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THESIS

PLANT FITNESS COMPARISON BETWEEN TRANSGENIC *PAPAYA RINGSPOT VIRUS* (PRSV) RESISTANT AND NON-TRANSGENIC PAPAYA UNDER SCREENHOUSE CONDITION

BENCHARONG PHUANGRAT

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Commercialization of genetically modified organisms (GMOs) commonly called transgenic organisms raises public concerns over possible adverse effects to the environment and human health. Among the environmental concerns, attention is focused on ecological risk of gene flow from GM plants to wild/cultivated species. There are two main factors contributing to transgene movement; plant fitness and reproductive characteristics especially in pollen. Plant fitness can be measured by determination of horticultural and agronomic characters such as plant morphological characteristic, yield and numbers of seeds. Reproductive character is determined via pollen viability and pollen germination at given time (pollen vigor). In this study, *papaya ringspot virus* (PRSV) resistant papaya lines p116/5 R_4 and R_5 previously shown to be homozygous line and highly resistant to almost all Thai PRSV strains was evaluated for fitness and reproductive characters in closed containment. The experiment was carried out for two consecutive seasons, with complete randomize design (CRD). The results showed that there was no significant difference in any agronomical characters including growth rate, fruits production as well as pollen biology. There was no significant difference in pollen viability and germination as well as in shape, size and weight among pollen grains from transgenic and non-transgenic papaya counterpart. In addition, sensitive polymerase chain reaction (PCR) based method was developed to detect transgene in pollen grains, a mobile male reproductive organ. It can be concluded that under closed containment condition both transgenic and non-transgenic papaya show similar plant fitness and pollen biology.

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Bencharong Phuangrat

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LIST OF ABBREVIATIONS

BC	=	back cross
bp	=	base pairs
^{o}C	=	degree Celsius
cm	=	centimeter
cm^2	=	square centimeter
CP	=	coat protein
CRD	=	complete randomized design
DAS	=	days after sowing to flowering
dH_2O	=	distilled water
DEPC	=	diethyl pyrocarbonate
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
dNTPs	=	deoxynucleotide triphosphate (s)
ELISA	=	enzyme link immunosorbent assay
g	=	gram
GMOs	=	genetically modified organisms
h	=	hour
ha	=	hectare
hg	=	hectogram
kb	=	kilobase
KCl	=	potassium chloride
K_2SO_4	=	potassium sulphate
kg	=	kilogram
kgf	=	kilogram force
l	=	liter
LiCl	=	lithium chloride
M	=	molar
Mbp	=	mega basepair
mg	=	milligram
min	=	minute

LIST OF ABBREVIATIONS (Continued)

ml	=	milliliter
mM	=	millimolar
μmol	=	micromolar
μW	=	microwatt
mRNA	=	messenger ribonucleic acid
MW	=	molecular weight
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
ng	=	nanogram
nm	=	nanometer
OD	=	optical density
PCR	=	Polymerase chain reaction
pmol	=	picomole
PRSV	=	*papaya ringspot virus*
RNA	=	ribonucleic acid
RNase	=	ribonuclease
rpm	=	round per minute
RT	=	room temperature
SDS	=	sodium dodecyl sulphate
SEM	=	scanning electron microscope
sec	=	second
TAE	=	tris-acetate EDTA
TMV	=	*tobacco mosaic virus*
TSS	=	total soluble solid
UV	=	ultraviolet

PLANT FITNESS COMPARISON BETWEEN TRANSGENIC *PAPAYA RINGSPOT VIRUS* (PRSV) RESISTANT AND NON-TRANSGENIC PAPAYA UNDER SCREENHOUSE CONDITION

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the most commonly grown fruit crop in tropical and sub-tropical regions. Although papaya is relatively easy to grow and manage, papaya is susceptible to a viral infection called papaya ringspot disease with more than 70% loss of papaya production. The disease is caused by *papaya ringspot virus* (PRSV). Common practices of disease control including roughing and zoning at an early stage of the infection can reduce the loss, however there is no natural resistant variety and hence making transgenic papaya with PRSV resistant character is a good alternation. In order to generate transgenic papaya for commercialisation, the plant is required to go through several stages of molecular, horticultural and agronomic character determination as well as food and environmental safety evaluation. In this study plant fitness and efficiency of male reproductive system of PRSV resistant transgenic papaya line p116/5 R_4 and R_5 are determined. Transgenic papaya and papaya Khak Nual (its counterpart) were grown under contained condition using complete randomized design (CRD). The data were collected for two consecutive seasons and statistically analysed using T-test. Growth rate and fruit production were determined for plant performance. Pollen biology including pollen morphology, pollen viability and germination rate was studied and observed by scanning electron microscopy (SEM) and light microscopy. The results indicated that there is no significant difference in plant performance and pollen biology between transgenic papaya and its counterpart. It can be concluded that PRSV resistant transgenic papaya line p116/5 and its counterpart has similar fitness including plant performance and male reproductive system under contained condition.

OBJECTIVES

1. To compare plant fitness including plant performance and male reproductive system between transgenic PRSV resistant papaya and its counterpart

2. To monitor and determine *Papaya ringspot virus* coat protein (*PRSV-CP*) and neomycin phospphotransferase II (*nptII*) selectable marker transgene expression in PRSV resistant transgenic papaya

LITERATURE REVIEW

1. Papaya

1.1 general information

Papaya (*Carica papaya* L.) belongs to family Caricaceae comprising 22 species in 6 genera; *Carica, Jacaratia, Jarilla, Cylicomorpha, Horovitzia* and *Vasconcellea* (Fitch, 2005). *Carica papaya* is the only species in genus *Carica* (Organisation for the Economic Cooperation and Development [OECD], 2010) which the nearest relative is *Vasconcellae* (Aradhya *et al.*, 1999; Van Droogenbroeck *et al.*, 2002). *C. papaya* was originated in tropical America (Aradhya *et al.*, 1999; OECD, 2005 and Office of the Gene Technology Regulator [OGTR], 2008) from eastern Central America, Mexico to Panama (Nakasone and Paull, 1998). Papaya was introduced into many areas including Caribbean, south-east Asia, India, also the Pacific and Africa (Villegas, 1997). Currently, papaya is cultivated throughout tropical and subtropical areas worldwide (Villegas, 1997; OGTR, 2008). Papaya is diploid species containing 372 Mbp of genome size (Ming *et al*., 2008) and 9 pairs of chromosomes (Bennett and Leitch, 2005). Papaya is an herbaceous perennial plant having hollow green or deep purple trunk which is usually single, straight and can reach 10 m height (OGTR, 2008). The trunk has prominent leaf scar and emerge spiral palmate lobed leaves which 25-100 cm long petioles. The leave normally divided into 5-9 main lobes with 25-75 cm in width (OGTR, 2008). Papaya is polygamous species having three sex types; male, female and hermaphrodite which producing staminate, pistillate and hermaphrodite flowers. Papaya fruit is fleshy berries (Villegas, 1997) having a central seed cavity. The exocarp is smooth and thick which is green at unripe stage and turn into yellow or orange when ripe. Shape of papaya fruit was varies depending upon sex types; globose, ovoid, obovoid or pyriform (OGTR, 2008) with 200 g to 9 kg in weight (Yon, 1994). The fruits from female are spherical to ovoid with large seed cavity whereas hermaphrodite fruits are cylindrical or pyriform with small seed cavity. In addition male plants sometimes can bear small elongated with poor quality fruit (Crane, 2005). Seeds of papaya are dark

brown or black in color enveloped by translucent sarcotesta mucilaginous (Yon, 1994; Paull *et al*., 2008). *Carica papaya* is classified into Eurosid II which sex chromosome is present (Tang *et al.,* 2008). Sex of papaya was controlled by a single gene comprising of 3 alleles; M, M^h and m for male, hermaphrodite and female plants. Male and hermaphrodite plants were controlled by the heterozygous alleles; Mm and M^hm whereas homozygous recessive (mm) conveying as female plants. Homozygous dominant alleles; MM, MM^h and M^hM^h were embryonic lethal supported by the present of 25% non-viable seed and lack of true-breeding hermaphrodite or male plants (Deputy *et al.,* 2002). Recent hypothesis suggested that papaya sex determination was considered as XY type base on their heterozygous male and homozygous female (Vyskot and Hobza, 2004; Ma *et al.,* 2004). Sex of papaya was controlled by primitive chromosome consisting small male-specific region on the Y chromosome (Chen *et al.,* 2007; Yu *et al.,* 2007, 2008a, 2008b). Y chromosome controlling male and hermaphrodite papaya was slightly different design as Y and Y^h respectively (Chen *et al.,* 2007; Ming *et al.,* 2007a; Yu *et al.,* 2007, 2008a). The genotypes of papaya male, hermaphrodite and female are XY, XY^h and XX (Chen *et al.,* 2007; Yu *et al.,* 2007), however the YY, YY^h and Y^hY^h are lethal (Chen *et al.,* 2007; Ming *et al.,* 2007b; Yu *et al.,* 2008a).

1.2 Papaya for human consumption

The major compositions of papaya fruit are water and carbohydrate. It is also rich in vitamins and minerals especially vitamin A and C, ascorbic acid and potassium with low calories (Manshardt, 1992). Papaya fruit can be consumed both unripe and ripe stage. Unripe fruit is cooked and utilized as vegetables, processed products and as a source of papain. Thai cuisine and other Asian cuisines consume unripe papaya as an ingredient in papaya salad and as cooked vegetable (Sone *et al*., 1998; Mendoza, 2007; Mano *et al*., 2009). Typically, unripe papaya must be washed or cooked prior to consumption to remove or denature the papain in the latex (Morton, 1987; Odu *et al.,* 2006). Ripe papaya generally is consumed as a fresh fruit by peeled, seeded and cut into pieces sometimes it can be cut into wedges and served with lime or lemon. Ripe papaya is also manufactured into puree, nectar, juice, frozen slices or chunks, mixed beverages, papaya powder, baby food, concentrated and candied items

(Mugula *et al.*, 1994; OECD, 2005; OGTR, 2008). Papain and chymopapain found in papaya latex are important proteolytic enzymes used in food and feed industries such as meat tenderizer, clarify beer and juice, produce chewing gum, coagulate milk, prepare cereals, and produce pet food (Morton, 1987; OECD, 2010). Furthermore, it involved in pharmaceutical and cosmetic industries (Pendzhiev, 2002; OGTR, 2008). Papaya seeds are sometimes used to substitute black pepper (Morton, 1987).

1.3 Papaya production

The world production of papaya in 2008 was estimated to be approximately 9,095,875 tonnes (FAOSTAT, 2010). The country with the largest papaya production in 2008 was India (2,685,900 tonnes) followed by Brazil (1,900,000 tonnes), Nigeria (765,000 tonnes), Indonesia (653,276 tonnes) and Mexico (638,237 tonnes). Within the top papaya producing countries, El Salvador had the highest yield at 1,103,441 hectogram/hectare (Hg/Ha). Nigeria had the greatest area of papaya harvested with 92,500 Ha. Mexico had the greatest papaya export value and quantity in 2007 by 55,327 million dollars and 101,306 tonnes respectively. The United States was a major papaya importing country in 2007 with an import value of 73,125 million dollars followed by the Netherlands (19,208), United Kingdom (18,231), Canada (17,987) and Germany (16,873). The 69% of papaya imported into the US market in 2006 came from Mexico followed by Belize (25%), Brazil (2.8%), Jamaica (1%) and elsewhere (2.2%) (Pollack and Perez, 2008). Small papaya farmers and commercial farmers in many countries grow papaya for both local and foreign markets. The local markets prefer medium- and large-fruited varieties whereas exported fruit are usually small or of medium size with yellow or red flesh (Medlicott, 2004). Generally, hermaphrodite fruits are more desirable over female fruits. However, the fruits have to be fresh, free from bruises and blemishes, and uniform in size and ripeness. The latest Codex Alimentarius standard for papaya, amended in 2005, includes standards regarding quality, size, uniformity, packaging, labelling, contaminants and hygiene (Codex, 2005). Papaya production of Thailand in 2008 was estimated by 201,099 tonnes with the exported quantity and value approximately 1,284 tonnes and 555,000 $, respectively (FOASTAT, 2010). However, Around 98% of papaya production in Thailand is for domestic consumption. The most famous

papaya variety growth commercially in Thailand included ‘Khak Dum’ and ‘Khak Nual’ varieties (Napasintuwong and Traxler, 2009).

1.4 Papaya disease

Papaya can be infected by pathogens at every stage of development from seedling to fruiting stage. Important diseases of papaya include fruit rot, anthracnose, yellow crinkle, mosaic, dieback and papaya ringspot. Papaya ringspot disease, one of the most economical important diseases is caused by *Papaya ringspot virus* (PRSV) (Davis and Ying, 2004). First report of PRSV infection was from Hawaii in 1940s. Afterward, the disease is found in other regions including the severe infection in many papaya plantation areas (Yeh *et al.*, 2006). PRSV is single-stranded positive sense RNA virus belonging to *potyvirus* family (Gonsalves, 1998). The PRSV was classified into two types; PRSV type P (PRSV-p) and PRSV type W (PRSV-w). PRSV-p infects both cucurbits and papaya whereas PRSV-w infects only cucurbits (OGTR, 2008). The disease can be transmitted non-persistently by many species of aphids, including *Myzus persicae*, *Aphis gossypii*, *A. craccivora*, and *A. maidis*. Although aphid is the major transmission of PRSV, seed transmission was reported in the Philippines (Bayot *et al.*, 1990). PRSV infected papaya shows severe mosaic and distortion of leaves, ringspots on fruit and water-soaking oily streaks on the upper stems and petioles. The infected plants are stunted and there is a drastic reduction of fruit size and quality (Yeh *et al.*, 2006). In some cases the infection of PRSV resulted in up to 100% losses of papaya production (Mendoza *et al.*, 2008).

There is no natural PRSV resistance in papaya gene pool, although a “mountain papaya” or *Vasconcellea spp.* shows the resistant character (Drew *et al.*, 2005; Dillon *et al.*, 2006). PRSV tolerant papaya varieties have been reported such as Florida ‘Cariflora’, Thailand ‘Thapra’, Taiwan ‘Red Lady’ and ‘Known You No. 1’ (Davis *et al.*, 2003; Teixeira da Silva *et al.*, 2007). The level of tolerance is varies depending upon severity, geographic origin of the virus and environmental conditions. There is no effective method in controlling the infection of the virus. Control methods include combination of quarantine and cultural practices such as restricted movement of papaya seedlings, scouting of orchards and the prompt removal of infected trees

rouging, quarantine, intercropping with corn as a barrier crop and protecting transplanted seedlings with plastic bags. These measures provide only temporary or partial solutions to the problem and only effective in regions where disease pressure is low (Yeh and Gonsalves, 1994). Cross-protection with mild strain of PRSV is also used to control the loss from PRSV virulent strain infection (Yeh and Gonsalves, 1994). This method, however, has several drawbacks, including the requirement for a large-scale inoculation program, reduction in crop yield and losses of cross-protected plants due to super-infection by virulent strains (Yeh and Gonsalves 1994). Hence, production of transgenic papaya is a rational alternative decision for controlling the distribution of the PRSV disease.

2. Generation and screening of transgenic PRSV resistance papaya

2.1 Generation of transgenic PRSV resistant papaya

Concept of pathogen derived resistance in which a plant transformed with DNA from its pathogen displaying resistant character to that pathogen was first developed by Sanford and Johnson (1985). Abel *et al*. (1986) demonstrated that transgenic tobacco expressing the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) indicating the resistance to TMV infection. The CP gene-mediated transgenic resistance has been proven effective for protecting tobacco, tomato, potato and other crops from many viral infections (Goldbach *et al.,* 2003). Thus, transgenic approach has become the most effective method to protect crops from virus infection.

PRSV was first discovered in 1950s in Oahu, Hawaii. It became a major cause of losses in papaya production in Hawaii. The severity of this disease was treaten to destroy papaya industry in Hawaii. It also spreads into other regions including Florida, Caribbean countries, South America, Africa, Asia as well as Australia (Davis and Ying, 2004). In Hawaii where papaya is a major fruit crop, papaya production was moved to PRSV free area, Puna (Gonsalves, 1998). However, PRSV disease reached Puna in 1994 and severely reduced Hawaii's papaya production (Gonsalves, 1998). Transgenic papaya was successfully developed in Hawaii in 1988 by transforming PRSV-CP strain HA 55-1 into embryogenic tissues

using particle bombardment (Gonsalves, 1998). The characterization and evaluation of PRSV resistant papaya were done under greenhouse condition (Lius *et al*., 1997). In 1991, transformant line 55-1 exhibited an excellent resistance to virulent PRSV HA was successfully developed in Hawaiian papaya cultivar Sunset (Lius *et al*., 1997) and also displayed some resistance against other PRSV strains (Lius *et al*., 1997). Commercialized transgenic papaya SunUp and Rainbow were cultivated in Puna in 1998. SunUp is a transgenic red flesh papaya which developed from Sunset cultivar whereas Rainbow is a transgenic yellow flesh papaya which developed by crossing SunUp and yellow flesh non-transgenic kapoho (Gonsalves, 1998; Ovesná and Hodek, 2009). Currently, transgenic papaya has been grown widely on Hawaii (Schwember, 2008). Transgenic papaya was approved for human consumption in Canada and recently in Japan (Ovesná and Hodek, 2009; Suzuki *et al.,* 2010). Orginally, transgenic papaya was strictly sold only in US. Transgenic papaya is now sold in other countries including Canada and Japan. Following the success of Hawaii transgenic papaya, other countries including Brazil, Jamaica, Venezuela, Thailand, China, The Philippines, Taiwan and Australia are also developing transgenic papaya using their own papaya varieties and PRSV genome (Lines *et al.,* 2002; Fermin *et al.,* 2004; Davidson, 2008).

