A CORRELATION BETWEEN DEFENCE-RELATED GENE EXPRESSION AND *XA21*-MEDIATED RESISTANCE AGAINST *XANTHOMONAS ORYZAE* PV. *ORYZAE* IN RICE RD47 x IRBB21 PROGENIES

PLOENPHIT PROMMA

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science Degree in Agricultural Biotechnology
May 2017

Thesis entitled "A correlation between defense-related gene expression and *Xa21*-mediated resistance against *Xanthomonas oryzae* pv. *oryzae* in rice RD47 x IRBB21 progenies "

by Ploenphit Promma

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science Degree in Agricultural Biotechnology of Naresuan University

Oral Defense Committee

Pongsopee Attasart ..... Chair

(Pongsopee Attasart, Ph.D.)

Kumrop Ratanasut ..... Advisor

(Assistant Professor Kumrop Ratanasut, Ph.D.)

C. Choopayak ..... Co – Advisor

(Assistant Professor Chonnanit Choopayak, Ph.D.)

Kawee Sujipuli ..... Internal Examiner

(Assistant Professor Kawee Sujipuli, Ph.D.)

Approved

.....

(Panu Putthawong, Ph.D.)

Associate Dean for Administration and Planning

for Dean of the Graduate School

3 MAY 2017

ACKNOWLEDGEMENT

First and foremost, I would like to express my greatest thank you to my advisor, Assistant Professor Dr. Kumrop Ratanasut, and co-advisor, Assistant Professor Dr. Chonnanit Choopayak for their guidance, valuable advice, and insight throughout the length of the study.

I would like to give my appreciation to Dr. Pongsopee Attasart and Assistant Professor Kawee Sujipuli for all their comments on the thesis. I also would like to give my sincere thank you to Phitsanulok Rice Research Center for kindly providing rice cultivar RD47 and IRBB21. I want to especially thank my family for their endless love, understanding and support. I thank my friends for their friendship and encouragement throughout the period of the study. I would like to acknowledge the Graduate School, Naresuan University for providing the necessary financial support for this research.

Finally, I would like to express my gratitude to all others for all support enabling me to complete this thesis but who are not named in this acknowledgement.

Ploenphit Promma

Title	A CORRELATION BETWEEN DEFENCE-RELATED GENE EXPRESSION AND *XA21*-MEDIATED RESISTANCE AGAINST *XANTHOMONAS ORYZAE* PV. *ORYZAE* IN RICE RD47 x IRBB21 PROGENIES
Author	Ploenphit Promma
Advisor	Assistant Professor Kumrop Ratanasut, Ph.D.
Co - Advisor	Assistant Professor Chonnanit Choopayak, Ph.D.
Academic Paper	Thesis M.S. in Agricultural Biotechnology, Naresuan University, 2016
Keywords	*Xa21*, *Xanthomonas oryzae* pv. *oryzae*, *Oryza sativa*, *Phenylalanine ammonia-lyase*

ABSTRACT

Bacterial Blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a serious disease of rice (*Oryza sativa* L.) in Thailand. The *Xa21* gene shows broad-spectrum resistance and has been widely used to improve BB disease resistance in rice worldwide. In this research, we demonstrated here the expression of *Xa21* and defense-related genes, *PR1b*, *PR10a* and *phenylalanine ammonia-lyase* (*PAL*), in rice cultivars RD47 and IRBB21 and their F_1 hybrid, F_2 and BC_2F_2 progenies. The results showed that *Xa21* expression was correlated with the homozygous and heterozygous *Xa21* genotypes identified by the pTA248 marker, and induced by wounding and *Xoo* infection. However, the expression of *Xa21* transcripts was not correlated with expression of *Xa21*-mediated disease resistance. The relative expression levels of *Xa21* transcripts responding to wounding were greater than *Xoo*-inoculation. The transcripts of *PR1b* and *PR10a* was expressed in both RD47 (no *Xa21*) and F_1 hybrid (with *Xa21*) which were wounded and infected with *Xoo* whereas the *PAL* expression was triggered either by wounding or *Xoo* infection only in rice carrying the *Xa21* gene and the relative expression levels of *PAL* transcripts responding to wounding were also greater than *Xoo*-inoculation. No *PAL* expression was detected in heterozygous-*Xa21* BC_2F_2 plants treated with either wounding or *Xoo*-inoculation. It is possible that the *PAL* expression is *Xa21*-dependent and required IRBB21 genetic background.

The evaluation of BB resistance in 6-leaf stage rice including RD47, IRBB21 and BC_2F_2 progenies at 21 days after inoculation with *Xoo* isolated Phisanulok by clipping method. The lesion lengths were measured in rice RD47 containing null-*Xa21* was moderately susceptible (MS) whereas IRBB21 carrying homozygous *Xa21* showed resistance (R). BC_2F_2 carrying null-*Xa21* showed MS phenotype as RD47 whereas BC_2F_2 carrying either homozygous or heterozygous *Xa21* showed R phenotype. Leaves of all tested plants clipped without *Xoo* showed similar lesions limited only at the clipped regions. These results indicated that only one copy of the *Xa21* gene (in heterozygous genotype) was effective to acquire resistance to *Xoo*. The resistance levels of BC_2F_2 carrying homozygous and heterozygous *Xa21* were not significantly different from IRBB21. Our results suggest that *Xa21*-mediated BB resistance in BC_2F_2 progenies of RD47 x IRBB21 may be acquired via the defense pathway different from IRBB21.

LIST OF CONTENT

LIST OF CONTENT (CONT.)

LIST OF CONTENT (CONT.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONT.)

CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple crop of the world. Most people depend on rice for food on a daily basis. Breeding technology has been a continuing effort to improve the potential yield and eating quality of rice. The transition from conventional way of breeding rice to the rise of modern day molecular genetics was the key player in the development of new and improved cultivars of rice. A non-glutinous and photoperiod insensitive rice cultivar "RD47" is one of the popular cultivars grown in lower Northern Thailand because of its high yielding and good seed quality. RD47 is resistant to both brown planthopper (BPH) and blast disease but is susceptible to bacterial blight disease (Bureau of Rice Research and Development, Thailand). Thus, bacterial blight poses a potential threat to the total production scale of a certain area.

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. oryzae (*Xoo*), is considered as one of the most disastrous rice diseases in Thailand. Yield loss due to BB can reach as much as 70% in infected growing areas (International Rice Research Institute, IRRI). It was first discovered in Japan in 1884, and have overspread in rainfed lowland ecosystems throughout Asia, Northern Australia, Mainland Africa, Southern United States and Latin America (Mew, 1987). In Thailand, BB was first discovered in 1963 (Eamchit, 1982). Breeding for BB resistant varieties is the most economic and the most effective way to control the disease. The identification of BB resistance genes has been the subject of many researches in rice genetics in Asia. To date, more than 20 BB resistance genes are already known. One of them is the *Xa21,* a dominant BB resistance gene originated from a wild rice *O. longistaminata* and was proven to be highly effective against BB races of South and Southeast Asia (Khush, et al., 1990).

Xa21 is the most widely used in rice breeding program because of its broad spectrum resistance to most strains of *Xoo* (Ikeda, et al., 1990; Khush, et al., 1989, 1990). *Xa21* was introgressed to *O. sativa* IR24 through backcross breeding. The near isogenic line IRBB21 containing *Xa21* with the background of IR24 exhibited

resistance to all physiological strains from the Philippines and India (Ikeda, et al., 1990). However, successful defense mechanism against BB infection of rice depends on the rapid recognition of pathogenic attack by resistant gene products and the rapid induction of appropriate plant defense reaction (Jones, et al., 2004).

Induced resistance is a systemic response of plant defense and can be either systemic acquired resistance (SAR) or induced systemic resistance (ISR). Induction of systemic resistance can lead to the direct activation of defense related genes, such as *pathogenesis-related* (*PR*) and *phenylalanine ammonia-lyase* (*PAL*) genes. SAR develops systemically in response to pathogen infection via a salicylic acid (SA)-dependent pathway (Walters, et al., 2005). Expression of PR proteins is generally pathogen- and host-specific pointing to the need to examine the relationship in each host-pathogen interaction (Muthukrishnan, et al., 2001; van Loon, et al., 2006).

PR1a, *PR1b* and *PR10* expression in rice has been examined in relationship to the specific response to jasmonic acid (JA). Results on the study of Ponciano, et al. (2007) revealed that *PR10* expression is induced by SA, H_2O_2, UV and $CaCl_2$, suggesting that *PR10* responds to both biotic and abiotic stresses. It has also been shown that *Xoo* infection induced the transient expression of *PR1a*, *PR1b* and *PR10* in the incompatible interaction compared to the compatible interaction (Schweizer, 1997; Jwa, et al., 2001).

PAL is a key enzyme in the phenylpropanoid pathway of higher plants producing soluble phenolics, flavonoids, phytoalexins and lignin contributing to disease resistance (Bowles, 1990; Bate, et al., 1994; Pellegrini, 1994; Camera, et al., 2004; Reichert, et al., 2009; Vogt, 2010; Gao, et al., 2012; Rawal, et al., 2013;). PAL is also a key enzyme for biosynthesis of SA, a plant hormone required to initiate SAR in plants (Lee, et al., 1995; Mauch-Mani and Slusarenko, 1996; Coquoz, et al., 1998; Achnine, et al., 2004; Beckers and Spoel, 2006; Gruner, et al., 2013; Roycewicz and Malamy, 2014).

The continuing studies on the expression of *PR* and *PAL* genes and their known activities in *Xa21*-mediated immunity poses a great impact on the development of new cultivars resistant to BB and the improvement of breeding technologies for rice. Thus in this study, we will report the expression of *Xa21* and defense-related gene in hybrid rice (RD47-IRBB21) subject to *Xanthomonas oryzae* pv. oryzae.

Objectives of the study

1. To characterize the expression of *Xa21* and defense-related genes in rice RD47 and IRBB21 used as female and male parental lines, respectively, in backcross breeding program for production of BB-resistance RD47, and their F_1 hybrid, F_2 and BC_2F_2 progenies carrying heterozygous *Xa21* and null *Xa21* genotypes.

2. To study the interaction of defense-related genes and the *Xa21* gene in response to *Xoo* developmental disease resistance in hybrid rice RD47-IRBB21 under wounding and *Xoo* infection conditions.

Hypothesis of the study

The *Xa21* gene could activate the defense mechanism and induce resistance to *Xoo* causing BB disease in rice RD47 background.

Expected outputs of the study

When this study completes, we will perceive the role of *Xa21* in activation of defense mechanism and induction of resistance to BB disease in rice RD47 background. This could be used to predict the success of rice RD47 improvement for BB resistance by a backcrossing method.

CHAPTER II

LITERATURE REVIEW

Economic importance of rice

Rice (*Oryza sativa* L.) have been cultivated since l0,000 years ago in the river valleys of South, Southeast Asia and China, Since then it was served as one of the most important staple foods in the world, feeding more than 60% of the population and contributing nearly 40% of total calorie intake (Cheng, et al., 2006). Asia is the major place of rice cultivation including China, India, Japan, Bangladesh, Indonesia, Burma and Thailand.

Although rice is the world's second largest cereal crop after wheat, it generates largest amount of crop residues. In 2015 annual world rice production was 478.14 million tons (World Rice Production 2015/2016) while rice production in Thailand was 34.3 million tons. Thailand produces rice not only for serving Thai population but also for exporting to many countries around the world. Table 1 shows export values of Thai rice in the last 10 years (2006-2015).

Table 1 Export values of Thai rice during 2006-2015

Year	Quantity (million tons)	Value (million baht)
2006	7.49	98.17
2007	9.19	119.21
2008	10.21	203.21
2009	8.61	172.20
2010	8.93	168.19
2011	10.71	193.84
2012	6.73	142.97
2013	6.61	133.83
2014	10.96	174.85
2015	9.79	155.91

Source: Office of Agricultural Economics Thailand, 2015

Rice classification and improvement trend

Rice belongs to the *Poaceae* or *Gramineae* family and the *Oryza* genus. There are 25 recognized rice species of which twenty three are wild species and two, *O. sativa* and *O. glaberrima*, are cultivated (Vaughan, 1994). The basic chromosome number of the genus *Oryza* is 12. *O. sativa*, *O. glaberrima* and fourteen wild species are diploids with 24 chromosomes, and eight wild species are tetraploids with 48 chromosomes. Rice genomes have been grouped into nine distinct genomes, e.g. genomes A, B, C, D, E, F, G, H and J (Table 2). There are more than 40,000 different varieties of *Oryza sativa*, classified into four major categories: indica, japonica, aromatic and glutinous. (Liu, et al., 1994). The japonica rice ecotype is mostly cultivated in temperate regions with high latitudes whereas the indica rice ecotype is mainly found in tropical and subtropical regions, with either low latitudes or altitudes (Vaughan, et al., 2008). The traditional method for identifying indica and japonica rice varieties relies essentially on variation in morphological characters including plant height, plant type, status of pubescence and type of grains, and in combination with some physiological and biochemical features such as winter hardiness and phenol response in rice grains (Wang, et al., 1997; Oka,et al., 1962).

During the 1960s and 1970s, the green revolution was the notable increase in cereal-grain production in Mexico, India, Pakistan, the Philippines, and other developing countries. This trend resulted from the introduction of hybrid strains of cereals including rice and the adoption of modern agricultural technologies, including irrigation and heavy doses of chemical fertilizer (mainly nitrogen) (Dawe, et al., 1998). However, biotic and abiotic stresses cause major yield losses in rice crop production and improvement in stress tolerance is a major breeding goal. Over seventy diseases of rice are caused by different pathogens including fungi, bacteria, viruses or nematodes (Zhang, et al., 2009). The major diseases in rice include sheath blight, bacterial blight, rice blast, yellow mottle virus, sheath rot, bakanae, brown spot, narrow brown spot, bacterial leaf streak and grassy stunt. Disease damage to rice can greatly reduce yield. Planting a resistant variety is the simplest and, often, the most cost effective for disease management.

The technological innovations and development of DNA based molecular markers have facilitated the transfer of genes that confer resistance to different biotic

and abiotic stresses. Marker-assisted selection (MAS) offers a simpler and more efficient and accurate way to improve rice cultivars. It is helpful for breeding disease resistance compared with selection-only based on phenotype screening (Zhang, et al., 2006; Gopalakrishnan, et al., 2008). MAS is extensively used in China to pyramid functional genes into cultivars of popular hybrid rice varieties for improving important agronomic traits such as disease resistance and grain quality (Zhou, et al., 2003).

Bacterial blight (BB) disease and BB resistance genes

The BB disease is caused by a gram-negative, rod-shaped bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Shen and Ronald, 2002), which enters either through wounds or hydathodes, multiplies in the epitheme and moves to the xylem vessels where active multiplication results in blight disease symptoms on rice leaves (Figure 1 and 2). The symptoms are observed at the tillering stage and disease incidence increases with plant growth of which disease severity peaks at the flowering stage (Mew, et al., 1979). BB was first discovered in Japan in 1884 and has been overspread since then in rainfed lowland ecosystems throughout Asia, northern Australia, mainland Africa, the southern United States and Latin America (Mew, 1987).

BB is one of the most disastrous rice diseases in Asia and Africa, implying huge economic consequences. BB disease occurrence and distribution in Asia are mostly in the rainy season and in typhoon path countries including China, Korea, Japan and the Philippines. As the monsoon season causes frequent rainstorms in Indonesia, Malaysia and Thailand, BB occurs more often. In Thailand, BB was first discovered in 1963 (Eamchit, 1982). Yield loss due to BB can be as much as 70% in susceptible varieties grown in environment favorable to the disease (Cruz, n.d.). The disease produces three types of symptoms: leaf blight, kresek and pale-yellow leaf (Ou, 1985). Especially for *Xoo*, rice plants were more susceptible at the seedling stage than at the adult stage, whereas the old senescent leaves were more resistant than younger leaves (Kaul and Shama, 1987). The disease is most likely to develop in areas that have weeds and stubbles of infected plants. It can occur in both tropical and temperate environments, the disease favors temperatures at 25-34°C, with relative humidity above 70%.

