												
	T1F4	**T1F7**	**T1F10**	**T1F14**	**T1F15**	**T1F27**	**T1F36**	**T25F3**	**T25F7**	**T25F16**	**T27F1**	**T27F3**	**T27F11**
26	0.126	-	-	-	-	-	0.124	-	1.15	-	-	1.145	-
27	0.13	-	-	-	-	-	0.245	-	0.847	-	-	1.005	-
28	1.055	-	-	-	-	-	0.134	-	0.942	-	-	0.123	-
29	0.121	-	-	-	-	-	0.131	-	1.055	-	-	1.096	-
30	0.124	-	-	-	-	-	0.343	-	0.123	-	-	0.126	-
31	1.027	-	-	-	-	-	0.127	-	0.432	-	-	0.125	-
32	0.11	-	-	-	-	-	0.648	-	0.527	-	-	0.126	-
33	0.118	-	-	-	-	-	0.129	-	0.16	-	-	0.131	-
34	0.108	-	-	-	-	-	0.123	-	0.471	-	-	1.572	-
35	0.107	-	-	-	-	-	0.13	-	0.127	-	-	0.127	-
36	0.108	-	-	-	-	-	0.512	-	0.13	-	-	0.471	-
37	0.14	-	-	-	-	-	0.227	-	1.055	-	-	0.128	-
38	0.113	-	-	-	-	-	0.952	-	0.426	-	-	0.134	-

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Appendix Table B1 (Continued)

#tested plant	A_{405} value 0f A44R5-												
	T1F4	T1F7	T1F10	T1F14	T1F15	T1F27	T1F36	T25F3	T25F7	T25F16	T27F1	T27F3	T27F11
39	0.123	-	-	-	-	-	0.129	-	0.412	-	-	0.122	-
40	0.346	-	-	-	-	-	1.483	-	0.39	-	-	0.171	-
41	0.109	-	-	-	-	-	0.133	-	0.372	-	-	0.134	-
42	0.109	-	-	-	-	-	0.31	-	0.136	-	-	0.132	-
43	0.418	-	-	-	-	-	0.123	-	0.128	-	-	-	-
44	0.108	-	-	-	-	-	0.365	-	0.123	-	-	-	-
45	0.303	-	-	-	-	-	0.406	-	0.121	-	-	-	-
46	0.105	-	-	-	-	-	0.283	-	0.846	-	-	-	-
47	0.108	-	-	-	-	-	1.055	-	0.429	-	-	-	-
48	0.143	-	-	-	-	-	0.639	-	0.192	-	-	-	-
49	1.173	-	-	-	-	-	0.679	-	0.796	-	-	-	-
50	0.154	-	-	-	-	-	0.426	-	0.148	-	-	-	-

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Appendix Table B1 (Continued)

#tested plant	A_{405} value 0f A44R5-												
	T1F4	T1F7	T1F10	T1F14	T1F15	T1F27	T1F36	T25F3	T25F7	T25F16	T27F1	T27F3	T27F11
51	0.879	-	-	-	-	-	0.132	-	0.226	-	-	-	-
52	0.657	-	-	-	-	-	0.115	-	0.195	-	-	-	-
53	0.274	-	-	-	-	-	0.122	-	0.546	-	-	-	-
54	0.749	-	-	-	-	-	0.688	-	0.305	-	-	-	-
55	1.568	-	-	-	-	-	1.055	-	0.978	-	-	-	-
56	0.211	-	-	-	-	-	0.122	-	1.005	-	-	-	-
57	0.922	-	-	-	-	-	0.12	-	0.49	-	-	-	-
58	0.751	-	-	-	-	-	0.104	-	0.193	-	-	-	-
59	0.237	-	-	-	-	-	0.101	-	0.222	-	-	-	-
60	0.193	-	-	-	-	-	0.098	-	0.213	-	-	-	-
61	1.233	-	-	-	-	-	0.122	-	0.298	-	-	-	-
62	0.455	-	-	-	-	-	0.113	-	0.181	-	-	-	-
63	0.757	-	-	-	-	-	0.114	-	0.837	-	-	-	-

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Appendix Table B1 (Continued)