In Thailand, PRSV resistant transgenic papaya was first successfully developed in 1997 in the collaboration between Department of Agriculture and Cornell University (Kosiyachinda and Srivatanakul, 2005) using two different varieties of Thai papaya; Khak Nual and Khak Dum. Concomitantly, PRSV resistant transgenic papaya was also developed from cooperation between National Center for Genetic Engineering and Biotechnology (BIOTEC) and Kasetsart University using coat protein (CP) gene of PRSV isolate Chaing Mai. *PRSV-CP* gene was inserted into p2CMCP vector and subsequently transformed into somatic embryo of immature papaya seed by particle bombardment. Transformed papaya were selected using antibiotic contiaining media, regenerated and root- and shoot-induced. Transgenic papaya plantlets harboring CP gene were acclimatized in the glass house. The overall process of generation of transgenic papaya was shown in Figure 1.

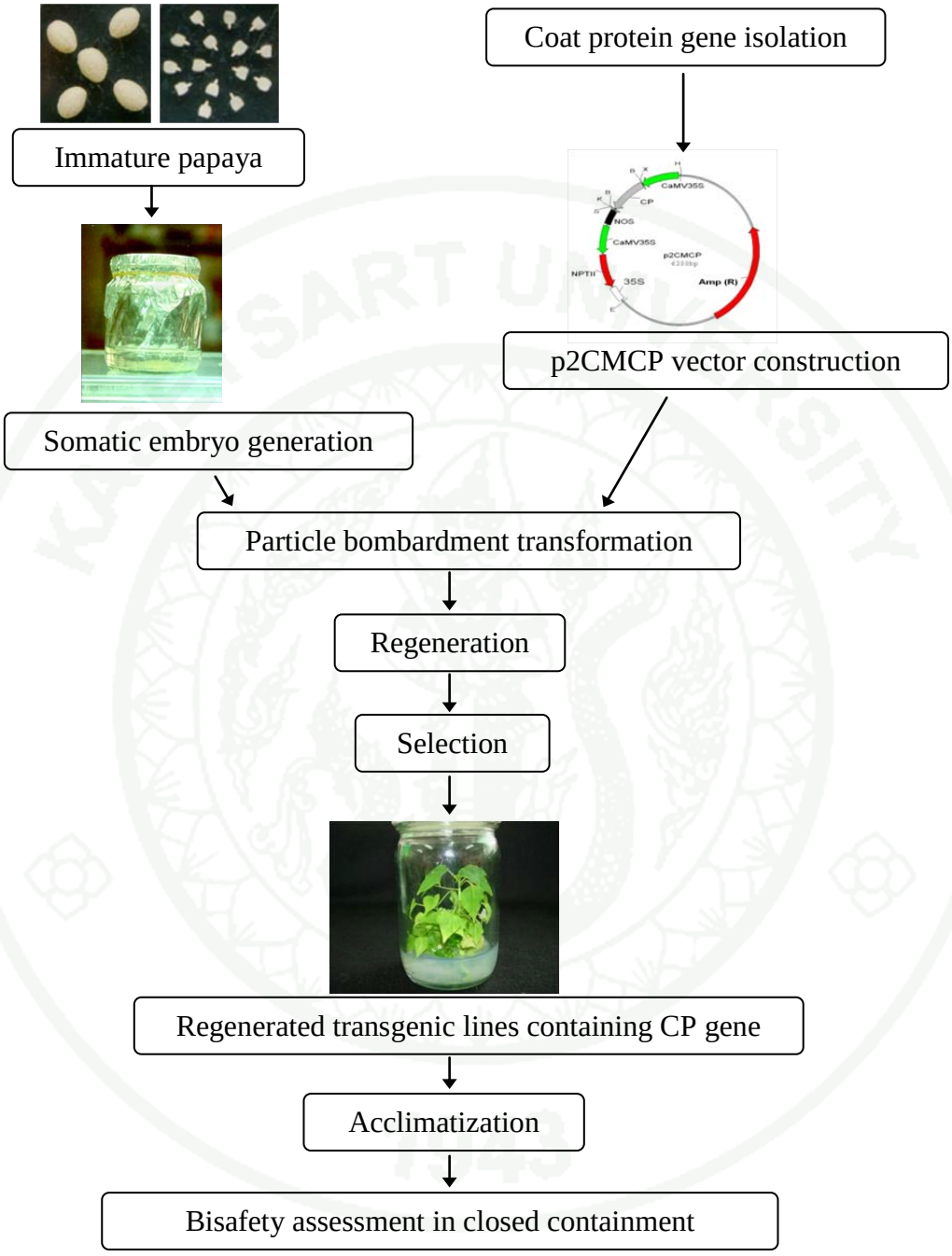

Figure 1 Overview process of transgenic papaya production

Source: modified from Burns *et al*. (2004)

2.2 Screening of PRSV resistant transgenic papaya

Transformed papaya plants must be evaluated to ensure the presence and integrity of introduced gene or DNA. Methods such as polymerase chain reaction (PCR) with specific primer, Southern blotting or real time PCR are commonly used to detected the presence and copy numbers of transgene, respectively (Fan *et al*., 2009). Automate sequencing is reassure the authenticity of DNA sequence. The acquired trait, PRSV resistance, was carried out by PRSV inoculation (Lines *et al*., 2002; Davis and Ying, 2004; Fermin *et al*., 2004). Agronomic and horticultural traits are determined under screen house and field condition to guarantee that those transgenic lines are suitable for commercialzation. Transgenic crops are required to have equal or better agronomic traits and show similar morphological appearance (Xiangdong *et al*., 2007).

3. Concerns over transgenic papaya

Transgenic plant production raises 2 major concerns; the environment safety and food safety. Environmental consideration of transgenic PRSV resistant papaya are likely to be similar to concerns over other transgenic plants including transgene movement (gene flow), possibility of weediness, horizontal gene transfer, effect on non-target organism and emerging of new virus (Stabinsky, 2000). Gene flow from transgenic plant onto relative species can occurred through pollen and seed resulting in the hybrid progeny harboring transgene which might have fitness advantage and feasibility to become weediness (Figure 2). Horizontal gene transfer is based on the possibility of transgene movement from transgenic plants to surrounding microorganisms and could raise antibiotic resistant character. In case of an effect on non-target organisms, the concern is intoxication of transgenic material to non-target organisms such as insects. Heteroencapsidation between coat protein producing by transgenic papaya and incoming virus could result in vector transmissibility in previously was non-transimissible virus. Three major possibilities of genome encapsidation in the first virus offspring are encapsidated by its own CP subunits, partially and fully encoded by CP transgene. Although newly formed virus can be insect transmitted, the second virus offspring will be alike to the challenge virus

(Figure 3). The recombination of transgene and incoming virus ensuing the creation of novel virus is one of environmental concern. If there is recombination between transgene and viral RNA during replication, modified RNA can be formed and encapsidated for subsequent vector acquisition and transmission (Figure 4). Synergism which is the interaction between viral protein and another challenge virus can enhance severity of plant symptom.

For human consumption, food safety of transgenic plant and its product is a major concern. Toxicity, allergenicity and nutritional analysis of transgene protein should be performed (Gonsalves, 1998; Fuchs and Gonsalves, 2007). In addition, the possibility of unexpected expression of transgene should also be considered.

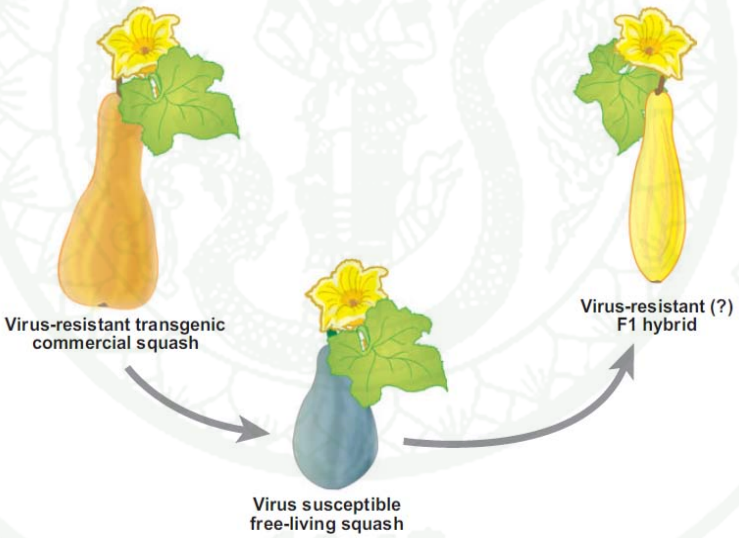

Figure 2 Transgene movement through hybridization.

Source: Fuchs and Gonsalves (2007)

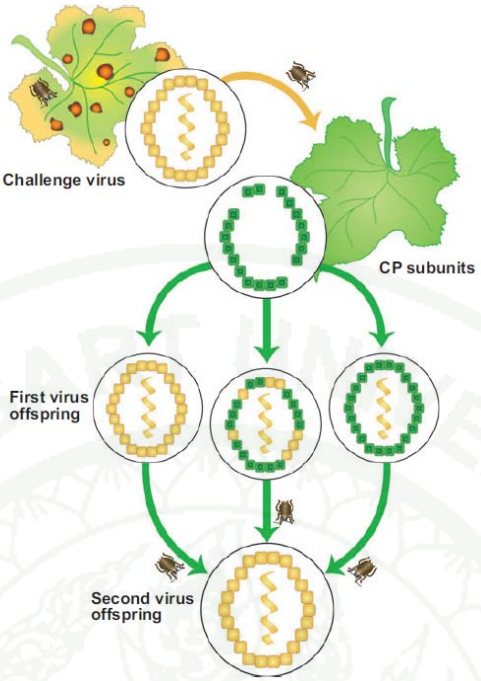

Figure 3 Heteroencapsidation between challenge virus and CP subunits of transgenic plant.

Source: Fuchs and Gonsalves (2007)

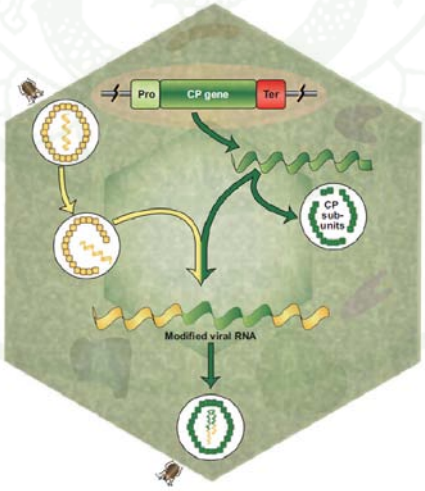

Figure 4 Model of recombination between transgene and viral RNA genome.

Source: Fuchs and Gonsalves (2007)

4. Biosafety assessment

Biosafety assessment of transgenic papaya using the same conception conducted in other transgenic plants. It is a process covering entire range of protocols needed to analyse, manage, regulate and control these risks in the development, release and utilization of transgenic plant. Biosafety assessment consists of three levels; laboratory or greenhouse, confined field trial and unconfined field trial conditions (Kosiyachinda and Srivatanakul, 2005; Technical Biosafety Committee [TBC], 2009). Transgenic crops and their non-transgenic counterpart will be evaluated simultaneously under the substantial equivalence, step by step and cases by case strategies (Traynor, 1999; Stewart *et al*., 2000; Shewry *et al*., 2007). Craig *et al*. (2008) and Raybould *et al*. (2010) summarized methods to evaluate environmental risk assessments of transgenic crops as 1) plant characterization in order to account the effects of genetic modification and unintended effects caused by transformation, 2) molecular characterization to identify insert DNA, undesirable change due to the insertion of transgene. Northern analysis and quantitative and semi-quantitative RT-PCR are widely practiced to determine transcriptional level of transgene. Western analysis and ELISA are routined for determination of protein (from transgene) production (Ovesná and Hodek, 2009). In 3), plant compositional analysis is determined if any difference in nutritional composition between transgenic and non-transgenic counterpart. Feeding study (4) is done by using tested animal to determine the unintended harmful nutrient. Phenotypic characterization (5); agronomic or horticultural traits are also performed in unconfined field trial for at least two seasons in an attempt to investigated agronomic or horticultural performance and ensured that transgenic plant will not have weediness and invasiveness possibility. Phenotypic characters can be accomplished in term of plant fitness. Lastly, 6) developmental expression study is investigated expressed protein in plant tissues such as leaf, root, pollen and seed.

Ferreira *et al*. (2002) investigated field trial experiment of PRSV resistant transgenic papaya ‘SunUp’ and ‘Rainbow’ in Hawaii. Plant performance and yield such as total fruit yield, average fruit weight and refractometer were examined. The results showed that transgenic papata did not exhibit the symptom of PRSV infection.

Moreover, fruit production was three times higher than infected papaya plants. Large scale field trial was done in Puna and showed acceptable horticultural quality and highly resistant to PRSV. Bau *et al*. (2004) carried out the field evaluation of transgenic papaya cultivar 'Tainung' in Taiwan by characterization virus resistant and fruit yield. The results indicated that transgenic papaya exhibits mild symptom in the both first and second seasons. However, there was no adverse effect on fruit yield which was 10.8-11.6 and 54.3-56.7 times greater than infected non-transgenic counterpart. Xiangdong *et al*. (2007) evaluated T4 generation of two transgenic papaya cultivars, Zhongkang1 and Zhongkang 2, in open field of Guangzhou, China. Horticultural traits including plant growth, fruit length, diameter, weight, flesh thickness and nutritional composition were performed using Zhongkang1 and Zhongkang 2 comparing to non-transgenic Meizhonghong and Guanghong cultivars. The results showed that transgenic papaya did not displayed any symptoms and had superior growth character and fruit quality.

Although several previous studies have been conducted the environmental safety of transgenic papaya under field conditions, Thailand biosafety assessment of transgenic papaya was restricted only in the laboratory or greengouse condition. Phironrit *et al*. (2007) investigated molecular and phenotypic characterization of PRSV resistant transgenic papaya in small scale. However, the experiment was done prior to the limitation of transgenic planting on confine field trials by the cabinet.

5. Determination of plant fitness

In ecological term, fitness in any living oganisms refers to the successful in reproduction which is determined by survival, productive or other life history (Pederson *et al*., 2005). In agricultural perspective, fitness defines as capability of plant to survive and produce profitable yield. The fitness is affected by several factors such as germination, survival, growth rate and lodging (Schlindwien, 2002; Pederson *et al*., 2005). An increase in fitness of transgenic plants raises environmental concern due to its relation to possibility of gene flow (Fuchs *et al*., 2004). Moreover, the enhanced fitness could cause superior competitive ability and enhanced tolerance of biotic or environmental stress (Farnum *et al*., 2007).

White (2002) suggested the fitness characters should be evaluated by comparing a transgenic plant with its wild relative in term of agronomic traits, economic traits and sexual reproductive systems. Agronomic traits include growth tendency, life span, vegetative vigor, ability to over-season, number of days for flowering and number of days until maturity while economic traits refer to fruit production and seed production. Reproductive system can be determined through seed dormancy, seedling emergence, seedling survival until reproduction, outcroosing frequency within species, pollinator species, pollen numbers, pollen viability, pollen longevity, pollen shape, pollen weight, fertility or infertility, self-compatibility or incompatibility, asexual reproduction and seed dispersion factors.

Snow *et al.* (1999) determined the fitness of transgenic rapeseed (*Brassica*) conferring glufosinate resistant gene. The results showed no significant difference in survival and number of seeds among transgenic and non-transgenic rapeseed in the absence of glufosinate. Burke and Rieseberg (2003) reported no fitness effect of disease resistant transgene in transgenic sunflower by measuring seed output in the absence of pathogen. Fei and Nelson (2004) determined reproductive fitness of transgenic creeping bentgrass conferring Roundup®-tolerant gene and its non-transformed line under greenhouse condition by measuring heading date, anthesis initiation, anthesis duration, inflorescence length, inflorescence, pollen size, pollen longevity and seed capacity. The results indicated that transgenic lines did not exhibited any differences in fitness-related reproductive traits from non-transgenic line except days for anthesis and pollen longevity. Di *et al.* (2009) investigated fitness and maternal effects including number of mature plants, number of days from sowing to flowering (DAS), plant height, canopy diameter, above-ground biomass, number of flowers, numbers of seeds and total seed weight in transgenic oilseed rape, wild brown mustard, their hybrids and non-transgenic *B. napus*. The results showed that hybrids had moderate composite fitness and least reproductive fitness whereas wild mustard had greatest fitness. Laughlin *et al.* (2009) determined impact of virus-resistant transgene on fitness of wild *Cucurbita pepo* under the presence and absence virus pressure in wild *C. pepo*, transgenic backcross, and non-transgenic backcross by measuring male flower number, female flower number, fruit number, seed number and total biomass of 2nd and 3rd generation of backcross (BC2 and BC3). The results

indicated that in the presence of virus, the fecundity of wild and non-transgenic backcross was decreased. However fitness effect of transgene in the absence of virus not detected.

MATERIALS AND METHODS

1. Plant Materials

Hermaphrodite transgenic papaya line p116/5 R_4 and R_5 which previously examined to be homozygous, having 2 copies of *PRSV-CP* gene and showing high resistance to PRSV and its non-transgenic counterpart (hermaphrodite papaya cultivar 'Khak Nual') were used in this study. One to two month old seedlings of both transgenic and non-transgenic papaya were transplanted into 0.8x1m cement pot with Complete Randomized Design (CRD) under 32 mesh screenhouse condition for 2 crop seasons between January 2006 – December 2007 and January 2008 – December 2009 (Figure 5).

