ANTISERUM PRODUCTION FOR *XANTHOMONAS AXONOPODIS* PV. *CITRI* DETECTION AND IDENTIFICATION OF CANKER RESISTANCE GENE ANALOGS IN THAI HYBRID LIME M33

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การผลิตแอนติเซรุ่มเพื่อตรวจสอบเชื้อ *Xanthomonas axonopodis* pv. *citri* และ การหายีนต้านทานโรคแคงเกอร์ในมะนาวไทยลูกผสม M33

นางสาวผ่องพรรณ ทรงวัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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ผ่องพรรณ ทรงวัฒนา: การผลิตแอนติเซรุ่มเพื่อตรวจสอบเชื้อ *Xanthomonas axonopodis* pv. *citri* และ การหายีนต้านทานโรคแคงเกอร์ในมะนาวไทยลูกผสม M33 (ANTISERUM PRODUCTION FOR *XANTHOMONAS AXONOPODIS* PV. *CITRI* DETECTION AND IDENTIFICATION OF CANKER RESISTANCE GENE ANALOGS IN THAI HYBRID LIME M33 อาจารย์ที่ปรึกษา: ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 82 หน้า.

โรคแคงเกอร์เป็นโรคที่สำคัญของมะนาวในประเทศไทย การทดลองนี้มีวัตถุประสงค์เพื่อทดสอบความต้านทานโรคแคงเกอร์ในมะนาว 3 พันธุ์ ได้แก่มะนาวลูกผสม M33 มะนาวแป้น และมะนาวน้ำหอมพบว่า มะนาวพันธุ์น้ำหอม และM33 มีความต้านทานมากกว่าพันธุ์แป้น เชื้อไอโซเลตที่ก่อโรคทั้งหมดสามารถตรวจสอบได้ด้วยคู่ไพเมอร์จำเพาะXAC01 และ XAC02 ด้วยวิธี PCR ผลการวิเคราะห์ลำดับเบสของ 16S rDNA ของเชื้อก่อโรครุนแรง (ไอโซเลต BP104 และ BP210) พบว่ามีความใกล้เคียงกับลำดับเบสของเชื้อ *Xanthomonas axonopodis* pv. *citri* (XAC) (AE008923.1) 99 % การทดลองนี้ได้นำเชื้อไอโซเลต BP210 ไปผลิตแอนติเซรุ่มในกระต่าย จากการทดสอบประสิทธิภาพและความจำเพาะเจาะจงของแอนติเซรุ่มที่เจือจาง 1:4,000 หรือน้อยกว่า พบว่ามีความจำเพาะเจาะจงต่อการตรวจสอบเชื้อXAC (BP210) ที่มีชีวิตในระดับความเข้มข้น 10^6 CFU/ml และเชื้อตายที่ความเข้มข้น10^5 CFU/ml อย่างไรก็ตาม แอนติเซรุ่มที่ความเข้มข้น 1:2,000 เหมาะสมต่อการตรวจสอบเชื้อ XAC มากที่สุด โดยเกิด cross-reaction กับเชื้อ *X. campestris* pv. *vesicatoria* เพียงชนิดเดียว จากการตรวจสอบเชื้อ XAC ที่ปลูกเชื้อบนใบมะนาวด้วยแอนติเซรุ่มความเข้มข้น 1:2,000 พบว่าสามารถตรวจสอบเชื้อได้ในวันที่4 หลังการปลูกเชื้อความเข้มข้น 10^5, 10^4 และ 10^3CFU/ml ซึ่งพบอาการของโรคบนใบที่ถูกปลูกเชื้อแล้ว ดังนั้นแอนติเซรุ่มที่ผลิตจึงไม่เหมาะสมต่อการตรวจสอบเชื้อ XAC บนใบก่อนการแสดงอาการของโรค

การพัฒนาเครื่องหมายโมเลกุลที่บ่งชี้ลักษณะความต้านทานโรคในพืชนั้นมีความสำคัญต่อการพัฒนาสายพันธุ์พืชให้มีความต้านทานในอนาคตได้ ในการทดลองนี้จึงได้ทำการศึกษายีนที่เกี่ยวข้องต่อความต้านทานโรคแคงเกอร์ในมะนาวลูกผสม M33 เปรียบเทียบกับมะนาวพันธุ์พ่อและแม่ โดยเริ่มศึกษาในกลุ่มยีน Nucleotide binding site (NBS) Leucine-rich repeat (LRR) ด้วยเทคนิค PCR ร่วมกับการตัดด้วยเอมไซม์ตัดจำเพาะ จากการประเมินระดับความต้านทานของM33 เทียบกับมะนาวแป้น และมะนาวน้ำหอม โดยการปลูกเชื้อXAC บนใบ พบว่า M33 และมะนาวน้ำหอม มีลักษณะการต้านทานต่อโรคโดยเกิด hypersensitive response ซึ่งให้ผลสอดคล้องกับผลของยีน

*Pt9/Alu*1, *Pt14/Bfa*1 และ *16R1-19/Tru*1I ว่ามีความเกี่ยวข้องกับลักษณะการต้านทานโรคแคงเกอร์ในมะนาว

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PONGPAN SONGWATTANA : ANTISERUM PRODUCTION FOR *XANTHOMONAS AXONOPODIS* PV. *CITRI* DETECTION AND IDENTIFICATION OF CANKER RESISTANCE GENE ANALOGS IN THAI HYBRID LIME M33. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 82 PP.

CANKER RESISTNCE/M33/HYBRID LIME/CITRUS CANKER/RESISTANCE GENE ANALOGS

Citrus canker is a serious disease of lime in Thailand. This study aims to evaluate the resistance characteristic of hybrid lime M33, Pan lime and Nam Hom lime. The results indicated that, Nam Hom and M33 showed higher resistance level than that of Pan lime. PCR amplification using *Xanthomonas axonopodis* pv. *citri* (XAC) specific primers (XAC01 and XAC02) could detect all pathogenic bacterial isolates. The 16S rDNA sequencing confirmed that the virulent bacteria (BP104 and BP210) were 99% identical with XAC (AE008923.1). Bacterial isolate BP210 was used for antiserum production by rabbit injection. The efficiency and sensitivity of antiserum from this study showed that, antiserum at the dilution of 1:4,000 or lower was able to detect XAC (BP210) bacteria at 10^6 CFU/ml for live cells and 10^5 CFU/ml for dead cells. However, the suitable dilution of this antiserum was 1:2,000 which could cross-react with only *X. campestris* pv. *vesicatoria* but not other *Xanthomonas* tested. The pathogen on infected leaves (10^5, 10^4 and 10^3 CFU/ml) was detected by 1:2,000 diluted antiserum. The results indicated that, this antiserum was able to detect the pathogen on infected leaves 4 days post-inoculation when symptom had already

appeared. Thus, this antiserum has low detection efficiency and cannot be used to detect low pathogen concentration or before canker lesion can be observed.

The development of molecular markers can help identify genes that linked to resistance characteristic on resistance plant. This methodology can be used to improve commercial favorable crop species to be disease resistant in the future. In this study, the citrus canker resistance (R) marker genes within M33 and its parents were screened using the Nucleotide binding site (NBS) Leucine-rich repeat (LRR) genes by PCR amplification in combination with restriction enzymes digestion. The resistant evaluation of hybrid lime and its parents was performed by inoculation with XAC on young leaves. The hypersensitive response phenotype on M33 and Nam Hom (resistant lime) confirmed that the marker *Pt9/Alu*1, *Pt14/Bfa*1 and *16R1-19*/*Tru*1I were closely linked with the citrus canker resistance genes.

School of Biotechnology Student's Signature _____

Academic Year 2010 Advisor's Signature _____

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LIST OF ABBREVIATIONS

ALP	=	alkaline phosphatase
Avr	=	avirulence
BP	=	Banpaew, Samut Sakhon
bp	=	base pair
°C	=	degree celcius
CFU	=	colony forming unit
DNA	=	deoxynucleotide
dNTP	=	deoxynucleotide 5’ triphosphate
ELISA	=	enzyme-linked immunosorbent assay
ETI	=	effector-triggered immunity
et al.	=	Et alia (and other)
Fig.	=	figure
g	=	gram
h	=	hour
HR	=	hypersensitive response
K	=	Kokkruad, Nakhon Ratchasima
L	=	liter
LRR	=	leucine-rich repeat
LPS	=	lipopolysaccharides
mAbs	=	monoclonal antibodies
mg	=	milligram

LIST OF ABBREVIATIONS (Continued)

mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
NBS	=	nucleotide-binding site
nm	=	nanometer
ng	=	nanogram
O.D.	=	optimal density
pAbs	=	polyclonal antibodies
PAMPs	=	plant-associated molecular patterns
PCR	=	Polymerase Chain Reaction
PJ	=	Phichit
PR	=	pathogenesis-related genes
PTA-ELISA	=	plate-trapped antigen ELISA
PTI	=	PAMP-triggered immunity
RGAs	=	resistance gene analogs
rpm	=	revolution per minute
rRNA	=	ribosomal RNA
sec	=	second
SUT	=	Suranaree University of Technology, Nakhon Ratchasima
Ta	=	annealing temperature
TIR	=	Toll and Interleukin-1 receptors

LIST OF ABBREVIATIONS (Continued)

U	=	unit
UV	=	ultraviolet
V	=	volume
v/v	=	volume per volume
w/v	=	weigth per volume
XAC	=	*Xanthomonas axonopodis* pv.*citri*
µg	=	microgram
µl	=	microlitre
µM	=	micromolar

CHAPTER I

INTRODUCTION

1.1 Background

Lime (*Citrus aurantifolia* Swingle) is a fruit tree in the family of Rutaceae, genus *Citrus*. It is an important economic crop in Thailand because it is often used to enhance the flavor of foods and beverages. Recently, lime has been planted in every regions of Thailand. The planted areas of lime in Thailand are about 116,000 rai dominating in the central plain (69,000 rai). The total yield of lime was around 163,000 tons with the value of 6,000 million baht (Office of agricultural economics, 2008).

Citrus canker is a serious disease in citrus plants and also in Thai lime. It is caused by *Xanthomonas axonopodis* pv.*citri* bacteria which is globally distributed. Moreover, this disease causes losses in yield and quality annually. It can infect leaves, twigs and fruit through stomata and wounds and induce callus-like lesion with water-soaked margins and often surrounded by a chlorotic halo. This bacterium can be dispersed by rain splash and wind to other area (Graham et al., 2004). Most commercial limes are susceptible host for citrus canker, including Pan lime.

Recently, the prevention and control of citrus canker are usually the use of copper-based spray programs or sometime complementary with antibiotics, especially in the young stages (Medina-Urrutia and Stapleton, 1986). However, copper

bactericides and antibiotics are disadvantages after long-term uses. Moreover, the accumulation of copper metal in soils causes phyto-toxic and environmental effects (McManus and Stockwell, 2001). This pathogenic bacterium can survive from one crop season to the next as latent infection in propagation organs or epiphytic populations on plant surfaces. These events often contain very low number of bacterial populations but they represent the primary source of inoculum for introduction of disease into subsequent crops. Therefore, the method for their detection is necessary.

Currently, many methods have been developed and are available in the laboratory. Polymerase chain reaction (PCR) method is a highly sensitive detection method (Cubero and graham, 2002; Cubero et al., 2001) but, laboratory equipment and specialized training are required. In contrast, immunodiagnostic is an easy method to use at the site where disease is suspected. They are not required neither special equipments nor training to perform the detection. The immunodiagnostic technique is based on the ability of an antibody to recognize and bind to a specific antigen, a substance associated with a plant pathogen. This method is known as enzyme-liked immunosorbent assay (ELISA). Therefore, this methodology can be used as diagnostic tool for pathogen detection in the field (Ward et al., 2004).

Resistance plant development is an alternative way that can be used to improve commercial favorable crop species to be resistant to disease. Resistant lime with commercially favor fruits has been successfully developed from Phichit research center (Thailand) by conventional breeding. This new resistant lime, named Phichit lime or M33 was crossed between canker susceptible Pan lime (female) and canker resistant Namhom lime (male) (Dangpium, 2003).

The development of molecular markers to identify the genes linked to the disease resistance characteristics would be helpful for further development of more resistant limes. This methodology can facilitate indirect selection of resistant plants and reduce the time required to identify resistant breeding line or segregating lines in the future. Generally, the activation of plant defense to pathogen attacks is often conferred by resistance (R) protein that controlled by *R* genes. These genes play important roles in improvement of crop species in the plant breeding programs. They also can lead to the understanding of the plant-pathogen interactions. However, the inheritance of citrus canker resistant traits are still unclear, the biochemical mechanism and the number of gene loci controlling this resistance have yet to be determined. An approach under consideration is cloning of *R* gene analogs (*RGAs*), from the resistant relative which includes cloning and screening diversity of *RGAs*, followed by functional tests of co-segregating analogs in transgenic plant and their expression (Hammond-Kosack and Kanyuka, 2007; Zimmerman et al., 2006; Manosalva et al., 2009).

Recently, many *RGA* sequences have been served as molecular makers for genetic mapping and molecular cloning of plant *R* genes for their tightly linkage or co-segregating with known disease resistant loci. *R* genes have been isolated and classified into several classes based on the structure of their predicted protein products. The largest *R* gene class in the plant genome is the nucleotide-binding site with leucine-rich repeats (NBS-LRR) class. Currently, a larger number of NBS-LRR genes have been cloned from different plant species such as citrus plant (Deng et al., 2000). They used PCR base method with degenerate primers designed from the conserved domains.

1.2 Objectives

This research aims to produce polyclonal antibody for citrus canker pathogen (*X. axonopodis* pv. *citri*) detection and also to identify molecular markers linked to the canker resistance gene(s) in Phichit lime (M33) and their parents using 12 specific primer pairs of citrus NBS-LRR class *RGAs* (Deng et al., 2000) in combination with restriction enzymes.

CHAPTER II

LITERATURE REVIEWS

2.1 The importance of canker disease

Lime is a small citrus fruit widely grown in the tropical and subtropical regions of the world especially, in Asia. Limes in Thailand have been planted in every regions dominating in the central plain. The main region for lime production in Thailand is Phetchaburi, Samut Sakhon, Ratchaburi and Phichit provinces (table 1.1).

Table 2.1 Harvested areas and production statistics of lime in Thailand (Office of Agricultural Economics, 2008).

Regions	**Harvested areas (Rai)**	**Production (Tons)**
North	19,600	26,900
Northeast	870	260
Central	69,000	124,000
South	16,000	12,000
Total	106,000	163,000
Provinces	**Harvested areas (Rai)**	**Production (Tons)**
Prachinburi	31,000	71,000
Samut Sakhon	16,000	24,000
Ratchaburi	31,000	12,000
Phichit	7,000	11,000

The sour limes are a great source of vitamin C (29 mg/100 ml; USDA National nutrient database, n.d.) and citric acid contents (1.38 g/oz; Kristina et al., 2008). Thus, it can be used as a fresh fruit for garnish and for processing into a wide range of value-added product, such as beverage, sauces and some kind of traditional drug.

However, the large problem of lime planting area is citrus canker disease from *X. axonopodis* pv. *citri* bacteria. This disease has a long history in Florida. It was first discovered by P J. Wester around 1910 when the disease was introduce into North Florida and other Gulf states on *Poncitras trifoliate* citrus rootstock material from Japan. This disease was declared eradicated from Florida and the adjacent states in 1933 (Schubert and Sun, 2003, Dopson, 1964). Currently, citrus canker has been spread worldwide into warm, moist, citrus-growing coastal regions. This disease cause losses in yield and quality annually by defoliation, blemished fruit, premature fruit drop, die-back of twigs and general debilitation of the tree (Goto and Yaguchi, 1979).

2.2 The pathogen description

The genus *Xanthomonas* are gram-negative, rod-shaped and polarly-flagellated bacteria. The fagella allow the bacteria to move throughout an infected plant. *Xanthomonas* can infect a wide variety of species including pepper, tomato (*X. axonopodis* pv. *vesicatoria)*, rice (*X. oryzae* pv. *oryzae)*, citrus (*X. axonopodis* pv. *citri*), cotton (*X. axonopodis* pv. *malvacearum*), brassicas (*X. campestris* pv. *campestris*), bean (*X. axonopodis* pv. *phaseoli*) and soybeans (*X. axonopodis* pv. *glycines*). *Xanthomonas* infection is dependent on the type III protein secretion

system, which relies on transport protiens, secreting several hypersensitive reaction proteins and outer proteins, causing an interaction with the plant (Gürlebeck, et al., 2006; Bonas, and Kay, 2009). Typical symptoms of the disease include lesions on the leaves, fruit, and stems as well as twig dieback. *Xanthomonas* colonies are normally yellow due to their presence of a particular carotenoid pigment. Reproduction in genus *Xanthomonas* is similar to other bacteria, occurring through asexual binary fission in which each dividing clonal daughter cell receives an identical copy of the parental genome. These bacteria are a heterotrophic bacterium that obtain nutrients from the plants tissue it's infects. This bacterium thrives in subtropical regions of warm, high humidity and heavy rainfall (Civerolo, 1984).