Table 2 Complexities of *Oryza* species and their geographical distribution

	Species Complex	Chromosome Number	Genome	Geographical Distribution
I.	**Sativa complex**			
	1. *O. sativa* L.	24	AA	Worldwide: originally South & Southeast Asia
	2. *O. nivara* Sharma et Shastry	24	AA	South & Southeast Asia
	3. *O. rufipogon* Griff.	24	AA	South & Southeast Asia, South China
	4. *O. meridionalis* Ng	24	AA	Tropical Australia
	5. *O. glumaepetula* Steud	24	AA	Tropical Australia
	6. *O. glaberrima* Steud	24	AA	Tropical West Africa
	7. *O. barthii* A. Chev. et Roehr	24	AA	West Africa
	8. *O. longistaminata* A. Chev. et Roehr.	24	AA	Tropical Africa
II.	**Officinalis Complex/ Latifolia complex**			
	9. *O. punctata* Kotschy ex Steud.	24	BB	East Africa
	10. *O. rhizomatis* Vaughan	24	CC	Sri Lanka
	11. *O. minuta* J.S.Pesl. ex C.B. Presl.	48	BBCC	Philippines, New Guinea
	12. *O. malamphuzaensis* Krishn. et Chandr.	48	BBCC	Kerala & Tamil Nadu
	13. *O. officinalis* Wall. ex Watt	24	CC	South & Southeast Asia
	14. *O. eichingeri* A. Peter	24	CC	East Africa & Sri Lanka
	15. *O. latifolia* Desv.	48	CCDD	Central & South America
	16. *O. alta* Swallen	48	CCDD	Central & South America
	17. *O. grandiglumis* (Doell) Prod.	48	CCDD	South America
	18. *O. australiensis* Domin.	24	EE	Northern Australia
	19. *O. schweinfurthiana* Prod	48	BBCC	Tropical Africa
III.	**Meyeriana Complex**			
	20. *O. granulata* Nees et Arn. ex Watt	24	GG	South & Southeast Asia
	21. *O. meyeriana* (Zoll. et Mor. ex Steud.) Baill.	24	GG	Southeast Asia
IV	**Ridleyi Complex**			
	22. *O. longiglumis* Jansen	48	HHJJ	Indonesia, New Guinea
	23. *O. ridleyi Hook. f*	48	HHJJ	Southeast Asia
V	**Unclassified (belonging to no complex)**			
	24. *O. brachyantha A. Chev. et Roehr*	24	FF	West & Central Africa
	25. *O. schlechteri* Pilger	48	HHKK	Indonesia, New Guinea

Source: Brar and Khush, 2003

BB can be severe in susceptible rice varieties under high nitrogen (N) fertilization. Breeding for BB resistant varieties is the most economic and effective way to control the disease. At least 38 major BB resistance genes, both dominant and recessive genes named *Xa1* to *Xa38*, have been mapped in rice, and six of them including *Xa1*, *xa5*, *xa13*, *Xa21*, *Xa26* and *Xa27*, have been cloned and characterized (Sattari, et al., 2014). The characterized *Xa* genes have been useful for rice breeding to gain BB resistance in susceptible elite rice varieties. However, different *Xa* genes give different effectiveness and levels of BB resistance against different *Xoo* strains or even isolates. Three of the cloned *Xa* genes including *xa5*, *xa13*, *Xa21* have been used for improving BB disease resistance in Thai cultivated rice. Luo and Yin (2013) pyramided *Xa21* and *Xa27* in T5105, they first generated backcross lines by marker-assisted selection using KDML 105 as the recurrent female parent, the potential to yield a broader spectrum and more durable resistance to bacterial blight pathogens than any single donor, and T5105 had higher yields than KDML 105 in the two field trials. Win, et al. (2012) showed that backcross introgression lines of the KDML105 with *Xa21* for seedling resistance were successfully developed through MAS, these rice are fragrant, have bacterial blight resistance and show broad adaptability to the target environments.

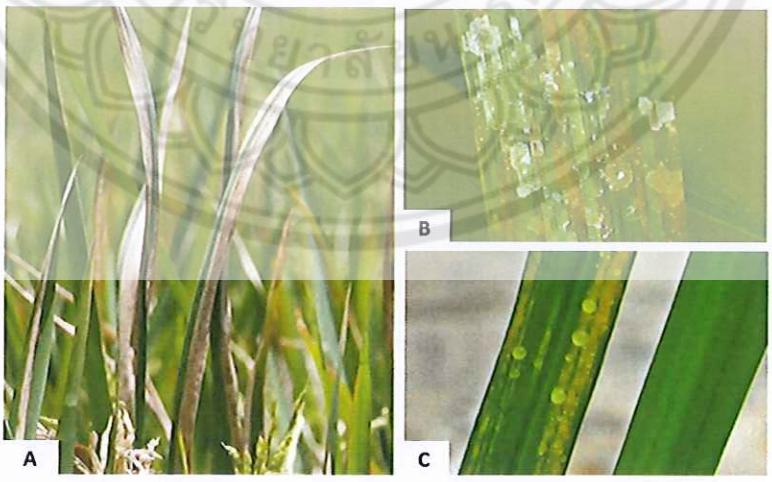


Figure 1 Lesions caused by *Xoo*

Note: (A) disease develop in the field (B) dried up bacterial ooze and (C) bacterial ooze
Source: http://www.knowledgebank.irri.org/index.

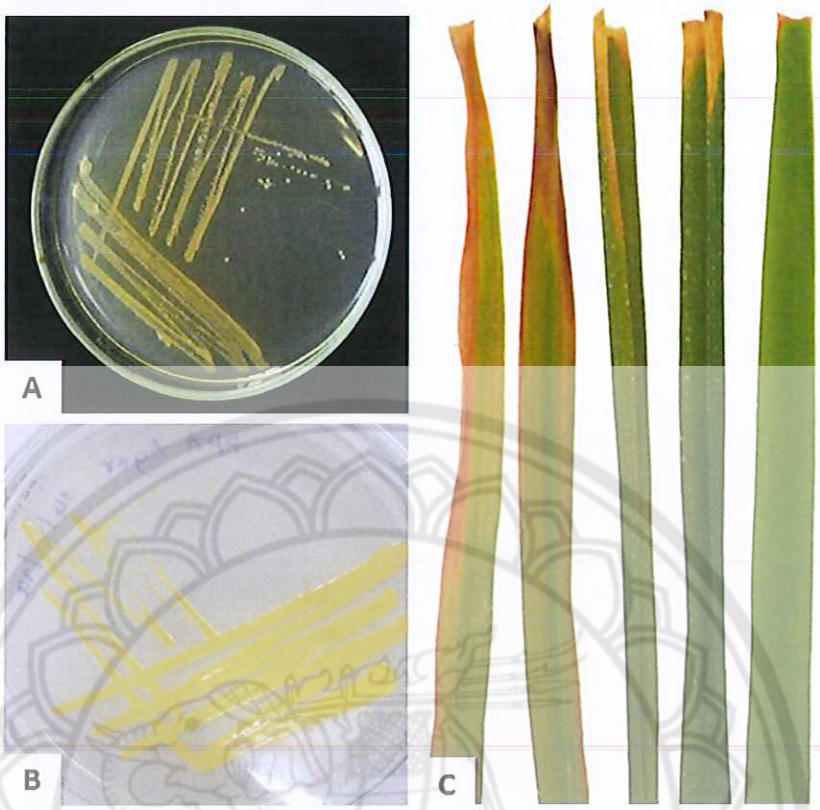


Figure 2 Yellow colony characteristic of *Xanthomonas oryzae* pv. *oryzae* and BB lesion length

Note: (A), (B) colonies of *Xanthomonas oryzae* pv. *oryzae* and (C) lesions on leaves after inoculation

BB resistance gene *Xa21*

Xa21 is a dominant BB resistance gene originated from the wild rice *O. longistaminata* and was proved to be highly effective against BB races of South and Southeast Asia (Khush, et al., 1990). The full length coding sequence of the *Xa21* gene has 3078 bp encoding deduced amino acid of 1026 residues (GenBank accession no U37133.1). There are many *Xa21* psuedogenes reported in GenBank database, for example , *O. longistaminata* receptor-like kinase protein *Xa21* pseudogene (accession no AH007024.1), complete cds and family member C pseudogene (accession no U72723.1), family member A2, pseudogene sequence (accession no U72727.1) and family member F, pseudogene sequence (accession no U72728.1). The *Xa21* gene was

classified into the LRR (Leucine-Rich Repeat) group of resistance (R) genes. This gene is the most widely used in rice breeding program because of its broad spectrum resistance to most strains of tested *Xoo* (Ikeda, et al., 1990, Khush, et al., 1989, 1990). The *Xa21* gene was first introgressed to *O. sativa* variety IR24 through backcross breeding at IRRI generating the near isogenic line IRBB21 containing the *Xa21* gene in IR24 genetic background (Ogawa, et al., 1991). IRBB21 exhibited resistance to all physiological strains from the Philippines and India (Ikeda, et al., 1990). *Xa21* also gave resistance to most *Xoo* strains collected from Sri Lanka in 1995 (Ochiai, et al., 2000) and from China in 2003 (Li, et al., 2009). The corresponding *Avr* gene AX21 (activator of *Xa21*-mediated immunity) was found to be conserved not only in *Xoo*, but also in *Xanthamonas* species of soybean, tomato, pepper, citrus, and brassica plant species, and even outside the genera in the plant bacteria *Xylella fastidiosa* (Chen and Roland, 2011). Transgenic rice expressing *Xa21* mutants with either a single or triple alanine-replacement mutant of these three sites display slightly compromised resistance compared with the wild-type *Xa21* (Xu, et al., 2006a). The *Xa21* gene could potentially be used to confer resistance in other crop species where *Xanthamonas* species are pathogens of agronomic importance. To maximize the effectiveness and durability of single and pyramided *Xa* genes, the improved resistant cultivars should be deployed along with continued monitoring for changes in pathotypes and genotypes of local *Xoo* population.

Plant-pathogen interaction

Many plant pathogens make special interaction with their hosts to suppress plant defenses and promote the release of nutrients. Pathogens are classified into two groups, biotroph and necrotroph, according to their pathogenic habits. Biotrophs are pathogens which keep their host alive and feed on living plant tissue, for example the bacterial rice pathogen *X. oryzae*. Necrotrophs often produce toxins or tissue-degrading enzymes that overwhelm plant defenses and promote the quick release of nutrients. If pathogens behave both biotrophic (during the early stages of infection) and necrotrophic (during the latter stages of disease) types they are called hemibiotrophs such as the fungus *Magnaporthe grisea* which causes rice blast disease. Most biotrophic and hemibiotrophic pathogens can only cause disease on a relatively small group of host plants because of the slightly different set of specialized genes and molecular mechanisms required for each host plant-pathogen interaction. Two possible responses of host plant-pathogen interaction are a compatible response which causes a disease and an incompatible response which causes no disease. However, some individuals of a particular plant species which is a susceptible host for a particular pathogen, may carry disease resistance genes that help recognize the pathogen and activate defenses. The incompatible interaction is controlled by the recognition of the products of the *avirulent* (*Avr*) and the corresponding resistance genes (Ellingboe, 1982). The compatible and incompatible interaction models for disease resistance are demonstrated in Figure 5.

Plants have developed a wide variety of constitutive and inducible defenses to protect themselves from diseases. Constitutive or continuous defenses include many anatomical barriers such as cell walls, waxy epidermal cuticles, and bark, which give the plant strength and rigidity, and protect the plant from invasion. Moreover, living plant cells have the ability to detect invading pathogens and respond with inducible defenses including the production of toxic chemicals, pathogen-degrading enzymes, defense-related proteins, and deliberate cell suicide. Plants generally produce toxic chemicals or defense-related proteins only when pathogens are detected because of the high fitness costs and nutrient requirements associated with their production and maintenance.

Pathogens have developed countermeasures that are able to suppress basal resistance that protects plants against entire groups of pathogens in certain plant species. If a pathogen is capable of suppressing basal defense, plants may respond with further defense mechanism.

For rice-*Xoo* interaction, resistance is governed by an interaction between the products of a single, dominant resistance gene in rice and a corresponding pathogen gene (*avr* gene) (Mew, 1987; Leach and White, 1995). For example, rice cultivars carrying *Xa7* and *Xa10* resistance genes were resistant to *Xoo* strains containing corresponding *avrXa7* and *avrXa10*, respectively (Bonas, et al., 1989).

Plant defense mechanism

Defense against infection depends on the recognition of a pathogen's attack by resistance gene products and the induction of appropriate plant defense response (Jones, et al., 2004). The immune defense response in plants is induced by elicitors which are very stable molecules released from polymeric precursors during infection (Ozeretskovskaya and Vasyukova, 2002; Zhao, et al., 2005; Holopainen, et al., 2009). The elicitor needs to be recognized on the plant by the corresponding receptor protein, which can be either abiotic or biotic elicitors (Ozeretskovskaya and Vasyukova, 2002). Abiotic elicitors involved in the stress responses in plants, such as wounding, drought, salinity stress or other commonly encountered environmental conditions (Zhao, et al., 2005). Biotic elicitors are the substances of biological origin that include the components of microbial cells, especially poly- and oligosaccharides (Chong, et al., 2005), and the signaling compounds in plant defense responses such as salicylic acid (SA) and methyl jasmonate (MJ) (Zhou and Wu, 2006; Smetanska, 2008).

Systemically induced resistance is activated following primary infection with a necrotizing pathogen, and renders distant, uninfected parts of the plant more resistant to broad spectrum of pathogens, including viruses, bacteria and fungi (Kuê, 1982). Induced resistance is a systemic response of plant defense and can be either systemic acquired resistance (SAR) or induced systemic resistance (ISR). Induction of systemic resistance can lead to the direct activation of defense-related genes including *pathogenesis-related* (*PR*) and *phenylalanine ammonia-lyase* (*PAL*) genes.

SAR is a natural occurrence whereby plants that are successfully resistant an attack by a pathogen can become highly resistant to a subsequence attack. Not only is SAR effective against microbial pathogens, but also it is involved in resistance to aphids and nematodes (Klessig and Malamy, 1994; Rossi, et al., 1998). SAR is long-lasting, broad-spectrum disease resistance that arises throughout a plant and develops systemically in response to pathogen infection via a SA-dependent pathway (Walters, et al., 2005) (Figure 3).

ISR requires an intact response to both jasmonic acid (JA) and ethylene (ET) (Pieterse, et al., 1998). A role of ISR plays in resistance to insects and necrotrophic fungal pathogens. ISR can be triggered by non-pathogenic rhizobacteria that are commonly found on plant roots to stimulate plant growth called plant growth-prmoting rhizobacteria (PGPR) (Van Loon, et al., 1998) (Figure 3).

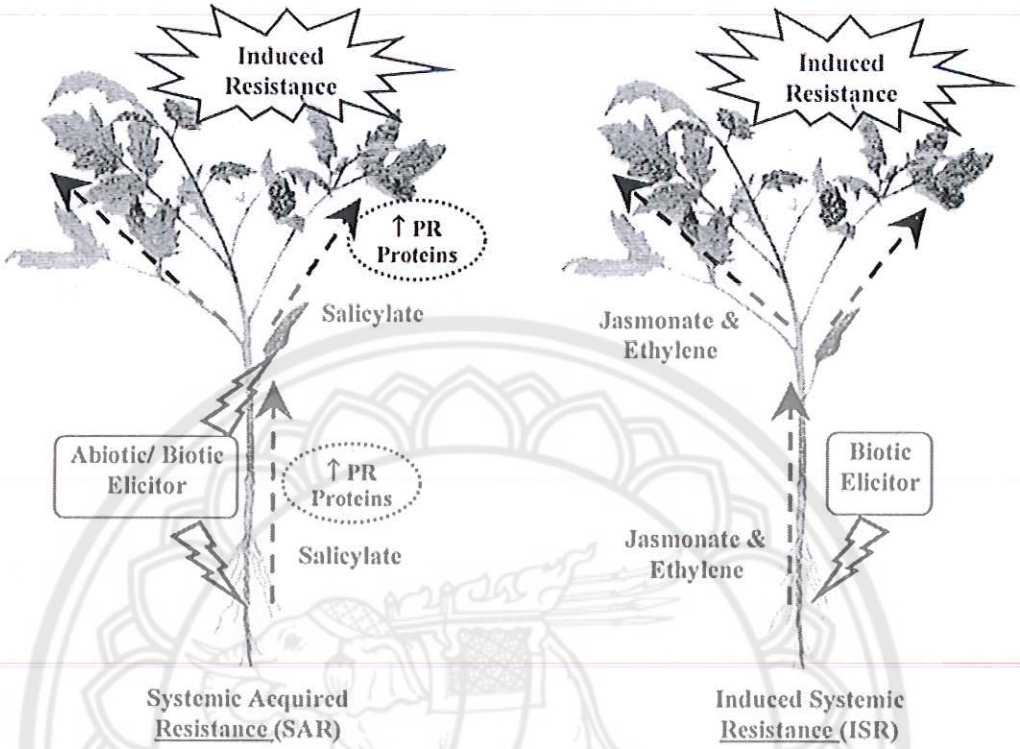


Figure 3 Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR)

Note: Systemic acquired resistance (SAR), induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of the salicylate (salicylic acid, SA), and associated with the accumulation of pathogenesis-related (PR) proteins. Induced systemic resistance (ISR), induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria (PGPR), is dependent of the jasmonate (jasmonic acid) and ethylene, independent of SA, and is generally not associated with the accumulation of PR proteins (or transcripts).

Source: Vallada and Goodman, 2004

Hypersensitive response

Hypersensitive response (HR) occurs in plants as an active defense system in response to infection by plant pathogenic fungi, bacteria and viruses, characterized by rapid cell death at the site of infection. HR is typically more pathogen-specific than basal resistance and is often triggered when gene products in the plant cell recognize the specific disease-causing elicitors introduced into the host by the pathogen. HR may limit pathogen access to water and nutrients by sacrificing a few cells in order to save the rest of the plant (Durrant and Dong, 2004). Cell death is induced by host signals called host-induced cell death. HR is a form of programmed cell death (PCD) around infection site accompanied by the induction of plant defense response that serves to confine the pathogen and protect the plant (Lam, et al., 2001). During HR, the plant cells dying strengthen cell wall by depositing different phenolic compounds, synthesize diverse the toxic compounds called phytoalexins, and accumulate proteins with an antimicrobial activity called pathogenesis-related (PR) proteins (Dangl, et al., 1996). The development of an effective HR relies upon rapid signal transduction system. The form of HR during different plant-pathogen interactions varies enormously in phenotype and timing at both macro and microscopic scales (Krzymowska, et al., 2007). No response is induced even when closely related pathogens lacking the particular elicitor infect the plant (Figure 4).