Figure 5 Transgenic and non-transgenic papaya plantation under screenhouse condition. In the first season, twenty transgenic and six non-transgenic papayas were planted as showed in (A) whereas in the second season papayas were grown both in (A) and (B).

2. Determination of plant morphological characteristics and horticultural traits

Plant morphological characteristics including plant height, height at first flower emergence and first fruit set, number of nodes at first flower emergence and first fruit set, trunk girth at first flower emergence and first fruit set were determined. Height was measured from the ground surface to shoot apex of papaya plant a week

interval until the fruit reaching mature stage. Height at first flower emergence and first fruit set was measured from ground level to the shoot apex (Figure 6A). Trunk circumference at first flower emergence and first fruit set was measured at 10 cm above ground level (Tennant *et al*., 2005) (Figure 6B). Number of nodes at first flower emergence and first fruit set was determined by counting all the nodes from ground surface to the node that differentiated the first flower and first fruit set, respectively (Figure 6C). The experiment was done for 6 replications in each transgenic generation comparing to their non-transgenic counterpart.

Horticultural traits including fruit numbers, fruit weight, fruit length, fruit width, pulp thickness and total soluble solid (TSS) were measured from 6 replications of transgenic and non-transgenic counterpart in each generations. Fruit number and weight was determined by counting all papaya fruit formed within 6-7 months after the commencement of flowering (Figure 7A). Fruit weight was the mean weight of all fruit in each tree. Fruit length was determined from pole to pole in longitudinal section of the fruit while fruit width was determined by measuring at the middle portion of vertical fruit length (Figure 7B). Pulp thickness was measured from the left and right portion of papaya fruit after longitudinal section. The TSS measurment from the top, middle and bottom portion from ripe papaya flesh, squeezing the juice and measuring the refractive index with a hand-held refractometer (Bellingham and Stanley Ltd., Lawrenceville, GA) (Figure 7C). Seed number was determined the total number of seed setting in each fruit (Subhadrabandhu and Nontaswatsri, 1997; Rimberia *et al*., 2007) (Figure 7D).

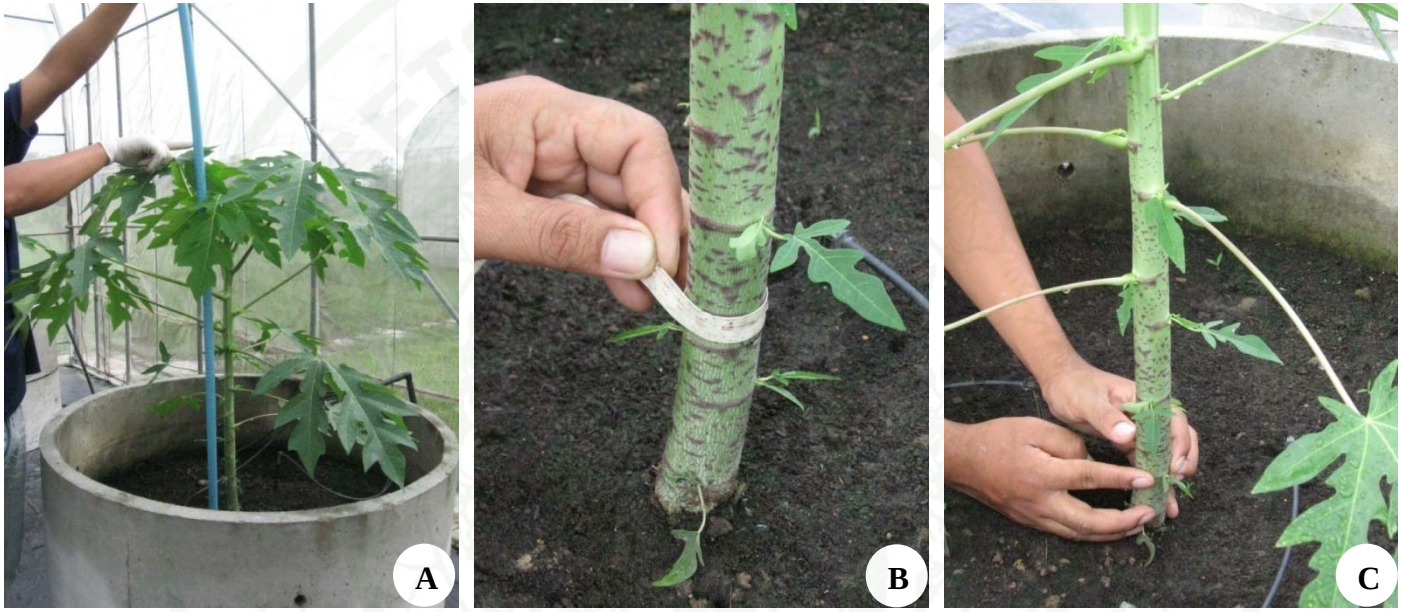

Figure 6 Methods to determine growth charactistics including height, trunk girth and number of node. Height was measured from ground level to the highest leaf blade (A) whereas trunk girth was assessed approximately 10 cm over the ground (B) while node number was determined by counting the node above ground level to the last leaf stalk (C).

Figure 7 Strategies to determine fruit numbers (A), fruits characters (B), total soluble solid (C) and seed number (D).

3. Determination of papaya male reproductive system

3.1 Pollen morphology

Fresh anthesis flowers were collected and pollen was brushed into petri dish containing distilled water and washed 3 times for 5 min each with agitation. The suspension was centrifuged at 3000 rpm for 5 min and supernatant was removed. The pollen was fixed in 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 for 2 h at 4°C with agitation. Fixed pollen was washed by rotating in 0.1M phosphate buffer, pH 7.2 then transferred to 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.2 for

2 h and washed with distilled water. The pollen was dehydrated in cold ethanol series (30%, 50%, 70%, 80%, 90% and 100%) and placed in 100% amyl acetate. The pollen was dried in critical point dryer (HITACHI, HCP-2, Japan) at 90-100 kgf/cm^2. The dried pollen was placed on stub and covered with gold particles using Ion sputter (FINE COAT, ion sputter JFC-1100, GEOL, Japan). Morphological characters including size, shape, aperture number and surface of pollen were determined under scanning electron microscope (SEM) **(**GEOL, model GSM5600LV, Japan**)** (Figure 8).

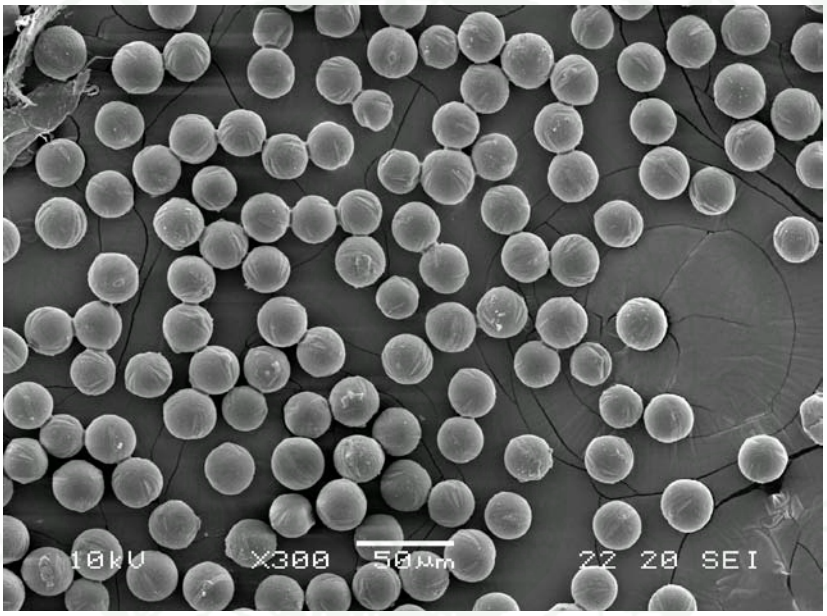

Figure 8 Pollen morphological study determining under scanning electron microscope.

3.2 Measurement of pollen physical characters

3.2.1 Pollen diameter

The photographs of pollen grains were taken from scanning electron microscope. One-hundred papaya pollen grains from transgenic and non-transgenic papaya pollen grains were randomly selected and measured. The calculation of pollen diameter was done by comparing with the standard bar.

3.2.2 Pollen weight

Fresh anthesis flowers were collected and pollen was brushed into petri dish and samples were taken to weigh with six decimal point balance (METTLER model MT 5, USA). The pollen grains were stained with 1% acetocarmine **(**1 g carmine in heated 100 ml of 45% acetic acid**)** solution and total number of pollen was counted under light microscope (Olympus model BX51, Japan) at 40x magnification. The weight of an individual pollen grain was calculated from total weight of pollen samples dividing with numbers of pollen grains. The experiments were done in six replications.

3.2.3 Pollen grain number

One to two days prior to anther anthesis, papaya flowers were collected and an anther was randomly selected from each flower. The experiment was done in 6 replications. Each anther was squashed in 1.5 ml microtube containing 100 ml of distilled water (modified from Koti *et al*., 2005). The pollen grains were stained with 1% acetocarmine solution. All pollen grains were counted on hemacytometer under light microscope (Olympus model BX51, Japan) at 40x magnification.

3.3 Pollen vigor

3.3.1 Pollen viability and pollen germination

Pollen viability was determined by acetocarmine staining and observation under light microscope. The stained pollen was considered as viable whereas unstained pollen considered as non-viable. The number of stained and unstained (transparent) pollen was calculated into percentage and compared between transgenic and non-transgenic papaya. Pollen germination was measured by incubating pollen at 28°C in germination medium (modified from Tuinstra and Wedal, 2000) containing 5% sucrose, 2.43 mM boric acid and 2.12 mM calcium nitrate for 5 h and stained with 1% acetocarmine. Germinated pollen was observed under light microscope. The pollen with pollen tube length greater than its diameter is

considered as germinated pollen (Tuinstra and wedal, 2000; Wang *et al*., 2004; Chen, 2009). The number of germinated pollen in total pollen count was calculated into percentage and compared between transgenic and non-transgenic papaya (Figure 9).

3.3.2 Pollen longevity

Fresh papaya pollen collected from anthesis flower was exposed to screenhouse condition (31-35°C, 53-65%RH). The pollen was collected at 0, 1, 3, 6, 12 and 24 h after exposure and incubated in germination medium. The viability and germination rate of the pollen from transgenic and non-transgenic counterpart were determined.

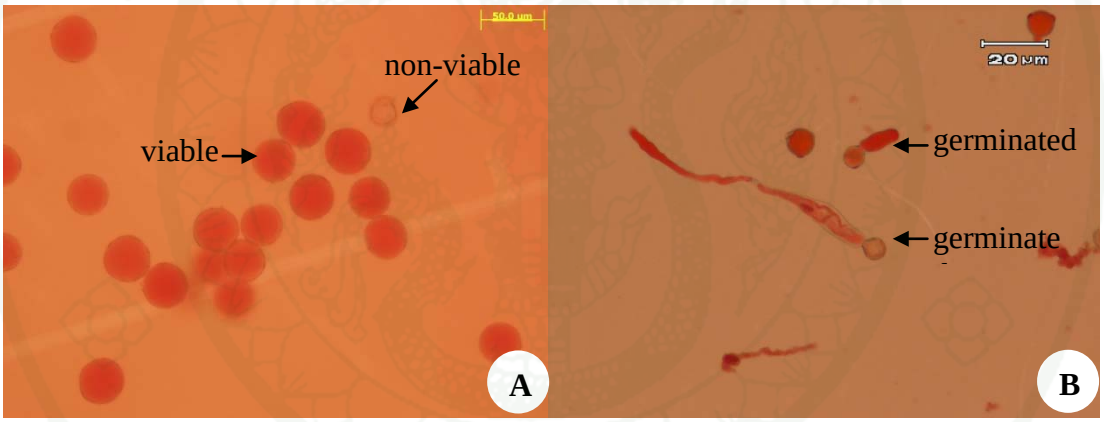

Figure 9 Description of viable (A), non-viable (A) and germinated (B) pollen observed under light microscope.

4. Effect of environmental stress on pollen germination

4.1 Temperature

In order to determined effect of temperature on pollen germination, pollen from transgenic and non-transgenic papaya was exposed to six different temperatures; 4°C, 25°C, 28°C, 32°C, 36°C and 40°C for 20 min before adding germination

medium. The exposed pollen was germinated in germination medium at 28°C in incubator for 5 h and the germinated pollen was counted under light microscope.

4.2 Relative humidity (RH)

Effect of RH on pollen germination was performed in chamber containing saturated chemical solution of $LiCl_2$, KCl, $NaCl_2$ and K_2SO_4 providing approximately 20%, 40%, 60% and 80% RH, respectively. Pollen was exposed to four different relative humidity for 20 min, then incubated in germination medium at 28°C for 5 h. The germinated pollen was counted under light microscope.

4.3 UVA and UVB radiation

The influence of UVA and UVB radiation was carried out by exposing the pollen both from transgenic and non-transgenic papaya to UV light (40 W, Sovenia, USA) at three different distances; 0 cm, 16 cm and 31 cm for 20 min. The UVA and UVB dose was approximately 15-30 $\mu W/cm^2$ determined by UV light meter (MANNIX, Taiwan). The exposed pollen was germinated in germination medium at 28°C in incubator for 5 h. The germinated pollen was counted under light microscope.

5. Detection of transgene in transgenic papaya

5.1 DNA extraction from pollen

Papaya pollen (0.005g pollen) was extracted with 700 µl of pollen extraction buffer (2%SDS, 100 mM Tris HCl (pH8.0), 20 mM EDTA) with 0.001mg/ml proteinase K (James *et al.*, 2001). The mixture was vortexed and heated at 65˚C for 10 min. The samples were extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new microtube. Two and a half volume of 100% ethanol and 0.1 volume of 3M sodium acetate were added and the sample was kept at -20 ˚C for 30 min. The suspension was centrifuged at 12,000 rpm for 10 min.

The supernatant was discarded and the pellet was washed in 70% ethanol, air-dried and re-suspended in 10 µl distilled water.

5.2 DNA extraction from leaves, unripe fruit and ripe fruit

Leaves, unripe fruit and ripe fruit was grounded with liquid nitrogen and extracted with 700 µl of CTAB buffer (2% CTAB, 0.1 M Tris HCl pH 8.0, 0.02 M EDTA, 1.4 M NaCl) (Doyle and Doyle, 1990). The mixture was vortexed and heated at 65˚C for 10 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new microtube. An equal volume of isoppropanol was added then centrifuged at 12,000 rpm for 10 min. The supernatant was discarded, the pellet was washed in 70% ethanol, air-dried and re-suspended in 100 µl distilled water.

5.2 PCR amplification

The *npt II* and *PRSV-CP* were amplified from genomic DNA of transgenic papaya using non-transgenic papaya as a negative comparator. *Papain and ACO* gene were amplified as endogenous gene positive control. Primers used in this study were shown in Table 1. The multiplex PCR amplification was performed in 20 µl volume reaction in the condition as followed: denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min and then a final 7 min extension at 72°C. For PCR amplification for *ACO* gene, the mixture was denatured at 94°C for 3 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min 30 s and then a final 10 min extension at 72°C. The PCR products were visualized on 1% agarose gel electrophoresis.

Table 1 Primers for amplification of transgenic PRSV-CP gene.

Primer name	**Gene**	**Sequence (5'→3')**	**Product size (bp)**	**Source**
SC104	*PRSV-CP*	ATTGCGCATACCTAGGAGAGAG TG	501	Phironrit *et al.*, 2010
SC501	*PRSV-CP*	AAAGTGGTATGAGGGAGTGAGG AA		
NPT1	*nptII*	ATGATTGAACAAGATGGATTGC	790	Phironrit *et al.*, 2007
NPT2	*nptII*	TCAGAAGAACTCGTCAAGAAGG		
Papain-A1	*papain*	GGCTCAATATGGTATTCACTACA GAAAT	363	Xu *et al.*, 2008
Papain-A2	*papain*	CATCGGTTTTGGCTGCATAA		

5.3 PCR Southern hybridization

Amplified DNA was separated on 1% agarose gel at 90 V for 30 min. The DNA was depurinated by soaking the gel in 0.25 M HCl for 10 min and neutralized by shaking in 0.4 N NaOH for 15 min at room temperature. The DNA was transferred to positively charge nylon membrane (Roche, USA). The membrane was prehybridized in 15 ml of standard hybridization buffer (5X SSC, 0.1% N-laurylsacosine, 0.02% SDS, 1% blocking reagent) at 65 °C for 1 h and hybridized overnight with 0.4 µg/ml interested probe. After hybridization, the membrane was washed with 2XSSC/ 0.1%SDS for 5 min twice and 0.1XSSC/ 0.1% SDS at 65 °C for 15 min twice. The blocking buffer was added onto the membrane and shaked at room temperature for 45 min. Anti-DIG was added to the membrane (1 µl of anti-DIG: 20,000 µl of blocking buffer) and shaked for 30 min. The membrane was washed twice in washing buffer (1X maleic acid pH 7.5/ 0.3% (V/V) Tween 20) for 15 min. Then was transferred to the detection buffer (0.1M Tris-HCl pH 9.5/ 0.1M NaCl) and shaked gently for 5 min. CDP star solution (800 µl of detection buffer + 4 µl CDP star) was added and the membrane was exposed to X-ray film.