The distinct types of citrus canker disease can cause by various pathovars and variants of the *X. axonopodis*. The separation of these froms from each other is based on host range and other phenotypic and genotypic characteristics of the bacterial strains. The most important strain in Asia canker disease is Canker A which caused by the Asian strain *X. axonopodis* pv. *citri* (Syn. *X. citri, X. campestris* pv. *citri*: Schaad et al., 2006*)*. It is the most widespread and severe form of the disease. This strain caused disease most often referred as "citrus canker". The B and C types are caused by *X. axonopodis* pv. *aurantifolia*. Pathotype B strains are most severe on lemons (*C. limon* (L.) Burm f.), while grapefruit (*C.paradisi* Macf.) and sweet orange (*C. sinensis* (L.) Osb.) are affected little only in the groves. This pathotype has been found only in Argentina, Uruguay, and Paraguay. Pathotype C strains or Mexican lime canker affects only Mexican lime (*C. aurantifolia* (Christm.) Swingle) which was found in Brazil (Gottwald, 1993).

Lime canker disease in Thailand are caused by *X. axonopodis* pv. *citri*-A* (Bui Thi Ngoc et al., 2007). This strain has also been discovered in Oman, Saudi Arabia, Iran and India. This *Xanthomonas* produces canker A-like lesions only on Mexican lime and appear to be distinct from the common A strains (Verniere et al., 1998). A similar strain has been isolated from Florida and designated as A^w (Sun et al., 2000). This strain has a restricted host rang that includes 'Key' lime and alemow (*C. macrophylla* Wester). Various cultivars of limes are found in Thailand. The most favorable commercial cultivars are Pan lime (*C. aurantifolia*) which has good quality of fragrant, thin skin and high amount of juice. This lime is in the group of west Indian or Mexican lime that is highly sensitive to canker disease. In contrast, citrus lemon group such as Tahiti lime (*C. aurantiun*), Nam Hom lime (*C. reticulata*) and Hnung kunturee lime (*C. limon*) are more resistance to canker but have lower quality and are unfavorable commercially (Graham et al., 1992, Graham, 2001). Thus, the controlling of disease is essential for prevention of yield devastation in Pan limes. Copper compound are standard control of the bacteria disease but dangerous to the farmers and environment. Disadvantage of copper treatment after long term used are accumulation of copper metal in soils and runoff water (McManus and Stckwell, 2001).

2.3 Symptoms and infection of citrus canker bacteria

The infection of citrus canker bacteria are mostly found on the above of ground leaf of citrus during the first half of the expansion phase growth. This

pathogen infective events often contain very low number of bacterial populations. But they represent the primary source of inoculum for introduction of disease into subsequent crops (Timmer et al., 1996). The *Xanthomonas* bacteria infection is like many other bacteria disease, infected through stomata of host plant tissues and wounds. The earliest symptoms on leaves appear as tinge, slightly raised blister-like lesions about 4-7 days after inoculation under optimum condition. The optimum condition are as followed present of water film and temperature between 20-30°C. Then, they turn light tan to brown when the lesion aged. The lesions are often surrounded by a chlorotic halo with spongy or corky on the centre of lesion (Fig 2.1A). Fruit and twigs lesions are similar, raised corky lesions surrounded by an oily or water-soaked margin (Fig 1B and C). The twig lesion do not contain chlorosis surround the lesion as occur on fruit lesions (Graham et al., 2004).

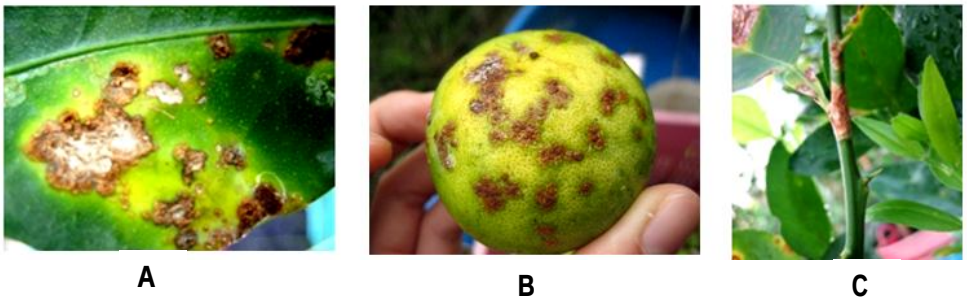

Figure 2.1 Canker lesion characteristic on lime leave (A), fruit (B), twig (C).

The canker bacterium has been spread through the transport of fruit, plants and equipment and also greatly dispersal by wind and rain (Myung et al. 2003). High wind speeds during rain causes water-soaking and facilitate entry of bacteria through stomatal openings into leaves. The canker bacterium remains alive in the margins of the lesions in leaves and fruit until they fall. The bacterial population has been

founded to be about 10^5 to 10^6 CFU/ml in rainwater under infected tree (Stall et al., 1980). However, under symptomless fruit tree, the bacterium has also been discovered in the range of 10^2 to 10^3 CFU/ml (Canteros et al., 2004). The bacteria can also survive for up to several years in lesions of woody branches. When lesions surface is free of moisture, bacteria are released from an extracellular polysaccharide matrix and dispersed to new growth by rain splash and force of windblown rain droplets (Goto and Hyodo, 1985).

2.4 Prevention and control of citrus canker

2.4.1 Screening tests of citrus canker bacterial

The spread of citrus canker to new countries can be prevented by screening the plant material and fresh fruit. Because, the pathogen has been spread through the transport of fruit and plant material. Moreover, the equipments for harvesting also the main cause of diseases spread. As of January 2006, more than 15 million commercial trees in groves and nurseries had been destroyed in Florida for citrus canker eradication (Bronson and Gaskolla, 2006). Recently, the transport of live citrus plants in and out of Florida is prohibited. Every citrus plant trees must purchase a certified symptom free citrus plant through nurseries registered with the state (FDACS, 2006)

The screening tests are necessary to prevent the citrus canker outbreak form plant material and fruit transported. Many methods for screening canker pathogen are available for diagnostic test. PCR screening test with specific primers is one of the reliable methods for rapid analysis of suspect samples. Since, high sensitivity of 10^2-

10^3 CFU/ml can be detected (Cubero et al., 2001, Cubero and Graham, 2002, Leon, 2008) via specific of primers complementary with only *X. axonopodis* pv. *citri* gene. Example of primer sets available for diagnosis of *X. axonopodis* pv. *citri* are primers XAC01 and XAC02 which amplified *rpf* gene encoded pathogenicity effector protein (Coletla-Filho, 2006) and XACF and XACR which amplified the *hrpW* gene (*h*ypersensitive *r*eaction and *p*athogenicity; Park et al., 2006). Several other primers have been reported and reviewed in the diagnostic of *X. axonopodis* pv. *citri* by Graham et al. (2004) and OEPP/EPPO (2005). However, PCR method required special reagents, equipments and skill for generating reliable diagnosis.

Serological or immune diagnostics are technique that uses antibodies to detect the presence of pathogens. Antibodies are produced in laboratory animals, typically mice (monoclonal antibodies) or rabbits (polyclonal antibodies), in response to plant pathogens. Polyclonal antibodies (pAbs) are produced by injecting extracts from the pathogen into animal, usually a rabbit. The serum collected from blood contains antibodies. These antibodies can be used directly or after further purification. Monoclonal antibodies (mAbs) are made by fusing antibody-producing cells (lymphocytes) from the spleens of an inoculated animal (usually mice or rats) with cultured myeloma cells. This generates many hybridoma cell lines which produce a different single (monoclonal) antibody in cell culture medium. mAbs are more specific but also more complicated and slow to produce. Moreover, it is more expensive to both produce and maintain. And occasionally the antibody hybridoma cell dies or stop producing the required antibody. A widely-used assay for detect antibody or antigen presences is enzyme-liked immunosorbent assay (ELISA). The principle aim of this assay is to detect or quantify the binding of the diagnostic

antibody with the target antigen. It involves an enzyme-mediated color change reaction to determine antibody binding against target antigen. This is usually done in a microtitre plate where the antigen or antibody is immobilized on. After the antigen formed a complex with the antigen, the specific antibody is added. The antibody binding can be determined by enzyme-mediated color change reaction between enzyme-linked antibody (e.g. p-nitrophenyl phosphate) and its substrate added. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample (Ward et al., 2004).

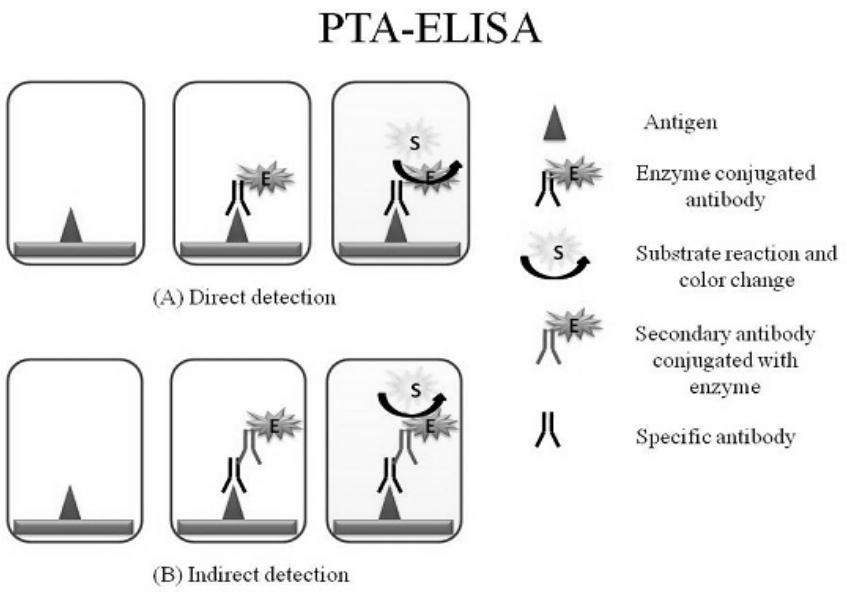

Figure 2.2 Schematic diagram illustrating of plate-trapped antigen (PTA-ELISA), here shown using an direct detection (A) and indirect detection systems (B) to detect coating antigen on wells (modified from Ward et al., 2004).

The simplest format of ELISA assay is the plate-trapped antigen ELISA (PTA-ELISA, Fig 2.2). In this assay, the test samples or the target antigens are directly coated to the microtitre plate wells. This is followed by incubation with specific antibody which binds to target antigen. In some assay, the specific antibody is conjugated to the enzyme (direct detection). In other, the specific antibody is detected by secondary antibody (such as anti-rabbit or anti-mouse) which is conjugated to the enzyme (indirect detection). This technique has been used as a diagnostic tool in several fields such as, medicine and plant pathology (Kevin, 1999; Ward et al., 2004; Kumar et al., 2008). The sensitivity of the pathogen detection of this method is about 10^5-10^6 CFU/ml (Jin et al. 2001; Alvarez, 2004; Leon et al., 2008; Kokoskova and Mraz, 2008).

2.4.2 Chemical and antibiotic control

In the regions where *X. axonopodis* pv. *citri* is endemic, copper based bactericides has been used as standard control of citrus canker world-wide. Copper based spray programs are effective when targeted to the spring leaf flush to protect leaves from the one-half to full expansion stage over a period of 2-4 weeks. While, fruit is susceptible as they grow from 2.0 to 6.0 mm in diameter for a period of 90-120 days, depending on citrus species. Thus, copper treatments are usually repeated during the summer months for continues the fruit expanded. However, the effectiveness of copper spray programmed is reduced by rain and wind. Moreover, copper bactericides are not available after long-term use, including resistance to copper in *Xanthomonas* populations and the accumulation of copper metal in soils and water can be phytotoxic and environmentally harmful.

The antibiotics in the group of streptomycin can also be effective with canker bacteria. However the possibility of antibiotic resistance development in *Xanthomonas* populations can occur (McManus and Stckwell, 2001). Therefore, the prevention and control of citrus canker by chemical and antibiotic treatments needed to be monitor carefully in dose and time used. However, early sprays in the season of slow bacterial growth and alternating with the treatment with copper might increase the effectiveness of citrus canker control.

2.5 Hybrid lime for canker disease resistance

Most commercial limes in Thailand are highly susceptible to canker disease especially, lime in the group of west Indian or Mexican lime, such as Pan lime, Egg-like lime, Hnung lime (*C. aurantifolia*). While, citrus lemon group such as Tahiti lime (*C. aurantiun*), Namhom lime (*C. reticulata*) and Hnung kunturee lime (*C. limon*) are more resistance to canker but, lower fruit quality and are unfavorable for consumer.

Conventional breeding program has been used by Dangpium et al. (2003) at Phichit research center to develop canker resistant lime. The hybrid lime (M33; Fig 2.3 C) was the product of crossed between Pan lime (female; Fig 2.3 A), a canker susceptible and Nam Hom lime (male; Fig 2.3 B), a canker resistant lime. F1 lime seeding were grown and selected for desirable characters including canker resistant efficiency, qualitative and quantitative of yield. The results of M33 evaluation showed high efficiency of canker resistance on leaves, twigs and fruits. The shape of fruit is round and flat with thin skin and high amount of juice and vitamin C.

A

B

C

Figure 2.3 Pan lime (A) and Nam Hom lime trees (B) planted at Suranaree University of Technology for field test; Phichit or M33 lime tree (C) planted at Phichit research center, Thailand.

2.6 Disease resistance (*R*) genes in plant

Plants have defense mechanisms to prevent themselves against pathogen infection. These mechanisms are controlled by *R* genes which are the specificity determinants of the plant immune response. The plant immune response system involves an allele specific genetic interaction between host *R* gene and pathogen avirulence (*Avr*) gene. This genetic interaction has been termed the gene-for-gene model (Flor, 1971). The model predicted that plant resistance will occur only when a plant dominant *R* gene complementary with pathogen *Avr* gene. Whereas, an

alteration or loss of the plant *R* gene (*R* changing to *r*) or of the pathogen *Avr* gene (*Avr* changing to *avr*) leads to successful pathogen and disease (Fig 2.4) (Bonas and Ackerveken, 1999).

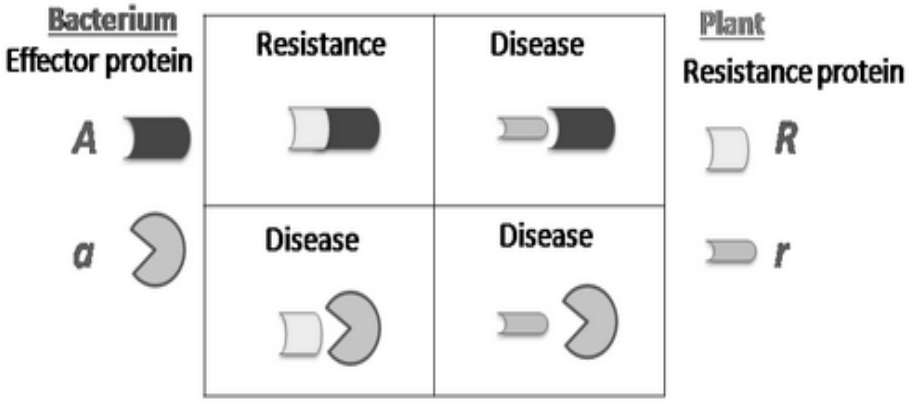

Figure 2.4 The gene-for-gene model of plant immunity (adapted from http://pseudomonas-syringae.org/outreach/Module_4_Lab.htm).

The defense response of genetic interaction is characterized by rapid calcium and ion fluxes, an extracellular oxidative burst, transcriptional reprogramming within and around the infection sites. In most cases, this response will be localized programmed cell death which is termed hypersensitive response (HR) (McDowell and Woffenden, 2003). However, plants also have basal defense response in the absence of specific recognition. These basal defense responses are triggered by plant-associated molecular patterns (PAMPs) such as flagellin and lipopolysaccharides (LPS) from the pathogen. The basal defense response does not prohibit pathogen colonization but does limit the extent of its spread. Thus, R-protein from the *R* gene apparently accelerates and amplifies the innate basal defense response (Chisholm et al., 2006, Grennan, 2006).