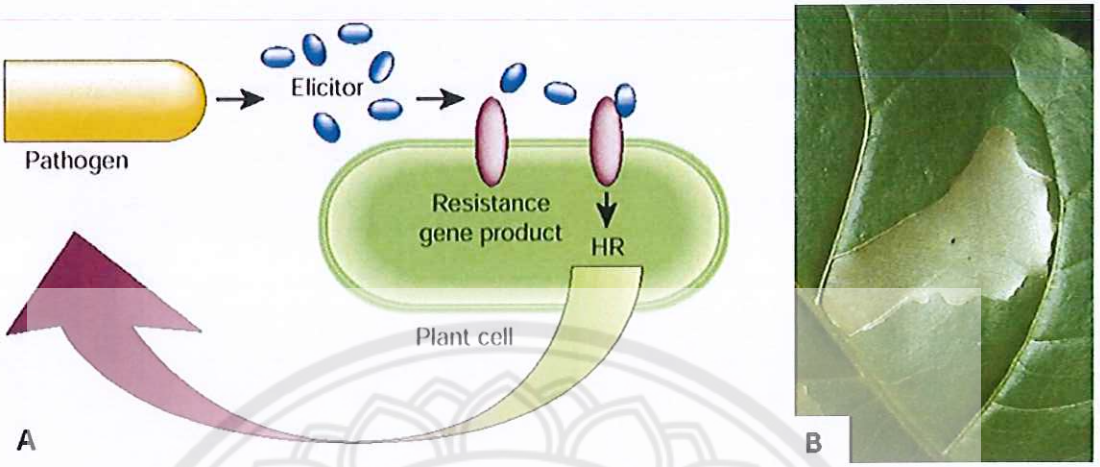


Figure 4 Hypersensitive response in plant

Note: (A) recognition of a pathogen attack by resistance gene products. (B) HR lesion on an Tobacco leaf.

Source: Stuiver and Custers, 2001
http://www.plantpath.cornell.edu/PhotoLab/Stills/TobaccoLeaf.htm

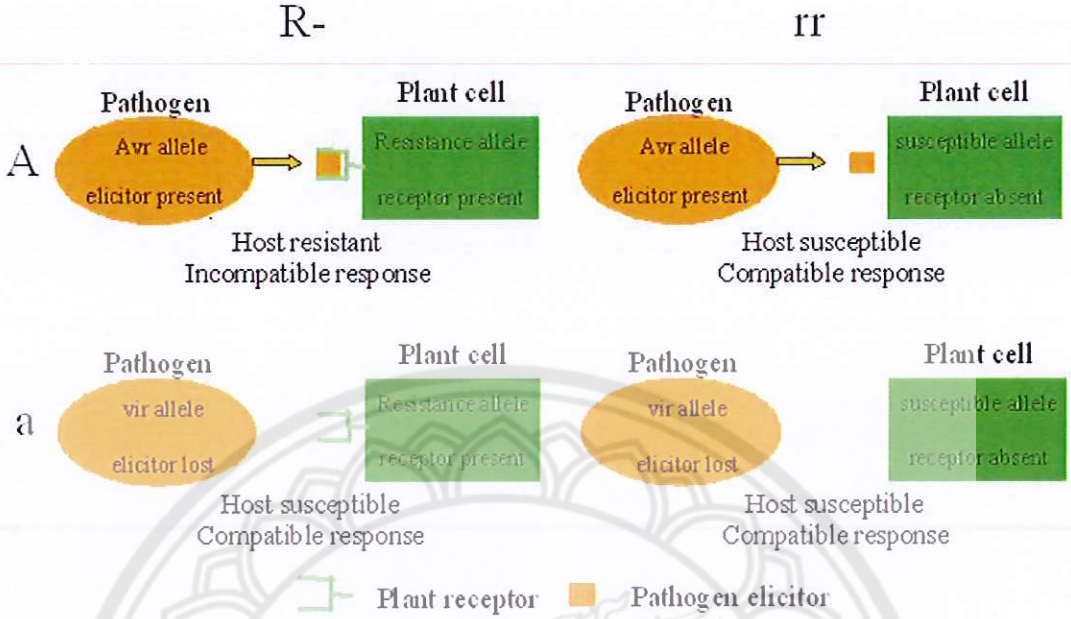


Figure 5 Guarding of pathogen virulence targets by plant R proteins

Note: AVR protein synthesised by a pathogen. AVR proteins often form part of a pathogen's attack on the plant. R proteins may bind directly to AVR proteins. R proteins may detect peptide fragments resulting from enzymatic attack by AVR proteins on target proteins. R proteins may detect changes in the conformation of plant proteins that are targeted and disabled by AVR proteins.

Source: McDonald, 2004

***Pathogenesis-related* genes (*PR* genes)**

Expression of PR proteins is generally pathogen- and host-specific pointing to the need to examine the relationship in each host-pathogen interaction (Muthukrishnan, et al., 2001, Van Loon, et al., 2006). PR proteins are induced by most pathogens including insect pests and nematodes (Gordon-Weeks, et al., 1997; Strobel, et al., 1996; Domngo, et al., 1994; Domngo, et al., 1994; Fidantset, et al., 1999; Rahimi, et al., 1996; Faize, et al., 2004). These proteins are also induced by growth regulators such as ethylene, indoleacetic acid and abscisic acid (Grillo, et al., 1995; Clarke, et al., 1998; Wilkinson, et al,. 2005), chemical treatments (Piggott, et al,. 2004), and biological products such as elicitors, toxins and enzymes (Halim, et al., 2004; Reiss and bryngelsson, 1996; Chang, et al., 1995). Several environmental factors such as

temperature, light and ozone (Gaudet, et al., 2000; Asselin, et al., 1985; Ernst, et al., 1992), and mechanical wounding (Piggott, et al., 2004) could also induce *PR* expression in various plants. The PR1 proteins have been detected in rice, wheat, barley, corn, tomato and several plants (Ergon, et al., 1998; Fidantsef, et al., 1999). They may be involved in cell wall thickening and offer resistance to the spread of pathogens in the apoplast (Benhamon, et al., 1991). Santén, et al. (2005) reported that the PR1 was accumulated on mesophyll cell walls in barley leaves infected by *Bipolaris sorokiniana.* In rice, it has been shown that infections induced the transient expression of *PR1a, PR1b* and *PR10* in the incompatible interaction compared to the compatible interaction (Jwa, et al., 2001; Schweizer, 1997). The expression of *PR1a*, *PR1b* and *PR10* genes in rice has been examined in relationship to the specific response to jasmonic acid (JA). The *PR10* gene was also induced by SA, H_2O_2, UV light and $CaCl_2$, suggesting that *PR10* responds to both biotic and abiotic stresses (Ponciano, et al., 2007). The previous study, *PR10a* gene is induced upon wounding in the potato after infection with the oomycete *Phytophthora infestans* (Després, et al., 1995). *PR1* is a defense-related marker gene which is known to be induced during the resistant response in *Arabidopsis,* rice, and other plant species (Kinkema, et al., 2000; Liu, et al., 2007). Sayari, et al. (2014) show the expression of *PR genes* were enhanced significantly in the rice Tarom after inoculation with *R. solani*, expression elevation commenced at 12 hours after inoculation (hai) or continued enhancing up to 24-48 hai after initial elevation, The *PR-10* expression level in the Tarom elevated significantly at 12 hai and highest level at this time point.

***Phenylalanine ammonia-lyase* gene (*PAL* gene)**

PAL is a key enzyme in the phenylpropanoid pathway of higher plants producing soluble phenolics, flavonoids, phytoalexins and lignin contributed to disease resistance (Bowles, 1990; Bate, et al., 1994; Gao, et al., 2012; La Camera, et al., 2004; Pellegrini, 1994; Rawal, et al., 2013; Reichert, et al., 2009; Vogt, 2010). Enhanced deposition of lignin can reinforce the plant cell wall providing a structural barrier to pathogen spread, and the toxic phenolic precursors produced during lignin biosynthesis or polymerization can directly inhibit pathogen multiplication and movement (Reimers and Leach, 1991; Naoumkina, et al., 2010). The phenylpropanoid pathway is activated

under the influence of different stress factors. An increase in PAL activity could be a marker of plant reaction to environmental stress (Dixon and Paiva, 1995; Sanchez-Ballesta, et al., 2000). PAL is involved in many biotic and abiotic stress process, such as pathogen infection, wounding and cold stress (Li and Steffens, 2002; Constabel, et al., 2000; Thipyapong and Steffens, 1997; Sanchez-Ballesta, et al., 2000). PAL is also a key enzyme for biosynthesis of SA, a plant hormone required to initiate SAR in plants (Lee, et al., 1995; Mauch-Mani and Slusarenko, 1996; Coquoz, et al., 1998; Achnine, et al., 2004; Beckers and Spoel, 2006; Gruner, et al., 2013; Malamy, et al., 2014). Four genes (*OsPAL1*, *2*, *3*, and *4*) are clustered on rice chromosome 2. Two of these genes (*OsPAL1*, *2*) have several alternatively-spliced forms as documented in the MSU rice annotation database (Ouyang, et al., 2007). Two *OsPAL* genes are arranged in tandem on chromosome 4 (*OsPAL5*, *6*), and single genes are found on chromosomes 5 (*OsPAL7*), 11 (*OsPAL8*), and 12 (*OsPAL9*). Most of the gene models have two exons and one intron, with the exception of genes *OsPAL3* and *OsPAL7*, which each contain one exon. Rice mutant line (*ospal4*) that harbors a deletion in the *OsPAL4* gene associated with increased susceptibility to three distinct pathogens, *X. oryzae* pv. *oryzae*, *R. solani* and *M. oryzae* (Bradley, et al., 2014). In rice, PAL genes accumulation and enzyme activity is induced by diverse types of rice pathogens (Giberti, et al., 2012; Gupta, et al., 2012; Li, et al., 2013). The PAL gene showed induction at 12 hai and continued to elevate till 48 hai in rice cultivars Tarom and Khazar after inoculation with fungus *R. solani*, the maximum level of PAL transcripts in infected tissues of the Tarom was 6 fold higher than the level observed in the Khazar at this time point (Sayari, et al., 2014). Overexpression of rice WRKY03 leads to constitutive expression of PAL and PR1b in rice and enhanced host resistance to bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Liu, et al., 2005).

Rice cultivar RD47

Rice cultivar RD47 is non-glutinous and photoperiod-insensitive, which have plant height 90-100 cm, short-harvesting time about 104-107 days and high yield as 793 kg per rai. This variety is one of the popular cultivars grown in Lower Northern Thailand. RD47 is derived from three-line cross between Suphanburi1, IR64 and CNT86074-25-9-1 at Chainat Rice Research Center, Thailand. RD47 cultivar is good seed quality and rather resistant to brown planthopper (BPH) and blast disease but susceptible to bacterial blight (BB) disease (Bureau of Rice Research and Development: Rice Department, Thailand).

Rice variety IRBB21

International Rice-Bacterial Blight (IRBB) lines contain different known *Xa* genes in the IR24 genetic background (Ogawa, et al., 1991). Between, 1998 and 2002, IRBB21 has been consistently resistant to the disease in both wet and dry seasons in the bacterial blight trap nursery where the effectiveness of different resistance genes under natural disease condition is evaluated, testing at the Central Rice Research Institute, Cuttack, India. In 2002, the disease trap nursery in wet season, the IRBB21 isolated plants exhibited susceptible reaction to the disease. To assess IRBB21 has succumbed to the disease, 20 of 150 plants expressing varied degrees of susceptible reaction to bacterial blight were collected at random and detected for the presence of *Xa21* using markers synthesized from the kinase domain of the gene (Ronald, et al., 1992)

Marker-assisted selection

Conventional rice breeding is a slow process, typically requires 10-15 years from initiation to varietal release. Conventional breeding mostly depends on environmental conditions. Breeding for new varieties takes long time, the release of improved varieties cannot be guaranteed (Werner, et al., 2005; Zhang, et al., 2006). Marker assisted selection (MAS) offer better selection strategies in rice breeding with a shorter period of time. MAS are more efficient, effective and reliable than phenotypic selection. MAS can shorten the development time of varieties significantly, so in some cases it will be more cost effective than selection based on phenotypes (Talukder, et al., 2004; Lopez-Gerena, et al,. 2006). Pan, et al. (2003)

reported selection against *Xa23* gene was used to select plants carrying *Xa23* gene prior to transplanting through MAS using a closely linked SSR marker (RM206) of 1.9 cm apart from the *Xa23* locus. Singh, et al. (2001) used MAS to pyramid genes for bacterial blight resistance into a high-yielding indica rice cultivar, PR106, which is susceptible to bacterial blight. MAS has been useful in pyramiding recessive genes for resistance to bacterial blight such as xa5 and xa13 with a dominant gene *Xa21*, which confers resistance to many races and thus masks the resistance conferred by recessive genes (Khush and Brar, 2001).

CHAPTER III

RESEARCH METHODOLOGY

All research work and experiments were carried out in the Agricultural biotechnology laboratory and rice plants for all experiments were grown inside the plant nursery of the department of Agricultural Science, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand.

Plant materials and growth condition

Rice (*Oryza sativa*, indica type) cultivars RD47 and IRBB21 (containing the *Xa21* gene with the background of IR24) obtained from Phisanulok Rice Research Center, Thailand. Before germination, the rice seeds were treated to break seed dormancy by incubation at 50 °C for 5 days (Agrawal and Nanda, 1969) and equilibration to room temperature for 2 days. For germination, seeds were soaked in water for 2 days and then moved to the moist paper until germination. The seedlings were allowed to grow up in a wet-clay tray for 2 weeks and then transplanted seedlings into the new wet-clay pot (three seedlings per pot). Seedlings were grown and maintained in the greenhouse, and treated with 30 kg/rai fertilizers containing nitrogen (N): phosphorus (P): potassium (K) ratio of 16-16-8 at the day before moving seedlings to pots and treated with 10 kg/rai ratio of 46-0-0 at flowering stage.

Rice breeding procedure

Rice cultivar RD47 carrying null *Xa21* (indicating as homozygous susceptibility, −/−) used as a recurrent parent (recipient) was crossed with rice cultivar IRBB21 carrying homozygous *Xa21* (indicating as homozygous resistance, +/+) used as a resistance donor (*Xa21* donor) to obtain F_1-hybrid carrying heterozygous *Xa21* (indicating as heterozygous resistance, +/−). The F_1-hybrid was self-pollinated to obtain F_2 or backcrossed with RD47 to obtain BC_1F_1 (+/−) and BC_2F_1 (+/−). The BC_2F_1 (+/−) was self-pollinated to obtain BC_2F_2 carrying three different genotypes including (−/−), (+/+) and (+/−) (Figure 6).

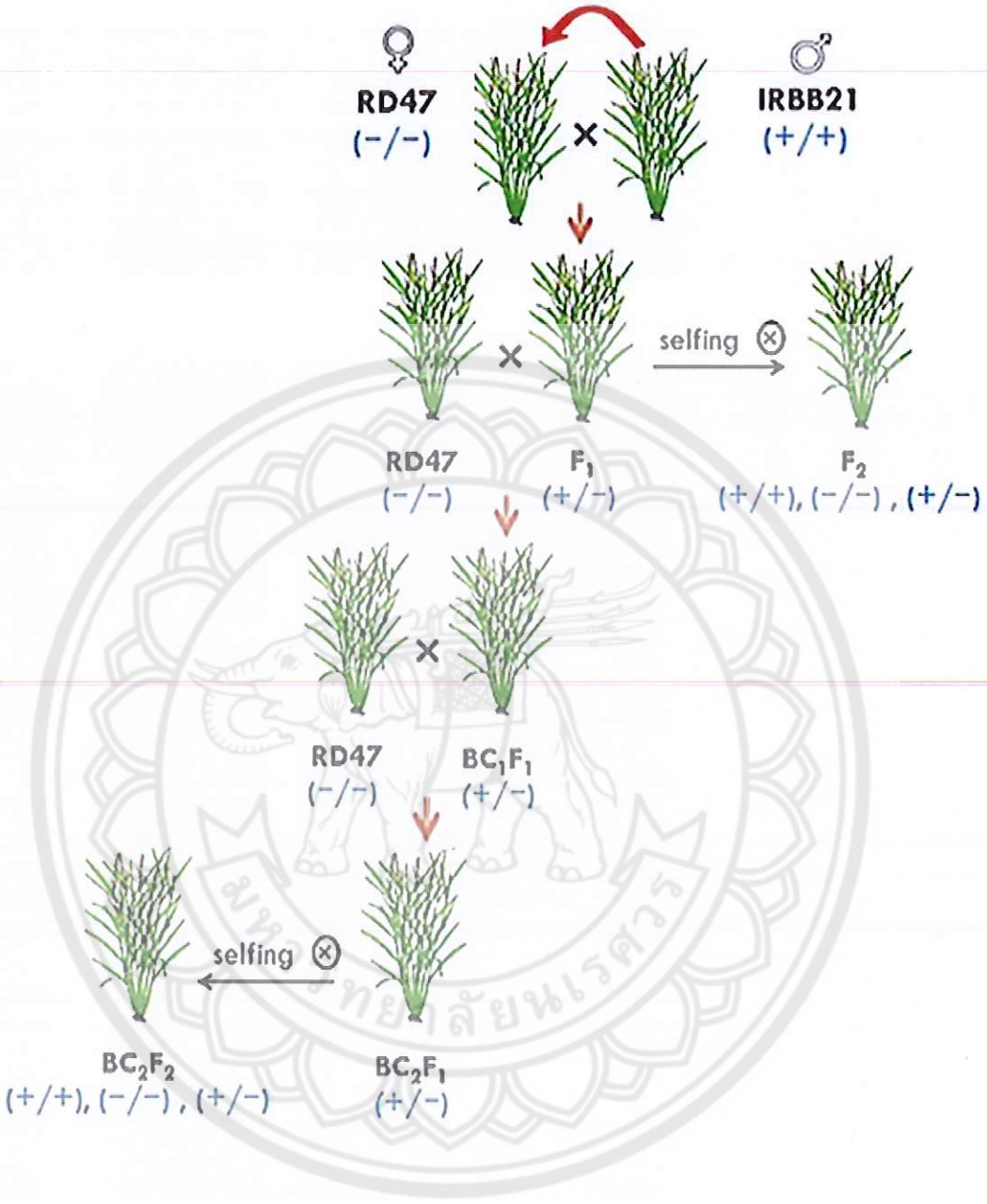


Figure 6 Rice backcross breeding program

Note: Rice cultivar IRBB21 used as a donor parent, Rice cultivar RD47 used as a recipient parent

Emasculation and hand pollination of rice flowers

At the appropriate flowering stage (25% of the panicle emerged from the flag leaf), the closed RD47 spikelet were carefully emasculated in the afternoon of the day before hand pollination. Selected rice spikelet were clipped the top off to remove all anthers. The panicles of the emasculated spikelet were covered with the labeled paper bag to protect natural cross-pollination. In the morning of the following day, the emasculated spikelet were pollinated with the IRBB21 pollens collected in petri dish with moist paper using the hand-dusting technique. The IRBB21 pollens were gently dusted on the stigma of the emasculated RD47 spikelet and then covered with paper bag. Seven days after pollination, the naked seeds started to form, and the F_1-hybrid seeds were then harvested within a month (Coffman and Herrera, 1980). The strategies of emasculation and hand pollination are shown in Figure 7.