6. Expression of transgene

6.1 Total RNA extraction

Total RNA was extracted by RNeasy® Plant Mini Kit according to the manufacturer's manual (QIAGEN, Germany). Papaya tissues (≤ 100 mg) were grounded with liquid nitrogen. Fine powder was transferred to a new microcentrifuge tube. Four hundred and fifty microliters of RLT buffer was added and vortexed vigorously. The lysate was pipetted directly onto QIAshredder spin column and centrifuged at 14,000 rpm for 2 min. Supernatant was transferred to a new tube and 0.5 volume of 100% ethanol was added. The mixture was immediately mixed by pipetting. The sample was applied to RNeasy mini column and centrifuged at 12,000 rpm for 15 sec. The flow-through was discarded and 700 µl of RW1 buffer was added to RNeasy column. The centrifugation was performed at 12,000 rpm for 15 sec. The RNeasy was transferred to a new collection and 500 µl of RPE buffer was added then centrifuged at 12,000 rpm for 15 sec. Five hundred microliters of RPE buffer was added and centrifuged at 12,000 rpm for 2 min. The column was transferred to a new microcentrifuge tube and RNA was eluted from RNeasy silica-gel membrane by adding 50 µl of RNase-free water and centrifuged at 12,000 rpm for 1 min. The concentration and purity of total RNA were determined using 1% agarose gel electrophoresis and spectrophotometer (Ultrospec® 500/1100) at 260 and 280 nm and kept at -20ºC until used.

6.2 Reverse-transcription-polymerase chain reaction (RT-PCR)

6.2.1 *DNase* I treatment

Total RNA was treated with with *DNase I* according to manufacturer instruction. One microgram of total RNA was *DNase I* treated in the reaction containing 1 µl of 10X reaction buffer with $MgCl_2$, 1 µl of *DNase* I (10 U/µl) and DEPC-treated water to total volume of 10 µl. The reaction was incubated at 37°C for 30 min and terminated by adding 1 µl of 0.1 M EDTA (pH 8.0) and incubated at 65°C for 10 min. The *DNase* I treated RNA was further used as template in RT reaction.

6.2.2 Reverse transcription-polymerase chain reaction (RT-PCR)

Multiplex reverse-transcription polymerase chain reaction (multiplex RT-PCR) was performed in order to determine transgene expression in leaf, unripe fruit, ripe fruit and pollen. *PRSV-CP* and *nptII* gene specific primer was used combining with internal control, *papain* gene. The reaction was carried out in 20 µl reaction and conducted as followed: 94°C for 5 min; 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min, 72°C for 1 min; final elongation at 72°C for 10 min. The PCR products were run on a 1% agarose gel 90 V for 30 min and further analysed using PCR Southern blotting.

First-strand cDNA was synthesized from 1 µg total RNA with gene specific primers according to the manufacturer's protocol (Invitrogen Life Technologies, USA). One microliter of reverse specific primer and 1 µl of dNTP was added into the *DNase* treated RNA and brought final volume of 13 µl with *RNase*-free water. The reaction was heated at 65°C for 5 min and quenched at least 1 min on ice. Four microliters of 5X First-Strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT (40 U/µl) and 1 µl of SuperScript^{TM} III RT (200U/µl) was added then incubated at 55°C for 30-60 min. Finally, the reaction was inactivated at 70°C for 15 min.

Expression patterns of genes in transgenic and non-transgenic papaya tissues including leaf, unripe fruit, ripe fruit and pollen grain were determined by RT-PCR analyses using the gene specific primer sets. The PCR mix consisted of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each gene specific primer, 2.5 U of *Taq* polymerase and 1x PCR buffer supplied with the *Taq* polymerase. The reaction was conducted under the following condition: 94°C for 10 min; 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min, 72°C for 1 min; final elongation at 72°C for 10 min. The PCR products were run on a 1% agarose gel 90 V for 30 min and further analysed using PCR Southern blotting.

6.2.3 Southern analysis

The DNA was depurinated by soaking the gel in 0.25 M HCl for 10 min and neutralized by shaking the gel in 0.4 N NaOH for 15 min at room temperature. The DNA was transferred to positively charge nylon membrane (Roche, USA). The membrane was prehybridized in 15 ml of standard hybridization buffer (5X SSC, 0.1% N-laurylsacosine, 0.02% SDS, 1% blocking reagent) at 65 °C for 1 h and hybridized overnight with 0.4 µg/ml DIG labelled probe. After hybridization, the membrane was washed with 2XSSC/ 0.1%SDS for 5 min twice and 0.1XSSC/ 0.1% SDS at 65 °C for 15 min twice. The blocking buffer was added onto the membrane and shaked at room temperature for 45 min. Anti-DIG was added to the membrane (1 µl of anti-DIG: 20,000 µl of blocking buffer) and rotated for 30 min. The membrane was washed twice in washing buffer (1X maleic acid pH 7.5/ 0.3% (V/V) Tween 20) for 15 min. The membrane was transferred to the detection buffer (0.1M Tris-HCl pH 9.5/ 0.1M NaCl) and shaked gently for 5 min. Finally, CDP star solution (800 µl of detection buffer + 4 µl CDP star) was added and the membrane was exposed to X-ray film.

7. Determination transgene protein

7.1 Protein extraction

Four different papaya tissue (leaf, unripe fruit, ripe fruit and pollen) were grounded with GEB and PEB extraction buffer according manufracturer instruction for *PRSV-CP* and *nptII* protein detection (Agdia, Elkhart, IN). Tissue weight and buffer ratio was 1:10 for GEB and 1:5 for PEB buffer (except for pollen). Grounded sample was transferred to a new microtube and centrifuged 10,000 rpm at room temperature for 5 min. Supernatant was transferred to a new tube and used for protein detection. The concentration of total protein was measured according to Bradford's method (Bradford, 1976). The 500 µg of total protein from each papaya tissue was further used for protein detection by DAS-ELISA.

7.2 DAS-ELISA

Protein expression of transgene in transgenic papaya was carried out using double antibody sandwich ELISA (DAS ELISA) method according to manufacturer instruction (Agdia, Elkhart, IN). The 500 mg of each protein sample was loaded into ELISA plate and incubated in moistured box for 2 h at room temperature. ELISA plate was washed 4-6 times with 1xPBST buffer then 100 µl of enzyme conjugate was added. After 2 h of incubation, ELISA plate was washed once before adding substrate solution. One hundred microliters of *p*-nitrophenyl phosphate (1 mg/ml in 1xPNP buffer) was added and incubated for 1 h at room temperature for *PRSV-CP*. The result was measured by 405 nm ELISA reader (Microplate Reader, Multiskan, Labsystem). For *nptII* protein detection, TMB substrate was added to *nptII* tested ELISA plate and incubated for 15 min before adding 50 µl of 3M sulfuric acid. The result was examined using ELISA reader at 450 nm (Microplate Reader, Multiskan, Labsystem). The experiment was done in triplicate.

8. Statistical analysis

The differences of pollen morphological studies including pollen diameter, pollen grain number per anther, pollen weight, pollen viability and pollen germination were statistical analysed using T-Test while effect of environmental stress on pollen germination was statistical analysed using Duncan's multiple range test by SPSS 15.0 (SPSS Inc, Chicago, USA). Each experiment was done as complete block design which differences were declared significant when $P<0.05$.

RESULTS

1. Plant morphological characteristics and horticultural traits

1.1 Plant morphological characteristics

Comparative analysis of papaya height was performed in two consecutive seasons. The first season indicated that height of non-transgenic was significantly lower than transgenic papaya line p116/5 R_4 in the first 9 weeks. After the 10^{th} weeks, the height of transgenic and non-transgenic papaya displayed no significant difference at $P<0.05$ (Figure 10A). In R_5 generation, height of both transgenic and non-transgenic papaya since the first week was determined (Figure 10B). For height at first flower emergence and first fruit set, number of nodes at first flower emergence and first fruit set, trunk girth at first flower emergence and first fruit set, the results showed no significant difference between transgenic and non-transgenic papaya determined within 2 generations. Each characters were resemble between generations (Table 2).

1.2 Horticultural traits

All of papaya fruit was collected and horticultural traits including fruit number per tree, fruit weight (kg) per tree, fruit weight, fruit length (cm), fruit radius (cm), fruit diameter (cm), pupl thick ness (cm), total soluble solid (TSS) as well as seed setting were measured. The first crop season, p116/5 R_4 generation of transgenic was compared to non-transgenic papaya counterpart. The statistical analysis showed that horticultural traits of transgenic papaya were not different from non-transgenic papaya. The differences of fruit length was found within first crop season, however it was not a critical character while the other characters was statistically identical. The average fruit weight was 1.78±0.1 kg and 1.51±0.4 kg with 26.19±5.8 kg and 25.33±16.5 kg of production in transgenic and non-transgenic, respectively. The result also showed the quantification of TSS in papaya fruit that the sweetest part of papaya came from the bottom part at 9.79±0.3 brix and 10.04±1.4 brix in transgenic and non-

transgenic, respectively. Comparison of seed setting also showed that there is no significant difference between pair comparison at 107.43±61.2 seed for transgenic and 138.45±60.1 seed for non-transgenic. However the difference was found in fruit length (Table 3).

In second season, comparative analysis of horticultural traits were also performed to repeat the first crop season. Although almost all characters was comparable when examined by statistical test, TSS displayed some differences though minimal. Comparative study of fruit characters including fruit form and flesh color indicated that both transgenic and non-transgenic papaya produced elongate fruit shape with a orange-red flesh color (28A) quantified by RSH color chart (Royal Horticultural Soceity, England) (Figure 11-Figure 13).

It could be indicated that transgenic papaya harboring coat protein gene of PRSV did not cause any adverse effect on growth rate and fruit quality and quantity of papaya plant.

2. Morphological and physical characters of papaya pollen

The study of papaya pollen morphology was done using both light microscopy and Scanning Electron Microscopy (SEM). Pollen grains from transgenic and non-transgenic papaya were spherical in shape consisting of three tricolporate apertures which was the combination between pore and elongate boat-shape structure. Aperture is an area that pollen tube will emerges during pollen germination. Papaya pollen surface was finely reticulate type (Figure 14-Figure 16). Physical characters of pollen grains were shown in table 4. The dimention of pollen was 25.52±1.2 µm and 25.18±1.2 µm in diameter with the average weigth of individual pollen grain at 13.23±3.1 ng and 11.76±1.5 ng for transgenic and non-transgenic papaya comparator. The average number of pollen grains from transgenic and non-transgenic papaya was 14,493±1,167 and 14,090±1,303 pollen grains per anther, respectively. These data showed no significant difference using T-Test at $P<0.05$. The result did not show any difference in morphological and physical characters of pollen grains from transgenic and non-transgenic papaya.

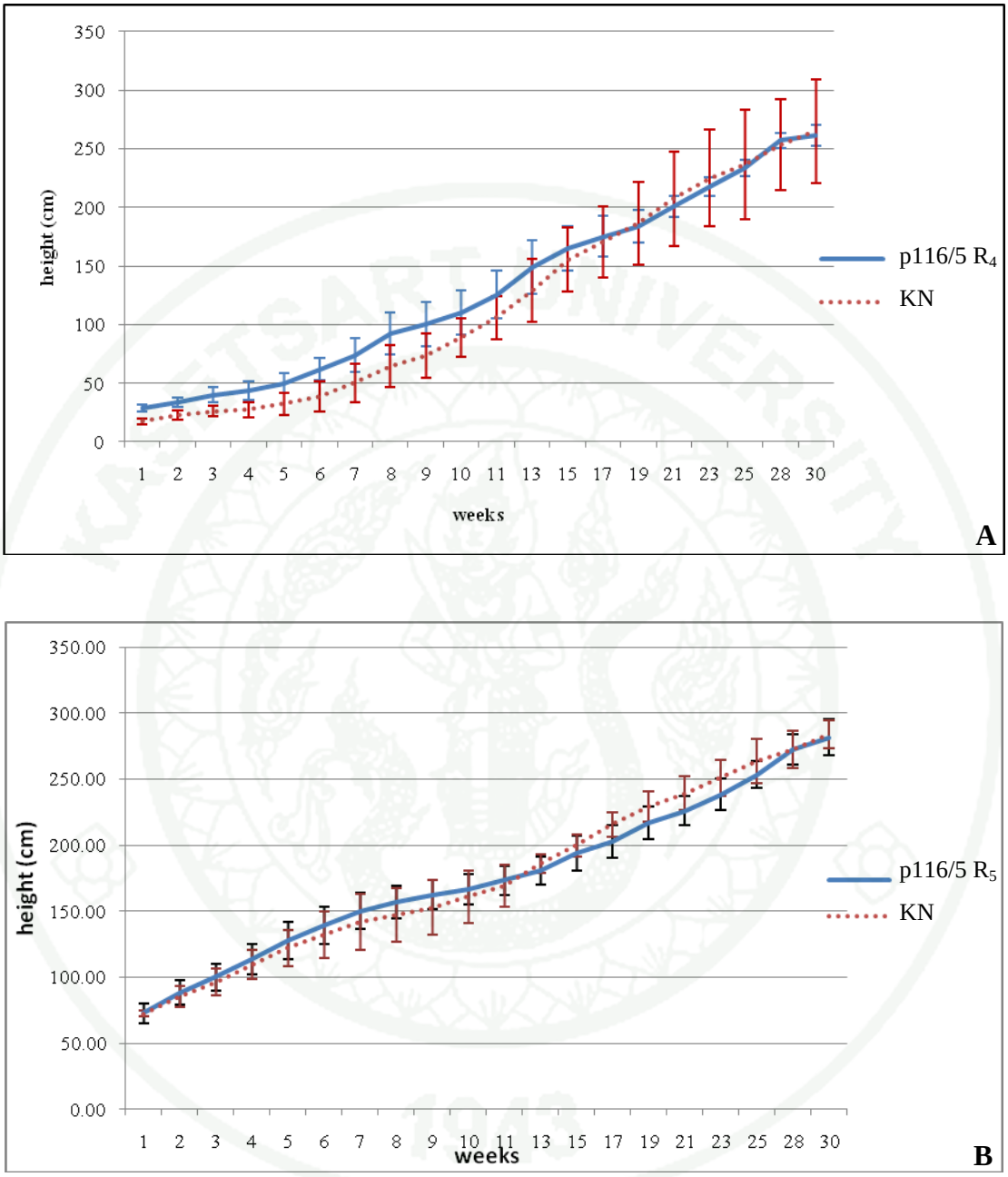

Figure 10 Height comparing between R_4 (A) and R_5 (B) generation of p116/5 tansgenic papaya and non-transgenic counterpart (KN) cultivated under screenhouse condition. Y axis represent height of papaya plant in cm unit whereas x axis represent time of measurement (week).

Table 2 Plant morphological characteristic of p116/5 transgenic papaya R_4 and R_5 generation comparing to non-transgenic papaya in two consecutive seasons.

Morphological characters	**R_4 generation**		**R_5 generation**	
	p116/5 R_4	**KN**	**p116/5 R_5**	**KN**
Height at first flowering (cm)	64.89±3.0[a]	64.67±7.0[a1/]	72.83±5.7[a]	79.83±5.3[a]
Height at first fruit set (cm)	74.28±5.3[a]	75.00±11.5[a]	89.33±14.5[a]	97. 83±8.1[a]
Number of node at first flowering	21.19±1.4[a]	21.5±1.1[a]	22.5±2.0[a]	24.0±2.0[a]
Number of node at first fruit	24.67±2.8[a]	24.50±2.4[a]	26.0±2.5[a]	27.83±1.9[a]
Girth length at first flowering (cm)	12.58±1.0[a]	12.67±1.6[a]	11. 5±2.1[a]	11.5±0.8[a]
Girth length at first fruit set (cm)	23.53±2.5[a]	27.50±4.4[a]	14.67±2.3[a]	16.5±2.6[a]

[1/]Mean values (±SD) are significantly different at $P<0.05$ when adjacent letters are different using T-Test.

The experiment was done in 6 replications.

Table 3 Horticultural traits of papaya fruit comparing between PRSV resistant transgenic (p116/5) and non-transgenic (KN) papaya in R_4 and R_5 generation.

Horticultural traits	R_4 generation		R_5 generation	
	p116/5 R_4	KN	p116/5 R_5	KN
fruit/tree	15.0±3.4[a]	17.83±6.2[a1/]	22.33±5.2[a]	21.67±5.3[a]
fruit weight (kg)	1.78±0.1[a]	1.51±0.4[a]	1.34±0.3[a]	1.21±0.2[a]
fruit weight/tree (kg)	26.19±5.8[a]	25.33±16.5[a]	29.28±6.9[a]	25.97±8.6[a]
fruit length (cm)	39.05±1.6[a]	32.91±4.5[b]	33.64±3.1[a]	32.2±2.1[a]
fruit radius (cm)	33.07±2.0[a]	33.44±1.6[a]	29.2±3.3[a]	29.16±1.3[a]
fruit diameter (cm)	10.08±0.7[a]	10.2±0.5[a]	8.66±0.9[a]	8.95±0.5[a]
left pulp thickness (cm)	2.64±0.1[a]	2.52±0.3[a]	2.27±0.1[a]	2.28±0.1[a]
right pulp thickness (cm)	2.63±0.1[a]	2.55±0.3[a]	2.30±0[a]	2.31±0.1[a]
top TSS (brix)	9.4±0.3[a]	9.43±1.1[a]	9.14±0.5[b]	10.10±0.5[a]
middle TSS (brix)	9.74±0.3[a]	9.94±1.2[a]	9.78±0.6[b]	10.75±0.6[a]
bottom TSS (brix)	9.79±0.3[a]	10.04±1.4[a]	10.05±0.5[b]	11.25±0.8[a]
seed setting/ fruit	107.43±61.2 [a]	138.45±60.1[a]	127.11±70.2[a]	151.89±85.6[a]

[1/]Mean values (±SD) are significantly different at $P<0.05$ when adjacent letters are different using T-Test

Figure 11 Overall fruit number and morphological characters comparing between transgenic (A) and non-transgenic papaya (B).