R genes have been isolated and classified to several classes based on the structure of their predicted protein product. The largest group of *R* genes carries leucine-rich repeats (LRRs) and nucleotide-binding site (NBS) domains. The NBS-LRR class of *R* genes can be further subdivided based on their ability to code for other recognizable domains. One subclass codes for a TIR domain (homology to the *Drosophila* Toll and mammalian Interleukin-1 receptors) at the N-terminus of the protein. NBS-LRR protein without TIR domain typically code for a coiled-coils structure near their N-terminus, sometimes in the form of a leucine zipper. The NSB sequence of this protein have conserved domains including consensus kinase 1a (P-loop), kinase 2, kinase 3a and GLPL motifs (Fig 2.5) while LRR domains play an important role in protein-protein interaction (McHale et al., 2006).

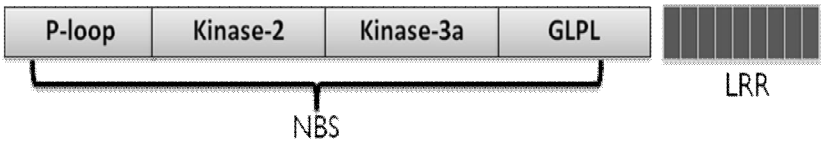

Figure 2.5 The composition of sub-domains in NBS-LRR protein class.

These common domains are the characteristic of various proteins with ATP/GTP binding activity (Moffett et al., 2002). Moreover, motifs of these domains are well conserved in several *R* genes, including *Arabidopsis RPS2* (Bent et al. 1994; Mindrinos et al. 1994), *RPP5* (Noel et al., 1999), tobacco *N* (Whithum et al. 1994) and Flax *L6* (Lawrence et al. 1995). The presence of these conserved domains have enabled rapid isolation of resistance gene analogs (*RGAs*) from different plant species using degenerate primers designed from these domains. Currently, NBS-LRR type *RGAs* have been cloned from different plant species by this approach. For examples, NBS-LRR classes of *RGAs* sequence from potato (Leister et al. 1996), soybean (Yu et

al. 1996), rice (Mago et al. 1999), wheat (Seah et al. 2000), cotton (He et al. 2004) sunflower (Radwan et al. 2003, 2004, 2008) and peanut (Radwan et al. 2010). NBS-LRR type *RGAs* in citrus plant have also been cloned (Deng et al., 2000). Cleaved amplified polymorphic sequence (CAPs) markers for resistance gene tagging and mapping has been developed. Some markers in the NBS-LRR class from Deng et al., 2000 were closely linked to citrus tristeza virus resistance (*Ctv*) gene and citrus nematode resistance (*Tyr1*) gene which are two important genes in *Poncitus* (Deng et al. 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 Antibody production for citrus canker detection

3.1.1 Bacteria isolation

Bacteria were isolated from infected lime leaves tissue by commonly used methods (OEPP/EPPO, 2005). Briefly, the infected leaves were washed with sterile water and surface sterilize by soaked in 1% sodium hypochlorite for 3 min. Then, the lesions were rinsed in sterile water several times and excised with scalpel. Bacteria isolated from Samut Sakhon and Nakhon Ratchasima were initially selected by streaked the water-soak tissue from the lesion margins on sterile semi-selective media (KCD medium, nutrient agar (NA) supplemented with Kasugamycin (16 μg/ml), Cephalexin (16 μg/ml) and Daconil (Chlorothalonil) (12 μg/ml) prior to enrichment on NA media without antibiotics. The bacteria isolated from Phichit were initially grew on NA medium and later on KCD medium. The bacteria were grown at 28-30°C for 24-48 h.

The bacterial colonies were collected for Gram staining and further used for pathogenicity tests. Other *Xanthomonas* strains included *X. axonopodis* pv. *vesicatoria*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *glycine*, *X. oryzae* pv. *oryzae* were obtained from Dr. Suvit Loprasert, Chulabhorn Research Institute (Thailand). They were used as negative control in strain identification and cross-reaction tests.

3.1.2 Specific primers identification and full length 16S rDNA sequencing

Single colony of *Xanthomonas* isolated and other *Xanthomonas* strains were resuspened in 1 ml DI-water and boiled for 10 min. Then, the suspension was used as template for PCR reaction, with specific XAC01 and XAC02 primers for *X. axonopodis* pv. *citri* identification (Coletta-Filho, 2006). The PCR reaction were performed with 50µl reaction mixture, containing 1µl of boiled cell suspension, 1X reaction buffer (Promega GoTaq), 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM primers (XAC01: CGC CAT CCC CAC CAC CAC CAC GAC, XAC02: AAC CGC TCA ATG CCA TCC ACT TCA), 1.25U *Taq* DNA polymerase (Promega). Unrelated bacterial DNA included *Eschericia coli*, *Sinorrhizobium* and *Agrobacterium* were also subjected to PCR reaction as negative control. PCR conditions were as followed; an initial cycle of 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec, with a final step of 72°C for 5 min. The PCR products were observed under UV light after electrophoresis through 1.0 % (w/v) agarose gels and stained with ethidium bromide. The sensitivity of PCR amplification was performed with *X. axonopodis* pv. *citri* (BP210) in tenfold dilution series (10^8 – 10^1 CFU/ml in 0.85% NaCl).

The full length 16S rDNA of BP104 and BP210 were sequenced by Macrogen (Korea) (http://dna.macrogen.com/eng/). The sequence results were applied to BLAST program for gene comparison with data in the GenBank (NCBI).

3.1.3 Pathogenicity test

The phenotype evaluation of the resistance and susceptible limes were performed by leaf inoculation. Half of M33, Nam Hom and Pan limes leaves were wounded with a needle and celite, the other halves were not wounded (healthy) and

then sprayed with 1 ml bacterial isolate suspension (10^8 CFU/ml). The plants were then covered with plastic bags overnight and leave in natural condition for 2-3 weeks. The virulent canker disease apparent on each leaf was evaluated.

3.1.4 Serological tests

3.1.4.1 Bacterial antigen preparation and antiserum production

The bacterial pathogen was cultured on NB (nutrient broth) at 28-30°C for 48 h. The bacterial cells were collected by low-speed centrifugation (1,430 xg) and washed once in steriled 0.85% NaCl. The bacterial suspension was adjusted spectrophotometrically to A_{600} 0.2-0.25 (about 10^8 CFU/ml) and heated to 85˚C for 15 min then used as antigen for antiserum production in two New Zealand white female rabbits. One ml of antigen mixed with Freund's complete adjuvant (1:1 v/v) was immunized to rabbit by subcutaneous injection on the first day. Then, 1 ml of antigen (mixed with Freund's incomplete adjuvant (1:1 v/v)) was injected subcutaneously 7 day later. After that, 1 ml of bacterial antigen without adjuvant was injected intravenous on day 14 and day 21. One week after last injection, the blood was drawn from the central ear artery and the serum was use as antiserum. This antiserum was used for indirect ELISA assay to consider the antibody titer, the sensitivity and the cross reaction of antiserum. The pre-immune serum was bleed before immunization to use as negative control in ELISA assay.

3.1.4.2 Indirect ELISA assay

The blood was kept overnight at 4˚C and the serum was collected after centrifugation at low speed for 15 min. This antiserum was aliquoted to several tubes and stored at -20˚C for future use. The bacterial concentrations were adjusted

from 10^3 – 10^8 CFU/ml in 0.05 M carbonate coating buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, 0.2 g/L NaN_3; pH 9.6). Half volume of each diluted bacterial suspension were treated at 85˚C for 15 min to kill the cells and used as dead cells antigen. One hundred µl of each diluted bacterial suspensions (both live and dead cells antigen) were coated in microtiter plate well at 4°C overnight. The plate was washed three times with PBS-0.05% Tween20 (PBST) and 200 µl of 2% skim milk were added and incubated for 1 h at 37°C. Then, the plates were washed 3 times as above and 100 µl of diluted antiserum were added to the wells. The ELISA plates were incubated at 37°C for 1 h. After that, three times washing were performed and 100 µl of dilution Anti-rabbit IgG Alkaline Phosphatase (ALP) conjugate (Sigma) (1:10,000 v/v) were added in each well and incubated for 1 h at 37°C. The wells were repeatedly washed and 100 µl of p-Nitrophenyl-phosphate (PNPP) substrate (Thermo Scientific) were added in each well. The reaction were stopped by adding equal volume of 0.75 M NaOH and the O.D. at 405 nm were measured after 15-30 min incubation in the dark at room temperature. The live and dead cells of *E. coli* (10^6 CFU/ml) and pre-immune serum were used as negative control.

3.1.4.3 Antibody titer and sensitivity tests

The antiserum was diluted to difference dilution (1:1,000, 1:2,000, 1:4,000, 1:8,000 and 1:1,600 v/v) and tested with both live and dead cells of bacterial antigen (10^4–10^8 CFU/ml) by ELISA assay to test the titer of the antibody. For sensitivity testing, the 1:2,000 (v/v) diluted antiserum was used to test both live and dead cells of bacterial antigen (10^3–10^8 CFU/ml) by indirect ELISA assay as described above. All experiments were done in duplicated and repeated at least 3 times.

3.1.4.4 Cross-reaction test

The diluted antiserum 1:2,000 (v/v) was used for *X. axonopodis* pv. *citri* (10^6 CFU/ml) and other *Xanthomonas* species (10^6 CFU/ml) detection (as mention in 3.1.1) by indirect ELISA assay as described above. The percent of cross-reaction was calculated by;{[A_{405} of individual other *Xanthomonas* species - A_{405} of negative control] / [A_{405} of positive control - A_{405} negative control]} x 100.

3.1.5 Detached leaf assay

Full expanded young leaves of lime were washed in running tap water and surface sterilized in 1% sodium hypochlorite for 1-4 min. The leaves were aseptically rinsed thoroughly with sterile distilled water. Each leaf was divided into 4 parts for treatment separation, included 10^5, 10^6 and 10^7 CFU/ml infected parts and one uninfected part. Each part of the leaf was wounded by puncturing with a small needle, through the lower surface (5 needle punctured wounds per part). Each wounded leaf was placed on 1% water agar in a Petri dish, with the back of the leaf up. Twenty µl of the different diluted bacterial suspension (10^5-10^7 CFU/ml *X. axonopodis* pv. *citri*) were dropped on wounds. The part of uninfected leaf, 20 µl of sterile 0.85% NaCl was dropped to used as negative control. The infected leaves were maintained in a lighted incubator at 25-30°C.

The canker pathogen on each part of infected leaf was detected by ELISA method. Each part of the infected leaf was ground in 150 µl coating buffer. One hundred µl of leaf extract was coated in microtiter plate well and incubated at 4°C for overnight before detection with 1:2000 (v/v) diluted antiserum. The steps were done as mention in the ELISA protocol described in 3.1.4.2. One µl of the leaf extract was

also performed in XAC specific PCR amplification as described in 3.1.2. The detection results were demonstrated as percentage (% detection), calculated by; [number of sample that can detect by XAC primers/total samples were tested] x 100.

3.1.6 Canker detection from field samples

Symptom and non-symptom plant materials (leaf and twig) from Pan, Nam Hom and M33 limes were taken from the field and washed in 25 ml 0.85% NaCl shaking for 30 min. Then, the plant material was removed, and the washing solution was boiled for 10 min. One µl of the boiled washing solution were used as template for specific primers (XAC01 and XAC02) amplification. The PCR condition was performed as described in 3.1.2. *Xanthomonas* BP210 isolate was used as positive control.

3.2 Canker resistance gene analogs in Thai hybrid lime M33

3.2.1 Bacterial isolation

The bacteria were isolated using the same method as in 3.1.1

3.2.2 Canker pathogen inoculation

The phenotype evaluation of the resistance and susceptible limes were performed by leaf inoculation as described in 3.1.3.

3.2.3 Plant samples

M33 limes (highly resistant to canker) were from Phichit research center, Samut Sakorn and Prachinburi (Thailand), Nam Hom limes (highly resistant to canker) were from Phichit research center and Pan limes (susceptible to canker) were

from Samut Sakorn. These lime plants were planted in the test field at Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.2.4 DNA isolation

Genomic DNAs were isolated from young leaflets using the modified 2.0% (w/v) cetyltrimethyl ammonium bromide (CTAB) protocol (Chen et al., 2003). Breifly, 2 gram of grounded leaves were add to 720 µl of warm (65˚C) CTAB extraction buffer (2% (w/v) CTAB, 1.4 M NaCI, 20 mM, EDTA, 100 mM Tris-Cl; pH 8.0) and incubated at 60˚C for 30 min with occasional gentle swirling. Then, 1 V of phenol: chloroform: isoamylalcohol (25:24:1) were added to the samples and rock gently to mix for 5-10 min. The top aqueous layer was transferred to a new tube after centrifugation at 16,000 xg for 10 min. Then, the genomic DNA was precipitated by added 1/10 V of 3M sodium acetate followed by 1 V of cool isoamyl alcohol and mix gently. DNA pellet was collected after centrifugation and washed with 300 µl of 70% ethanol. Then, DNA pellet was dried and resuspended in 50 µl TE buffer (100 mM Tris-Cl and 1 mM EDTA; pH 8.0) and stored at -20˚C. The quality and concentration of DNA were checked by agarose gel electrophorosis and nanodrop-spectrophotometer, respectively.

3.2.5 PCR amplification combination with restriction enzymes

Resistance gene candidates of Pan, Nam Hom and M33 limes were screened by PCR amplification using 12 specific primers (table 3.1). The PCR amplifications were performed in 25 µl reaction volume; containing 1X reaction buffer (Promega GoTaq), 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primers, 1.25U of *Taq* polymerase (Promega) and 100~150 ng genomic DNA. The initial denaturation

was done at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 47-67°C (depended on primers; described in table 3.1) and 30 sec at 72°C. The final step was performed at 72°C for 5 min for the last extension. One µl each of PCR products were digested in 10 µl reaction volume with 1U of *Eco*RI, *Tru1*I, *Bfa1*, *Hinf*I, *Alu*I or *Taq*I, separately. The digested incubation time and condition were described in table 3.2. The PCR products and their digested products were separated on 2% agarose gels, visualized by staining with ethidium bromide and UV illumination.

3.2.6 DNA sequencing and analysis

The PCR products of resistance gene analogs of *Pt9*, *Pt14* and *16R1-19* from each plant were cloned into Promega pGEM T ® -Easy Vector and transformed to DH5α *E. coli* cells. The DNA sequencing was performed by Macrogen (Korea) (http://dna.macrogen.com/eng/) using SP6 and T7 primers. Resulting sequences were applied to BLAST program (blast.ncbi.nlm.nih.gov) for gene comparison with data in GenBank (NCBI). The alignment and phylogenetic tree were created by DNA analysis program (CLC Main Workbench 5).

Table 3.1 Primers name, sequence of forward and reverse primers and annealing temperature (Ta) used for canker *RGAs* screening.

RGAs primers [a]	Forward primers [b]	Reverse primers [b]	Ta (°C)
Pt6	GACTTGATCTCTCATGAATTTGAA	CACCGACATACCGTAGAACACC	59
Pt14	GACAATATCTCTTATCAGTTTGATG	GAGCTCAAAATAACCATCTGTAG	47
11P31	TGCTCGGGAGGTCTACAATGACAG	CCTCCGCACTTTCCATCAATCTT	63
Pt3	CCACAACAATGATGTCAAGAATAA	GTCCCTTTTCAGCCTTAGAGTTAC	59
Pt7	TTACGGCGACAAAGATGTCAG	TCCCAACTTCTCCAATCCTTTATTA	49
Pt18	TAAGCATAAATTCGATTGTTGTG	TCCAACTATTATCTGGCCTTAGAA	55
Pt8	ATTCGCGGAAAGATGATTTTGA	ACACTCTTTCGTCACGGTTTCAG	55
Pt9	AGCTTCTTGGTGCACCAAATGGTT	CCCTTTAGCTACACTTCTGGCTAGTTCA	65
18P33	AAGTCAACAACAACTTCCGCTATCA	GGTTTCGGCTAGCTCTGGAATACT	67
Pt19	AGGAAATTCAGAAGCAAGCAAAAG	ATCCGTCAGCCACCTCTCTT	67
16R1-19	TCTGCAGTGAGGAGCATGATTTTGAT	ATGACACTGACGTGCCACATGCT	67
11P33	GCAAGCTGCAGGTTGTGGTGTTTA	AGGCCGACCTGGTTGAGTTTG	63

[a]: *RGAs* primers sequence derived from Deng et al. (2000); [b]: primer sequences were arranged from 5'- to 3'-end.