Genotyping using marker assisted selection

Twenty-day old seedlings were screened by PCR-based marker pTA248 closely linked to *Xa21* gene (Ronald et al., 1992) using forward primer, 5'-AGACGCG GAAGGGTGGTTCCCGGA-3', and reverse primer, 5'-AGACGCGGTGTAATCGAA AGATGAAA-3'. PCR was carried out according to the Phire plant direct PCR master mix (Thermo scientific, EU). Amplification reactions were carried out in a 20 µl volume mixture containing 1 µl of DNA template prepared following the manufacturer's instructions, 10 µl of 2X Phire Plant Direct PCR Master Mix, 0.5 µM of each primer and 8 µl of RNase-Free ddH_2O. Template denaturation was conducted for 5 min at 98°C, followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 60°C for 10 s and extension at 72°C for 22 s (modified from manufacturer's instructions).

Selected F_1-hybrid plants carrying heterozygous *Xa21* gene (+/−) were rice breeding backcrossed to RD47 to obtain BC_2F_1. Heterozygous *Xa21* genotype of backcrossed progenies were confirmed using PCR-based marker pTA248 through Phire plant direct PCR master mix, and then self-pollinated to obtain BC_2F_2 progenies.

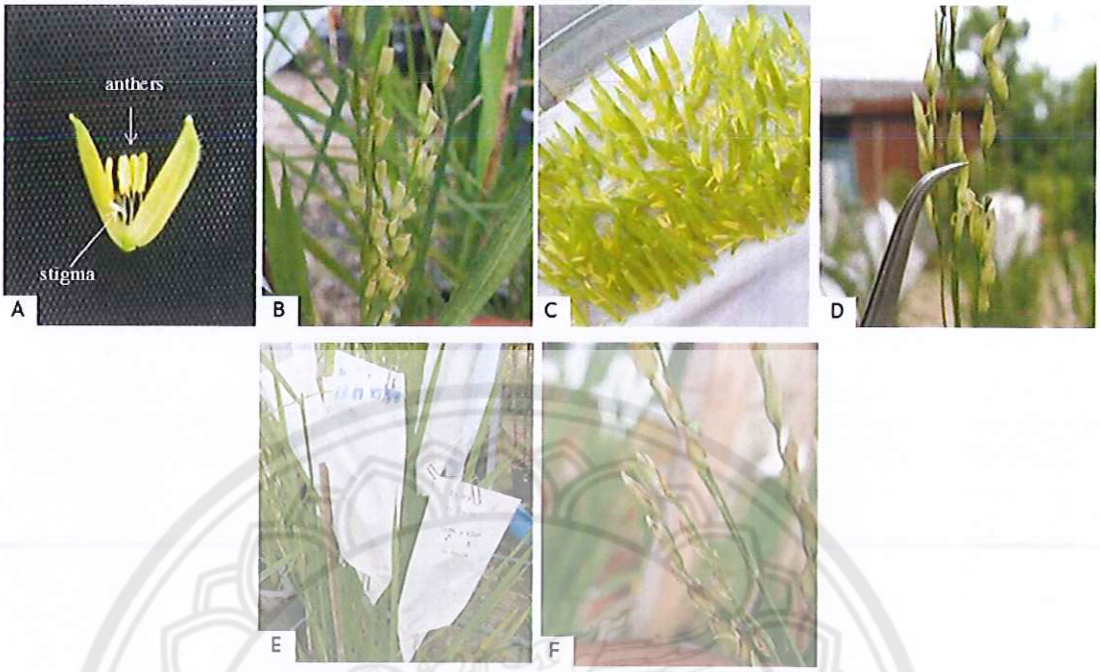


Figure 7 Emasculation and hand pollination of rice flowers

Note: (A) Rice spikelet with 6 anthers and 2 stigmas. (B) Opened spikelet of RD47 (C) Collection of matured anthers from IRBB21 in petri dish with moist paper. (D) Pollination by gently dusting pollens on the stigma of RD47 with curved forceps (E) Pollinated spikelet covered with paper bag. (F) The naked seeds ready to harvest after a month.

***Xanthomonas oryzae* pv. *oryzae* (*Xoo*) culture preparation**

The *Xoo* culture was kindly provided by Phitsanulok rice research center, Phitsanulok, Thailand. Inoculum was prepared by streaking *Xoo* on a nutrient agar (NA) plate (Figure 8). After incubated at 28°C for 24 h, a single colony was selected from the culture plate and transferred into 1 ml of sterile distilled water (SDW). Then 200 µl of the *Xoo* culture was spread on a NA plate. After incubation at 28°C for 48 h, the *Xoo* culture was collected from the spread plate by resuspending with 150 ml of SDW, and homogenous bacterial suspension was adjusted to 0.6 OD at 600 nm.

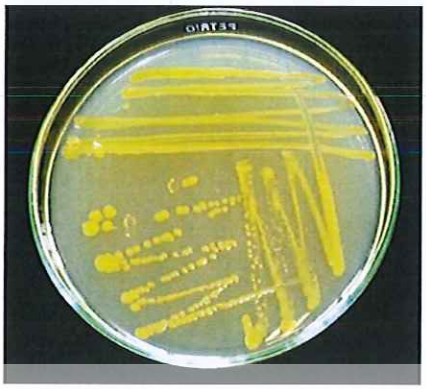


Figure 8 Yellow colony characteristic of *Xanthomonas oryzae* pv. *oryzae*

***Xoo* infection by the clipping method**

At the 6-leaf stage (45-60 days), the rice leaves of RD47, IRBB21 and BC_2F_2 progenies were inoculated with 10 ml of *Xoo* inoculum containing 3 drops of Tween-20 by the clipping method (Kauffman, et al., 1973). Fully opened leaves were cut (approximately 2 cm from the leaf tip) using sterile scissors dipped into *Xoo* inoculum for 10 s (Figure 9). The inoculated leaves were collected at 0, 24, 48, 72 h and 7 days after clipping for RNA analysis. The mock treatment was performed by clipping with *Xoo* free scissors.

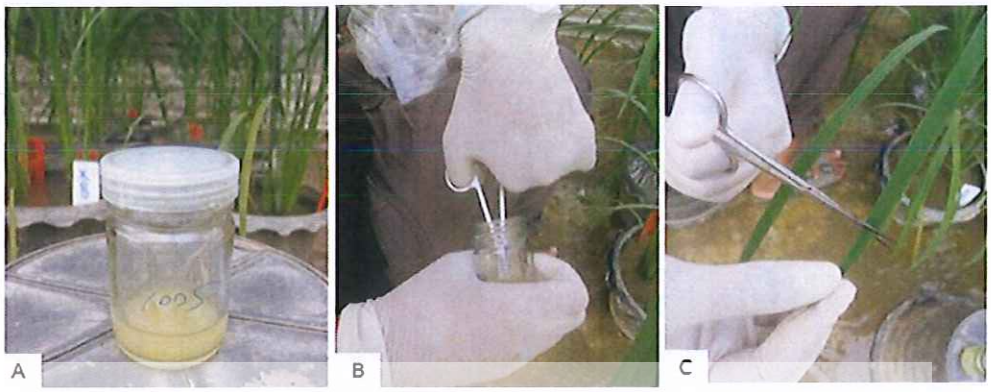


Figure 9 Strategy of the clipping method for *Xoo* infection test

Note: (A) 10 ml of *Xoo* inoculum containing 3 drops of Tween-20. (B) Sterile scissors dipped into *Xoo* inoculum for 10 s. (C) The leaves were cut 2 cm from the leaf tip.

Lesion length measurement and *Xoo* resistance scoring system

The lesion length was measured at 21 days after *Xoo* inoculation. *Xoo* resistance was assessed on rice cultivars RD47 and IRBB21, and BC_2F_2 progenies. The *Xoo* resistance scoring system was based on the bacterial blight (BB) resistance scoring system of International Rice Research Institute or IRRI (Table 3).

Table 3 IRRI scoring system for BB resistance (IRRI, 1996)

Greenhouse Test	
Lesion Length (cm)	**Description**
0-5	Resistance (R)
>5 – 10	Moderately Resistance (MR)
>10 – 15	Moderately Susceptible (MS)
>15	Susceptible (S)

RNA extraction and quality analysis

Total RNA was isolated from rice leaf samples. Up to 100 mg of fresh samples were ground in liquid nitrogen. RNA was extracted with the Extraction kit (RBC Real Genomic, Taiwan) following manufacturer's instructions. RNA was stored at -80 °C until used.

Total RNA was measured using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, US). The ratio of absorbance at 260/280 nm (generally the pure ratio of ~2.0) was used to assess the purity of RNA. To confirm RNA quality, 1 µl extracted RNA was run on 1.5% (w/v) TAE buffer agarose gel electrophoresis.

First stand cDNA synthesis

In total volume of 20 µl, the priming premix was prepared on ice using Tetro cDNA Synthesis Kit (Bioline, USA) containing 1 µl of random hexamer, 1 µl of 10mM dNTP mix, 4 µl of 5x RT Buffer, 1 µl of RiboSafe RNase Inhibitor, 1 µl of Tetro Reverse Transcriptase (200u/µl) and Total RNA (1µg), The reactions were mixed gently by pipetting and incubated as followed ; 25°C for 10 min, 45°C for 30 min and 85°C for 5 min. And using qScript™ cDNA Synthesis Kit (Quanta BioSciences, USA) In total volume of 20 µl, containing 4 µl of qScript Reaction Mix (5X), 1 µl of qScript RT, and Total RNA (1µg), The reactions were vortexed gently, and then centrifuged 10 sec, place the tubes in a thermal cycler program as followed; 22°C for 5 min, 42°C for 30 min and 85°C for 5 min. The reactions were stored at -20°C for long term storage, or proceed to PCR immediately.

Cloning of the *Xa21* and *PAL* fragments from rice cultivar IRBB21

1. PCR amplification

To amplify the *Xa21* and *PAL* fragments, cDNAs from resistance rice cultivar IRBB21 were used as templates in PCR using MyTaq™ HS Mix (Bioline, USA). For *Xa21*, the specific primers based on the *Xa21* accession number U37133, forward primer 5'-CAGAGTATGGCGTGGGCT-3' and reverse primer 5'-CGGGTC TGAATGTACTG TCA-3', were designed to give the 114-bp PCR product. For *PAL*, the specific primers reported by Jain et al. (2006), forward primer 5'-GCACATCTTG GAGGGAAGCT-3' and reverse primer 5'-GCGCGGATAACCTCAATTTG-3', gave the 141-bp PCR product. PCR was carried out in a 20 µl volume mixture containing 12.5 µl of My Taq HS Mix, 0.2 µM of each primer, 1 µl of cDNA template, and 4.5 µl of RNase-Free ddH2O. Template denaturation was conducted for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR products were electrophoresed on 1.5% (w/v) TAE agarose gel visualized with ethidium bromide staining.

2. Purification of PCR products

To recover PCR products from the agarose gel, HiYield™ Gel/PCR Fragments Extraction Kit (RBC Bioscience, Taiwan) was used according to the manufacturer's instruction. The purified PCR products were electrophoresed on 1.2% (w/v) TAE agarose gel.

3. PCR product cloning

The purified PCR products were cloned into the pGEM®-T Easy Vector (Promega, USA) according to the manufacturer's protocol. The molar ratio used for ligation reactions was 1:3 of vector:insert. The ligation reactions were incubated overnight at 4°C.

The ligation reaction was transformed into high-efficiency competent cells ($\geq 1 \times 10^8$cfu/µg DNA). Five microliters of ligation reaction were mixed with 100 µl of competent cells and then the mixture was left on ice for 30 min. The competent cells with ligation products were heat-shocked for 45 s at 42°C and then immediately placed on ice for 2 min. One milliliter of LB broth was added to the heat-shocked reaction and incubated at 37 °C for 1 h. The transformed cells were spread on the LB agar plate containing 100 µg/ml ampicillin, 0.002% X-Gal (5-bromo-4-chloro-3-indolyl-β-d-

galactopyranoside, 0.02 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The transformation plates were incubated overnight at 37°C. The white colonies were selected and confirmed positive clones by colony PCR.

***E. coli* competent cell preparation**

The competent cells were prepared by growing a single colony of *E. coli* strain DH5α in 5 ml of LB broth overnight at 37°C, 250 rpm. One milliliter of the cell culture was re-cultured in 100 ml of fresh LB broth at 37°C, 250 rpm for 2-3 h until OD600 reached 0.4. The cell culture was placed on ice for 10 min before centrifugation at 4°C, 4000 rpm for 10 min (cells must remain cold all times). Cell pellets were resuspended in 2 ml of cold 0.1M $CaCl_2$ solution containing 30% (v/v) glycerol. The competent cells were used for transformation immediately or made 200 µl aliquots and stored at -80 °C.

Extraction of plasmid DNA using the alkaline lysis method (Bimboim and Doly, 1979)

A single white colony was grown in 5 ml of LB broth with ampicillin 100 µg/ml overnight at 37 °C (250 rpm). One milliliter of the cell culture was centrifuged at 13,000 rpm for 5 min. Cell pellets were suspended in 200 µl of solution I (50 mM Tris-HCl pH 8.0, 10 mM EDTA and RNaseA 100 µg/ml) and mixed by vortexing. Two hundred microliters of solution II (0.2 M NaOH and 1% SDS) were added and mixed gently by pipetting. Two hundred microliters of solution III (potassium acetate (MW 98.14) 29.4 g and 1.1 ml glacial acetic acid, pH 5.5) were then added and mixed by inverting several times before centrifugation at 13,000 rpm for 10 min. The supernatant was mixed with 900 µl of absolute ethanol and centrifuged at 13,000 rpm for 20 min. The DNA pellets were washed with 70 % ethanol and air-dried for 5-10 min. The DNA pellets was resuspended with 20 µl of TE buffer and electrophoresed on 1.2% (w/v) TAE agarose gel. Stored at -20 °C until needed.

Real-Time PCR (RT-qPCR)

The qPCR was carried out using PerfeCTa® SYBR® Green FastMix, ROX™, the 20-µl PCR reaction contained 10 µl of PerfeCTa SYBR Green FastMix, ROX (2X),10 µM of each primer, 1 µl of cDNA template, and 7 µl of RNase-Free ddH_2O, using PCRmax Eco™ 48 Thermal Cyclers (PCRmax, UK) according to the manufacturer's protocol The PCR reactions followed: 95 °C for 3 min, 40 cycles at 95 °C for 8 sec, 60°C for 10 sec and 72°C for 20 sec. followed by melt curve analysis. The data was normalized using 18s rRNA as endogenous control and analyzed to relative transcript levels were calculated values using ΔCt method determination and as the means of three biological replicates were performed for each sample and standard error was calculated.

Statistical analysis

The baseline correction and automatically calculated the threshold for determining cycle threshold (Ct) values for each reaction, Comparative CT method for relative quantification has been used with *18s rRNA* as control and the sample taken before inoculation (0 h) as calibrator. Relative quantity ($RQ = 2^{-\Delta\Delta CT}$) of any gene is given as fold change related to 0 h. The data were statistically assessed using analysis of variance accompanied by Duncan's range test ($p < 0.05$) were analyzed using the SPSS statistics 17.0 software. Graphs were constructed using the means and standard errors for each data point.

CHAPTER IV

RESULTS AND DISCUSSION

Identification of the *Xa21* genotypes in experimental rice plants using the pTA248 marker

Resistance breeding with marker-assisted selection (MAS) has been employed to develop broad spectrum bacterial bright (BB) resistance in rice. As a tightly linked, functional marker named pTA248 (Ronald, et al., 1992) is available for *Xa21*, which has been successfully introgressed into several elite rice varieties and hybrid rice parental lines.

In this research, rice cultivar RD47 (female parent) was cross-pollinated with rice variety IRBB21 (male parent). F_1 progenies were used as recurrent parents to backcross with RD47 to generate BC_1F_1. Then BC_1F_1 plants carrying *Xa21* gene were used as recurrent parents to backcross with RD47 to generate BC_2F_1. F_2 and BC_2F_2 were produced from self-pollination of F_1 and BC_2F_1, respectively.