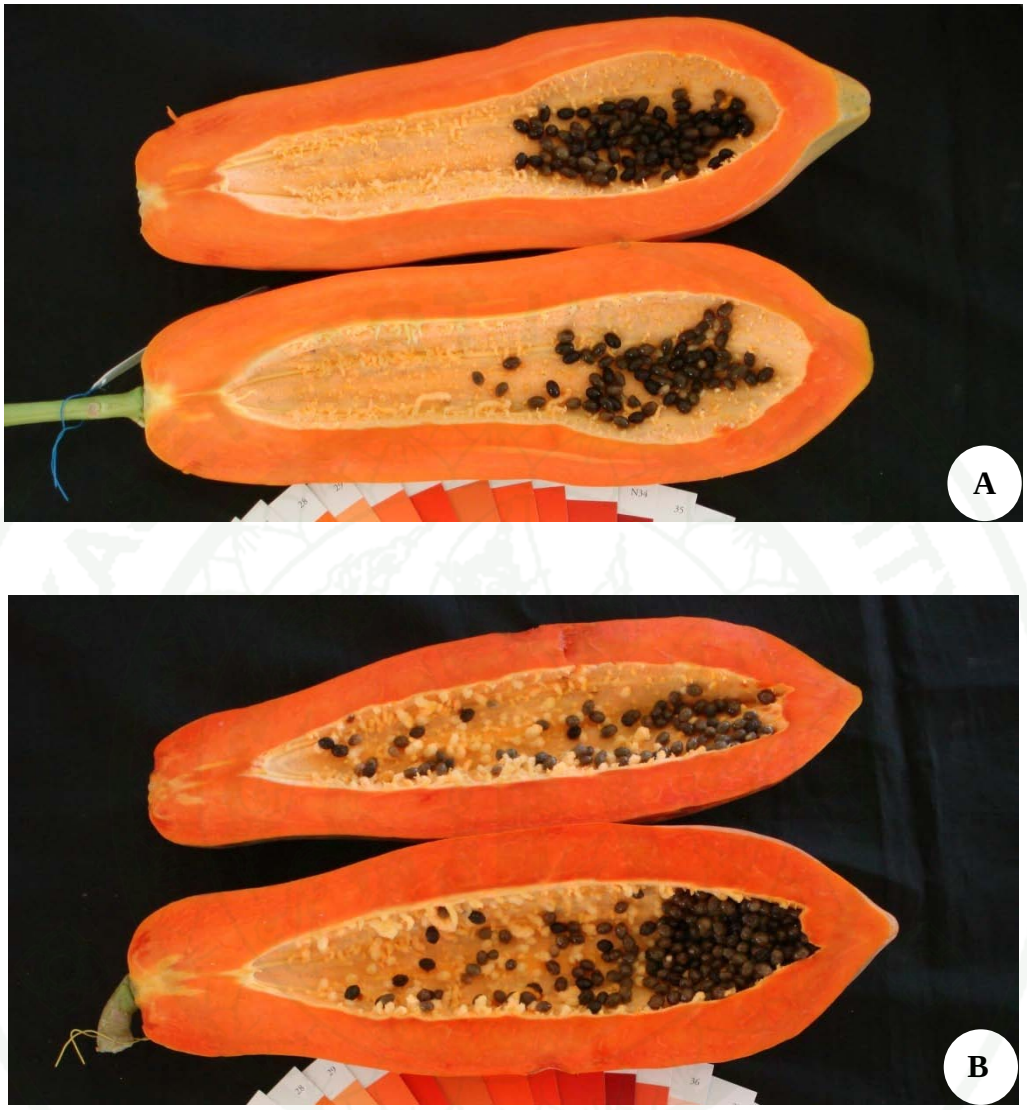

Figure 12 Fruit character of transgenic papaya (A) and non-transgenic papaya (B) afer cut vertically showed pulp thickness and seed number.

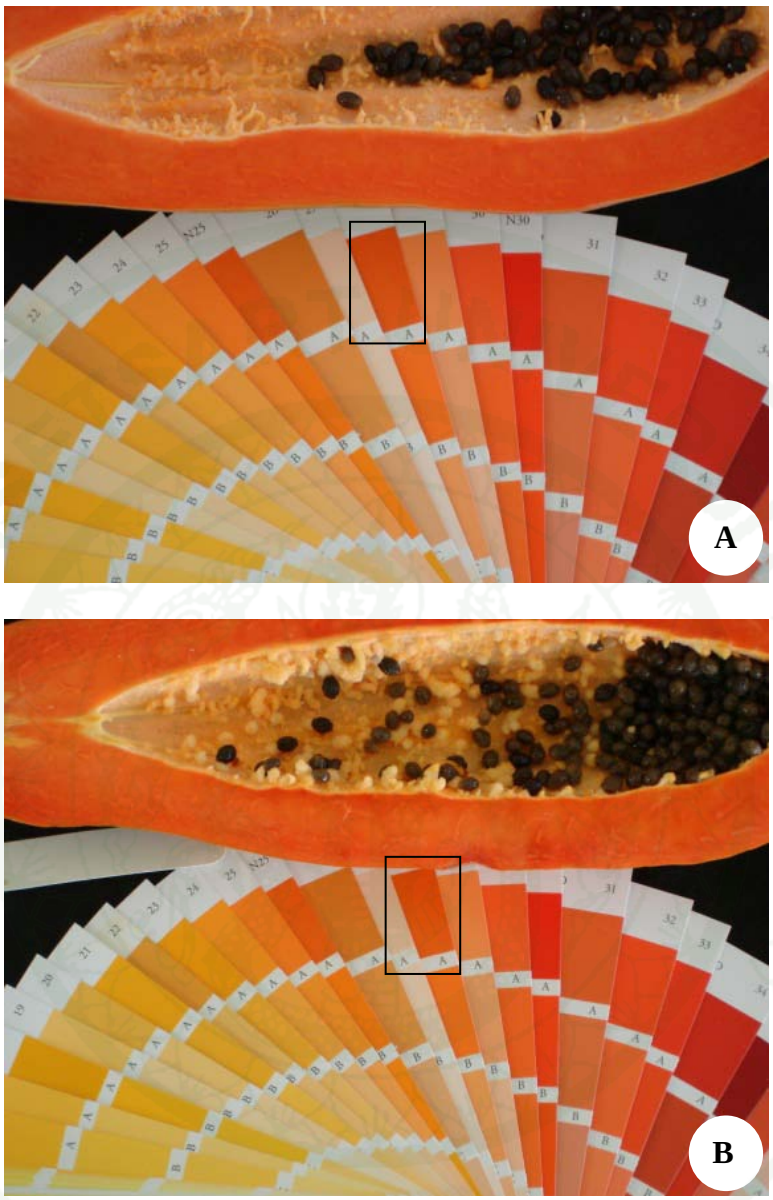

Figure 13 Flesh color of transgenic (A) and non-transgenic papaya (B) was determined by comparing to the color chart. The range of each papaya flesh color was represent in a square.

3. Pollen biology

Pollen biology is an important criterion to determine plant fitness. In this study, pollen viability, pollen germination and pollen longevity were measured. Viability of mature pollen grains from transgenic and non-transgenic papaya was

94.27±2.3 and 94.76±1.4%, respectively. The germination was 60.42±9.7 and 64.73±3.4%, respectively (Table 4). There was no significant difference between transgenic and non-transgenic papaya pollen viability and germination using T-test at p=0.05. The investigation of pollen longevity indicated that pollen grains from both transgenic and non-transgenic papaya lost almost their viability after 24 h at (31-35°C, 53-65%RH) (Figure 17).

4. Determination of environmental stress effect on pollen germination

In order to investigated the influence of physical factors on the capability of pollen germination, both transgenic and non-transgenic papaya pollen were exposed to three different factors; temperature, relative humidity and UVA and UVB radiation. The results showed that 28ºC condition supported highest germination at 73.02% and 49.73% for transgenic and non-transgenic papaya, respectively. At high temperature (> 32ºC), pollen germination tend to be decreased and when comparing the germination capacity between transgenic and non-transgenic papaya at the same temperature the results showed that there is no significant difference among pollen germination percentage from two types of papaya (Figure 18). Examination the effect of relative humidity (RH) on pollen germination also displayed no difference. Among four relative humidity levels, 80% RH given the best pollen germination percentage while 20%, 40% and 60% RH showed similar results. Moreover, the comparison of pollen germination between transgenic and non-transgenic papaya at the same relative humidity level also showed no different at 20% and 40% RH whereas 60% and 80% RH exhibited some differences (Figure 19). The effect of UVA and UVB radiation on pollen germination was also observed. The results indicated that UVA and UVB had an effect on pollen germination. The negative correlation between the distance from UVA and UVB radiation and pollen germination was observed with the pollen located far away from UV source had higher germination than when closing. In addition, germination of transgenic and non-transgenic pollen at the same UV distance also investigated and did not found any different between transgenic and non transgenic pollen (Figure 20).

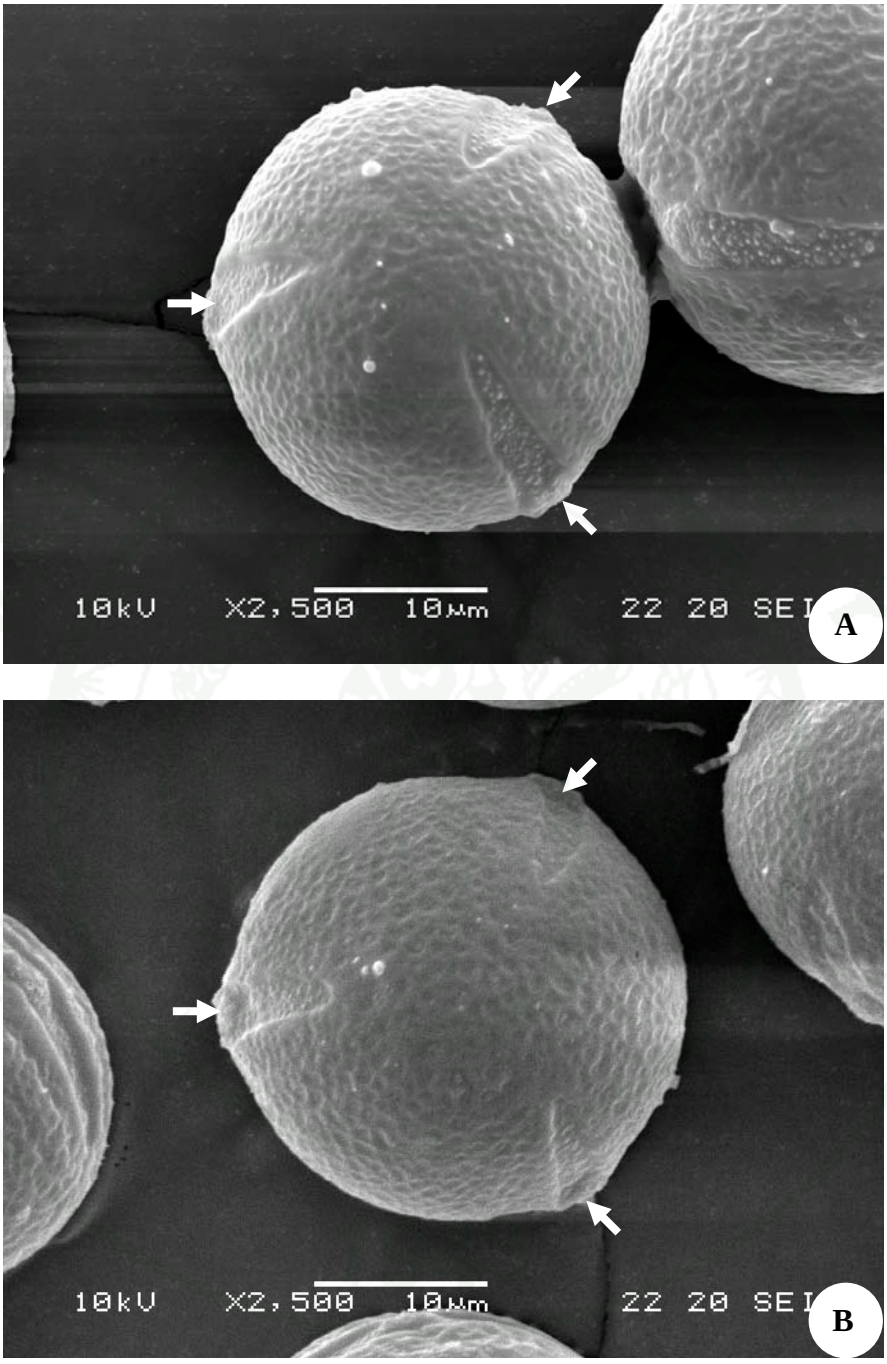

Figure 14 Pollen configuration of transgenic (A) and non-transgenic papaya (B) showed that pollen was spherical in shape comprising three apertures (white arrow).

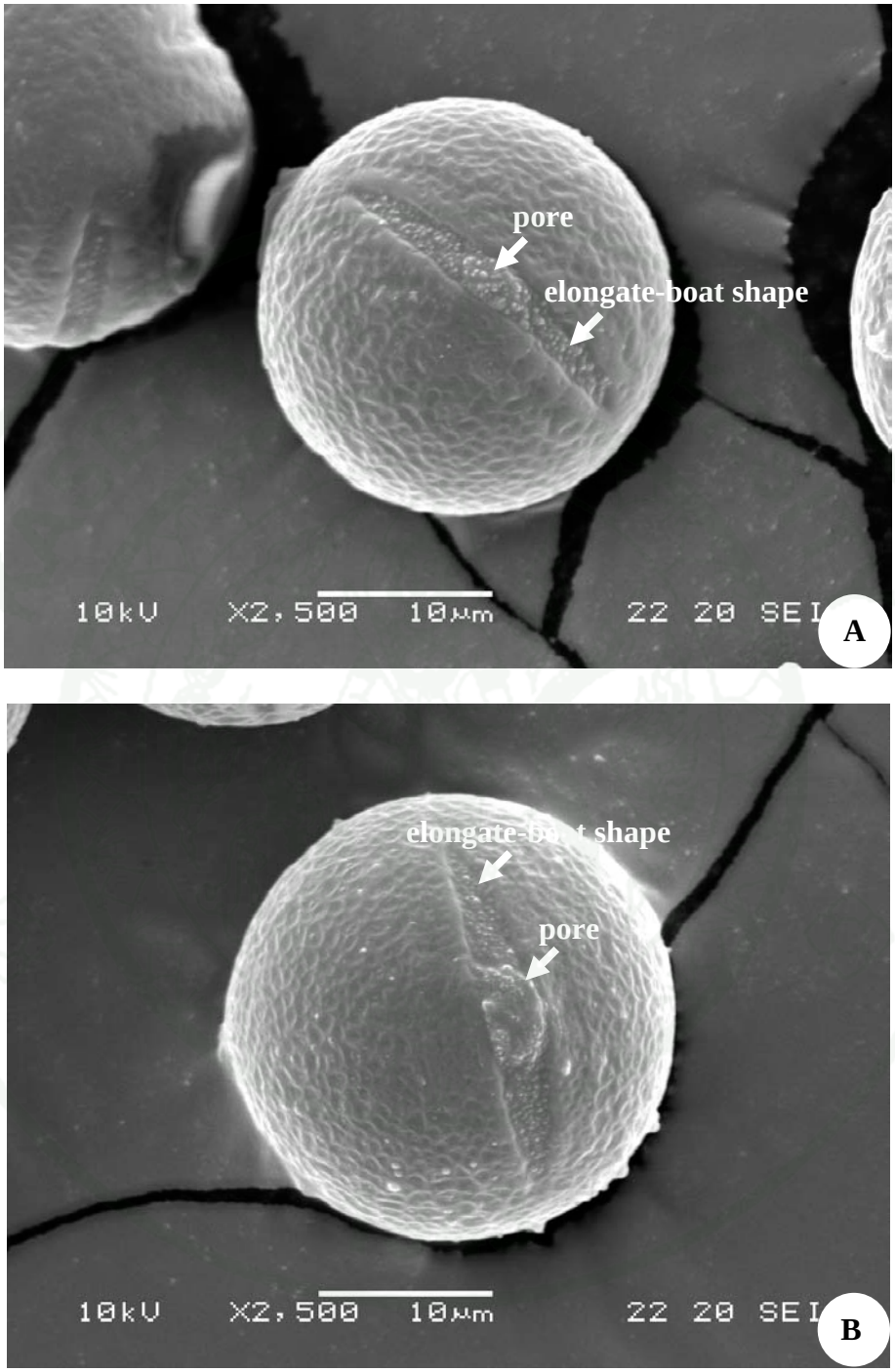

Figure 15 Pollen apertures of both transgenic (A) and non-transgenic papaya (B). Papaya pollen aperture is the combination between pore and elongate-boat shape furrow.