Table 3.2 Restriction enzymes used in the experiment.

Restriction enzyme	Restriction site	Optimal temperature	Incubation time	Company
***EcoR*I**	G↓AATTC	37°C	Overnight	Fermentas Ltd.
***Tru*1I**	T↓TAA	65°C	Overnight	Fermentas Ltd.
***FspB*I (*Bfa*I)**	C↓TAG	37°C	Overnight	Fermentas Ltd.
***Hinf*I**	G↓ANTC	37°C	Overnight	Fermentas Ltd.
***Alu*I**	AG↓CT	37°C	Overnight	Sib Enzyme Ltd.
***Taq*I**	T↓CGA	65°C	Overnight	Sib Enzyme Ltd.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Antibody production for citrus canker (*X. axonopodis* pv. c*itri*) detection

4.1.1 Bacteria isolation

Nineteen bacterial isolates from three different regions in Thailand; Samut Sakhon, Nakhon Ratchasima and Phichit were isolated. The bacteria isolated from Samut Sakhon and Nakhon Ratchasima were initially selected on KCD semi-selective medium. The bacteria isolated from Phichit were initially grew on NA medium and later on KCD medium.

The results showed that, 3 isolates from Phichit (PJ02, PJ04 and PJ05) did not grow on KCD medium. The colony morphology of bacteria isolated from Samut Sakhon on NA medium were circular in shape, convex, mucoid and shiny with yellow color (Fig. 4.1A). After long incubation period, barely visible small white colony appeared spontaneously which is a general colony characteristic of *Xanthomonas* bacteria similar to the report of Dai et al., 1991. The colonies on KCD medium were smaller and have less color (Fig. 4.1B), especially colony of PJ01 and PJ03 from Phichit were white on KCD medium. Bacteria isolated from Nakhon Ratchasima (SUT02, SUT06, K01 and K02) showed orange-yellow colony on both NA and KCD media but the colonies size on KCD medium were smaller than on the NA medium (Figs. 4.1C and D).

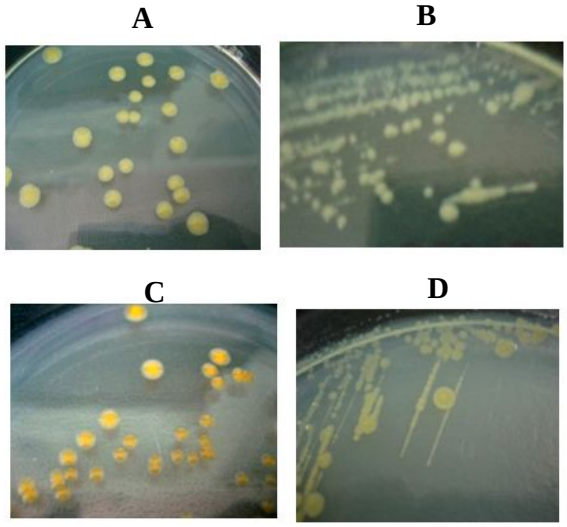

Figure 4.1 Colony morphology of bacteria isolated from lime leaves. A: yellow colony of *Xanthomanas* (BP102) on NA media, B: light-yellow colony of *Xanthomonas* (BP102) on KCD medium, C: orange-yellow colony of bacteria (SUT02) on NA medium, D: yellow colony of bacteria (SUT02) on KCD medium.

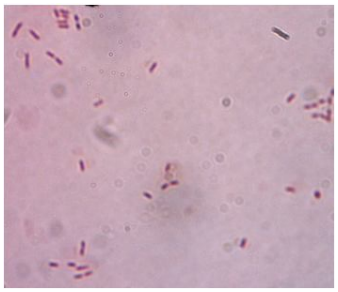

Figure 4.2 Gram staining of *Xanthomonas* bacteria (BP210).

The cell morphology of the bacteria were also demonstrated by Gram staining and observed under microscope. The Gram stain indicated that, all isolates were Gram-negative rod (Fig. 4.2) bacteria.

4.1.2 Specific primers identification and full length 16S rDNA sequencing

The isolated bacteria were identified by XAC specific primers (Coletta-

Filho, 2006). The results indicated that, all isolated from Samut Sakhon and 2 isolated from Phichit contain 581 bp Xac *rpf* gene (Fig. 4.3 lanes 2-10, 16 and 17). But this gene was not detected in samples from Nakhon Ratchasima, other Xanthomonas species and unrelated bacteria, such as *E. coli, Sinorhizobium* and *Agrobacterium* that were used as negative control (Figs. 4.3 and 4.4). Non-target sizes DNA bands were detected in isolated from Nakhon Ratchasima and some from Phichit. Two pathogenic *Xanthomonas* strains isolated from Samut Sakhon (BP104 and BP210) were identified by 16S rDNA sequencing. The 16S rDNA sequence confirmed that both strains were *X. axonopodis* pv. *citri* (Appendix A Figs. 2a and b).

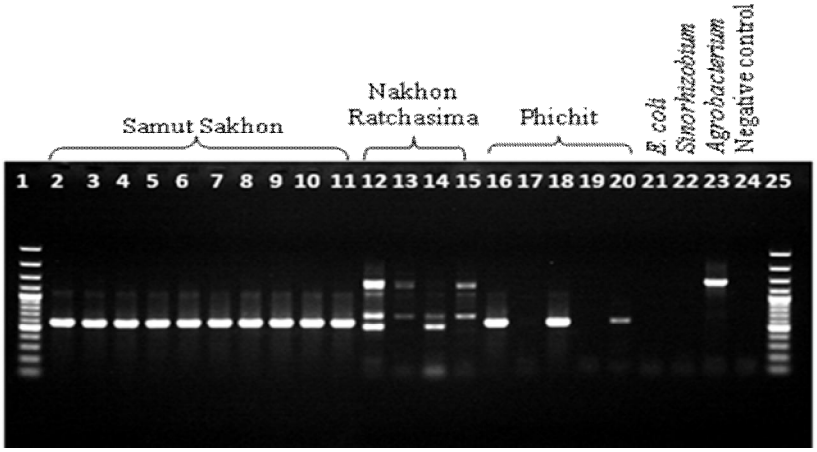

Figure 4.3 Specific amplification of XAC target (581 bp fragment) by XAC01 and XAC02 specific primers. Lane 1 and 25: 100 bp marker; Lane 2-11: bacterial isolated from Samut Sakhon; Lane 12-15: Nakhon Ratchasima; Lane 16-20: Phichit; Lane 21-23 unrelated bacteria consist of *E. coli, Sinorhizobium* and *Agrobacterium*, respectively. Lane 24: negative control.

4.1.3 Pathogenicity tests

All bacterial isolates (19) from different regions were inoculated to leaves of

Pan, Nam Hom and M33 limes for canker resistance evaluation. The results showed that all isolates from Samut Sakhon with yellow colonies on NA medium can infect wounded lime leaves. However, isolates from Nakhon Ratchasima (SUT02, SUT06, K01, K02) with orange yellow colonies cannot infect neither wound or unwounded leaves (Appendix A table 1). The 581 bp XAC specific PCR products were also not detected in these samples (Fig. 4.3 lanes 12-15).

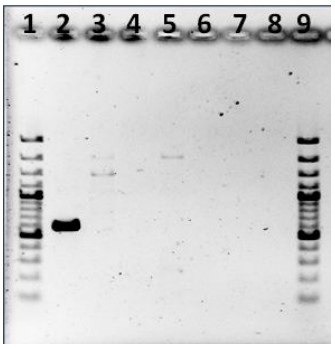

Figure 4.4 Specific amplification of XAC target by PCR. Lane 1 and 9: 100 bp marker; lance 2: *X. axonopodis* pv. *citri* (BP210); Lane 3: *X. axonopodis* pv. *vesicatoria;* Lane 4: *X. axonopodis* pv. *phaseoli*; Lane 5: *X. campestris* pv. *campestris*; Lane 6: *X. axonopodis* pv. *glycine;* Lane 7: *X. oryae* pv. *oryzae*, respectively. Lane 8: negative control.

This indicated that they might not be *Xanthomonas*, even if they can grow on KCD medium. Some isolates from Phichit (PJ02, PJ04 and PJ05) also have similar phenotype and the XAC specific PCR product were not detected as the isolates from Nakhon Ratchasima. However, isolate PJ01 and PJ03 should be *Xanthomonas* species since XAC specific genes product were seen (Fig. 4.3 lanes 16 and 18) and similar colonies morphology as the Samut Sakhon isolates were observed. Pathogenic bacteria isolates can infect wounded and unwounded lime

leaves. The earliest symptoms on Pan lime leaves were characterized by water-soaked slightly raised blister-like lesions surrounded by chlorotic halo. Subsequently, the spongy or cocky on the centre of lesions appeared and turned brown 3 weeks post-infection. Whereas, the lesion on Namhom and M33 lime leaves were smaller and turned brown within 2 weeks post-infection (Fig. 4.5). This is the characteristic of hypersensitive response of resistant lime.

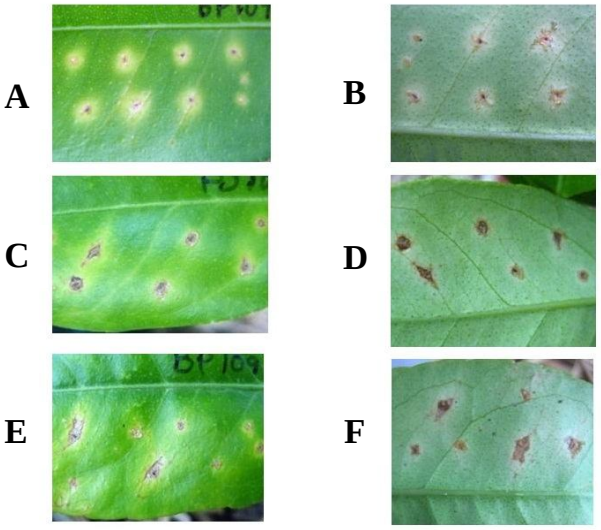

Figure 4.5 Canker lesion on upper and under leaf of Pan (A and B), Nam Hom (C and D) and M33 limes (E and F) by bacterial isolate BP109 inoculation.

The lesion caused by isolated BP104 and BP210 on both susceptible (Pan) and resistance limes (Nam Hom and M33) did not turn brown after 3 week post-infection (Fig. 4.6). Thus, isolated BP104 and BP210 could be virulence pathogen for citrus plant. These results demonstrated that *Xanthomonas* BP104 and BP210 isolate is most virulent bacteria that are pathogenic to the resistant lime. In this experiment, *Xanthomonas* BP210 isolate was used as bacterial antigen for further antibody production.

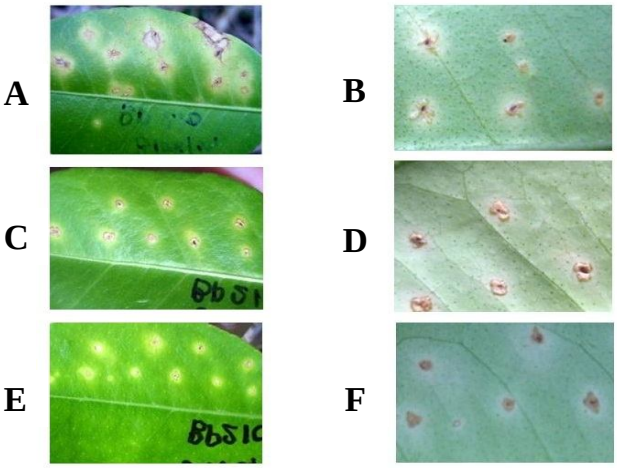

Figure 4.6 Canker lesion on upper and under leaf of Pan (A and B), Nam Hom (C and D) and M33 limes (E and F) by bacterial isolate BP210 inoculation.

4.1.4 Antiserum titer test

Antiserum were produced for *X. axonopodis* pv. *citri* (XAC) detection by rabbit injection. The XAC isolated from Banpaew, Samut Sakhon were used as antigen to produce antiserum in duplicated (pAb1 and pAb2). Titer of antiserum were tested using indirect ELISA assay. The results showed that, 1:16,000 or lower dilution of both antiserum (pAb1 and pAb2) were able to detected dead cells of XAC at 10^5 CFU/ml or more. Whereas, 1:4,000 diluted antiserum were reactive against live cells of 10^6 CFU/ml and dead cells at 10^5 CFU/ml or more. The highly concentrate antiserum (1:2,000 and 1:1,000 dilution) strongly reacted against both live and dead cells (10^5 CFU/ml) of XAC. However, pAb1 showed stronger reaction than pAb2 (Figs. 4.7 and 4.8 or Appendix A table 2). These results indicated that, at least 1:2,000 dilution of these pAbs were able to detected both live and dead cell of XAC at 10^5 CFU/ml. Nevertheless, dead cells of bacteria showed stronger reaction with pAbs than live cells. Since these pAbs were produced from injection with heat killing cells might have broken some cells.

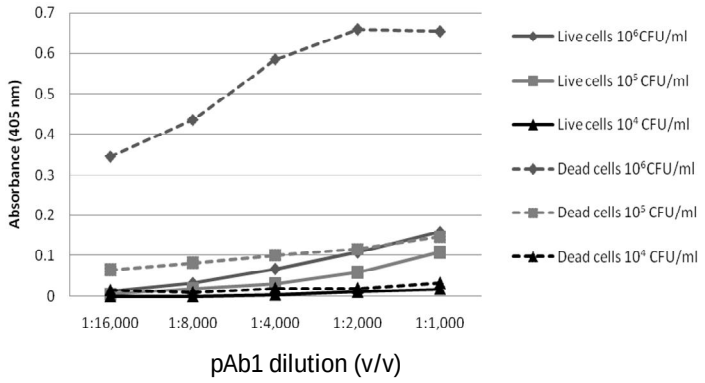

Figure 4.7 ELISA for pAb1 titer test. Different pAb1 dilutions (1:16,000, 1:8,000, 1:4,000, 1:2,000 and 1:1,000) were used to detect live and dead cells of *X. axonopodis* pv. *citri* suspension at different concentrations (10^4, 10^5 and 10^6CFU/ml). A $_{405}$ data was indicated in Appendix A table 2.

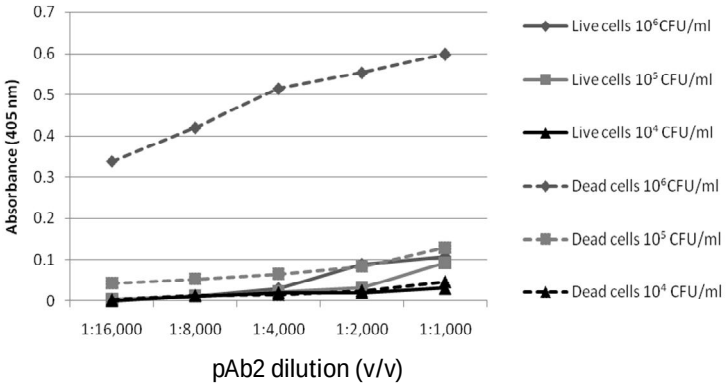

Figure 4.8 ELISA for pAb2 titer test. Different pAb2 dilutions (1:16,000, 1:8,000, 1:4,000, 1:2,000 and 1:1,000) were used to detect live and dead cells of *X. axonopodis* pv. *citri* suspension at different concentrations (10^4, 10^5 and 10^6CFU/ml). A $_{405}$ data was indicated in Appendix A table 2.

4.1.5 Sensitivity of *X. axonopodis* pv. *citri* detection

pAb1 and pAb2 at the dilution of 1:2,000 were tested for the sensitivity with live and dead cells of *X. axonopodis* pv. *citri* at different concentrations (10^3-10^8

CFU/ml). The positive result is the number which is at least double the reaction intensity of negative control (*E. coli* and pre-immune serum reaction).

Table 4.1 Sensitivity of ELISA in detecting live and dead cells of *X. axonopodis* pv. *citri*.

Antiserum	Cell status	*X. axonopodis* pv. *citri* antigen (CFU/ml)						
		10^8	10^7	10^6	10^5	10^4	10^3	*E. coli* (10^6 CFU/ml)
pAb1 (1:2,000)	Live cell	++ [a]	++	++	--	--	--	--
	Dead cell	++	++	++	++	--	--	--
pAb2 (1:2,000)	Live cell	++	++	++	--	--	--	--
	Dead cell	++	++	++	++	--	--	--

[a]: The result was taken as positive (+) when A_{405} was twice greater than that of the negative (-). The A_{405} data was indicated in Appendix A table 3.