#tested plant	A$_{405}$ value 0f A44R5-												
	T1F4	T1F7	T1F10	T1F14	T1F15	T1F27	T1F36	T25F3	T25F7	T25F16	T27F1	T27F3	T27F11
64	1.07	-	-	-	-	-	0.111	-	0.781	-	-	-	-
65	0.631	-	-	-	-	-	0.576	-	0.221	-	-	-	-
66	0.13	-	-	-	-	-	0.112	-	0.824	-	-	-	-
67	1.364	-	-	-	-	-	0.273	-	0.135	-	-	-	-
68	0.154	-	-	-	-	-	0.234	-	0.487	-	-	-	-
69	0.124	-	-	-	-	-	0.105	-	1.254	-	-	-	-
70	0.45	-	-	-	-	-	0.124	-	0.604	-	-	-	-
71	-	-	-	-	-	-	0.417	-	1.301	-	-	-	-
72	-	-	-	-	-	-	0.11	-	0.418	-	-	-	-
73	-	-	-	-	-	-	1.03	-	0.151	-	-	-	-
74	-	-	-	-	-	-	1.038	-	1.165	-	-	-	-
Healthy							0.125						
Disease							0.854						

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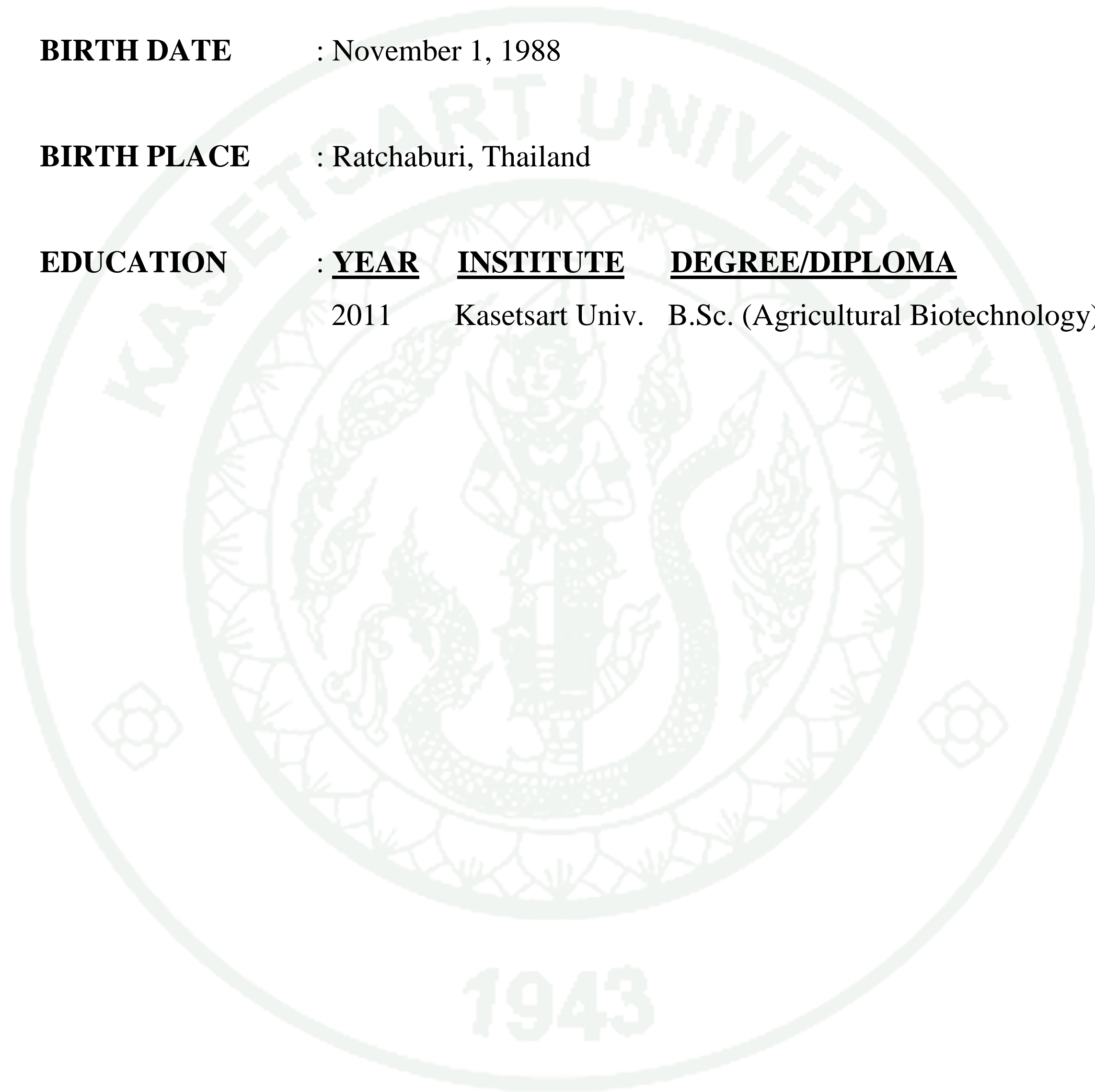

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