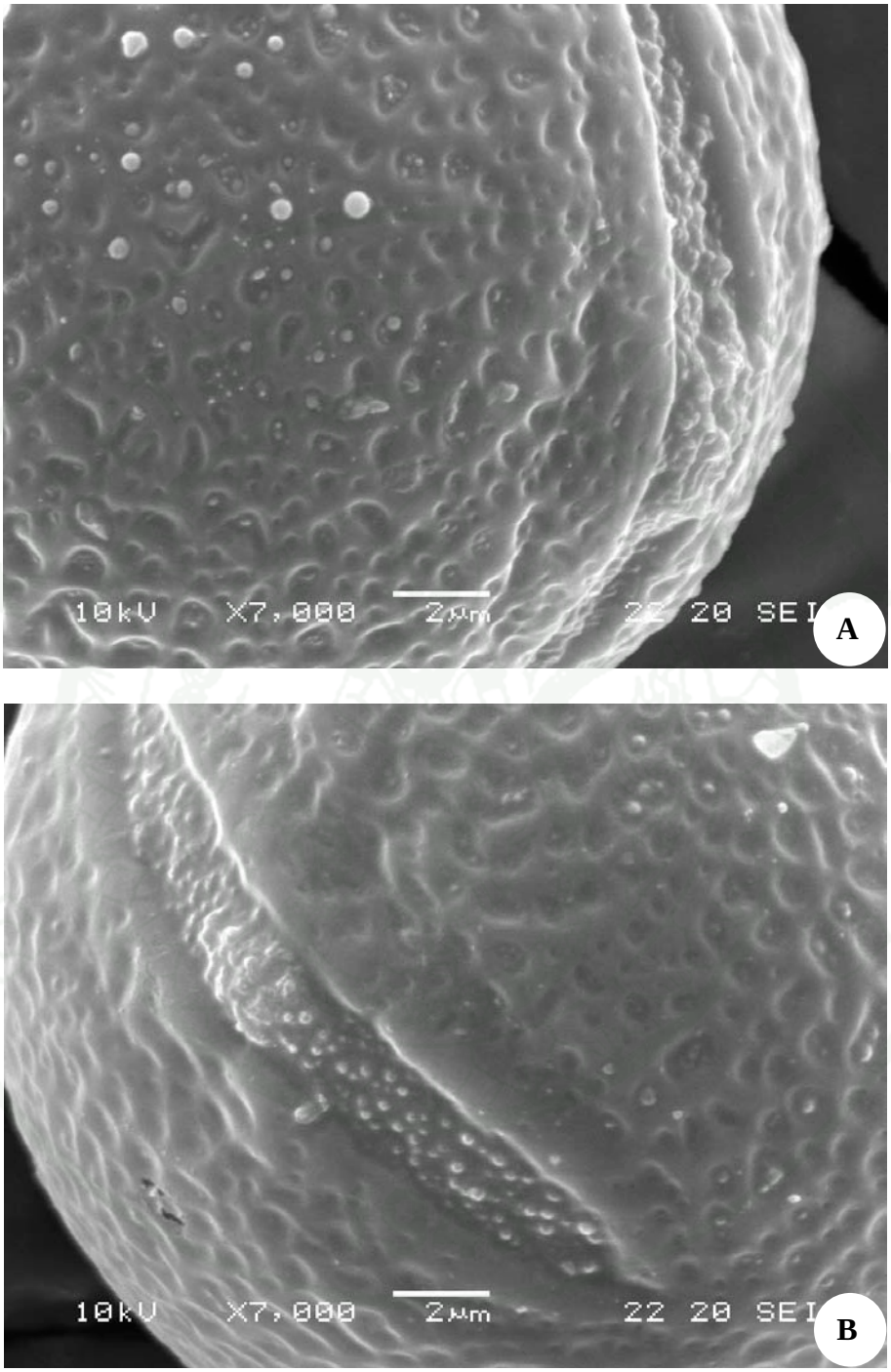

Figure 16 Exine sculpture (surface) of transgenic (A) and non-transgenic pollen (B) showed that it was finely reticulate type.

Table 4 Mean values (±SD) of pollen morphological and physical characters of p116/5 R_5 transgenic and non-transgenic papaya (KN).

Character	**p116/5 R_5**	**KN**
Shape	Spherical	Spherical
Aperture type	Tricolporate	Tricolporate
Surface	Finely reticulate	Finely reticulate
Number of aperture	3	3
diameter (µm)	25.52 ± 1.2[a]	25.18 ± 1.2[a1/]
weight (ng)	13.23 ± 3.1[a]	11.76 ± 1.5[a]
Pollen per anther (pollen)	14,493 ± 1167[a]	14,090 ± 1,303[a]
Viability (%)	94.27 ± 2.3[a]	94.76 ± 1.4[a]
Germination (%)	60.42 ± 9.7[a]	64.73 ± 3.4[a]

[1/]Mean values are significantly different at $P<0.05$ when adjacent letters are different using T-Test.

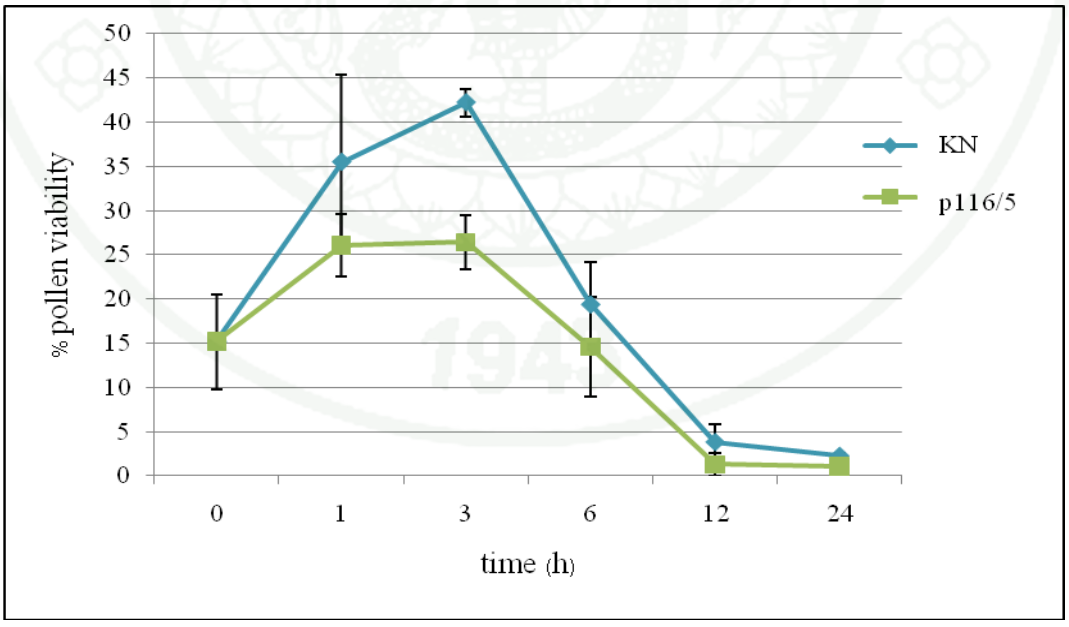

Figure 17 Pollen longevity comparing between transgenic (p116/5) and non-transgenic papaya (KN) was determined since pollen shedding till almost lack of ability to germination.

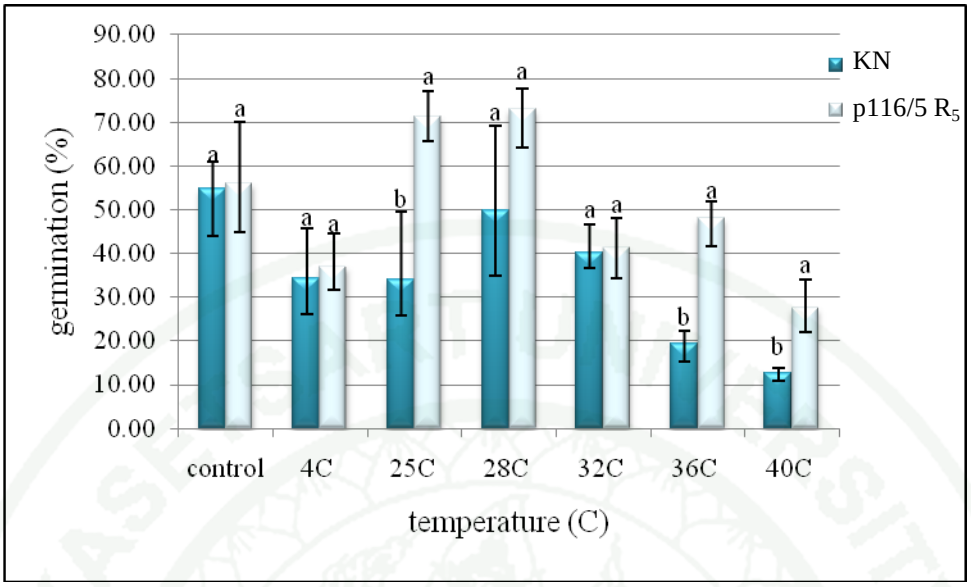

Figure 18 Investigation the effect of temperature on pollen germination comparing between transgenic (p116/5 R_5) and non-transgenic papaya (KN).

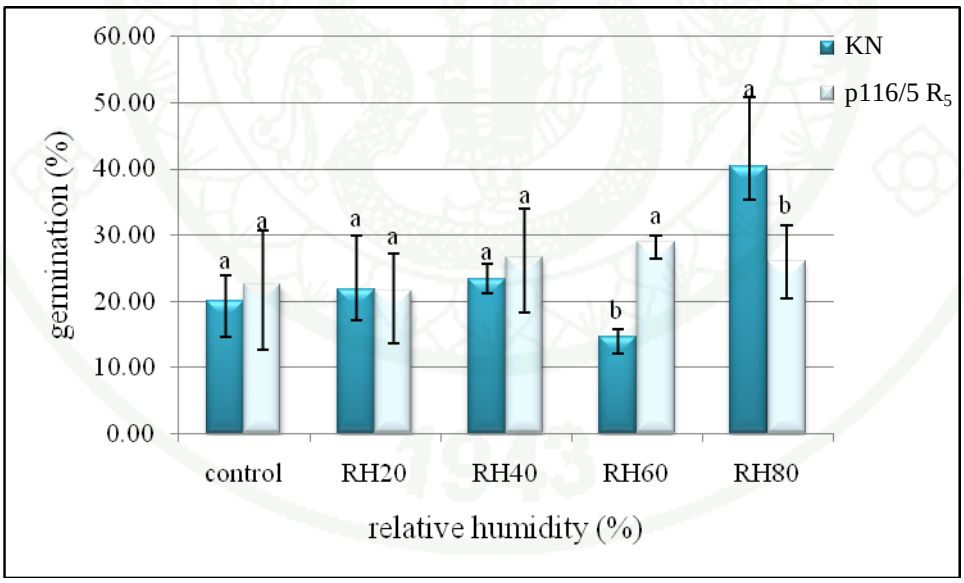

Figure 19 Comparative study the influence of relative humidity on pollen germination of transgenic (p116/5 R_5) and non-transgenic counterpart (KN) pollen.

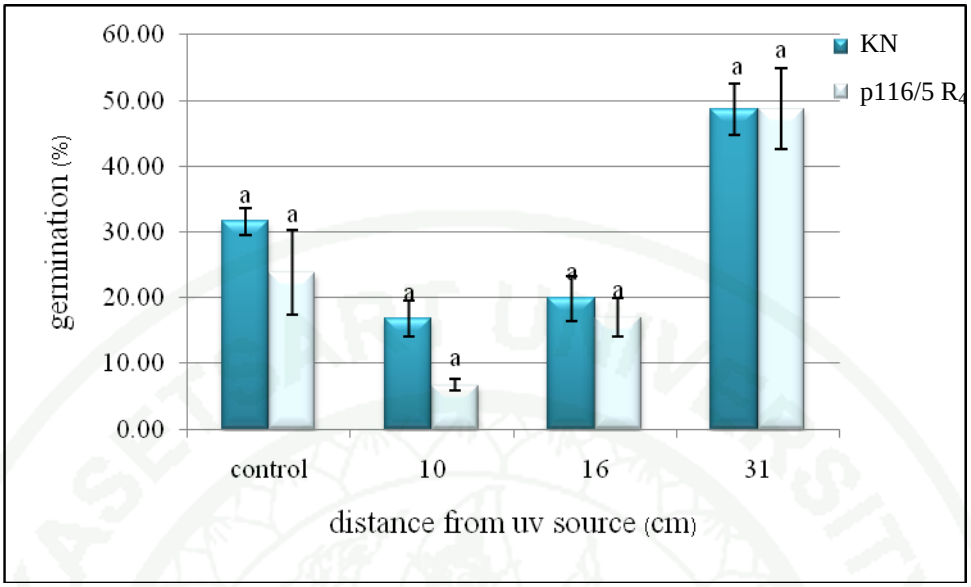

Figure 20 Comparison of transgenic (p116/5 R_4) and non-transgenic (KN) papaya pollen germination affected by the explosion under UVA and UVB radiation**.**

5. Presence of transgene and transgene expression in transgenic papaya

5.1 Development of an effective method for pollen genomic DNA extraction

In this study, an effective method for pollen extraction was developed. The liquid nitrogen extraction without adding of proteinase K in any buffer gives the better results comparing to the extraction without liquid nitrogen (Figure 21). The results showed that pollen extracted through CTAB buffer provided the best result and was used for further studies. The same method was further used for DNA extraction in another plant tissue include leaf, unripe fruit and ripe fruit. The extracted DNA was verified the quality and quantity by 1% agrorose gel electrophorsis. The results showed that papaya leaf tissue gave highest yield comparing to the others. There was no difference in quality and quantity of genomic DNA from transgenic and non-transgenic papaya tissue (Figure 22).

5.2 Presence of transgene in transgenic papaya

Two transgenes, *PRSV-CP* and *nptII* in transgenic papaya were determined by multiplex PCR amplification using gene specific primers. The 501 bp of CP gene and 790 bp of *nptII* gene were found only in tested tissues of transgenic papaya but not in any tissues of non-transgenic papaya. An endogenous gene, *papain*, was used as internal control. PCR product with 363 bp in size, was amplified from any papaya samples (Figure 23 and Figure 24).

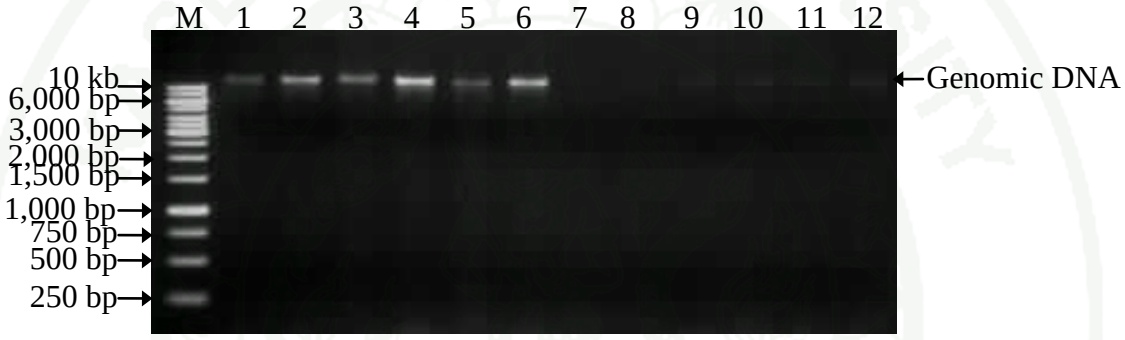

Figure 21 Genomic DNA extraction from papaya pollen with (lane 1-6) and without homogenization (lane 7-12) using CTAB buffer (lane1-2 and 7-8), pollen extraction buffer (lane3-4 and 9-10), and TLES buffer (lane 5-6 and 11-12). The odd numbers represent the extraction combining proteinase K whereas the even numbers represent the extraction without combining proteinase K. The 1kb DNA ladder was showed on the left (lane M).

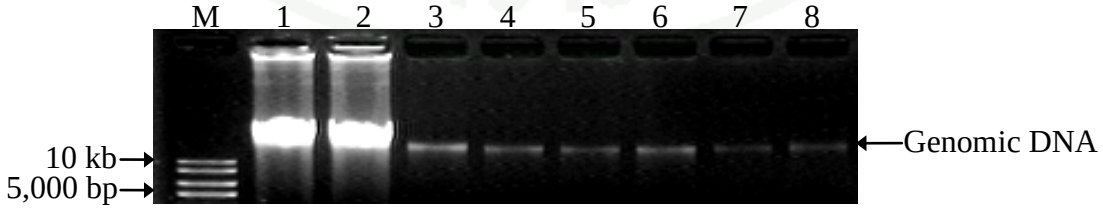

Figure 22 Genomic DNA extraction from leaves (lane 1-2), unripe fruit (lane 3-4), ripe fruit (lane 5-6) and pollen (lane 7-8). Odd and even number represent tissue from non-transgenic and transgenic papaya, respectively. The 1kb DNA ladder was showed on the left (lane M).