The bacterial suspension of live cells at 10^6 CFU/ml and dead cells at 10^5 CFU/ml showed positive reaction in ELISA (table 4.1). The reaction intensities of serological reaction increased with the increased bacterial densities in both pAbs tested. These results indicated that, both pAb1 and pAb2 have the sensitivity of detection at 10^6 CFU/ml for live cell and 10^5 CFU/ml for dead cells. The sensitivity of ELISA for bacterial pathogen has been reported to be at the level of 10^5-10^6 CFU/ml (Jin et al. 2001; Alvarez, 2004; Leon et al., 2008; Kokoskova and Mraz, 2008). The detection efficiency of ELISA is limited by the level of the pathogen population and dependent upon the immunological properties of antiserum used. Anyhow, the pAbs produced in this study have the sensitivity as high as pAb produced in other reports.

4.1.6 Cross-reaction of antiserum to other *Xanthomonas sp.*

Antiserum dilutions (1:1,000 and 1:2,000) were tested for cross-reacted against live cells of other five *Xanthomonas sp.* and *E. coli* at 10^6 CFU/ml. The results showed that, dilution of 1:1,000 antiserum (both pAb1 and pAb2) strongly cross-reacted against *X. axonopodis* pv. *vesicatoria* and weakly reacted against *X. axonopodis* pv. *phaseoli* and *X. campestris* pv. *campestris*. While, more diluted antiserum (1:2,000) were cross-reacted with only *X. axonopodis* pv. *vesicatoria* but not with others (table 4.2). These results suggested that, antiserum diluted to 1:2,000 is the suitable condition for detection of target bacteria of at least 10^6 CFU/ml. Although, these antiserum showed cross-reaction with *X. axonopodis* pv. *vesicatoria* but this bacteria does not infect citrus plant. They only infect tomato and *Capsicum* pathogen (Kim et al., 2010).

Therefore, the cross-reaction detection should not be alarm. Bacterial cell surfaces display a variety of antigenic molecules, including protein, lipopolysaccharides and extracellular polysaccharides. Thus, polyclonal antibodies (or antisera) from bacterial species induces are mixtures of antibodies with multiple specificities. Moreover, these antibodies are obtained from different B cell resources. Although the dominant antibodies showed acceptable specificity for a given pathogen but cross-reactions with unrelated species can be detected. On the other hand, monoclonal antibodies (mAbs) can be more specific than pAbs because it provides a single epitope to recognized target epitope which performed from individual hybrid cell line (hybridoma cell). But mAbs are time-consuming to produce. The entire process of producing mAbs takes 3-4 months for each fusion experiment. Moreover, monoclonals against conformational epitopes on native proteins may lose reactivity

with antigens. Due to these limitations, mAbs might miss the important cross-reactive determinants.

Table 4.2 Cross-reaction of ELISA to other *Xanthomonas* species and unrelated microorganism.

Bacterial strain tested (10^6 **CFU/ml)**	**Diluted antiserum**			
	pAb1		**pAb2**	
	(1:1,000) (%)	**(1:2,000) (%)**	**(1:1,000) (%)**	**(1:2,000) (%)**
X. axonopodis pv. *citri* (positive control)	100 [a]	100	100	100
X. axonopodis pv. *vesicatoria*	100	91	80	85
X. axonopodis pv. *phaseoli*	33	0	39	0
X. campestris pv. *campestris*	47	0	27	0
X. axonopodis pv. *glycine*	0	0	0	0
X. oryzae pv. *oryzae*	0	0	0	0
E. coli (negative control)	0	0	0	0

[a]: The percent of reaction was calculated from formula in 3.1.4.4.

However, mAbs also used ELISA assay as detection procedures to determine. Many studies reported that, the ELISA sensitivity 10^5-10^6 CFU/ml is sufficient for identification of bacteria pathogens from symptomatic plants and colonies on selective media (Jin et al. 2001; Alvarez, 2004; Leon et al., 2008; Kokoskova and Mraz, 2008). However, boiling the bacteria samples cloud improved the sensitivity of detection (Figs. 4.7 and 4.8). Jones et al. (1997) have reported that, the sensitivity of ELISA can be increased tenfold by using an extraction buffer

containg EDTA (ethylenediaminetetraacetic acid) and lysozyme. This buffer extracted lipopolysaccharide (LPS) into solution, thereby enhancing the antibody–antigen reaction without increasing background readings.

4.1.7 Detach leaf assay

Different concentrations of canker bacteria (10^7, 10^6 and 10^5 CFU/ml) were inoculated to Pan lime leaves using detach leaf assay. The pathogen on the infected leaves were detected by ELISA method every 2 days after inoculation. The results showed that, infected leaves detection on first day of inoculation (day zero) showed negative reactions in ELISA whether the bacterial cells were killed by heat treatment or not (table 4.3). The inoculation of this experiment used 20 µl bacterial suspensions dropped on leaves. Thus, bacteria population on leaves has lower 100 fold than bacterial suspension used, 10^7, 10^6 and 10^5 CFU/ml inoculation indicated 10^5, 10^4 and 10^3 CFU on the inoculated leaves. Moreover, the sensitivity of ELISA (10^5 CFU/ml or 10^4 CFU/well for dead cells) should be sufficient for dead cells detection but protein molecules from crushing leaf may influenced to bacteria binding to the plate which caused low detection of target bacteria. Infected leaves on day two showed positive reaction with infected leaves that was inoculated with 10^5 CFU of bacteria. Consequently, infected leaves after 4 days inoculation showed positive reaction in all bacteria concentration (10^5, 10^4 and 10^3 CFU) whether the cells were treated with boiling water or not. The boiled bacteria from infected leaves showed higher reaction in ELISA test than living cells. These results were in accordance with the results of antibody titer and sensitivity test (4.1.4 and 4.1.5). After day 4 of inoculation, symptom on the infected areas was observed as slightly raised blister-like lesions

(Fig. 4.9). Thus, these results indicated that, ELISA assay is a well-established method for identification of bacterial pathogens from symptomatic plants.

Table 4.3 ELISA detection of bacteria on infected leaf using detached leaf assay.

Day after inoculation	Initial bacteria density (CFU)	Cell status		PCR detection (XAC specific primers)
		Live	Dead	
0	10^5	- - - [a]	- - -	+
	10^4	- - -	- - -	+
	10^3	- - -	- - -	+
2	10^5	+ + +	+ + +	+
	10^4	- - -	- - -	+
	10^3	- - -	- - -	+
4	10^5	+ + +	+ + +	+
	10^4	- + +	+ + +	+
	10^3	- + +	+ + +	+
6	10^5	+ + +	+ + +	+
	10^4	+ + +	+ + +	+
	10^3	+ + +	+ + +	+
8	10^5	+ + +	+ + +	+
	10^4	+ + +	+ + +	+
	10^3	+ + +	+ + +	+
10	10^5	+ + +	+ + +	+
	10^4	+ + +	+ + +	+
	10^3	+ + +	+ + +	+

[a]: The positive result (+) was indicated when A_{405} was twice greater than that of the negative (-).

However, enrichment techniques can enhance ability of pathogen infection on natural samples but more time is needed for the culturing period (Jin et al., 2001).

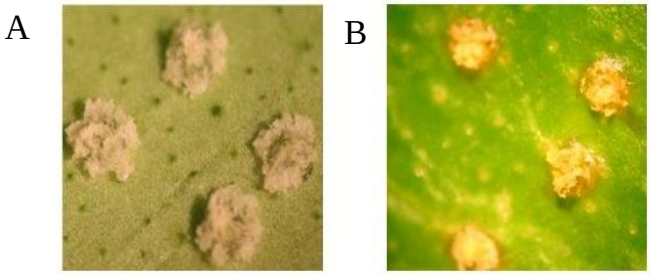

Figure 4.9 Canker lesions on lime leaf infection using detached leaf assay. Lesion on under (A) and upper leaf (B) showed callus-like lesion with water-soaked margins.

4.1.8 Amplification of XAC from canker lesions

Canker pathogen on symptom and non-symptom plant materials (leaf and twig) in infected areas were detected by PCR amplification using XAC specific primers. The results showed that, the XAC specific PCR products were detect in all samples of symptom plant materials and some non-symptom plant materials (table 4.4).

These specific primers were also detected canker pathogen on infected leaves from detach leaf experiment. The results showed that, canker pathogen were detected by these specific primers on first day inoculation while ELISA was not available (table 4.3). Since, Amplification assay using specific primers allowed detecting at low population of pathogen (10^2-10^3 CFU/ml) (Cubero et al., 2001, Cubero and Graham, 2002, Coletla-Filho, 2006, Leon, 2008, Park et al., 2006). The sensitivity of PCR amplification of this study showed high sensitivity at 10^3 CFU/ml

or 1 CFU (Appendix A Fig. 3). Moreover, this methodology was not cross-reacted with other *Xanthonomas* species (Fig. 4.4). However, PCR method required laboratory equipment, special reagents and skill for preformed.

Table 4.4 XAC specific amplification of canker pathogen from symptom and non-symptom plant materials.

Plant materials	Plant status [a]		PCR detection [b] (% detection)	
	Symptom	Non-symptom	Symptom	Non-symptom
Leaf	10	10	100%	40%
Twig	6	6	100%	33%

[a]: Number of plant material samples that used in this study.

[b]: The percentage of detection was calculated from formula in 3.1.6.

4.2 Canker resistance gene analogs in Thai hybrid lime M33

4.2.1 Response to *X. axonopodis* pv. *citri* inoculation

M33 and its parents (Pan and Nam Hom) were inoculated with citrus canker bacteria on leaves. Blushed-like lesions surrounded by chlorotic halo with water-soaked were seen on both wounded and unwounded Pan leaves. Whereas, the lesions on M33 and Nam Hom leaves were smaller and turned brown within 2 weeks post infection (table 4.5) which is the characteristic of hypersensitive response of resistance lime. From these responses, we can hypothesize that Nam Hom and M33 could have defense response mechanisms to prevent themselves from XAC infection.

Table 4.5 The host-pathogen interaction of virulent bacteria with susceptible and resistance plant.

Pathogen	Effectors interact with specific host targets • Virulent bacterial isolates	
Plant	Susceptible (Pan lime)	Resistance (Nam Hom and M33 lime)
Plant defense response	• Compatible • Basal defense only	• Incompatible • Basal defense and hypersensitive response
Out come	 Large lesions, cocky with water-soaked margin and chlorotic halo	 Small and brown lesions (dead cell response)

Defense responses in resistance plants can be divided into 2 types, including basal defense response and hypersensitive response. Basal defense response can be activate within minutes of attack through PAMP-triggered immunity (PTI) and induced by Pathogen-Associated Molecular Patterns (PAMPs) which are common molecules expressed by all bacteria such as flagellin or bacterial lipopolysaccharides (LPS) (Chisholm et al., 2006; Reina-Pinto and Yephremov, 2009). The perception of

PAMPs by plant extracellular receptors activated signal-transduction cascades that turn on basal defenses, including cellulose and silicone deposition to reinforce the cell wall, production of reactive oxygen species and ethylene, transcriptional induction of a large suite of defense genes, including pathogenesis-related genes (*PR*) and post-transcriptional suppression of the auxin-signalling pathway (Alfano and Collmer, 1996). Basal defense does not prohibit pathogen colonization but it does limit the extent of its spread (Glazebrook et al., 1997). However, hypersensitive response (HR) or programmed cell death activation is a second layer of defense that can overcome pathogen invasion. HR is activated by incompatibility between dominant 'resistance gene' (*R*) of plant and dominant 'avirulence gene' (*Avr*) of pathogen which produce effector proteins for infection. This interaction is known as the 'gene-for-gene model.

The infected plant cell used programmed cell death to create a protective zone of dead cells (browning) around the site of pathogen invasion (as seen in the infection of virulent bacteria to Namhom and M33 limes (table 4.5). This R protein-mediated resistance is referred to as effector-triggered immunity (ETI) (Ade and Innes, 2007). Nevertheless, some *Xanthomonas* isolated from this experiment can infect the lime known to be resistant to canker disease (M33 and Namhom limes). This may be due to the compatibility of the *Xanthomonas Avr* gene with the *R* gene (Hammond-Kosack and Jones, 1997) of the resistance plant that caused the susceptibility of M33 and Namhom limes with virulence bacteria (BP210).

4.2.2 Citrus canker resistance gene analogous discovery

The hybrid lime (M33) and it's parents (Pan and Nam Hom) were screened using 12 NBS-LRR genes specific primers in combination with restriction enzymes

(Appendix B table1). The results indicated that, only three specific amplicons combination with three different restriction enzymes were closely linked to canker resistance gene within M33 and Nam Hom limes, including *Pt9*/*Alu*1, *Pt14*/*Bfa*1 and *16R1-19*/*Tru*1I (Appendix B table 2 and 3). These results correspond with the resistance phenotype (hypersensitive respond) of M33 and Nam Hom limes after inoculation with *Xanthomonas*.

4.2.2.1 *Pt9*/*Alu*1 marker gene discovery

The *Pt9* NBS-LRR gene fragment combination with *Alu*1 showed 2 bands linked with the canker resistance of M33 and Nam Hom limes (Fig. 4.10 lanes 6-9). The PCR products of *Pt9* amplification from all limes cultivars were approximately to 450 bp (Fig. 4.10 lanes 1-3). After the PCR products were digested with *Alu*1, 2 smaller bands of 320 and 130 bp were seen from DNA of M33 and Nam Hom limes (Fig. 4.10 lanes 6-9). Whereas, PCR products from Pan lime followed by *Alu*1 digestion showed three bands consisted, 320 and 130 bp as seen from M33 and Nam Hom limes digestion results and another band of 250 bp (Fig. 4.10 lane 5).

These results indicated that, the *Pt9* amplicon from Pan lime contains only one allele with the *Alu*1 restriction site similar to the *Pt9* amplicon from M33 and Nam Hom. Another allele of *Pt9* amplicon from Pan lime can be digested with *Alu*1 at different position therefore, different band pattern was observed (Fig 4.10 lane 5 and Fig. 4.11). Since citrus plant are diploid (2n), these results can assumed that, R_{Pt9} gene on the chromosomes of M33 and Nam Hom genomes should be two dominant *R* gene (RR_{Pt9}) that can be completely digested by *Alu*1 enzyme and generated two specific bands of 320 and 130 bp. Whereas, Rr_{Pt9} gene on Pan lime

chromosomes should be one dominant *R* gene (R_{Pt9}) and one recessive *r* gene (r_{Pt9}) that contains different position of restriction site of *Alu*1 (Fig. 4.11). This RR_{Pt9} gene were also founded on other resistance lime (Puang) (Fig. 4.10 lane 10) which confirmed the result from M33 and Nam Hom that the *Pt9*/*Alu*1 genotype is linked to the *Xanthomonas* resistant phenotype in limes.

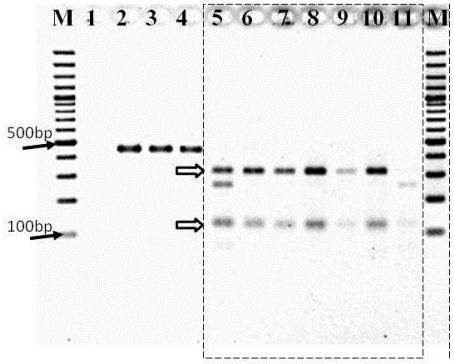

Figure 4.10 *Pt9*/*Alu*1 marker gene screened on M33 and their parents (Pan and Nam Hom); M: 100 bp marker; Lane 1: negative control; Lane 2-4: PCR product of *Pt9* from Pan, Nam Hom and M33, *Alu1* digested *Pt9* amplicon from Pan (lane 5), Nam Hom (lane 6), M33 from Phichit (lane 7), M33 from Samut Sakhon (lane 8), M33 from Prachinburi (lane 9), Puang resistant lime (lane 10) and Giant resistant lime (lane 11).

The *Pt9* fragments from Pan, Nam Hom and M33 were cloned and sequenced (Appendix B table 2). The sequencing results confirmed the *Alu*1 restriction sites on R_{Pt9} and r_{Pt9} from Pan, Nam Hom and M33 genomes (Appendix B table 2). *Alu*1 restriction sites on the *Pt9* sequences gave the fragment size similar to what were observed on the agarose gel. The digestions by these enzymes also resulted to some small pieces that were not observed on the gel (Appendix B table 2 and Fig. 4.10).