The twenty-day old seedling stage of RD47, IRBB21, F_1, F_2 and BC_2F_2 plants were inspected for the *Xa21* genotypes by the pTA248 co-dominant marker. The RD47 maternal plant exhibited a single 730-bp PCR product whereas the IRBB21 paternal plant exhibited a single 925-bp PCR product. This indicated that the genotype of RD47 was null *Xa21* (homozygous susceptibility) whereas the genotype of IRBB21 was homozygous *Xa21* (homozygous resistance, *Xa21*/*Xa21*). All F_1 progenies exhibited both 730-bp and 925-bp fragments indicating the heterozygous *Xa21* genotype (heterozygous resistance, *Xa21*/-). For F_2 and BC_2F_2 progenies, three different patterns of PCR products were detected. F_2 plants exhibiting the single 730-bp and the 730-bp and 925-bp PCR product patterns and BC_2F_2 plants exhibiting the 730-bp and 925-bp PCR product pattern were selected for further analysis. The *Xa21* genotypes of selected RD47, IRBB21, F_1, F_2 and BC_2F_2 plants are shown in Figure 10

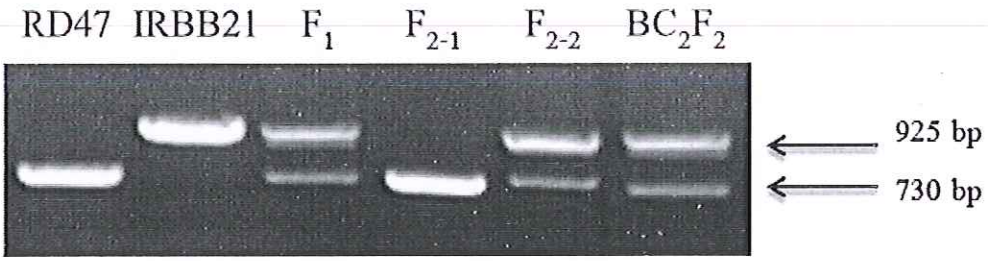


Figure 10 The *Xa21* genotypes of selected RD47, IRBB21, F_1, F_2 and BC_2F_2 plants identified by the pTA248 marker

Note: The 730-bp and 925-bp fragments indicate null-*Xa21* and *Xa21* alleles, respectively. The single 730-bp pattern indicates homozygous null-*Xa21* (homozygous susceptibility). The single 925-bp pattern indicates homozygous *Xa21* (homozygous resistance). The 730-bp and 925-bp pattern indicates heterozygous *Xa21* (heterozygous resistance). RD47, a recurrent parent; IRBB21, a donor parent; F_1, RD47xIRBB21 hybrid progeny; F_{2-1} and F_{2-2}, F_1 self-pollination progenies; BC_2F_2, BC_2F_1 self-pollination progeny.

Cloning and sequencing of the *Xa21* and *PAL* fragments from rice IRBB21

The cDNA synthesized from RNA of rice cultivar IRBB21 was used as a template to amplify the designed 114-bp fragment of *Xa21* using specific primers based on accession number U37133 and the 141-bp of *PAL* using specific primers as described in Jain, et al. (2006). The PCR products corresponding to the expected sizes of the *Xa21* and *PAL* fragments were obtained as shown in Figure 11. To confirm the sequences of the *Xa21* and *PAL* genes, the PCR products were purified and cloned into pGEM®-T Easy Vector (Promega, USA) for DNA sequencing. The candidate clones of *Xa21* (114 bp) and *PAL* (141 bp) were sequenced by the service of first BASE Laboratories Sdn Bhd, Malaysia. The deduced nucleotide sequences of the 114-bp *Xa21* and the 141-bp *PAL* were obtained from the Clustal Omega nucleotide sequence alignment of four clones (Figure 12). Blastn analysis revealed that the 114-bp *Xa21* sequence had 100% nucleotide identity to *O. longistaminata* strain IRBB21 receptor kinase-like protein (AH005614.2), *O. sativa* Indica Group *Xa21* gene for receptor kinase-like protein (AB212798.1), *O. longistaminata* receptor kinase-like protein (*Xa21*) gene (U72723.1) and *O. sativa* receptor kinase-like protein (*Xa21*) gene (U37133.1) (Figure 13A). The 141-bp *PAL* sequence had 100 % nucleotide identity to *O. sativa* Japonica Group *phenylalanine ammonia-lyase* (XM_015771243.1) and *O. sativa* Japonica Group cultivar Zhonghua 11 *phenylalanine ammonia-lyase* (*PAL06*) gene (KF556681.1) (Figure 13B). These results confirmed that the 114-bp *Xa21* and the 141-bp *PAL* fragments were the segments of the *Xa21* and *PAL* genes, respectively. However, it is possible that the 141-bp *PAL* fragments were a member of the *PAL06* gene.

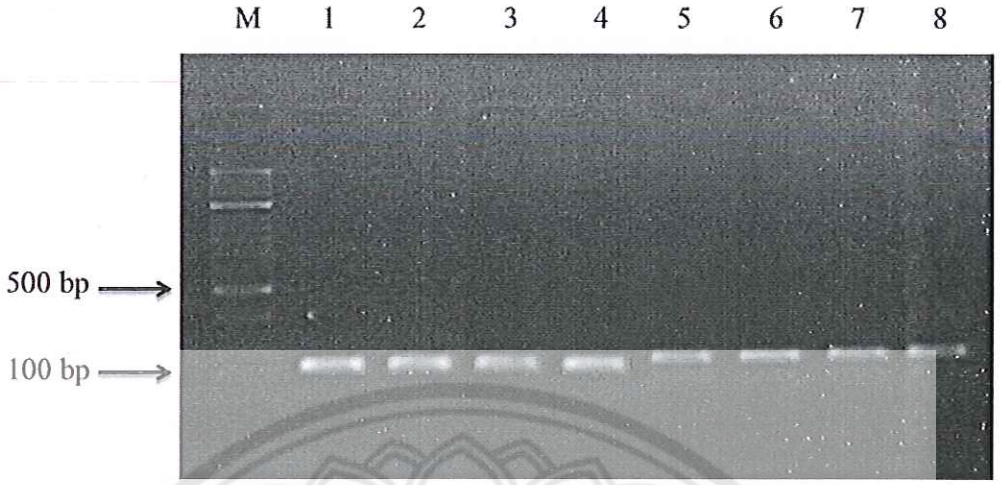


Figure 11 RT-PCR products of the 114-bp *Xa21* and the 141-bp *PAL* from IRBB21

Note: lane M, 100bp DNA Ladder H3 RTU; lanes 1-4, PCR products for the 114-bp *Xa21*; lanes 5-8, PCR products for the 141-bp *PAL*.

Figure 12 Clustal omega nucleotide sequence alignment of the *Xa21* and *PAL* of the sequenced PCR products

Note: (A) nucleotide sequence alignment of the 114-bp *Xa21* fragments, (B) nucleotide sequence alignment of the 141-bp *PAL* fragments

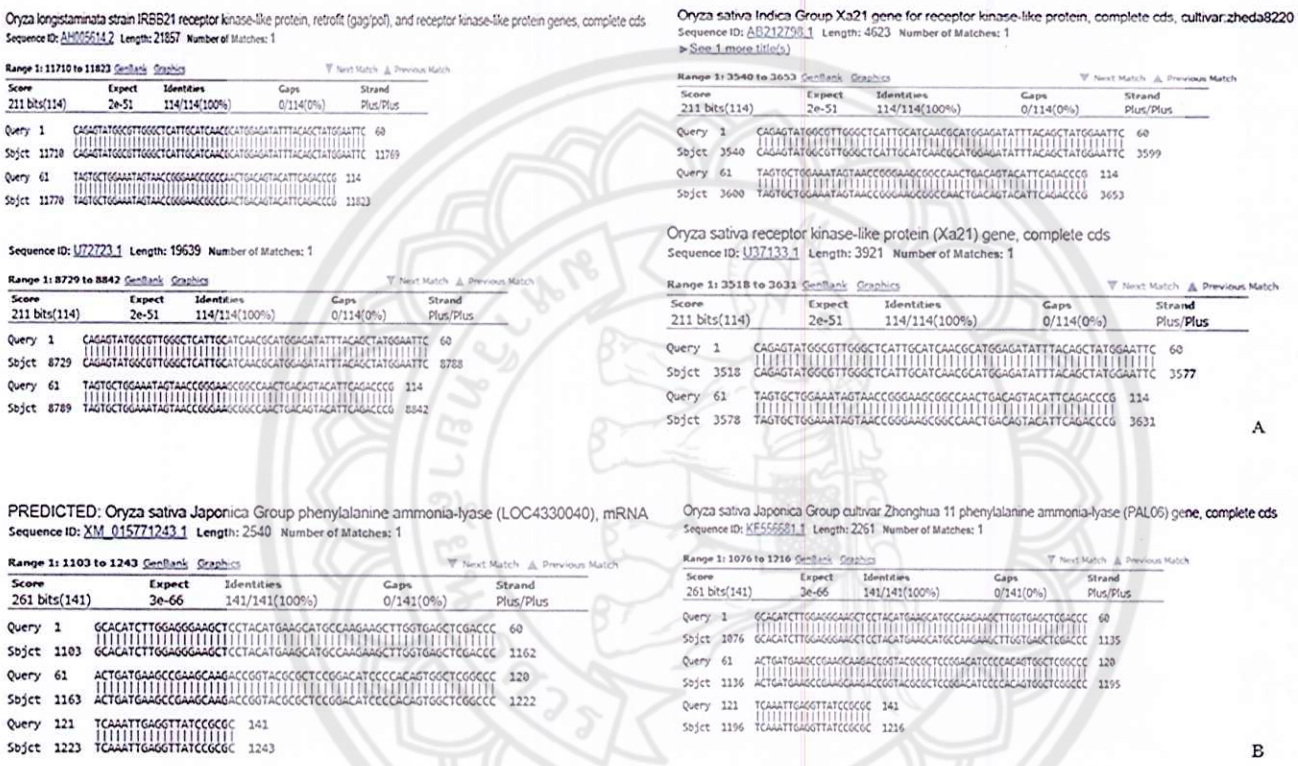


Figure 13 Blastn analysis of *Xa21* and *PAL* nucleotide sequence identities

Note: (A) The 114-bp *Xa21* sequence nucleotide identity, (B) the 141-bp *PAL* sequence nucleotide identity

Expression analysis of *Xa21* in wounded and *Xoo*-infected RD47, IRBB21 and F_2 plants by RT-PCR

Determination of *Xa21* expression in RD47 and IRBB21 plants given wounded and *Xoo* inoculated conditions revealed that *Xa21* transcripts were detected only in IRBB21 plants even at 0 h post wounding and *Xoo* inoculation. The *Xa21* transcript levels in IRBB21 plants increased to the maximum level at 24 h post wounding and *Xoo* inoculation. However, the expression of *Xa21* significantly decreased at 48 h post wounding while the expression of *Xa21* was continuously induced at 48 h post *Xoo* inoculation (Figure 14). Expression analysis of *Xa21* in wounded F_2 carrying null-*Xa21* ($F_{2\text{-}1}$) and heterozygous *Xa21* ($F_{2\text{-}2}$) genotypes showed similar expression patterns to RD47 and IRBB21 parental plants, respectively. No *Xa21* transcript was detected in *Xoo* inoculated $F_{2\text{-}1}$ carrying null-*Xa21*. In *Xoo*-inoculated $F_{2\text{-}2}$ carrying heterozygous *Xa21* genotype, the expression of *Xa21* was continuously induced after inoculation and the maximum level of the *Xa21* transcripts was detected at 48 h post inoculation (Figure 14). The *Xa21* expression in IRBB21 and heterozygous *Xa21* $F_{2\text{-}2}$ plants indicated that the *Xa21* gene was constitutively expressed and induced by wounding and *Xoo* inoculation. However, the *Xa21* expression induced by wounding was not long-lasting or immediately dropped after the induction peak if no *Xoo* infected the plant while the prolonged induction of the *Xa21* expression was observed in the *Xoo*-infected plant. This indicates that *Xa21* responds to *Xoo* infection. Park, et al. (2010) was also found that the *Xa21* gene in transgenic rice Nat XA21 plant wounded by cutting the leaf tip with scissors was induced to a moderate level of expression after wounding while the *Xa21* gene in the transgenic rice Nat XA21 plant infected with *Xoo* was induced to maximum expression at 72 h after inoculation. The previous study of the *Xa21* gene showed that it was effective in controlling resistance to diverse isolates of *Xoo* when expressed in susceptible rice cultivars (Wang, et al., 1996). Recently, there was also reported that the *Xa21* gene gave *Xanthomonas campestris* pv. *musacearum* (*Xcm*) resistance to Banana Xanthomonas wilt (BXW) in the transgenic banana (Tripathi, et al., 2014).

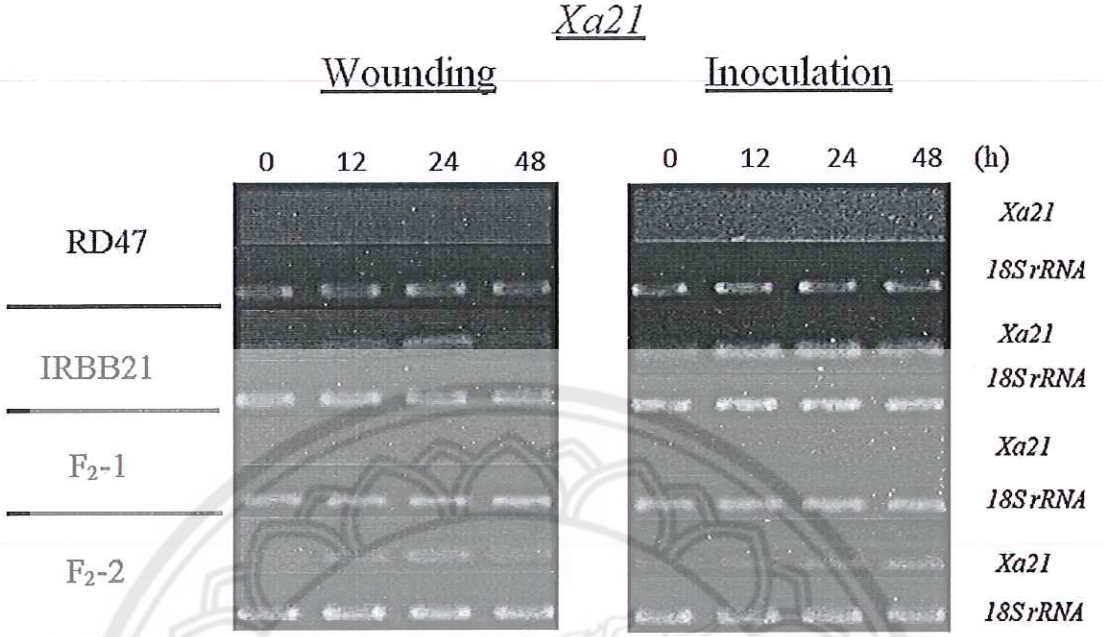


Figure 14. RT-PCR analysis of the *Xa21* expression in wounded and *Xoo*-infected RD47, IRBB21 and F_2 plants

Note: Expression analysis of *Xa21* was carried out at 0, 12, 24 and 48 h post wounding and *Xoo* inoculation. RD47, a recurrent parent; IRBB21, a donor parent; $F_{2\text{-}1}$ and $F_{2\text{-}2}$, F_1 self-pollination progenies carrying null-*Xa21* and heterozygous *Xa21* genotypes, respectively. The *18S rRNA* was used as the internal control.

Expression analysis of defense genes in F_1 hybrid (RD47 x IRBB21) infected with *Xoo*

To study the interaction of defense genes and the *Xa21* gene in response to *Xoo* developmental disease resistance in the F_1 hybrid (RD47 x IRBB21), expression of *Xa21*, *PR1b*, *PR10a* and *PAL* was determined at 21 days post inoculation when the symptom of the BB disease was obviously noticed in RD47 but not in the F_1 plants. At 21 days after wounding and *Xoo* inoculation, the *Xa21* transcript levels were similar between wounded and *Xoo*-infected F_1 plants (Figure 15). Together with the previous result, it is possible that the *Xa21* expression was induced at the initial stage of *Xoo* infection to trigger a defense mechanism and then decreased to a background level. However, a previous report showed that the *Xa21* expression was independent of wounding or *Xoo* infection (Century, et al., 1999).

Comparing to wounded RD47 and F_1 plants, the transcript levels of *PR1b* and *PR10a* were insignificantly induced in *Xoo*-infected RD47 plants but were significantly induced in *Xoo*-infected F_1 plants (Figure 15). This suggests that *Xa21* could enhance resistance to *Xoo* via induction of *PR1b* and *PR10a* expression. Ponciano, et al. (2006) also reported that the expression of *PR1b* and *PR10a* in IRBB21 plants was induced by *Xoo* but only *PR1b* expression was important for mounting the resistance response to *Xoo*. Previously, study on rice mutants with high *PR1b* expression showed enhanced race non-specific resistance to *Xoo* (Yin, et al., 2000) while another study showed that the constitutive expression of *PR1b* and *PR10a* in rice was correlated with enhanced resistance to *Magnaporthe grisea* and *Burkholderia glumae* (Xiong and Tang, 2003).

For *PAL* expression analysis, the *PAL* transcripts were slightly induced in wounded F_1 plants but highly induced in *Xoo*-infected F_1 plants. The *PAL* transcripts were not detected in both wounded and *Xoo*-infected RD47 (Figure 15). Tonnessen, et al. (2015) reported that *OsPAL4* mutant rice which exhibited low expression level of *OsPAL4* and unlinked *OsPAL6* genes showed a higher level of susceptibility to *Xoo*, *Rhizoctonia solani* causing sheath blight and *Magnoporthe oryzae* causing rice blast than a wide type. *OsPAL4* and *OsPAL6* co-localized with quantitative trait loci (QTL) for rice bacterial blight and sheath blight, and rice blast, respectively. They suggested that *OsPAL4* and possible *OsPAL6* were key contributors to board-spectrum disease

resistance in rice. Wound-induced *PAL* expression in rice was also demonstrated in the previous report that the expression level of *PAL* was significantly higher in resistant Kasalath rice than in the susceptible Wuyujing3 rice in response to small brown planthopper (SBPH) feeding (Duan, et al., 2014).