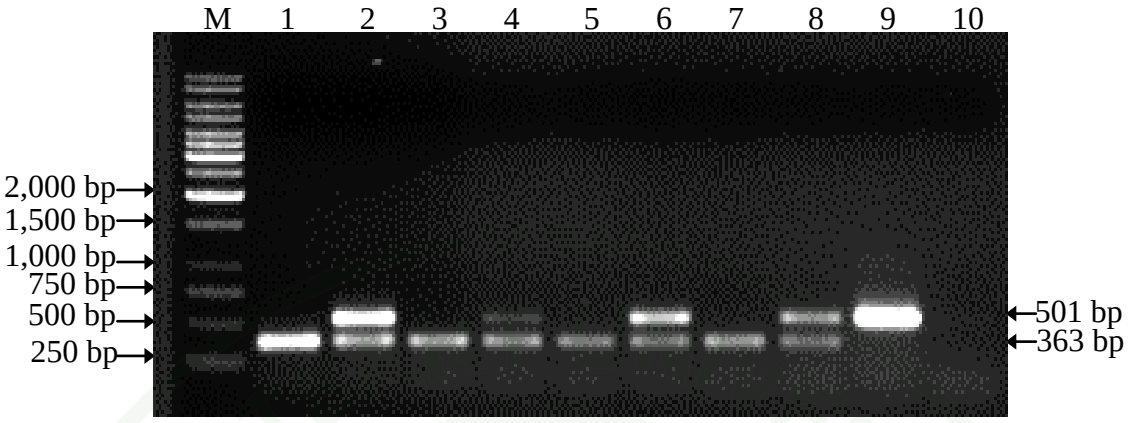

Figure 23 Multiplex PCR of *PRSV-CP* and *papain* gene from leaves (lane 1-2), unripe fruit (lane 3-4), ripe fruit (lane 5-6) and pollen (lane 7-8) of non-transgenic (lane 1, 3, 5 and 7) and transgenic papaya (lane 2, 4, 6 and 8). The p2CMCP plasmid (lane 9) and H_2O (lane 10) was used as positive and negative control, respectively. The 1kb DNA ladder was showed on the left (lane M).

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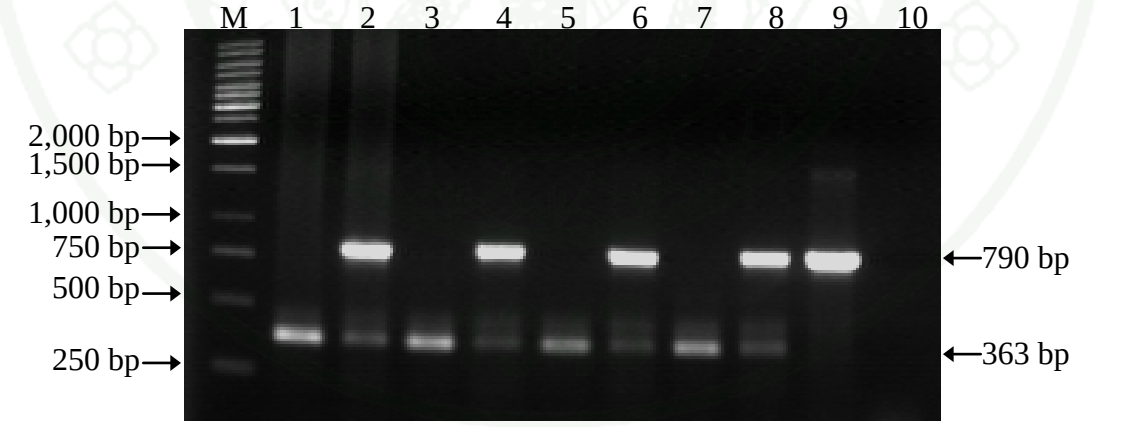

Figure 24 Multiplex PCR of *nptII* and *papain* gene from leaves (lane 1-2), unripe fruit (lane 3-4), ripe fruit (lane 5-6) and pollen (lane 7-8) of non-transgenic (lane 1, 3, 5 and 7) and transgenic papaya (lane 2, 4, 6 and 8). The p2CMCP plasmid (lane 9) and H_2O (lane 10) was used as positive and negative control, respectively. The 1kb DNA ladder was showed on the left (lane M).

5.3 Sensitivity of transgene detection in transgenic papaya pollen

Both transgenes (*PRSV-CP* and *nptII*) and an endogenous gene (*ACO*) can be amplified from genomic DNA from papaya pollen. The PCR and PCR Southern analysis showed similar sensitivity of detection except for *PRSV-CP* which PCR Southern analysis was 1000 times more sensitive. Pollen count and mathematics calculation indicated that approximately 30-40 pollen grains could be detected for the presence of transgene. Moreover, PCR amplification could detect 3-4 pollen grains for an endogenous gene, ACC oxidase (*ACO*). In conclusion, we were successfully developed a simple and sensitive method to detect transgene in transgenic papaya pollen (Figure 25-27 and Table 5).

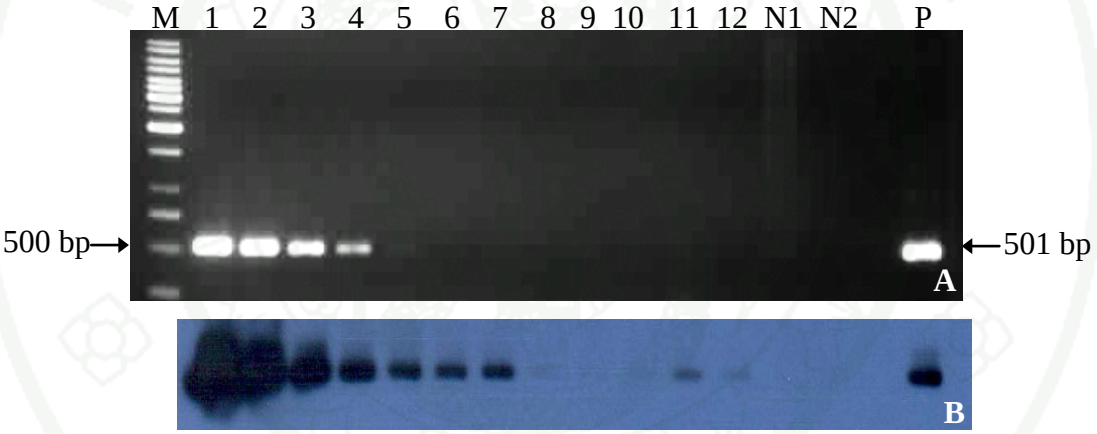

Figure 25 PCR amplification (A) and PCR Southern analysis (B) of the *PRSV-CP* gene from transgenic papaya pollen. Lane1-12 represent ten-fold serial dilution of genomic DNA from pollen. Non-transgenic papaya leaves and H_2O (lane N1 and N2) was used as negative control, respectively while transgenic papaya leaf (lane P) was used as positive control. The 1kb DNA ladder was showed on the left (lane M).

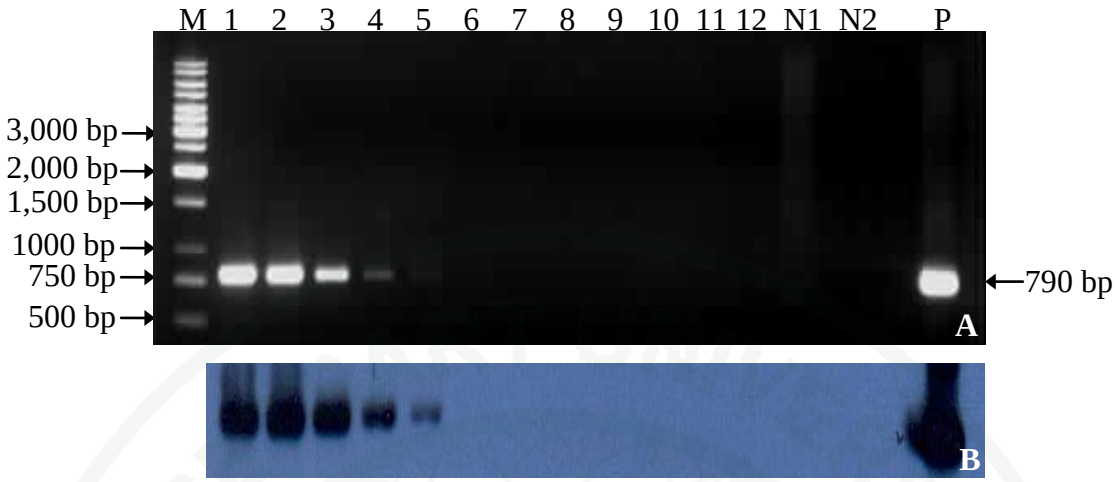

Figure 26 PCR amplification (A) and PCR Southern analysis (B) of *nptII* gene from transgenic papaya pollen. Lane1-12 represent ten-fold serial dilution. Leaves of non-transgenic papaya and H_2O (lane N1 and N2) was used as negative control, while leaves of transgenic papaya (lane P) was used as positive control. The 1kb DNA ladder was showed on the left (lane M).

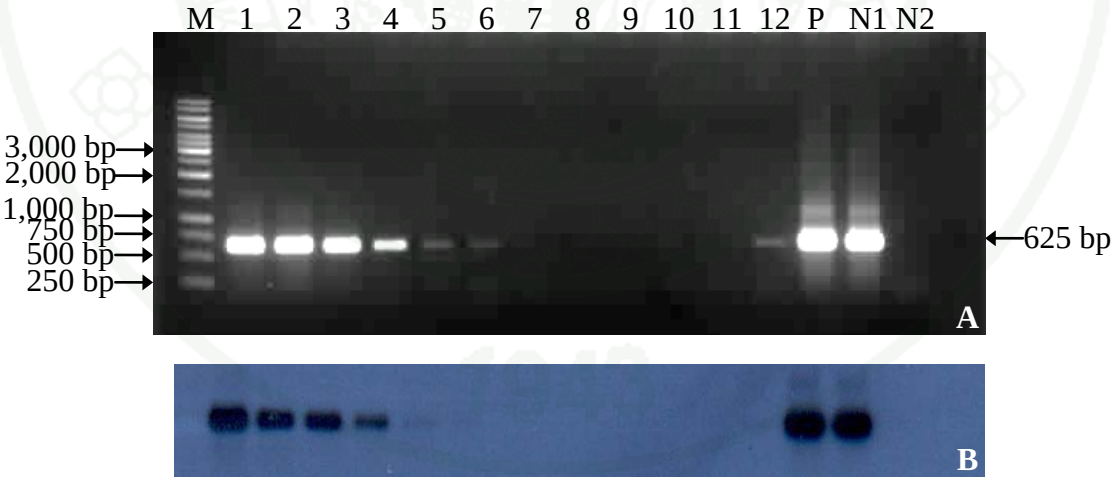

Figure 27 PCR amplification (A) and PCR Southern analysis (B) of the *ACO* gene from transgenic papaya pollen. Lane1-12 represent ten-fold serial dilution of genomic DNA from pollen. Leaves of non-transgenic and transgenic papaya (lane P and N1) was used as positive control, while and H_2O (lane N2) was used as negative control. The 1kb DNA ladder was showed on the left (lane M).

Table 5 Number of papaya pollen grains in ten-fold serial dilution.

Dilution of papaya pollen	No. of pollen
10^{0}	376,562.5
10^{-1}	37,656.3
10^{-2}	3,765.6
10^{-3}	376.6
10^{-4}	37.7
10^{-5}	3.8
10^{-6}	0.4
10^{-7}	0

5.4 Expression of transgene in transgenic papaya

5.4.1 Transcriptional level

At transcriptional level, the expression of *PRSV-CP*, *nptII* and *papain* gene was determined by one step RT-PCR amplification. The results indicated that there were transcripts of transgenes in tested transgenic plant tissue except pollen. The level of *PRSV-CP* and *nptII* transcripts was highest in leave followed by ripen and unripe fruit (Figure 28 and Figure 29). Expression of endogenous gene, *papain*, was detected in every tested transgenic and non-transgenic papaya tissue except pollen. Further, hybridization with papaya probe indicated that *papain* gene was expressed at low level. In addition, papain like gene, isoform of papain, was also found (Figure 30).

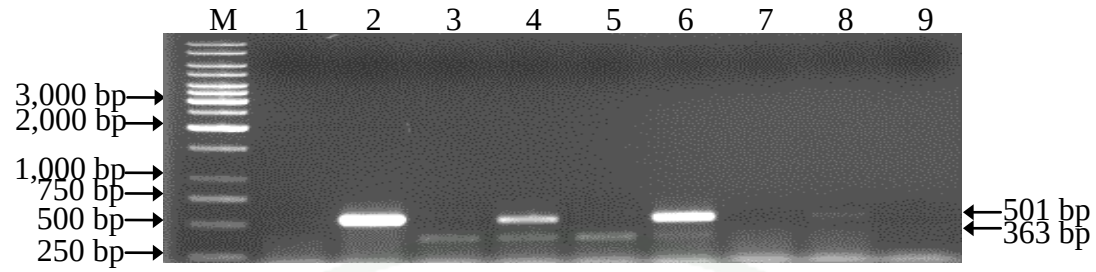

Figure 28 Multiplex RT-PCR of *PRSV-CP* gene from leaves (lane 1-2), unripe fruit (lane 3-4), ripe fruit (lane 5-6) and pollen (lane 7-8) of non-transgenic (lane 1, 3, 5 and 7) and transgenic papaya (lane 2, 4, 6 and 8). The H_2O was use as negative control while 1kb DNA ladder was showed on the left (lane M).

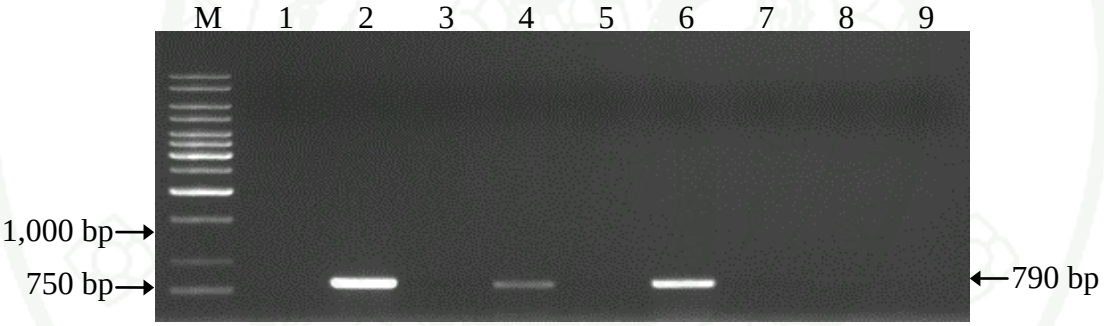

Figure 29 RT-PCR of *nptII* gene from leaves (lane 1-2), unripe fruit (lane 3-4), ripe fruit (lane 5-6) and pollen (lane 7-8) of non-transgenic (lane 1, 3, 5 and 7) and transgenic papaya (lane 2, 4, 6 and 8). H_2O was use as negative control. The 1kb DNA ladder was showed on the left (lane M).

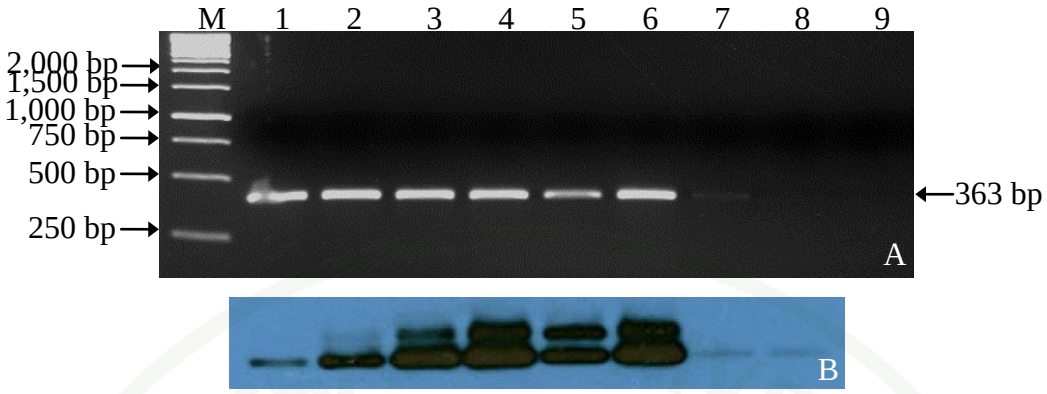

Figure 30 RT-PCR amplification (A) and RT-PCR Southern analysis (B) of *papain* gene in leaves (lane 1-2), unripe fruit (lane 3-4), ripe fruit (lane 5-6) and pollen (lane 7-8). The odd lane represent tissue from non-transgenic papaya while even lane represent tissue from transgenic papaya. H_2O was use as negative control whereas 1kb DNA ladder was showed on the left (lane M).

5.4.2 Detection of transcripts transgenes in pollen using hybridization

The expression of *PRSV-CP* and *nptII* genes in pollen was further studied by two step RT-PCR and hybridization. The results showed that minimum amount of 0.1 µg of total RNA from pollen was sufficient for detection of *PRSV-CP* by two step RT-PCR. For *nptII*, however, even at 1 µg of total RNA from pollen *nptII* expression was not detected. When a more sensitive step, hybridization, was added, Southern RT-PCR can detect the presence of both *nptII* and *PRSV-CP* transcripts at 0.01 µg and 0.1 µg of total RNA from pollen (Figure 31 and Figure 32).