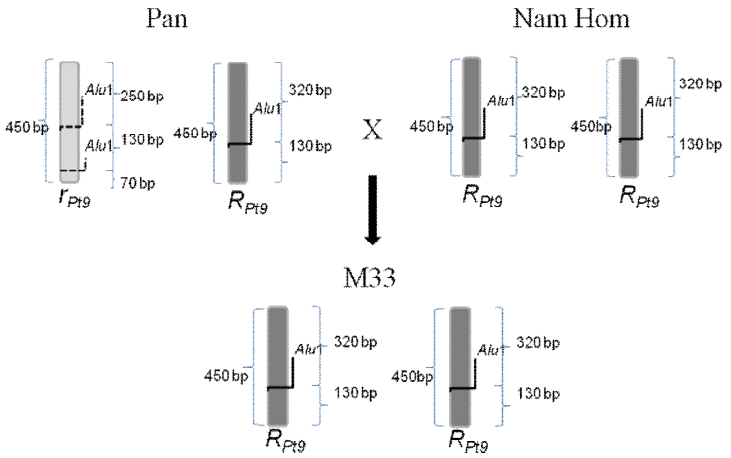

Figure 4.11 *Alu*1 restriction enzyme map of R_{Pt9} and r_{Pt9} gene on Pan, Nam Hom and M33 amplicons. Rr_{Pt9} of Pan crossing with RR_{Pt9} of Nam Hom, resulted in RR_{Pt9} on M33 genome.

The *Pt9* sequences could be translated to polypeptide without any stop codons. Using the BLASTX program to search the GenBank database to the results indicated that, all Pt9 clones showed strong overall similarities (>90% identity) to *C. grandis* x *P. trifoliate* Pt9, Pt11 (Deng et al., 2000) and RGA 24 sequence recently cloned from *C. triloliata* (Shen et al., 2004).

Multiple alignment was performed with the translated sequence of Pt9 and the 3 most similar *RGAs* gene sequences and the partial resistance protein of *Arabidopsis* RPS2 (Bent et al. 1994; Mindrinos et al. 1994), RPP5 (Noel et al., 1999), tobacco N (Witham et al. 1994), and flax L6 (Lawrence et al. 1995). The similarity was especially high at the two NBS motifs (kinase-2 and kinase-3a) (Appendix B Fig. 1). Deng et al. (2000) and Meyers et al. (1999) had performed extensive analyses of the NBS domains of the plant NBS-LRR class *R* genes and a vast number of *RGAs*. They found that, *RGAs* could be classified into either TIR (Toll/Interleukin-1

receptor) or non-TIR groups. In their analysis, N and L6 belong to the TIR group, while RPS2 fall into the non-TIR group. The translated Pt9 peptide sequences from this study indicated that, the Pt9 clones formed a major cluster with RPS2. A Tryptophan residues (W) were found at the end of kinase 2 motif which often seen in the non-TIR-LRR class proteins. Thus, Pt9 proteins from our limes belong to the non-TIR-LRR class (Appendix B Fig. 1). Two differential amino acid sequences between R_{Pt9} and r_{Pt9} were founded, valine (V) replaced alanine (A) and glutamate (E) replaced lysine (K) (Appendix B Fig. 1). These 2 amino acid mutations might be involved in the malfunction of r_{Pt9} on susceptible lime.

4.2.2.2 *Pt14*/*Bfa*1 marker gene discovery

Pt14 NBS-LRR gene amplification in combination with *Bfa*1restriction digest showed 2 bands linked with the canker resistance of M33 and Nam Hom limes (Fig. 4.12 lanes 6-9). The PCR products of *Pt14* amplification were 430 bp in all lime cultivars (Fig. 4.12 lanes 2-4). After the PCR products were digested with *Bfa*1, 2 smaller bands of 300 and 130 bp were seen from DNA of M33 and Nam Hom limes (Fig. 4.12 lanes 6-9) indicating that both alleles of *Pt14* can be digested with *Bfa*1. These results correspond with the resistance phenotype (hypersensitive respond) of M33 and Nam Hom limes after inoculation with *Xanthomonas*. Whereas, the results of *Pt14* gene from Pan lime followed by *Bfa*1 digestion showed 3 bands of 430 bp, 300 bp and 130 bp (Fig. 4.12 lane 5).

These results indicated that, only one allele the *Pt14* amplicon from Pan lime can be digested with *Bfa*1 and the other allele does not contain the *Bfa*1 restriction site. Since citrus plant are diploid (2n), these results can assumed that, R_{Pt14}

gene on the chromosomes of M33 and Nam Hom genomes should be two dominant *R* gene (RR_{Pt14}) that can be completely digested by *Bfa*1 enzyme. Whereas, R_{Pt14} gene on Pan lime chromosomes should be one dominant *R* gene (R_{Pt14}) and one resessive *r* gene (r_{pt14}) that cannot be cut with *Bfa*1 (Fig. 4.13).

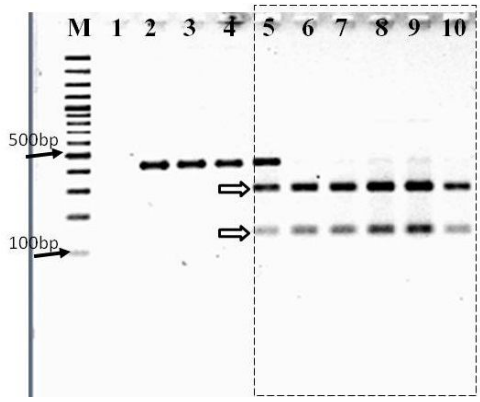

Figure 4.12 *Pt14/Bfa1* marker gene screened on M33 and their parents (Pan and Nam Hom); M: 100 bp marker; Lane 1: negative control; Lane 2-4: PCR product of *Pt14* from Pan, Nam Hom and M33, Lane 5-10: *Bfa*1 digestion of *Pt14* from Pan (5), Nam Hom (6), M33 from Phichit (7), M33 from Samut Sakhon (8), M33 from Prachinburi (9) and Puang resistant lime (10).

This RR_{Pt14} gene were also founded on other resistance lime (Puang) (Fig. 4.13 lane 10) which confirmed the result from M33 and Nam Hom that the *Pt14/Bfa1* genotype is linked to the *Xanthomonas* resistant phenotype in lime. The *Pt14* fragments from each plant were cloned and sequenced. The sequencing results confirmed the *Bfa*1 restriction sites on R_{Pt14} from Pan, Nam Hom and M33 genomes (Appendix B table 2).

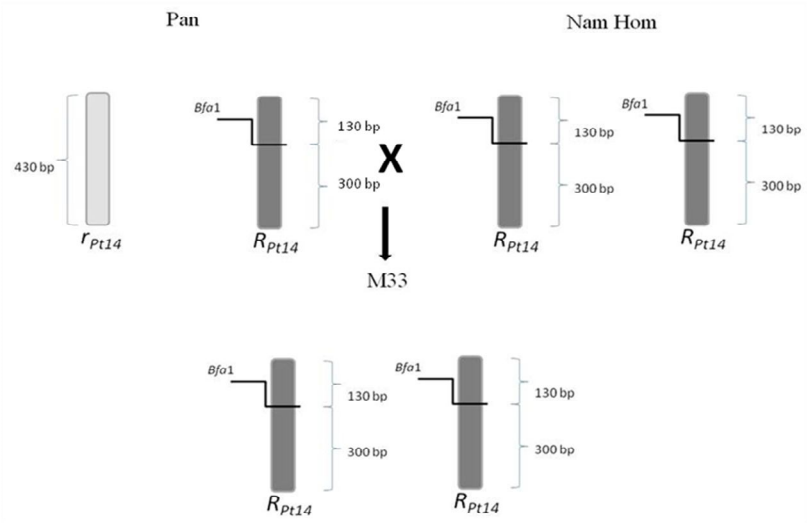

Figure 4.13 *Bfa*1 restriction enzyme map of R_{Pt14} and r_{Pt14} gene on Pan, Nam Hom and M33 amplicons. Rr_{Pt14} of Pan crossing with RR_{Pt14} of Nam Hom. resulted in RR_{Pt14} on M33 genome.

The sequence results also confirmed that, r_{Pt14} from Pan did not contain *Bfa*1 restriction site in the sequence. The *Bfa*1 digestion in the R_{Pt14} sequences were close to the fragment size seen on the gel. The *Pt14* sequences could be translated to polypeptide without any stop codons. The BLASTX program search indicated that, all *Pt14* clones showed strong overall similarities (>90% identity) to *C. grandis* x *P. trifoliate* Pt14 cloned by Deng et al. (2000).

Multiple alignment with the translated Pt14 and the most similar RGAs peptide sequences (*C. grandis* x *P. trifoliate* Pt14) were performed with the partial sequence of the resistance protein of *Arabidopsis* RPS2 and RPP5, tobacco N, and flax L6. The similarity was especially high at the two NBS motifs (kinase-2 and kinase-3a) class (Appendix B Fig. 2). Pt14 peptide sequences from this study indicate

that, the Pt14 protein formed a major cluster with *Arabidopsis* RPP5, N and L6. They all have an aspartic acid residue (D) at the final residue position of the kinase-2 motif that is often seen in the TIR group (Appendix B Fig. 2). Thus, Pt14 protein should belong to the TIR-LRR class. One different amino acid was found between R_{pt14} and an r_{Pt14} protein, which is asparagine (N) replaced the threonine (T) at the internal kinase-3a motif (Appendix B Fig. 2). This amino acid mutation might be responsible for the malfunction of r_{Pt14} on the Pan susceptible lime.

4.2.2.3 *16R1*/*Tru*1I marker gene discovery

16R1-19 NBS-LRR gene amplification in combination with *Tru*1I restriction digest showed 2 bands links with the canker resistance of M33 and Nam Hom limes (Fig. 4.14 lanes 6-9). The PCR products of *16R1-19* amplification were 450 bp in all lime cultivars (Fig. 4.14 lanes 2-4) whiles, M33 showed one smaller band about 420 bp. However, when the *16R1-19* amplicons were digested with *Tru*1I, 2 dominant bands of 2x200 and 50 bp were seen from DNA of Pan, M33 and Nam Hom limes (Fig. 4.14 lanes 5-9). Whereas, *16R1-19*/*Tru1*I from Pan lime showed a bigger band of 250 bp above the 2 dominant bands (200x2 and 50 bp).

These results indicating that, the *16R1-19* amplicon from Pan lime contains two different allele. One is similar to the amplicon of *16R1-19* from M33 and Nam Hom. The other allele of *16R1-19* amplicon from Pan lime can be digested with *Tru*1I that gave different band patterns on gel. These results can assumed that, $R_{16R1\text{-}19}$ gene on the chromosomes of M33 and Nam Hom genomes should be two dominant *R* gene ($RR_{16R1\text{-}19}$) that can be completely digested by *Tru*1I enzyme and generated specific bands of 2x200 and 50 bp on gel.

Whereas, $R_{16R1\text{-}19}$ gene on Pan lime chromosomes contain one dominant *R* gene ($R_{16R1\text{-}19}$) and one recessive *r* gene ($r_{16R1\text{-}19}$) that contains different restriction site of *Tru*1I (may be 250 and 200 bp) (Fig. 4.15).

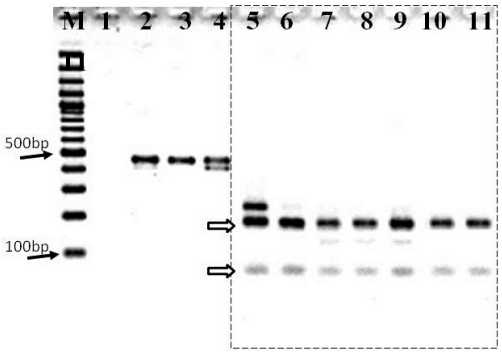

Figure 4.14 *16R1-19/Tru1I* marker gene screened on M33 and their parents (Pan and Nam Hom); M: 100 bp marker; Lane 1: negative control; Lane 2-4: PCR product of *16R1-19* from Pan, Nam Hom and M33, Lane 5-10: *Tru*1I digestion of *16R1-19* from Pan (5), Nam Hom (6), M33 from Phichit (7), M33 from Samut Sakhon (8), M33 from Prachinburi (9) Puang resistant lime (10) and Giant resistance lime (11).

This $RR_{16R1\text{-}19}$ gene were also founded on other resistance lime included Puang and Giant limes (Fig. 4.14 lanes 10 and 11) which confirmed the result from M33 and Nam Hom that the *16R1-19*/*Tru1*I genotype is linked to the *Xanthomonas* resistant phenotype in lime.

The amplicon of *16R1-19* from 3 lime cultivars were cloned and sequenced. The sequencing results confirmed the present of *Tru11* restriction sites on $R_{16R1\text{-}19}$ and $r_{16R1\text{-}19}$ from Pan, Nam Hom and M33 (Appendix B table 3).

*Tru*1I restriction site on $R_{16R1-19}$ and $r_{16R1-19}$ sequences were similar to the fragment size seen on the gel. The *16R1-19* sequences could be translated to polypeptide without any stop codons.

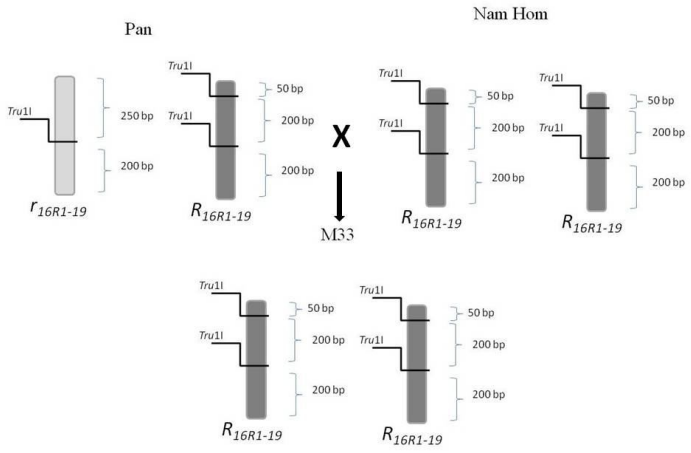

Figure 4.15 *Tru*1I restriction enzyme map of $R_{16R1-19}$ and $r_{16R1-19}$ gene on Pan, Nam Hom and M33 amplicons. $Rr_{16R1-19}$ of Pan crossing with $RR_{16R1-19}$ of Nam Hom, resulted in $RR_{16R1-19}$ on M33 genome.

The BLASTX search program of NCBI indicated that, all *16R1-19* peptides showed strong overall similarities (>90% identity) to *C. grandis* x *P. trifoliate* 16R1-19, 16R1-13 (Deng et al., 2000) and many RGA sequences recently cloned from *C. triloliata* included, RGA 12, RGA 20 and RGA 21 (Shen et al., 2004). Multiple sequences alignment with the translated 16R1-19 and the five most similar RGAs peptide sequences were performed with the resistance protein of *Arabidopsis* RPS2 and RPP5, tobacco N, and flax L6. The similarity was especially high at the two NBS motifs (kinase-2 and kinase-3a) (Appendix B Fig. 3). The 16R1-19 peptide sequences from this study indicate that, 16R1-19 fragments formed a

major cluster with RPS2 and they all have a tryptophan residue (W) at the final residue position of the kinase-2 motif that is often seen in the non-TIR group. Thus, 16R1-19 protein should belong to the non-TIR-LRR class (Appendix B Fig. 3). Two different amino acids sequence between $R_{16R1-19}$ and $r_{16R1-19}$ proteins were founded. A methionine (M) replaced a leucine (L) and a serine (S) replaced an arginine (R) (Appendix B Fig. 3). These amino acid mutations might be responsible for the malfunction of the $r_{16R1-19}$ on susceptible Pan lime.