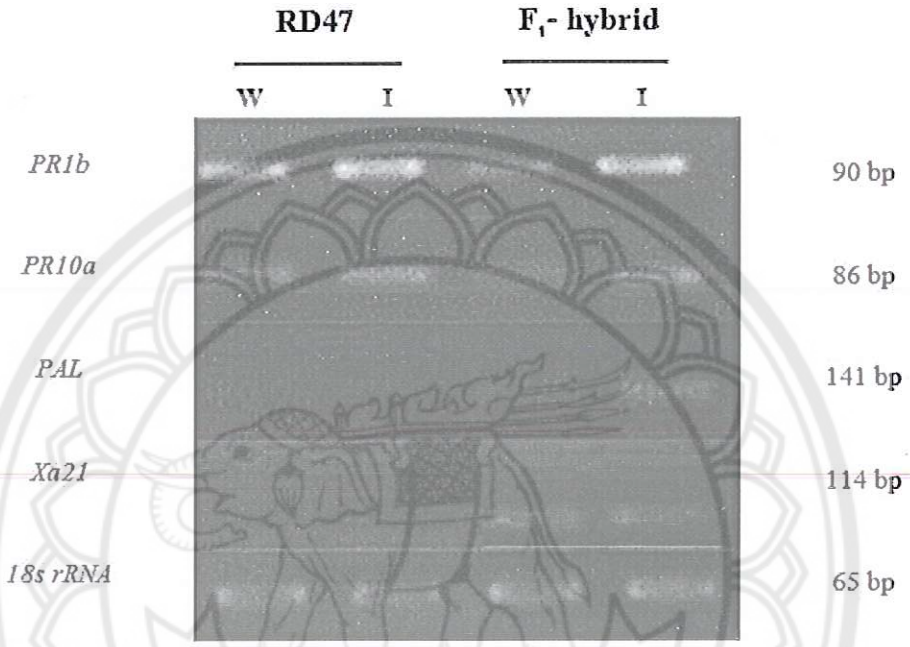


Figure 15 RT-PCR analysis of *Xa21*, *PR1b*, *PR10a* and *PAL* expression in wounded and *Xoo*-infected RD47 and F_1 (RD47 x IRBB21) plants

Note: Expression analysis of *Xa21*, *PR1b*, *PR10a* and *PAL* was carried out at 21 days post wounding and *Xoo* inoculation. RD47, a maternal plant; F1, RD47 IRBB21 hybrid; I, inoculation; W, wounding. The *18S rRNA* was used as the internal control.

Expression analysis of *PAL* in wounded and *Xoo*-infected F_2 plants carrying null-*Xa21* and heterozygous *Xa21* genotypes by RT-PCR

The previous result showed that expression of *PAL* was induced in F_1 carrying the *Xa21* gene but not in RD47, the maternal plant without the *Xa21* gene, in response to wounding and *Xoo* inoculation. To confirm whether *Xa21* expression was related to induction of *PAL* expression, the *PAL* transcripts were determined at the initial stage (0-48 h) of wounding and *Xoo* inoculation in F_2 plants carrying null-*Xa21* and heterozygous *Xa21* genotypes compared to RD47 and IRBB21. The results revealed that no *PAL* transcripts were detected in RD47 and F_2 carrying null-*Xa21* plants. The *PAL* transcript levels in IRBB21 and F_2 carrying the heterozygous *Xa21* genotype rapidly increased to the maximum level at 12 h post wounding before decreasing after 24 h post wounding (Figure 16). In response to *Xoo*, *PAL* in IRBB21 was highly expressed at 12 and 24 h post *Xoo* inoculation before decreasing at 48 h whereas the *PAL* expression levels in F_2 carrying the heterozygous *Xa21* genotype was insignificantly different at 0-48 h post *Xoo* inoculation (Figure 16). These results suggested that *Xa21* in IRBB21 and F_2 plants may involve in induction of *PAL* expression after wounding and *Xoo*-inoculation. However, RT-PCR might not be sensitive enough to detect very less *PAL* transcripts in RD47 and F_2 carrying null-*Xa21* plants.

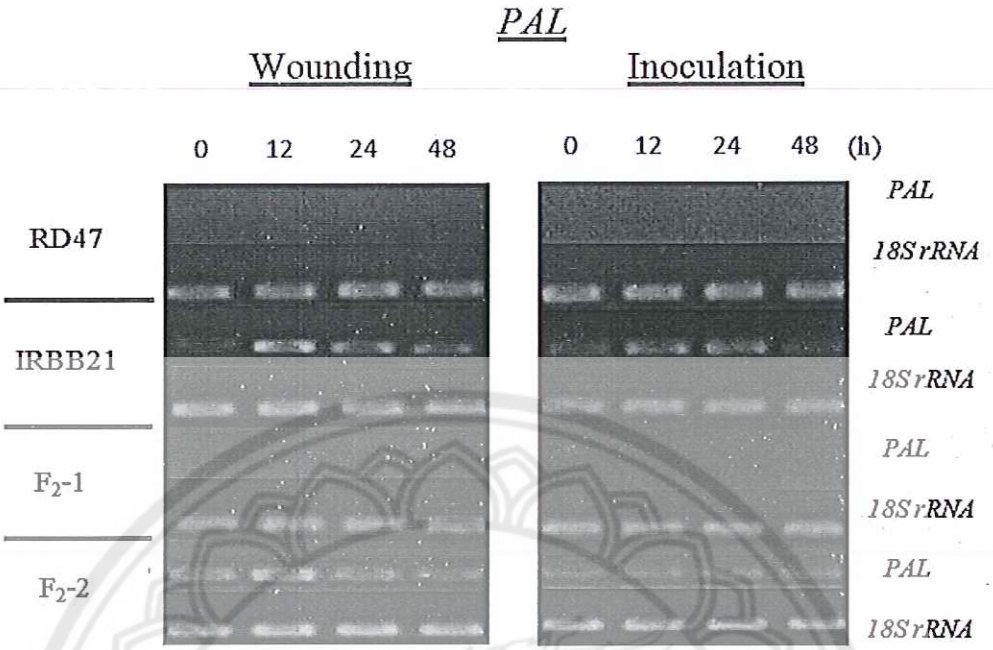


Figure 16 RT-PCR analysis of *PAL* expression in wounded and *Xoo*-infected RD47, IRBB21 and F_2 carrying null-*Xa21* and heterozygous *Xa21* genotypes

Note: Expression analysis of *PAL* was carried out at 0, 12, 24 and 48 h post wounding and *Xoo* inoculation. RD47, a recurrent parent; IRBB21, a donor parent; $F_{2\text{-}1}$ and $F_{2\text{-}2}$, F_1 self-pollination progenies carrying null-*Xa21* and heterozygous *Xa21* genotypes, respectively. The 18S rRNA was used as the internal control.

Evaluation of BB resistance in BC_2F_2 inoculated with *Xoo*

BC_2F_2 plants with different *Xa21* genotypes including homozygous null-*Xa21*, heterozygous *Xa21* and homozygous *Xa21* were evaluated the BB resistance phenotype in the greenhouse with *Xoo* controlled conditions. The 6-leaf stage of RD47, IRBB21 and BC_2F_2 plants were inoculated with *Xoo* by the clipping method, and infected leaves were evaluated at 21 days after inoculation. The lesion length was measured following the scoring system of International Rice Research Institute (IRRI, 1996) (Appendix A). The disease severity is assessed based on lesion length measurement of percent diseased leaf area. The lesion lengths of infected BC_2F_2 plants compared to RD47 and IRBB21 are shown in Table 4. Rice RD47 containing null-*Xa21* was moderately susceptible (MS) whereas IRBB21 carrying homozygous *Xa21* showed resistance (R). BC_2F_2 carrying null-*Xa21* showed MS phenotype as RD47 whereas BC_2F_2 carrying either homozygous or heterozygous *Xa21* showed R phenotype (Table 4 and Figure 17). Leaves of all tested plants clipped without *Xoo* showed similar lesions limited only at the clipped regions (Table 4 and Figure 18). These results indicated that only one copy of the *Xa21* gene (in heterozygous genotype) was effective to acquire resistance to *Xoo* isolated from Phitsanulok. The resistance levels of BC_2F_2 carrying homozygous and heterozygous *Xa21* were not different. Zhang, et al. (2006) showed that rice plants containing both the *Xa21* and *Xa7* genes have the same strong resistance regardless of whether they are heterozygous or homozygous to BB resistant and very useful in hybrid rice breeding. The combination of *Xa21* and *xa13* was most effective with short lesion length. *Xa21* in combination with either *xa5*, *xa13* or both had shown promise advocating the utility of *Xa21* in achieving higher levels of resistance in rice (Huang, et al.,1997; Sridhar,et al., 1999; Sanchez, et al., 2000; Singh, et al., 2001). Singh, et al. (2001) attempted to pyramid dominant or recessive BB resistant genes to improve conventional inbred varieties. Recessive genes are not suitable for improving hybrid rice because they can only be expressed when the alleles are homozygous. Dominant genes were found to be highly efficient for the improvement of resistance and other favourable agronomic traits in hybrid rice.

Table 4 Lesion length scoring in RD47, IRBB21 and BC_2F_2 inoculated with *Xoo*

	Lesion Length (cm)		Description
	Wounding	**Inoculation**	**Inoculation**
RD47	0.30±0.12 c	13.60±1.41 a	MS
IRBB21	0.30±0.17 c	0.80±0.23 c	R
BC_2F_2 (null-*Xa21*)	0.30±0.20 c	10.20±1.68 b	MS
BC_2F_2 (homozygous *Xa21*)	0.30±0.30 c	1.00±0.29 c	R
BC_2F_2 (heterozygous *Xa21*)	0.30±0.10 c	1.10±0.22 c	R

Note: The data shows the average of five leaves for each tested plant at 21 days after wounding and inoculation. The values represent the means ± standard deviation (SD) of 5 replicates. Means accompanied by different letters are significantly different at $P < 0.05$ (Duncan's test).

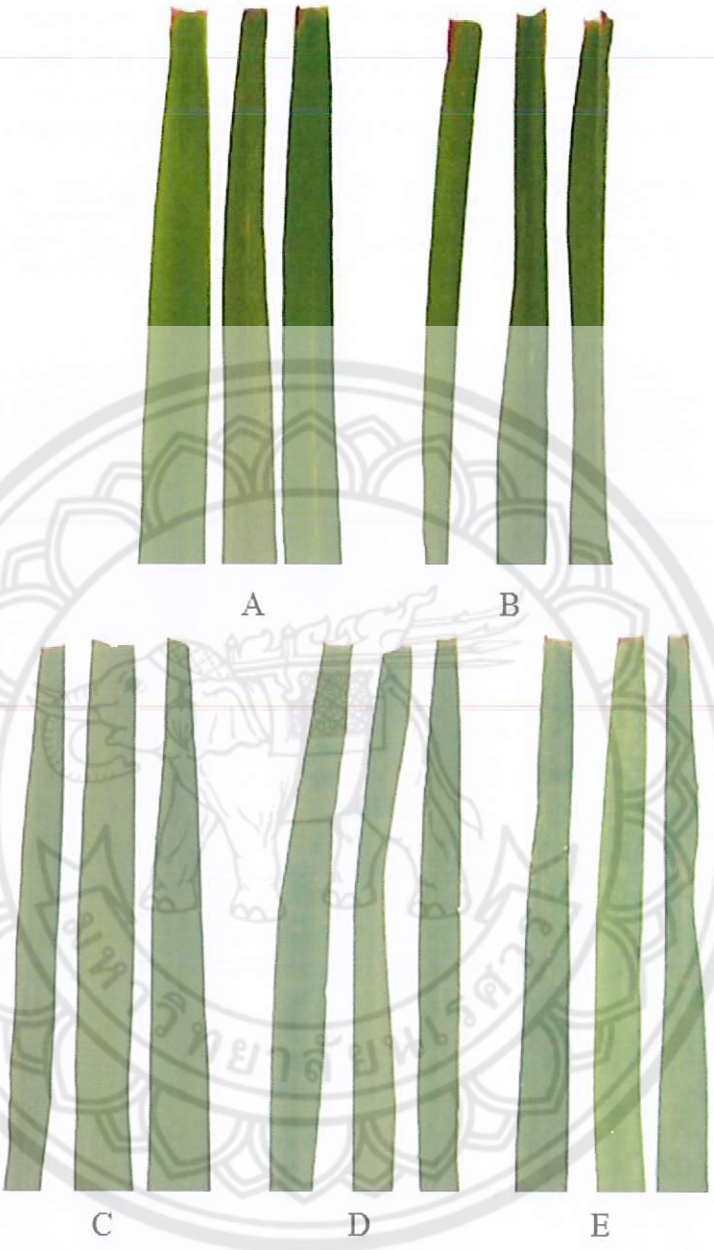


Figure 17 Lesion phenotypes of RD47, IRBB21 and BC_2F_2 leaves clipped without *Xoo*

Note: The lesion phenotypes were examined at 21 days after clipping. (A) RD47, (B) IRBB21, (C) BC_2F_2 carrying null-*Xa21*, (D) BC_2F_2 carrying homozygous *Xa21*, (E) BC_2F_2 carrying heterozygous *Xa21*.

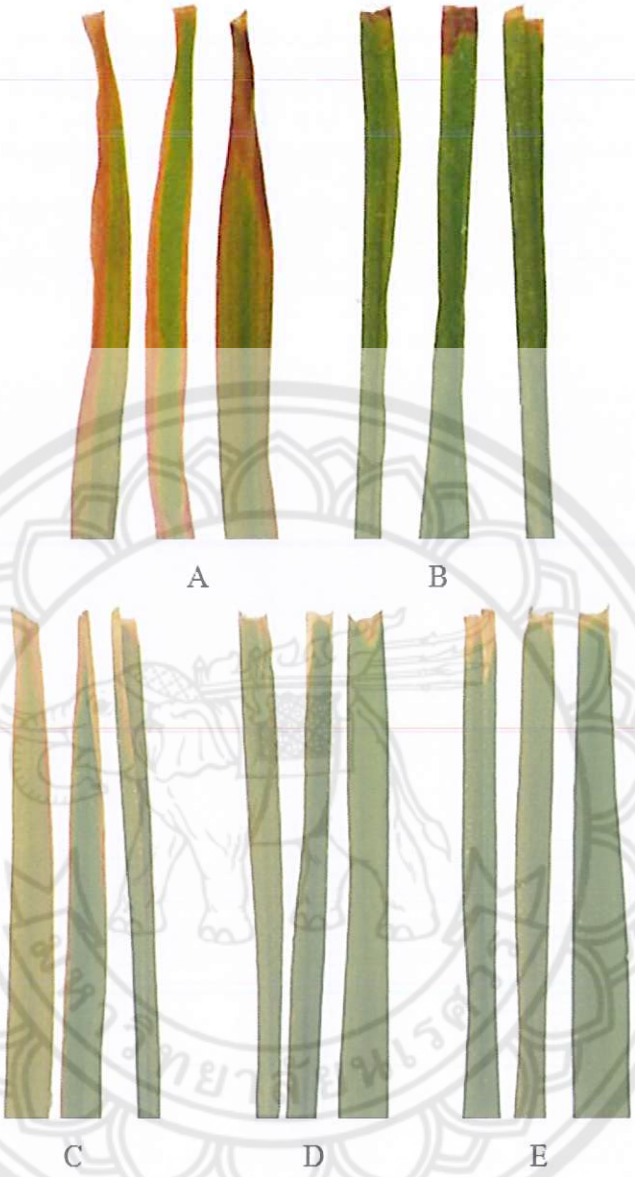


Figure 18 Lesion phenotypes of RD47, IRBB21 and BC_2F_2 leaves clipped with *Xoo*

Note: The lesion phenotypes were examined at 21 days after clipping. (A) RD47, (B) IRBB21, (C) BC_2F_2 carrying null-*Xa21*, (D) BC_2F_2 carrying homozygous *Xa21*, (E) BC_2F_2 carrying heterozygous *Xa21*.

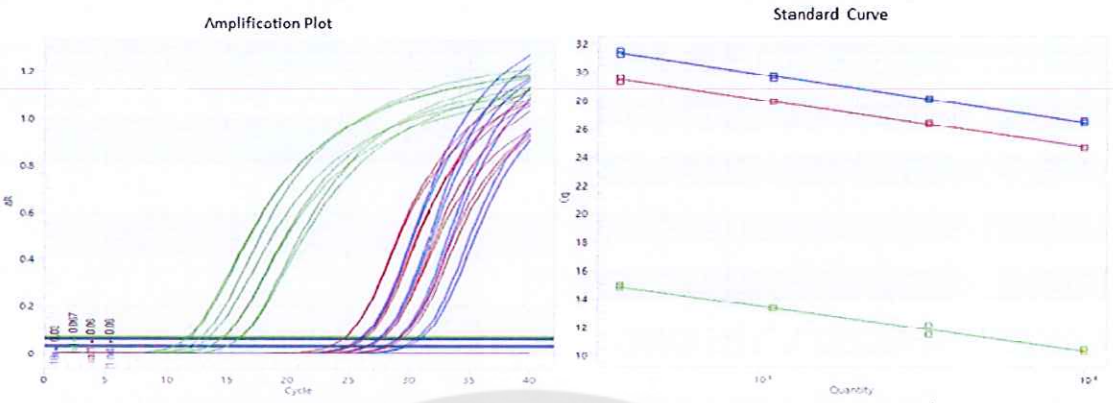
Expression analysis of *Xa21* in wounded and *Xoo*-infected RD47, IRBB21 and BC_2F_2 plants by real-time quantitative PCR (qPCR)

1. Optimization of qPCR assay

To determine an optimized qPCR assay, 4-fold serial dilutions of cDNA templates from RD47, IRBB21 and BC_2F_2 progenies were run to check the efficiency of the reaction and used the results to generate a standard curve. The standard curve is constructed by plotting the log of the starting quantity of template against the Ct value obtained during amplification of each dilution (Figure 19). The coefficient of determination (R^2) of the linear regression line and amplification efficiency (E) for *18s rRNA*, *PAL* and *Xa21* genes shown in Figure 19C indicate that the qPCR assay was optimized ($R^2 > 0.980$ and E = 90–110 %) (Ish-Shalom and Lichter, 2010; Freeman, 2013; Wagner, 2013).