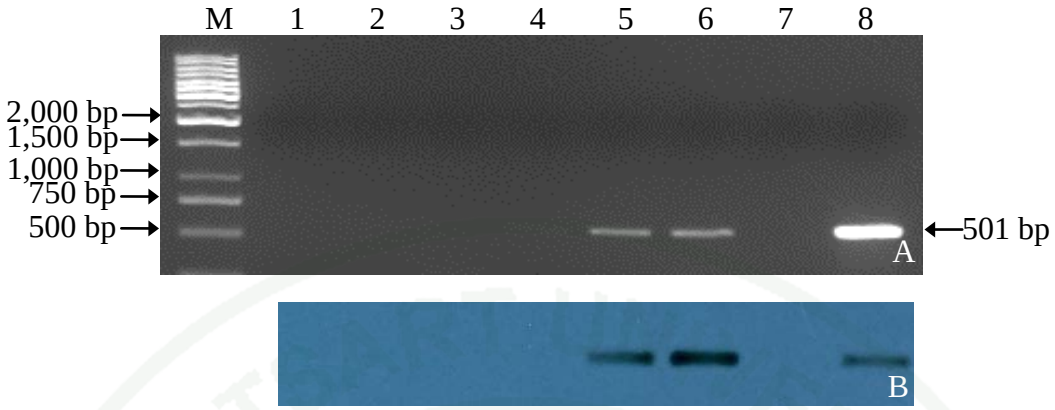

Figure 31 RT-PCR amplification (A) and RT-PCR Southern analysis (B) of *PRSV-CP* gene in papaya pollen. Lane 1-3 represent 0.01 µg, 0.1 µg and 0.5 µg of total RNA from non-transgenic papaya. Lane 4-6 represent 0.01 µg, 0.1 µg and 0.5 µg of total RNA from transgenic papaya. Lane 7 was H_2O while lane 8 was p2CMCP plasmid used as negative and positive control, respectively.

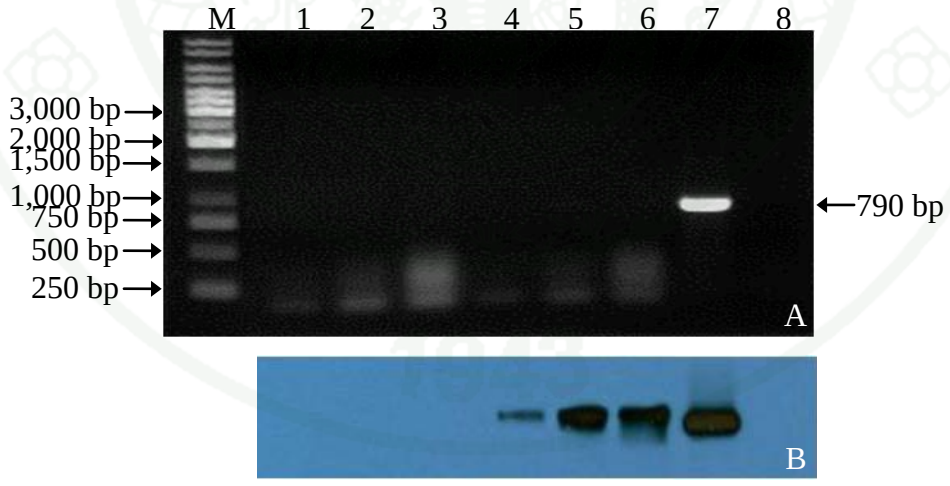

Figure 32 RT-PCR amplification (A) and RT-PCR Southern analysis (B) of *nptII* gene in papaya pollen. Lane 1-3 represent 0.01 µg, 0.1 µg and 0.5 µg of total RNA from non-transgenic papaya. Lane 4-6 represent 0.01 µg, 0.1 µg and 0.5 µg of total RNA from transgenic papaya. Lane 7 was H_2O while lane 8 was p2CMCP plasmid.

5.5 Evaluation of transgene protein in transgenic papaya tissue

The expression of *PRSV-CP* and *nptII* transgene in transgenic papaya was carried out by ELISA according to manufracturer instruction (Agdia Inc. Elkhart, IN). The results considered positive when the absorbence was two times greater than negative sample (Zehnder *et al*., 1999). The results showed that nptII protein expressed only in leave and pollen while unripe and ripe did not found nptII protein expression. In contrast with CP, the expression of *PRSV-CP* gene did not detected in any samples (Table 6).

Table 6 Expression of CP and nptII protein in transgenic papaya comparing to non-transgenic papaya with DAS-ELISA method.

protein	Papaya type	ELISA absorbence[1]				Positive control[2]
		leaves	Unripe fruit	Ripe fruit	pollen	
PRSV-CP	p116/5 transgenic papaya	0.160±0.010	0.096±0.002	0.097±0.002	0.122±0.009	3.094±0.088
	non-transgenic papaya	0.149±0.041	0.091±0.002	0.094±0.001	0.134±0.009	
nptII	p116/5 transgenic papaya	0.677±0.097	0.065±0.006	0.085±0.015	0.428±0.237	0.665±0.004
	non-transgenic papaya	0.093±0.020	0.054±0.002	0.066±0.007	0.085±0.008	

[1]PRSV-CP protein and nptII protein were measured at 405 nm and 450 nm, respectively.

[2]purified PRSV-CP protein and 2purified nptII protein were used

The experiment was done in 3 replications

Reaction will be positive when absorbance is 2 time greater than negative control

DISCUSSION

1. Plant fitness comparison between p116/5 PRSV resistant and non-transgenic papaya under screenhouse condition.

Plant fitness was measured in term of plant morphological characteristics during vegetative stage and at flowering and fruit setting stage. Additionally, horticultural traits were determined when fruit reached maturity. From our results, morphological characteristic of transgenic papaya was identical to non-transgenic papaya under screenhouse condition. Although in p116/5 R_4 generation, height of transgenic papaya was different to non-transgenic in the first 9 weeks with a higher growth rate, eventually non-transgenic papaya could rise to the same growth rate from 10^{th} week on. The divergence during developmental stage of plants between transgenic and non-transgenic plants was suggested by Tennent *et al.* (2005). However the difference of morphological characteristics did not found in p116/5 R_5 generation. The other growth measurements determined during first flowering and first fruit setting stage, height, numbers of node and trunk girth were comparable among transgenic and non-transgenic papaya.

Horticultural traits showed no significant difference between transgenic and non-transgenic papaya in the p116/5 R_4 and p116/5 R_5 generation excepted for TSS content. Production was ranging from 25-29 kg of fruit per tree within first 6 months which quite minute but was resemble to previously reported by Rimberia *et al.* (2007) for dwarf cultivar 'Wonder blight' at 27.4 kg. The experiment was done under closed containment and free of PRSV hence the study was primary information demonstrating that introduction of transgene to transgenic papaya did not cause any effect on plant fitness. Although comparative study of papaya plant fitness in screenhouse is lacking, many countries accomplished field evaluation of transgenic papaya compare to non-transgenic papaya. Generally, transgenic papaya achieved greater PRSV resistant and higher fruit production while non-transgenic papaya usually revealed severe PRSV symptom (Ferreira *et al*., 2002; Bau *et al*., 2004; Xiangdong *et al*., 2004; Tennant *et al.,* 2005; Phironrit *et al*., 2007).

With our investigation on plant morphological characteristics and horticultural traits indicated that introduction of transgene into transgenic papaya did not increased fitness advantage especially invasiveness or weediness. It is likely that when growing outside screenhouse condition, transgenic papaya would perform in a similar fashion to non-transgenic papaya with introduced character of PRSV resistance. Although some characters such as TSS content were dissimilar, it did not reflect environmental concern.

2. Pollen fitness comparison between p116/5 PRSV resistant and non-transgenic papaya under screenhouse condition.

Pollen is a vital factor contributing to transgene movement, its morphological and physical characters that influence gene flow were determined. Our investigation found that morphological character of transgenic papaya pollen was very similar to non-transgenic papaya pollen. Pollen shape and surface (scrulpture) was spherical comprising three apertures (tricolporate) and finely reticulate type as previous reported by Fisher (1980). Pollen from transgenic and non-transgenic papaya was similar in size and was slightly smaller than previous reports by Fisher (1980) and Watanabe *et al*. (2006) at 32-39 μm and 28-30 μm, respectively. The number of pollen produced in an anther from transgenic and non-transgenic papaya showed no significant difference at 14,493 and 14,090 pollen. The data was higher than previously reported in 'Golden' and 'Tainung 01' at 10,805 and 12,195 pollen grains per anther (Damasceno *et al*., 2009). Many factors were reportedly affected variation in pollen grain numbers including variety, climate and season (Damasceno *et al*., 2009; Khanduri and Sharma, 2009). Average weight of non-transgenic papaya pollen grains was lighter than those of transgenic pollen, however there was no statistical difference. The relatively small size, its round and fine recticulate surface and physical characters indicated that papaya pollen was mobile and can be transferred by both wind and animals. The presence of nectar and wax in papaya flower and large numbers of pollen producing flowers (reduced elongate) further supported the importance of animal pollination (Ingrouille and Eddie, 2006).

Pollen biology particularly pollen viability and germination is associated to vertical gene transfer by encouraging opportunity of gene flow in environmental competition and also important in terms of quality because of its link to higher fruit weight, fruit volume and number of seed (Cohen and Roy, 1989). In this study, viability and germination of pollen of transgenic and non-transgenic was determined. The results did not found any difference in pollen viability and germination between transgenic and non-transgenic papaya. Although pollen viability was relatively high at 94.27% and 94.76% but pollen germination was lower level at 60.42% and 64.73%, respectively.

Longevity of papaya pollen has not been previously reported. Longevity of pollen enhances the opportunity in reproductive successes. Pollen longevity is generally considered as duration in which pollen grains still have ability to germinate (Dafni and Firmage, 2000). Pollen grains with longer longevity have better chance for gene transfer. The results showed that almost all of transgenic and non-transgenic papaya pollen lost their viability after 24 h. It can be point out that when exposed to the environmental condition, both transgenic and non-transgenic pollen have the identical fitness. Comparing to other plants such as cotton, maize, sorghums and Sudan grass, pollen longevity reported to be 90 min, 2 h, 5 h and 5 h, respectively (Wang *et al*., 2004; Luna *et al*., 2001; Stephens and Quinby, 1934; Hogg and Ahlgren, 1943) while papaya pollen have extended longevity.

Determination of pollen fitness could be indicated that transgene inserted into papaya genome did not enhance adverse effect on male reproductive fitness. Pollen morphological and biological characters investigated here indicated that there was no evidence regarding to environment concern that transgenic papaya had better chance of gene transfer comparing with non-transgenic papaya. With the similar pollen morphology, biology and longevity, it could be indicated that both transgenic and non-transgenic pollen have an equal chance for fertilization in the environment.

3. Determination of environmental stress effect on pollen germination

Environmental stresses including UV, relative humidity (RH) and temperature are influent the maturity and overall pollen fitness. After releasing from anther, pollen was directly exposing to ambient environmental condition before reaching receptive stigma. Temperature stress is currently become prominent because of global climate change. The impact of high temperature is based on intensity, duration and rate of temperature change (Zinn *et al*., 2010). The extreme temperature can effect reproductive tissues in 4 steps; 1) early or delay flowering, 2) asynchrony of male and female reproductive development, 3) defects in parental tissue. In this study we investigated the last step; 4) defects to male and female gametes. Environmental stresses resulting in the reduction of pollination and fertilization and therefore change in quantity and quality of seed (Demchik and Day, 1996; Van de Staaij *et al*., 1997).

In our results showed that high temperature (40°C) caused a sharp reduction in germination of both transgenic and non-transgenic papaya pollen. Although there was significant difference between germination of transgenic and non-transgenic papaya pollen at 25°C, 36°C and 40°C, reports suggested that pollen could survived only for a short period of time particularly at high temperature (higher than 30°C). Wang *et al*. (2004) reported that germination rate of tall fescue was almost vanished after 20 min at 40ºC. Studies in tomato, canola and maize suggested that the impact of high temperature was more severe to pollen (male gamete) particularly during development than to ovule (female gamete) (Peet *et al.,* 1998; Young *et al.,* 2004; Dupuis and Dumas, 1990). In high temperature, pollen germination was low could cause by pollen dehydration. This result could explain the lack of seed in papaya fruit during summer season with high temperature for long period, which was in accordance with Weaver and Timm (1988) sensitivity of pollen to high temperature resulting deficient in fertilization. Low temperature also influences pollen germination. Kakani *et al.* (2005) suggested that high and low temperature cause the reduction of pollen germination and tube growth. In this study, pollen was stored at -20°C which could explain lower pollen germination than previously reported.

Unlike temperature effect, the impact of relative humidity (RH) is diverse. Wang *et al.* (2004) reported that RH from 40-99% had no effect to pollen germination percentage of tall fescue. In cotton and canola, on the other hand, RH around 50-80% resulted in highest pollen germination percentage (Sato *et al*., 1998; Burke *et al.*, 2004). Our results showed that RH from 20-80% had no effect on pollen germination percentage from transgenic papaya. High RH (60-80%), however, had an impact on germination of pollen grains from non-transgenic papaya. Replication of this condition in various seasons will be required to confirm this outcome. In case of UVA and UVB treatment, pollen of transgenic and non-transgenic papaya was exposed to UVA and UVB source at the dose of 15-30 $\mu W/cm^2$ in various distance. The results indicated that closed distance to UVA and UVB, corresponding to high dosage of UVA and UVB, resulting in low germination. The UV dose of 15-30 $\mu W/cm^2$ can affect pollen germination even if it was a minute dose comparing with natural condition (~500-700 $\mu W/cm^2$ in sunny day). Previous reports suggested that UVB affecting *in vitro* pollen germination and tube growth in *Scrophularia peregrina, Geranium viscosissimum, Papaver rhoeas* and *Cleome lutea, Nicotiana tabacum* and *Petunia hybrida* (Flint and Caldwell, 1984; Feder and Shrier, 1990). UVA causes damage to pollen nucleic acids and proteins (Bohrerova *et al*., 2009) whereas UVB causes DNA damage by generate mutagenic and cytotoxic DNA lesions (Sinha and Hader 2002). This outcome was supported by studies in tall fescue and soybean (Wang *et al.*, 2004; Koti *et al.*, 2005). The UVA and UVB radiation was also found to positively affect pollen germination as reported here at longer distance from UVA and UVB source (30 cm) and in alpine meadow (Wang *et al*., 2006).

4. Determination the expression of transgene in transgenic papaya.

Stability of transgene (*PRSV-CP* and *nptII*) was determined in leaf, unripe fruit, ripe fruit and pollen of transgenic papaya comparing to non-transgenic papaya by PCR based method using specific primers. We found that both *PRSV-CP* and *nptII* gene were present in every parts of transgenic papaya. Furthermore, expression of transgene in transcriptional and translational level was also determined. The results of transcriptional expression exhibited the 501 bp and 790 bp PCR product of *PRSV-CP* and *nptII* gene in all parts of transgenic papaya with low level of expression found in

pollen. The nptII protein was detected by DAS-ELISA while PRSV-CP protein was not found. Our results were in accordance with previously reported in other transgenic papaya lines (Pancharoonrat, 2006; Kasemsin, 2007). It indicated that the PRSV resistance in our transgenic papaya was controlled by post-transcriptional gene silencing mechanism (Tennant *et al*., 2001; Ruanjan *et al.*, 2007).

Although nptII protein was detected in leaf tissue and pollen, it was absent in both unripe and ripe fruit. It was possible that mRNA was degraded by post-transcriptional gene silencing mechanism in fruit tissue resulting in lower than limitation threshold of to DAS-ELISA detection (Fermin *et al*., 2010). The differential expression of nptII in various plant tissues was also reported in pepper and grapevine. In transgenic pepper, the expression of nptII protein was highest in leaf tissue but not to found in pollen (Kim *et al.*, 2010). Eom and Reisch (2008) suggested that tartaric and ellagic acids are factors opposing nptII detection in grapevine leaf tissue. Moreover, polyphenolics and low pH also influence efficiency of ELISA detection. This finding could decrease public concern on possibility of producing unintended protein, even through nptII protein had been proven for food safety.

CONCLUSIONS

Study on fitness comparison between PRSV resistant and non-transgenic papaya under screenhouse condition can be summarized as followed:

1. Transgene introduced into transgenic papaya did not has effect on plant fitness (plant morphological characteristic and horticultural traits) and pollen fitness (morphology, physical character and effect of environmental factors). Therefore transgenic papaya did not influence environmental concern especially weediness and invasiveness.

2. Monitoring and determination of transgene expression in transgenic papaya could be conducted using PCR based method and DAS-ELISA. RT-PCR, a reliable and sensitive method, could detect transgene even in minute amount. The absence of PRSV-CP protein in transgenic papaya indicated that mechanism of resistance in transgenic papaya line p116/5 was post-transcriptional gene silencing.

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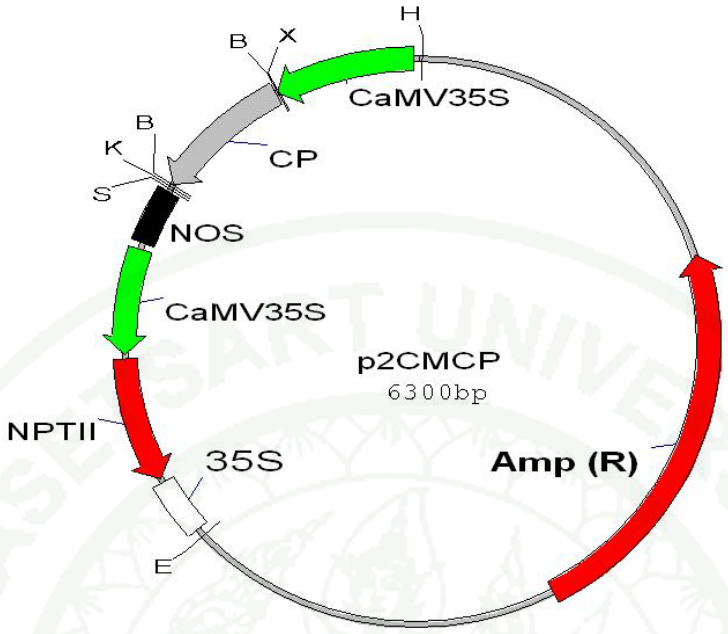
APPENDIX



Appendix Figure1 Plasmid p2CMCP containing *PRSV-CP* gene and *nptII* selectable marker gene

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