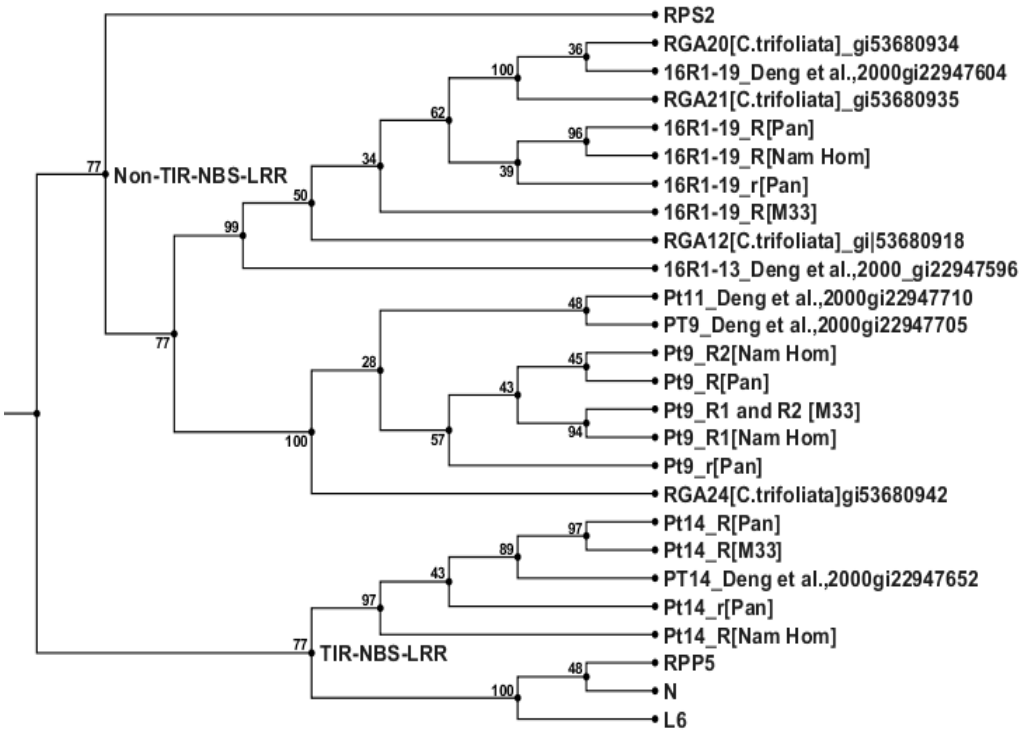

Figure 4.16 Phylogenetic analysis of Pt9, Pt14 and 16R1-19 peptide sequences from Pan Nam Hom and M33 limes, their most similar RGAs peptide sequences and the partial resistance protein of *Arabidopsis* RPS2, RPP5, tobacco N, and flax L6.

4.2.2.4 Phylogenetic tree analysis

All of the predicted Pt9, Pt14 and 16R1-19 peptides from this experiment, their most similar RGAs peptide sequences and the partial sequence of resistance protein of *Arabidopsis* RPS2 and RPP5, tobacco N, and flax L6 were used to create phylogenetic tree for sub-family classification. The results confirmed that, Pt9, 16R1-19 and their similar proteins were grouped with *Arabidopsis* RPS2 which classified to non-TIR-NBS-LRR class. While, Pt14 protein and their similar proteins were grouped within *Arabidopsis* RPP5, tobacco N, and flax L6 which classified to TIR-NBS-LRR class (Fig 4.16).

CHAPTER V

OVERALL CONCLUSSION

5.1 Antibody production for citrus canker detection

Nineteen canker bacteria isolated from three different provinces in Thailand; Samut Sakhon, Nakhon Ratchasima and Phichit. Some of the bacteria isolates identified as *X. axonopodis* pv. *citri* could infect both sensitive (Pan) and resistant (Namhom and M33) limes but with different symptom indicating that the resistant limes have some defense response mechanisms to prevent themselves against *Xanthomonas* infection. Polyclonal antibody specific to virulence *X. axonopodis* pv. *citri* (BP210) can detect live target bacteria at 10^6 CFU/ml and 10^5 CFU/ml for dead cells. Antibody dilution of 1:2,000 is the suitable concentration for *Xanthomonas* BP210 detection which cross–react with only *X. axonopodis* pv. *vesicatoria*. The sensitivity of this antibody was not sufficient for canker bacteria detection before the symptom can be observed but was able to detect on day 4 post infection which the symptoms can be observed.

5.2 Canker resistance gene analogs in Thai hybrid lime M33

The citrus canker resistance (R) marker genes within M33 and its parents were screened utilized the Nucleotide binding site (NBS) Leucine-rich repeat (LRR) genes

by PCR amplification in combination with restriction enzymes digestions. The resistant phenotype evaluation showed that, the marker *Pt9/Alu*1, *Pt14/Bfa*1 and *16R1-19*/*Tru*1I were closely linked to the citrus canker resistance genes in M33 and Nam Hom lime. These *R* genes were founded as two dominant genes on the resistance lime (RR_{Pt9}, RR_{Pt14} and $RR_{16R1\text{-}19}$). Whereas, only one single dominant R gene and one recessive *r* gene (Rr_{Pt9}, Rr_{Pt14} and $Rr_{16R1\text{-}19}$) were found in Pan limes which give sensitive phenotype. These RR_{Pt9}, RR_{Pt14} and $RR_{16R1\text{-}19}$ genotype was also observed in Puang lime which is another resistance lime. The predicted Pt9 and 16R1-19 protein can be classified in to the non-TIR-NBS-LRR subfamily. The Pt9 protein belongs to the family of resistance protein *Pt9* and *Pt11* from *C. grandis* x *P. trifoliate* and RGA 24 from *C. triloliata*. While, 16R1-19 protein belongs to the family of resistance protein RGC20 and RGC2 from *C. trifoliata* and 16R1-19 and 16R1-13 from *C. grandis* x *P. trifoliate.* Predicted Pt14 protein can be classified to the TIR-NBS-LRR subfamily and belongs to the resistance protein Pt14 family from *C. grandis* x *P. trifoliate*. The Pt9 and 16R1-19 and their similar proteins were clustered with the *Arabidodsis* RPS2 protein. While, Pt14 protein and the most similar RGAs peptide sequences (*C. grandis* x *P. trifoliate* pt14) belong to a major cluster of the resistance protein of *Arabidopsis* RPP5, tobacco N, and flax L6. Some amino acid mutation on recessive r protein might be involved in the malfunction of R protein in the susceptible limes.

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APPENDICES

APPENDIX A

Figure 1 Alignment 16S rDNA sequence from bacteria BP104 and BP210 isolates with *X. axonopodis* pv. *citri* strain 306 (GenBank: AE008923

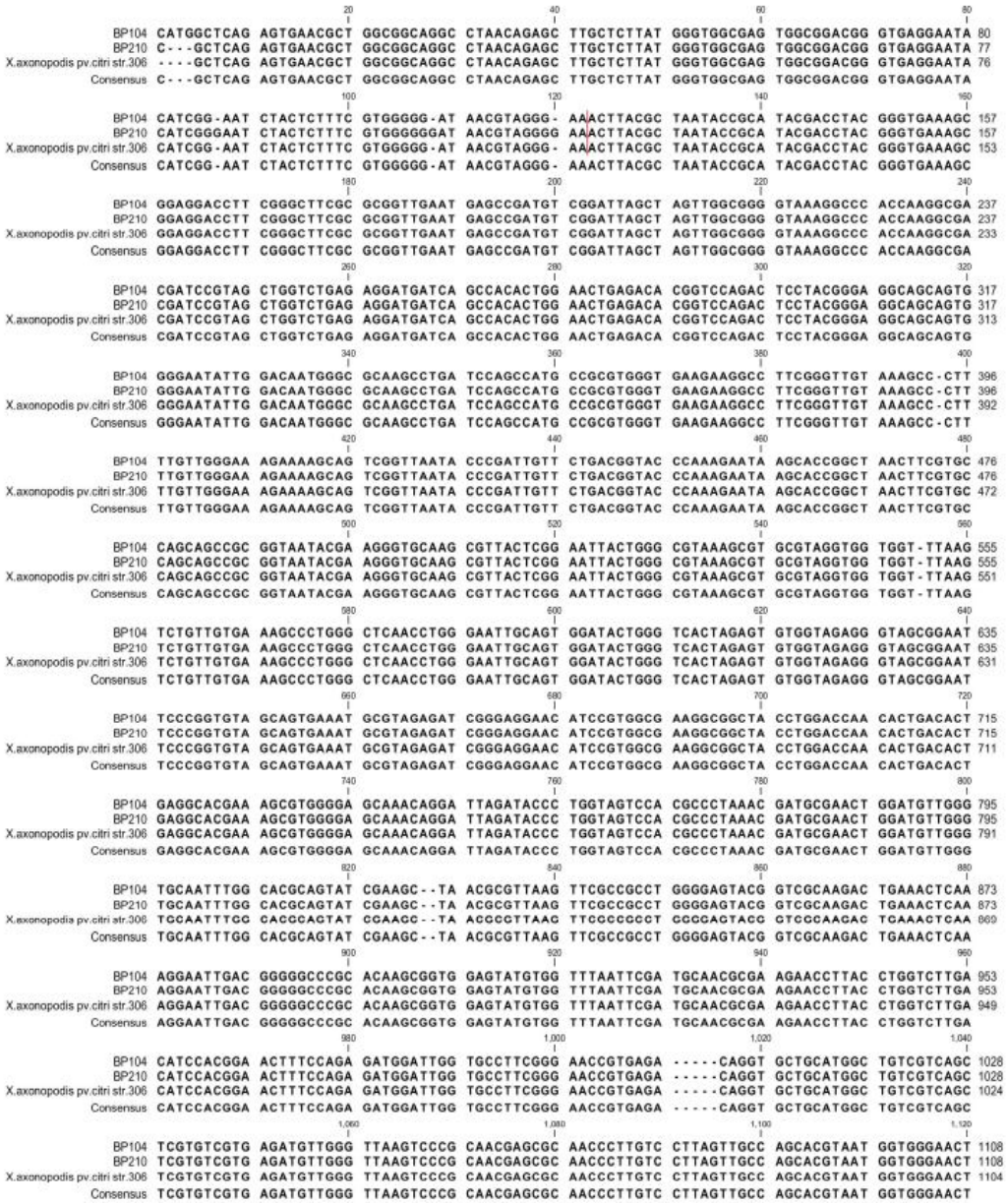

Figure 2 Blastn results of 16S rDNA from bacteria BP104 (A) and BP210 (B) isolate.

a

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AE008923.1	Xanthomonas axonopodis pv. citri str. 306, complete genome	2579	5158	98%	0.0	98%	
DQ991194.1	Xanthomonas axonopodis pv. citri strain XCW 16S ribosomal RNA gene,	2579	2579	98%	0.0	98%	
HQ264097.1	Uncultured Xanthomonas sp. clone SL37 16S ribosomal RNA gene, parti	2573	2573	98%	0.0	98%	

b

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AE008923.1	Xanthomonas axonopodis pv. citri str. 306, complete genome	2676	5353	98%	0.0	99%	
DQ991194.1	Xanthomonas axonopodis pv. citri strain XCW 16S ribosomal RNA gene,	2676	2676	98%	0.0	99%	
HQ264097.1	Uncultured Xanthomonas sp. clone SL37 16S ribosomal RNA gene, parti	2671	2671	98%	0.0	99%	

Figure 3 XAC specific PCR amplification from a tenfold dilution series of *X. axonopodis* pv. *citri* cultured cells. M: 100 bp marker; Lane 1: negative control; Lanes 2-7: 10^5 CFU, 10^4 CFU, 10^3 CFU, 10^2 CFU, 10^1 CFU and 1 CFU; lanes 8-9: <1 CFU respectively.

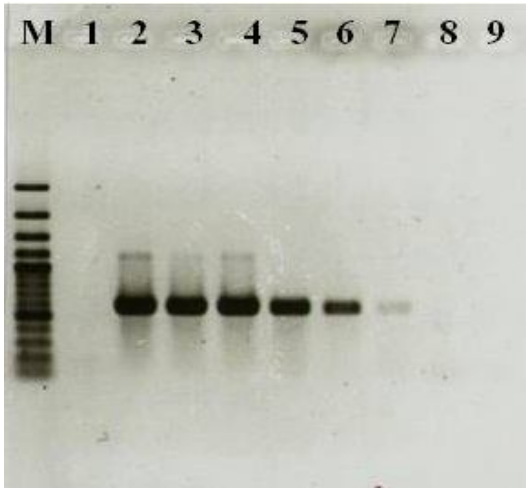

Table 1 Colony morphology of bacterial isolates from canker lesion on lime, present and absent of XAC specific PCR product and diseases reaction on Pan, Namhom and M33 limes after inoculation.

Bactertial isolates	Colony morphology [a]		XAC specific products (581 bp) Fig. 4.3[b]	Pan		Nam Hom		M33	
	NA medium	KCD medium		wound	Not wound	wound	Not wound	wound	Not wound
BP 102	Y, C	LY, C	+	+ [c]	- [d]	+	+	+	-
BP 104	Y, C	LY, C	+	+	+	+	-	+	-
BP 105	Y, C	LY, C	+	+	+	+	-	+	+
BP 107	Y, C	LY, C	+	+	-	-	-	+	-
BP 109	Y, C	LY, C	+	+	+	+	-	+	-
BP 201	Y, C	LY, C	+	+	-	+	-	+	-
BP 202	Y, C	LY, C	+	+	+	+	-	+	-
BP 203	Y, C	LY, C	+	+	-	+	-	+	-
BP 205	Y, C	LY, C	+	+	-	-	-	+	-
BP 210	Y, C	LY, C	+	+	+	+	-	+	-
SUT02	OY, C	OY, C	-	-	-	-	-	-	-
SUT06	OY, C	OY, C	-	-	-	-	-	-	-

Table 1 (Continued).

Bactertial isolates	Colony morphology [a]		XAC specific products (581 bp) Fig. 3[b]	Pan		Namhom		M33	
	NA medium	KCD medium		wound	Not wound	wound	Not wound	wound	Not wound
K01	OY, C	OY, C	-	-	-	-	-	-	-
K02	OY, C	OY, C	-	-	-	-	-	-	-
PJ01	Y, C	W	+	+	-	+	-	+	-
PJ02	OY, C	-	-	-	-	-	-	-	-
PJ03	Y, C	W	+	+	-	+	-	+	-
PJ04	OY, C	-	-	-	-	-	-	-	-
PJ05	OY, C	-	-	-	-	-	-	-	-

[a]: Colony morphologies were indicated as Yellow (Y), Ligt-yellow (LY), Orange yellow (OY), White (W) in color and Circular (C) in shape; [b]: The results of XAC specific amplification were taken as positive (+) with 581bp detected and negative (-) with non detected or non-target detected; The present of symptoms after inoculation was taken as positive ([c]+), whereas, symptomless was be negative ([d]-); The name of the bacterial isolate are from the acronym of the location isolated; Banpaew, Samut Sakhon (BP), Phichit (PJ), Suranaree University of Technology, Nakhon Ratchasima (SUT), Kokkruad, Nakhon Ratchasima (K)

Table 2 ELISA for pAb1 and pAb2 titer test. Different antiserum dilutions (1:16,000, 1:8,000, 1:4,000, 1:2,000 and 1:1,000) were used to detect live and dead cells of XAC suspension at different concentrations (10^4, 10^5 and 10^6CFU/ml).

Antiserum dilution	Cell status	Density (CFU/ml)	pAb1		pAb2	
			A_{405}	Reaction	A_{405}	Reaction
1:1,000	Live	10^6	0.249	+[a]	0.199	+
		10^5	0.198	+	0.183	+
		10^4	0.098	-[c]	0.121	-
	Dead	10^6	0.743	+	0.689	+
		10^5	0.236	+	0.220	+
		10^4	0.123	-	0.137	-
1:2,000	Live	10^6	0.198	+	0.180	+
		10^5	0.150	-	0.123	-
		10^4	0.101	-	0.110	-
	Dead	10^6	0.748	+	0.644	+
		10^5	0.205	+	0.174	+
		10^4	0.110	-	0.115	-
1:4,000	Live	10^6	0.158	+	0.120	-
		10^5	0.121	-	0.112	-
		10^4	0.094	-	0.110	-
	Dead	10^6	0.674	+	0.605	+
		10^5	0.191	+	0.156	-
		10^4	0.110	-	0.105	-

Table 2 (Continued)

Antiserum dilution	Cell status	Density (CFU/ml)	pAb1		pAb2	
			A_{405}	Reaction	A_{405}	Reaction
1:8,000	Live	10^6	0.123	-	0.103	-
		10^5	0.110	-	0.102	-
		10^4	0.090	-	0.102	-
	Dead	10^6	0.525	+	0.511	+
		10^5	0.173	-	0.143	-
		10^4	0.100	-	0.103	-
1:16,000	Live	10^6	0.103	-	0.094	-
		10^5	0.095	-	0.094	-
		10^4	0.090	-	0.090	-
	Dead	10^6	0.434	+	0.429	+
		10^5	0.156	-	0.133	-
		10^4	0.105	-	0.093	-
	Control		0.085[b]			

[a]: The positive reaction (+) was indicated when A_{405} was twice greater than that of the negative control ([b]).

[c]: The negative reaction (-) was indicated when A_{405} was lower than twice of the negative control ([b]).