2. Real time quantification

To analyze the expression of *PAL* and *Xa21* genes to *Xoo* inoculation, at the 6-leaf stage (45-60 days), the rice leaves of RD47, IRBB21 and BC_2F_2 progenies were inoculated with *Xoo* inoculum by the clipping method (Kauffman, et al., 1973). The inoculated leaves were collected at 0, 24, 48, 72 h and 7 days after clipping for RNA analysis. The mock treatment was performed by clipping with *Xoo*-free scissors. Real-time qPCR was performed in triplicate on cDNA of RD47, IRBB21 and BC_2F_2 progenies along with a no template control in parallel for each gene. The melting-curve analysis was performed by the PCR machine after 45 cycles of amplification. The Ct mean values for the *18s rRNA* which was a reference gene on the rice samples under study were used to compare the expression rates between *PAL* and *Xa21* within the set of samples. All genes showing a single pick (Figure 20).



A B

Assay	Efficiency	Equation	R^2
18s	99.28 ± 0%	y=-3.34x+46.57	0.996
pal	100.1 ± 0%	y=-3.32x+29.11	0.990
xa21	101.79 ± 0%	y=-3.28x+44.46	0.997

C

Figure 19 A standard curve to assess reaction optimization of qPCR assay

Note: A standard curve was generated using a 4-fold dilutions of a template. (A) Amplification curves of the dilution series, (B) Standard curve with the Ct plotted against the log of the starting quantity of templates for each dilution. (C) % efficiency for each primer

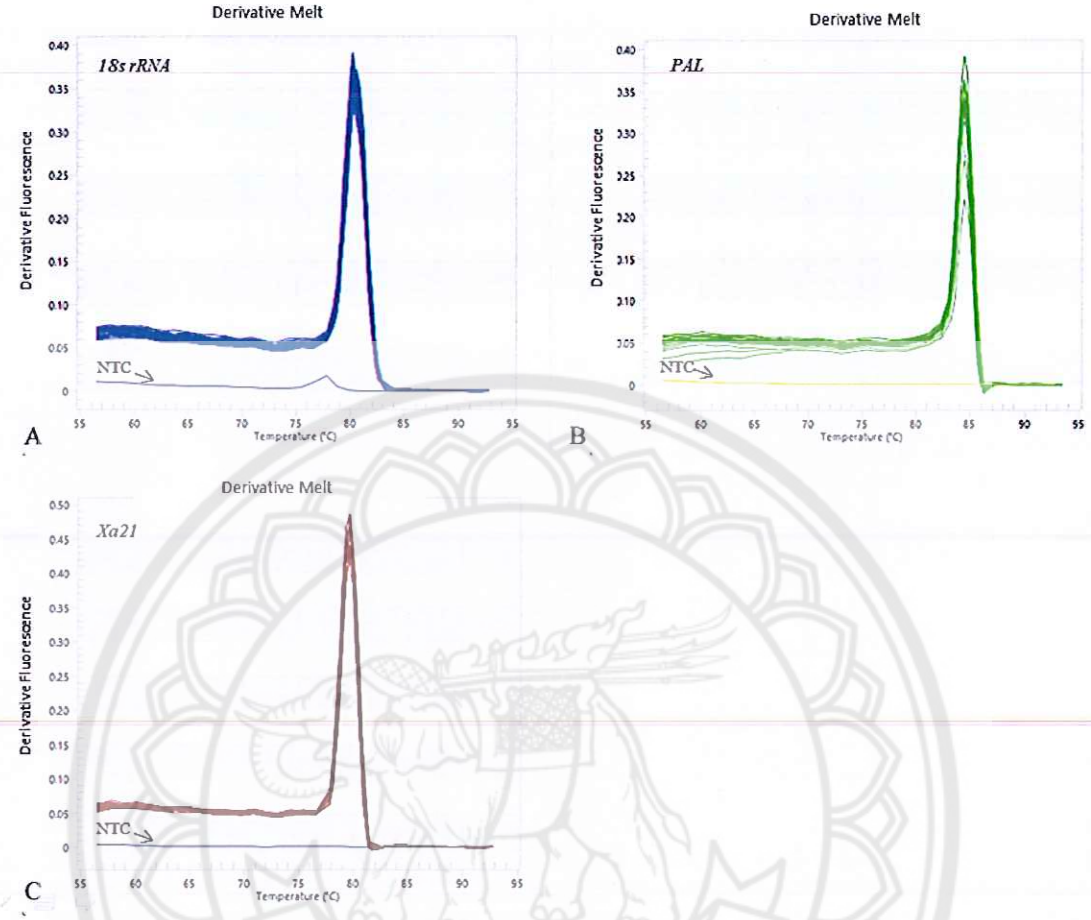


Figure 20 Specificity of real-time PCR amplification

Note: Dissociation curves for three genes with a single peak obtained from technical replicates along with a no template control (NTC). (A) Melting curve of *18s rRNA*, (B) Melting curve of *PAL*, (C) Melting curve of *Xa21*.

3. Differential expression of *Xa21* in wounded and *Xoo*-infected IRBB21 and heterozygous-*Xa21* BC_2F_2 plants

Differential expression in IRBB21 and heterozygous-*Xa21* BC_2F_2 plants after wounding and *Xoo*-inoculation at 24 h, 48 h, 72 h and 7 days relative to IRBB21 plants after wounding and *Xoo*-inoculation at 0 h was measured by qPCR on the *Xa21* gene (Table 5 and Figure 21). The expression of *Xa21* in IRBB21 was induced by wounding and *Xoo*-inoculation as expected. The expression profiles of *Xa21* investigated by qPCR were different from the expression profiles of *Xa21* previously shown in RT-PCR. The expression of *Xa21* was induced within 24 h after wounding and *Xoo*-inoculation. The wide range of *Xa21* expression levels between biological replicates could be, if not technical errors, an effect of different efficiencies of cDNA synthesis in each replicate causing unequal amounts of cDNA templates. The *Xa21* expression profiles of individual replicates (Appendix B) demonstrated that the expression patterns were inconsistent between replicates. However, induction of *Xa21* was lasting at least a few days after wounding and *Xoo* infection. Surprisingly, the relative expression levels of *Xa21* transcripts responding to wounding were greater than *Xoo*-inoculation. The recognition of *Xoo* elicitors by *Xa21* may be a signal to suppress the *Xa21* expression once the defense cascade is induced.

Comparing to IRBB21, the expression of *Xa21* was also induced in wounded heterozygous-*Xa21* BC_2F_2 but the relative expression levels of *Xa21* transcripts were 2-3 folds lower. This could be an effect of plant genotypes with different *Xa21* copy numbers. Unexpectedly, the expression of *Xa21* in heterozygous-*Xa21* BC_2F_2 was burst at 0 h after *Xoo* inoculation and gradually declined after that (Figure 22). This expression profile of *Xa21* contradicted with the gradual increase of *Xa21* transcript levels after *Xoo* inoculation in heterozygous-*Xa21* F_2 (Figure 14). Induction of *Xa21* expression by other unknown elicitors might be initiated before the selected heterozygous-*Xa21* BC_2F_2 plants were inoculated with *Xoo*. Inconsistency of the *Xa21* expression pattern was also observed between replicates (Appendix B).

Although the expression profiles of *Xa21* demonstrated by RT-PCR and qPCR were rather different in details, they exhibited similar trend in that *Xa21* was constitutively expressed and induced at early periods (0-72 h) after wounding and *Xoo* infection. These results contradict the previous report showing that the *Xa21*

expression was independent of wounding or *Xoo* infection (Century et al., 1999). However, the expression levels of *Xa21* after induction were regulated individually in each plants.

Table 5 The relative expression levels of *Xa21* and *PAL* genes in rice IRBB21 and heterozygous-*Xa21* BC_2F_2 after wounding and *Xoo* inoculation

		PAL		*Xa21*	
	Time (h)	Wounding	*Xoo* inoculation	Wounding	*Xoo* inoculation
IRBB21	t=0	1.00±0.00 a	1.00±0.00 a	1.00±0.00 a	1.00±0.00 c
	t=24	2.54±2.06 a	1.16±0.77 a	3.07±0.86 a	1.07±0.06 bc
	t=48	2.64±0.96 a	1.26±0.94 a	3.22±1.49 a	2.09±0.32 a
	t=72	5.35±5.31 a	1.99±1.69 a	3.99±3.10 a	1.99±0.64 ab
	t=7d	2.96±2.34 a	1.56±1.28 a	2.05±0.92 a	1.46±0.87 abc
BC_2F_2	t=0	n/a	n/a	1±0.00 a	1±0.00 a
	t=24	n/a	n/a	1.99±0.72 a	0.61±0.04 ab
	t=48	n/a	n/a	2.44±0.71 a	0.67±0.30 ab
	t=72	n/a	n/a	2.16±0.66 a	0.44±0.17 b
	t=7d	n/a	n/a	1.51±0.78 a	0.30±0.06 b

Note: Values are means of three replications ± SD. Different lowercase letters indicate significant difference at the $p < 0.05$ in Duncan's tests. n/a = not applicable.

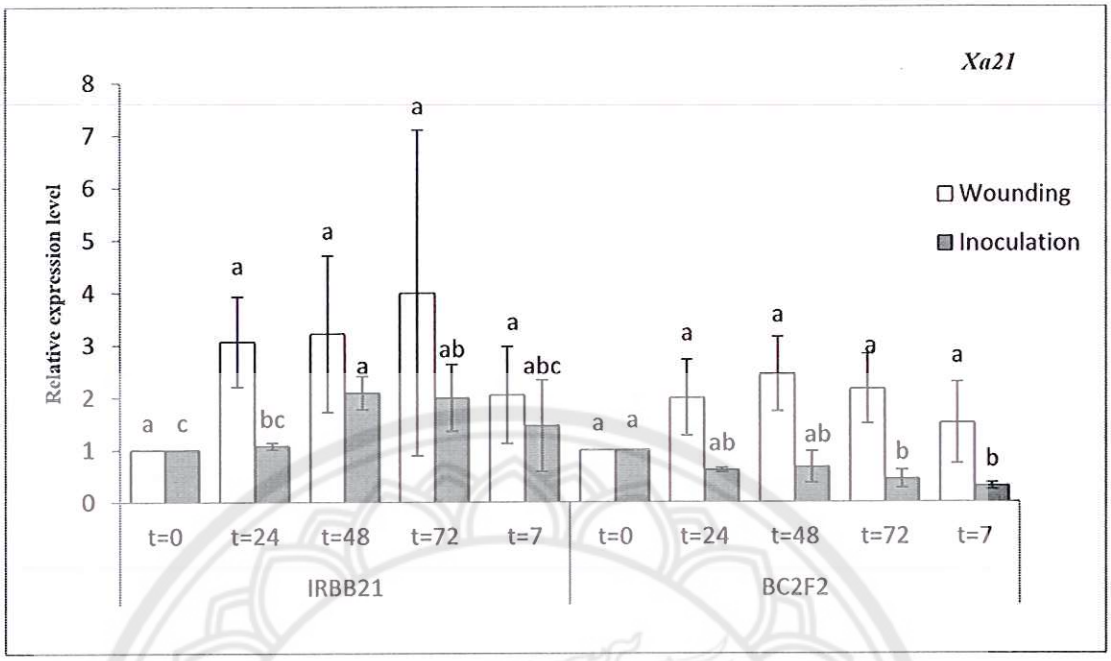


Figure 21 The relative expression levels of *Xa21* in rice IRBB21 and heterozygous-*Xa21* BC_2F_2 after wounding and *Xoo* inoculation

Note: Expression analysis of *Xa21* was carried out at 0, 24, 48 72h and 7d post wounding and *Xoo* inoculation. IRBB21, a donor parent; BC_2F_2, backcross progenies with the heterozygous-*Xa21* genotype. The expression level was calculated relative to the expression level in T=0 : the control was set to 1 and the relative fold of increase/decrease was calculated. The data were normalized by using *18S rRNA* expression. Values are means ± SD (n = 3)

4. Differential expression of *PAL* in wounded and *Xoo*-infected IRBB21 and heterozygous-*Xa21* BC_2F_2 plants

Differential expression in IRBB21 and heterozygous-*Xa21* BC_2F_2 plants after wounding and *Xoo*-inoculation at 24 h, 48 h, 72 h and 7 days relative to IRBB21 plants after wounding and *Xoo*-inoculation at 0 h was measured by qPCR on the *PAL* gene (Table 5 and Figure 22). In IRBB21, the expression of *PAL* was also induced after wounding and *Xoo*-inoculation as expected. The expression profiles of *PAL* investigated by qPCR were different from the expression profiles of *PAL* previously shown in RT-PCR. The expression of *PAL* was induced within 24 h and lasting for 72

h after wounding and *Xoo*-inoculation. The wide range of *PAL* expression levels between biological replicates was also observed. The *PAL* expression profiles of individual replicates (Appendix B) demonstrated that the expression patterns were quite similar but highly different in the expression levels between replicates. As found in the *Xa21* case, the relative expression levels of *PAL* transcripts responding to wounding were also greater than *Xoo*-inoculation. However, the expression pattern of *PAL* either in response to wounding or *Xoo* infection corresponded to the expression pattern of *Xa21*. This may imply that the expression of *PAL* and *Xa21* was similarly induced at initial stage of wounding and *Xoo* infection.

Although the expression of *PAL* was detected in F_1 and $F_{2\text{-}2}$ carrying heterozygous-*Xa21* by RT-PCR (Figure 15 and 16), no *PAL* expression was detected in heterozygous-*Xa21* BC_2F_2 plants treated with either wounding or *Xoo*-inoculation. The result was consistent in all three biological replicates (Appendix B). F_1, $F_{2\text{-}2}$ and BC_2F_2 plants contained a single copy of the *Xa21* gene in different IRBB21 genetic background. BC_2F_2 plants contained only 12.5% IRBB21 genetic background. The previous RT-PCR results also showed that no *PAL* expression was detected in null-*Xa21* RD47 and $F_{2\text{-}1}$ plants. Taken these results together, if not technical errors, it is possible that the *PAL* expression is *Xa21*-dependent and required IRBB21 genetic background. However, BC_2F_2 carrying either homozygous *Xa21* or heterozygous *Xa21* exhibited similar BB resistance phenotype (Figure 18). Tonnessen, et al. (2015) reported that *OsPAL4* QTL contributed to BB resistance in rice. *Xa21*-mediated BB resistance in BC_2F_2 may induce alternative defense pathway.

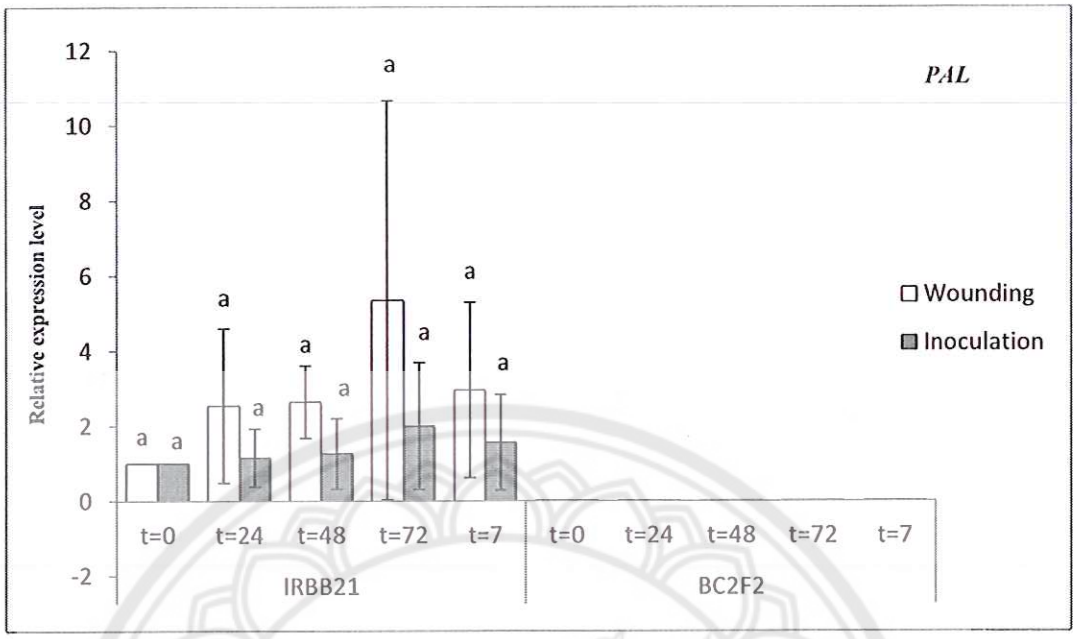


Figure 22 The relative expression levels of *PAL* in rice IRBB21 and heterozygous-*Xa21* BC_2F_2 after wounding and *Xoo* inoculation

Note: Expression analysis of *PAL* was carried out at 0, 24, 48 72h and 7d post wounding and *Xoo* inoculation. IRBB21, a donor parent; BC_2F_2, backcross progenies with the heterozygous *Xa21* genotype. The expression level was calculated relative to the expression level in T=0 : the control was set to 1 and the relative fold of increase was calculated. The data were normalized by using *18S rRNA* expression. Values are mean ± SD (n = 3).