Table 3 Sensitivity of ELISA in detecting live and dead cells of *X. axonopodis* pv. *citri.*

Antiserum	Cell status	*X. axonopodis* pv. *citri* antigen (CFU/ml)						
		10^8	10^7	10^6	10^5	10^4	10^3	*E. coli* [b]
pAb1 (1:2,000)	Live	1.300 [a]	0.692	0.231	0.154	0.102	0.100	0.090
	Dead	1.990	1.630	0.739	0.212	0.123	0.102	0.090
pAb2 (1:2,000)	Live	0.543	0.472	0.183	0.120	0.101	0.098	0.090
	Dead	0.803	0.763	0.633	0.180	0.100	0.096	0.090

[a]: The results was indicated as A_{405} by the ELISA reader.

[b]: *E. coli* at 10^6 CFU/ml was used as negative control.

APPENDIX B

Table 1 Difference restriction enzymes digestion of 12 *R* gene analogs from Pan, Nam Hom and M33 limes.

RGCs	Amplicon	Digestion					
		***EcoR*I**	***Tru*1I**	***Bfa*I**	***Hinf*I**	***Alu*I**	***Taq*I**
Pt3	√ [a]	X [b]	√	X	X	√	√
Pt6	√	X	√	X	X	X	X
Pt7	X	- [d]	-	-	-	-	-
Pt8	√	X	X	X	√	X	√
Pt9	√	X	√	*** [c]	√	***	X
Pt14	√	X	X	***	√	√	√
Pt18	√	X	X	X	X	X	√
Pt19	√	X	√	√	√	√	√
11P31	√	X	√	X	X	√	X
11P33	X	-	-	-	-	-	-
18P33	√	X	√	√	√	√	√
16R1-19	√	X	***	√	√	X	X

[a] √: able to amplify and digest but similar results in all limes.

[b] X: unable to amplify or digest.

[c]***: was able to distringuish between susceptible and resistant limes.

[d]-: was notperformed.

Table 2 Restriction sites of dominant $R_{pt9,}$ R_{pt14} and recessive $r_{pt9,}$ r_{pt14} clones from Pan, Nam Hom and M33 DNA used *Alu*1I and *Bfa*1enzymes.

***RGAs* name**	***R* clones**	**Sequences length (bp)**	**Digestion enzymes**	**Digested positions**	**Fragment size (bp) [a]**	**Observed band**
Pt9	Pan-*r*	446	*Alu*1I (Fig. 4.10)	439, 316, 66, 3	250, 123, 63, 7, 3	– 313 – 123,126
	Pan-*R*	446		439, 316, 3	313,126, 7, 3	
	Nam Hom-*R*1	443		436, 316, 3	313,120, 7, 3	– 313x2 – 120,123
	Nam Hom-*R*2	446		439, 316, 3	313,123, 7, 3	
	M33-*R*1 and –*R*2	443		436, 316, 3	313,120, 7, 3	– 313 – 120
Pt14	Pan-*r*	426	*Bfa*1 (Fig. 4.12)	None [c]	426	– 426 – 288 – 138
	Pan-*R*	426		288	288, 138	
	Nam Hom- *R*1	425		288	288, 137	– 288x2 – 138, 137
	Nam Hom- *R*2	426		288	288, 138	
	M33-*R*1 and –*R*2	426		288	288, 138	– 288 – 138

[a]: the underline number is fragment size of observed band.

Table 3 Restriction sites of dominant $R_{16R1\text{-}19}$ and recessive $r_{16R1\text{-}19}$ clones from Pan, Nam Hom and M33 DNA used *Tru*11 enzymes.

***RGAs* name**	***R* clones**	**Sequences length (bp)**	**Digestion enzymes**	**Digested positions**	**Fragment size (bp) [a]**	**Observed band**
16R1-19	Pan-*r*	455	*Tru*11 (Fig. 4.14)	401, 223	223, 178, 54	– 223 – 178x2, 163 – 57, 54x2
	Pan-*R*	452		163, 220, 398	178, 163,54, 57	
	Nam Hom-*R*1 and -*R*2	455		166, 220, 401	178, 166,54, 57	– 178, 166 – 57, 54
	M33-*R*1 and –*R*2	452		163, 220, 398	178, 163, 54, 57	– 178, 163 – 54, 57

[a]: the underline number is fragment size of observed band.

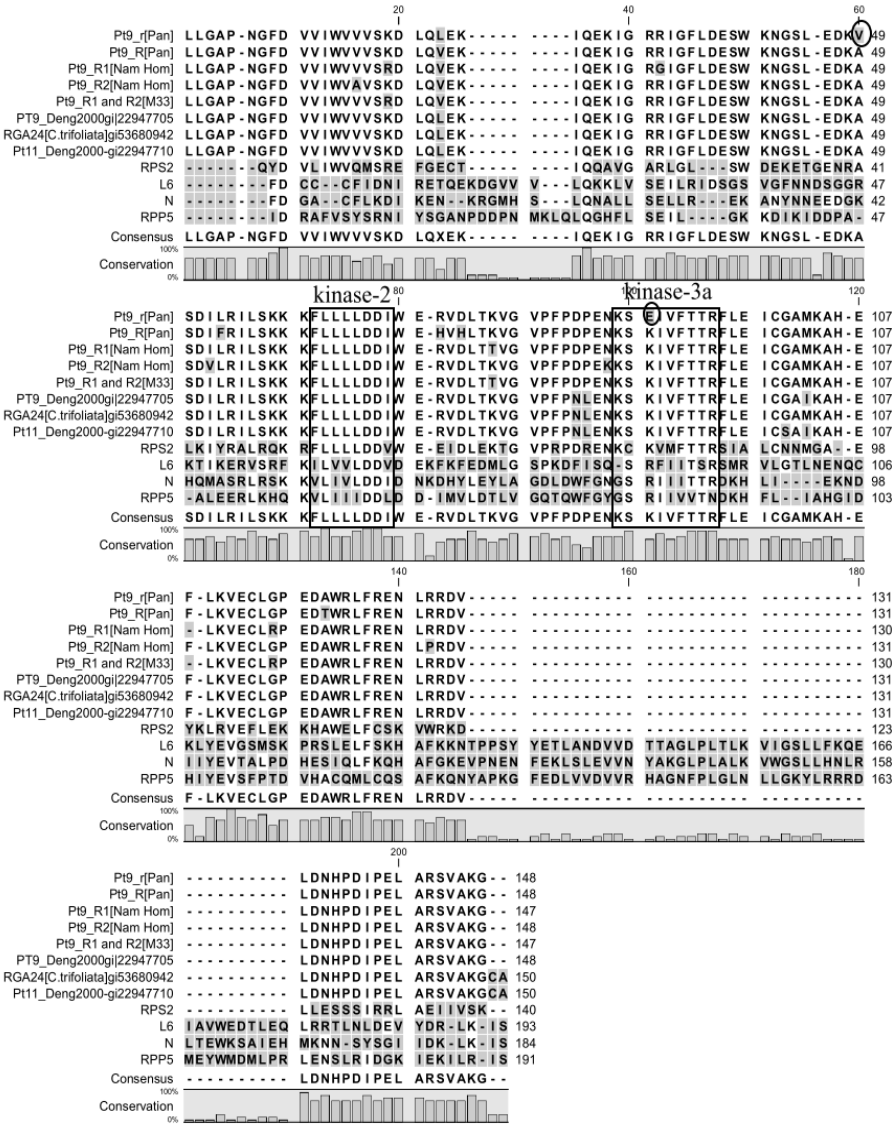

Figure 1 Alignment of predicted Pt9 amino acid sequences from Pan, Nam Hom and M33 limes compared with the NBS domains of other R proteins: *C. grandis* x *P. trifoliate* Pt9, Pt11 (Deng et al., 2000), *C. triloliata* RGA 24 (Shen et al., 2004), RPP5 (Noel et al., 1999), tobacco N (Witham et al. 1994), *Arabidopsis* RPS2 (Bent et al. 1994; Mindrinos et al. 1994) and flax L6 (Lawrence et al. 1995). Consensus kinase-2 and kinase-3a motifs are square. The amino acid mutations in Pt9-r Pan are circled.

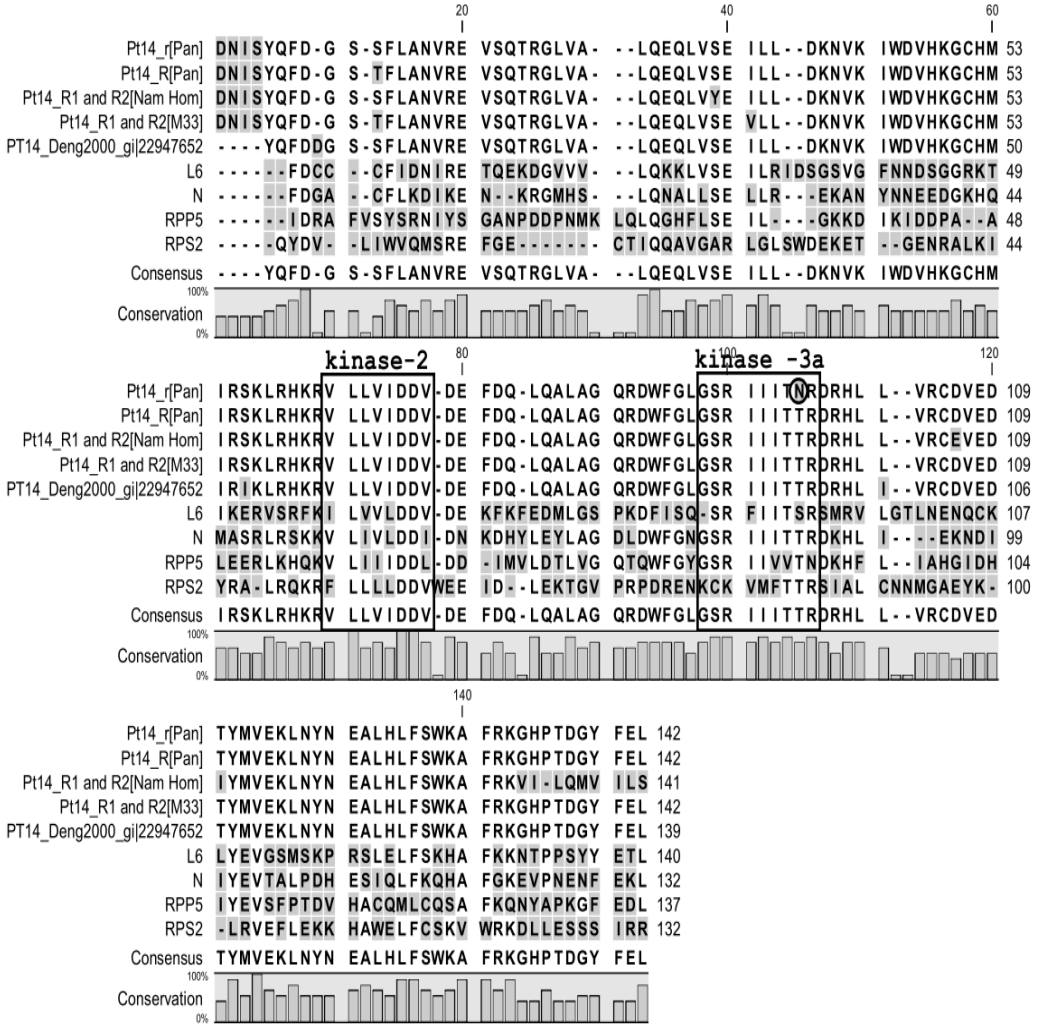

Figure 2 Alignment of predicted Pt14 amino acid sequences from Pan, Nam Hom and M33 limes compared with the NBS domains of other R proteins: *C. grandis* x *P. trifoliate* Pt14 (Deng et al., 2000), RPP5, tobacco N, *Arabidopsis* RPS2 and flax L6. Consensus kinase-2 and kinase-3a motifs are square. The mutated amino acid is circled.

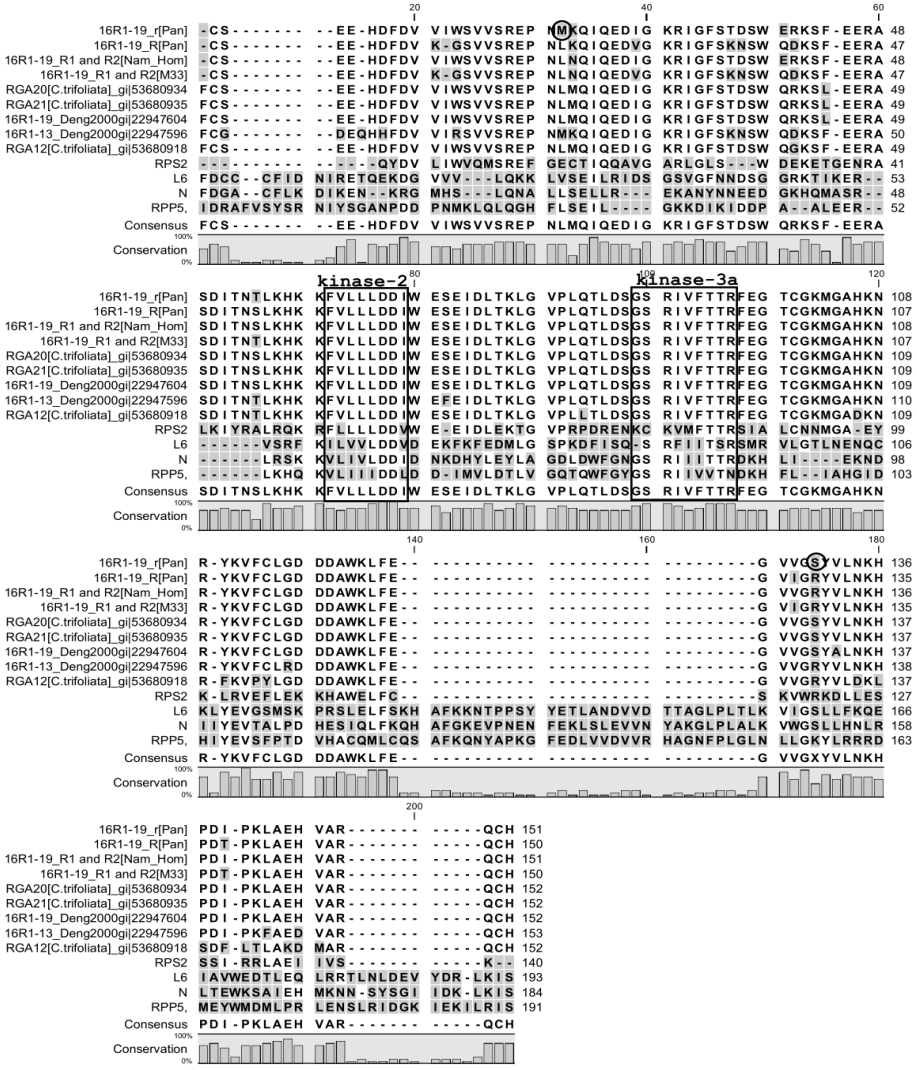

Figure 3 Alignment of predicted 16R1-19 amino acid sequences from Pan, Nam Hom and M33 limes compared with the NBS domains of other R proteins: *C. grandis* x *P. trifoliate* 16R1-19, 16R1-13 (Deng et al., 2000), *C. triloliata* RGA 12 RGA 20, RGA21(Shen et al., 2004), *Arabidopsis* RPS2 and RPP5, tobacco N, and flax L6. Consensus kinase-2 and kinase-3a motifs are square. Hypothesis amino acid mutation is circled.

BIOGRAPHY

Ms. Pongpan Songwattana was born on August 15th, 1985 in Samut Sakhon , Thailand. She graduated with a bachelor degree of Crop production of technology from Suranaree University of Technology in Year 2007. She applied to study Master degree course in school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology whit Asst. Prof. Dr. Mariena Ketudat-Cairns. While studying, she received a Graduate scholarship from SUT to support her tuition and fee. Her research topic was production of polyclonal antibody for citrus canker detection and identification of resistance gene analogs in Thai hybrid lime. The results from some part of this study have been presented as poster and oral presentation at (1) The 15th National Graduated Research Conference, Rajaphat Nakhon Ratchasima 14-15th Dec 2009. (2) 8th International Symposium on Biocontrol and Biotechnology, King Mongkut's Institute of Technology Ladkrabang (KMITL) and Khon Kaen University, Nongkhai Campus, 4-6th October 2010. (3) The 3rd SUT Graduate Academic Seminar 2010 Suranaree University of Technology, Nakhon Ratchsima, 21-23th November 2010.