CHAPTER V

CONCLUSION

Rice (*Oryza sativa* L.) is one of the most widely consumed staple crops in the world, feeding about half of the world population. Bacterial Blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a serious disease of rice. The *Xa21* gene shows broad-spectrum resistance and has been widely used to improve BB resistance in rice worldwide.

In this study, we demonstrated here the expression of *Xa21* and defense-related genes, *PR1b*, *PR10a* and *PAL*, in rice cultivars RD47, IRBB21 and their cross progenies including F_1, F_2 and BC_2F_2.

1. Expression of defense-related and *Xa21* genes in response to wounding and *Xoo* infection in F_1 hybrid rice (RD47 x IRBB21) by RT-PCR.

The transcripts of *PR1b* and *PR10a* were expressed in both RD47 and F_1 plants. They were insignificantly induced in *Xoo*-infected RD47 plants but were significantly induced in *Xoo*-infected F_1 plants. The *PAL* expression was triggered either by wounding or *Xoo* infection in F_1 plants, not in RD47 plants, but highly induced in *Xoo*-infected F_1 plants.

2. Characterization of *Xa21* and *PAL* expression in rice RD47, IRBB21 and F_2 plants by RT-PCR.

The *Xa21* gene was constitutively expressed and induced by wounding and *Xoo* infection in rice carrying either homozygous or heterozygous *Xa21*. The *Xa21* expression induced by wounding was not long-lasting or dropped after the induction peak if no *Xoo* infected the plant while the prolonged induction of the *Xa21* expression was observed in the *Xoo*-infected plants. The expression of *PAL* was induced in IRBB21 and F_2 carrying heterozygous *Xa21* plants but no *PAL* transcripts were detected in RD47 and F_2 carrying null-*Xa21* plants. The *Xa21* gene in IRBB21 and F_2 plants may involve in induction of *PAL* expression after wounding and *Xoo*-inoculation.

3. The expression of *PAL* and *Xa21* in response to wounding and *Xoo* infection in IRBB21 and BC_2F_2 carrying heterozygous *Xa21* by qPCR

The relative expression levels of *Xa21* transcripts in response to wounding were greater than *Xoo*-inoculation in both IRBB21 and heterozygous-*Xa21* BC_2F_2. The relative expression levels of *PAL* transcripts in response to wounding were also greater than *Xoo*-inoculation in IRBB21. No *PAL* expression was detected in heterozygous-*Xa21* BC_2F_2 plants treated with either wounding or *Xoo*-inoculation. However, the expression pattern of *PAL* either in response to wounding or *Xoo* infection corresponded to the expression pattern of *Xa21* in IRBB21. It is possible that the *PAL* expression is *Xa21*-dependent and required IRBB21 genetic background (Figure 23).

Evaluation of BB resistance in BC_2F_2 inoculated with *Xoo* isolate Phitsanulok showed that the resistance levels of BC_2F_2 carrying homozygous and heterozygous *Xa21* were not significantly different from IRBB21. This suggests that *Xa21*-mediated BB resistance in BC_2F_2 may be induced by the alternative defense pathway (Figure 23).

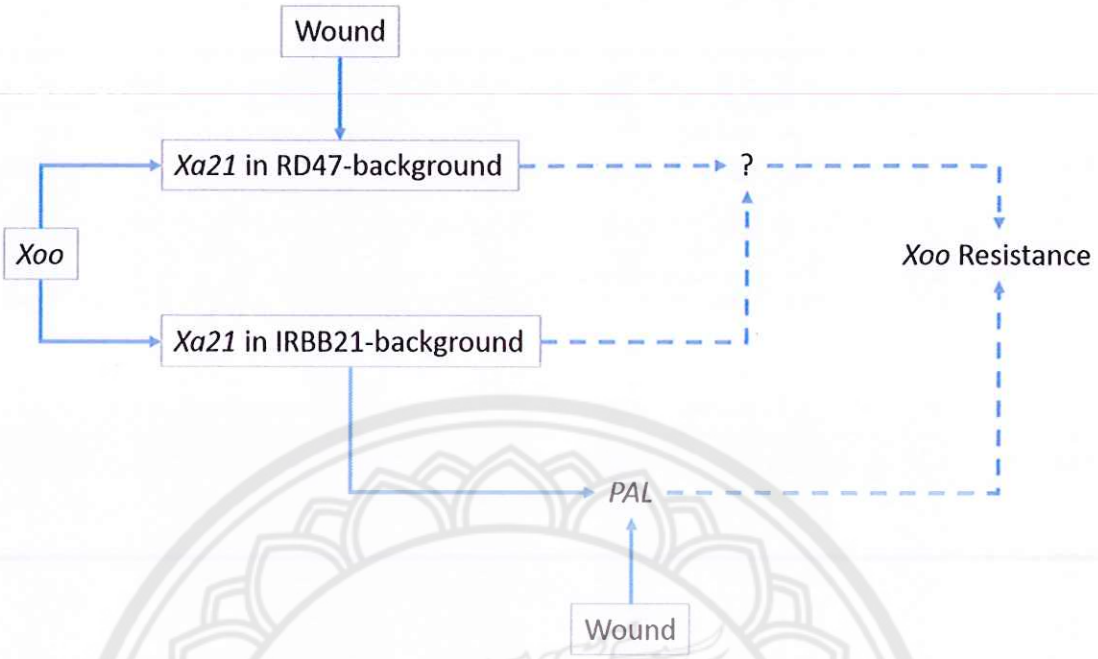


Figure 23 The deduced *Xa21*-mediated *Xoo* resistance pathway in BC_2F_2 progenies of RD47 x IRBB21 and IRBB21

Note: The expression of *Xa21* is induced either by *Xoo* infection and wounding in both BC_2F_2 progenies of RD47 x IRBB21 and IRBB21. The expression of *PAL* is also induced either by *Xoo* infection and wounding but it is *Xa21*-dependent and required IRBB21 genetic background. The dash lines indicate possible pathways of *Xa21*-mediated *Xoo* resistance in BC_2F_2 progenies of RD47 x IRBB21 and IRBB21.

REFERENCES

REFERENCES

Achnine, L., Blancaflor, E. B., Rasmussen, S., & Dixon, R. A. (2004). Colocalizationof L-phenylalanine Ammonia-lyase and Cinnamate 4- Hydroxylase for Metabolic Channeling in Phenylpropanoid Biosynthesis. *The Plant Cell*, *16*, 3098-3109.

Asselin, A., Grenier, J., & Côté, F. (1985). Light-influenced extracellular accumulation of *b* (pathogenesis-related) proteins in *Nicotiana* green tissue induced by various chemicals or prolonged floating on water. *Canadian Journal of Botany*, *63*, 1276-1283.

Bate, N. J., Orr, J., Ni, W., Meroni, A., Nadler-Hassar, T., Doerner, P, W., … Elkind, Y. (1994). Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate determining step in natural product synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 7608-7612.

Beckers, G. J. M., & Spoel, S. H. (2006). Fine-Tuning Plant Defence Signalling: Salicylate Versus Jasmonate. *Plant Biology*, *8*, 1-10.

Benhamou, N., Grenier, J., & Asselin, A. (1991). Immunogold localization of pathogenesis-related protein P14 in tomato root cells infected by Fusarium oxysporum f. sp. radicis-lycopersici. *Physiological and Molecular Plant Pathology*, *38*(4), 237-253.

Bonas, U., Stall, R. E., & Staskawicz, B. J. (1989). Genetic and structural characterization of the avirulence gene avrBs3 from Xanthomonas campestris pv. vesicatoria. *Molecular Genetics and Genomics, 218*, 127-136.

Bowles, D.J. (1990). Defense-related proteins in higher plants. *Annual Review of Biochemistry, 59*, 873-907.

Brar, D. S., & Khush, G. S. (2003). Utilization of wild species of genus *Oryza* in rice improvement. In Nanda J.S., & Sharma, S.D. (eds.), *Monograph on Genus Oryza* (pp. 283-309). Enfield, NH: Science Publishers.

Bureau of Rice Research and Development: Rice Department. (2016). *Rice Knowledge Bank Varieties*. Retrieved March 10, 2017, from http://www.brrd.in.th/rkb/varieties/index.php.htm

Camera, S. L., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M., & Heitz, T. (2004). Metabolic Reprogramming in plant innate Immunity: The Contributions of Phenylpropanoid and Oxylipin Pathways. *Immunological Reviews*, *198*, 267-284.

Century, K. S., Lagman, R. A., Adkisson, M., Morlan, J., Tobias, R., Schwartz, K., … Whalen, M. C. (1999). Short communication: developmental control of *Xa21*-mediated disease resistance in rice. *Plant Journal*, *20*, 231-236.

Chen, X., & Roland, P. C. (2011). Innate immunity in rice. *Trends in Plant Science*, *16*, 451-459.

Constabel, C.P., Yip, L., Patton, J. J., & Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar: cloning and expression in response to wounding and herbivory. *Plant Physiology*, *124*, 285-296.

Coffman, W. R., & Herrera, R. M. (1980). Rice. In: Fehr W. R. and Hadley H. H. (eds.) *Hybridization of Crop Plants* (pp. 511-522). Madison, Wisconsin, USA: American Society of Agronomy.

Coquoz, J. L., Buchala, A., & MeÂtraux, J-P. (1998). The biosynthesis of salicylic acid in potato plants. *Plant Physiology*, *117*, 1095-1101.

Dangl, J. L., Dietrich, R. A., & Richberg, M. H. (1996). Death don't have no mercy: Cell death programs in plant-microbe interactions. *Plant Cell*, *8*, 1793-1807.

Dawe, D. (1998). Re-Energizing the Green Revolution in Rice. *American Journal of Agricultural Economics*, *80*, 948-953.

Despres, C., Subramaniam, R., Matton, D. P., & Brission, N. (1995). The activation of the potato PR-10a gene requires the phosphorylation of the nuclear factor PBF-1. *Plant Cell*, *7*, 639-647.

Dixon, R. A., & Pavia, N. L. (1995). Stress-induced phenylpropanoid metabolism. *Plant Cell*, *7*, 1085-1097.

Duan, C., Yu, J., Bai, J., Zhu, Z., & Wang, X. (2014). Induced defense responses in rice plants against small brown planthopper infestation. *The Crop Journal*, *2*, 55-62.

Durrant, W. E., & Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology*, *42*, 185-209.

Eamchit, S., & Mew, T. W. (1982). Comparison of virulence of *Xanthomonas Campestris* pv. oryzae in Thailand and the Philippines. *Plant Disease*, *66*, 556-559.

Ellingboe, A. (1982). Genetical aspects of active defense. In Wood R.K.S. (Ed.), *Active Defense Mechanisms in Plants* (pp. 179-192). New York: Plenum Press.

Ergon, A., Klemsdal, S. S., & Tronsmo, A. M. (1998). Interactions between cold hardening and *Microdochium nivale* infection on expression of pathogenesis-related genes in winter wheat. *Physiological and Molecular Plant Pathology*, *53*, 301-310.

Ernst, D., Schraudner, M., Langebartels, C., & Sandermann, H. Jr. (1992). Ozone-induced changes of mRNA levels of β-1,3-glucanase, chitinase and 'pathogenesis-related' protein 1b in tobacco plants. *Plant Molecular Biology*, *20*, 673-682.

Freeman, L. A. (2013). Lipoproteins and Cardiovascular Disease: Methods and Protocols. In Freeman L. A. (Ed.), *Northern analysis of gene expression* (pp. 85-121). New York: Humana Press.

Fidantsef, A. L., Stout, M. J., Thaler, J. S., Duffey, S. S., & Bostock, R. M. (1999). Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. *Physiological and Molecular Plant Pathology*, *54*, 97-114.

Gao, Z. M., Wang, X. C., Peng, Z. H., Zheng, B., & Liu, Q. (2012). Characterization and primary functional analysis of phenylalanine ammonia-lyase gene from Phyllostachys Edulis. *Plant Cell Reports*, *31*, 1345-1356.

Gaudet, D. A., Laroche, A., Frick, M., Davoren, J., Puchalski, B., & Ergon, A. (2000) Expression of plant defense-related (PR-protein) transcripts during hardening and dehardening of winter wheat. *Physiological and Molecular Plant Pathology*, *57*, 15-24.

Gopalakrishnan, S., Sharma, R. K., Anand Rajkumar K., Joseph, M., Singh, V. P., & Singh, A. K. (2008). Integrating marker assisted background analysis with foreground selection for identification of superior bacterial blight resistant recombinants in Basmati rice. *Plant breeding*, *127*, 131-139.

Gordon-Weeks, R., Sugars, J. M., Antoniw, J. F., & White, R. F. (1997). Accumulation of a novel PR1 protein in *Nicotiana langsdorfii* in response to virus infection or treatment with salicylic acid. *Physiological and Molecular Plant Pathology*, *50*, 263-273.

Gruner, D. S., & Mooney, K. A. (2013). Green grass and high tides: Grazing lawns in terrestrial and aquatic ecosystems. *Oikos Journal*, *122*, 313-316.

Halim, V. A., Hunger, A., Macioszek, V., Landgraf, P., Nürnberger, T., Scheel, D., Rosahl, S. (2004). The oligopeptide elicitor Pep-13 induces salicylic acid-dependent and -independent defense reactions in potato. *Physiological and Molecular Plant Pathology*, *64*(6), 311-318.

Holopainen, J. K., Heijari, J., Nerg, A. M., Vuorinen, M., & Kainulainen, P. (2009). Potential for the use of exogenous chemical elicitors in disease and insect pest management of conifer seedling production. *The Open Plant Science Journal*, *2*, 17-24.

Ikeda, R., Khush, G. S., & Tabien, R. E. (1990). A new resistance gene to bacterial blight derived from *O. longistaminata*. *Jpn. J. Breed*, *40*, 280-281.

International Rice Research Institute: IRRI. (2002).*Growth and Morphology of the rice plant*. Manila: Rice Knowledge Bank.

International Rice Research Institute: IRRI. (1996). *Growth and Morphology of the rice plant*. Manila: Rice Knowledge Bank. (The lesion length was measured following the scoring system)

Ish-Shalom, S., & Lichter, A. (2010). Analysis of fungal gene expression by real time quantitative PCR. *Molecular and Cell Biology Methods for Fungi*, *368*, 103-114.

Jain, M., Tyagi, A. K., & Khurana, J. P. (2006). Molecular characterization and differential expression of cytokinin-responsive Type-aResponse Regulators in Rice (*Oryza sativa*). *Plant Biology*, *6*, 1-11.

Jones, D. A., & Takemoto, D. (2004). Plant innate immunity direct and indirect recognition of general and specific pathogen-associated molecules. *Current Opinion in Immunology*, *16*, 48-62.

Jwa, N. S., Agrawal, G. K., Rakwal, R., Park, C.H., & Prasad, A. V. (2001). Molecular cloning and characterization of a novel jasmonate inducible pathogenesis-related class 10 protein gene, JIOsPR10, from rice (*Oryza sativa* L.) seedling leaves. *Biochemical and Biophysical Research Communications*, *286*, 973-983.

Kaul, M. L. H. & Sharma, K. K. (1987). Bacterial blight of rice. *A Review Biol. Zent. Bl.*, *106*, 141-167.

Klessig, D. F., & Malamy, J. (1994). The salicylic acid signal in plants. *Plant Mol Biol*, *26*, 1439-1458.

Khush, G. S., Bacalangco, E. & Ogawa, T. (1990). A new gene for resistance to bacterial blight from *O. longistaminata*. *Rice Genetics Newsletter*, *7*, 121-122.

Khush, G. S., Mackill, D. J., & Sidhu, G. S. (1989). Breeding Rice for Resistance to Bacterial Leaf Blight. In IRRI. (Ed.), *Bacterial Blight of Rice* (pp. 207-217). International Rice Research Institute (IRRI), Philippines: Manila.

Krzymowska, M., Konopka-Postupolska, D., Sobczak, M., Macioszek, V., Ellis, B. E., & Hennig, J. (2007). Infection of tobacco with different *Pseudomonas syringae* pathovars leads to distinct morphotypes of programmed cell death. *The Plant Journal*, *50*, 253-264.

Kuc, J. (1982). Induced immunity to plant disease. *Bioscience*, *32*, 854-860.

Lam, E., Kato, N., & Lawton, M. (2001). Programmed cell death, mitochondria and the plant hypersensitive response. *Nature*, *411*, 848-853.

Leach, J. E., & White, F. F. (1996). Bacterial a virulence genes. *Annual Review of Phytopathology*, *34*, 153-179.

Lee, K. H., Qi, G. H., & Sim, J. S. (1995). Metabolizable energy and amino acid availability of full-fat seeds, meals, and oils of flax and canola. *Poultry Science*, *74*, 1341-1348.

Li, L., & Steffens J. C. (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta*, *215*, 239-247.

Li, G., Song, C. F., Pang, X. M., Yang, Y., & Wang, J. S. (2009). Analysis of pathotypic and genotypic diversity of *Xanthomonas oryzae* pv. oryzae in China. *J. Phytopathol.*, *157*, 208-218.

Lopez-Gerena, J. (2006). *Mapping QTL controlling durable resistance to rice blast in the cultivar Oryzica Llanos 5* (Doctoral dissertation). Manhattan: Kansas State University.

Luo, Y., & Yin, Z. (2013). Marker-assisted breeding of Thai fragrance rice for semi-dwarf phenotype, submergence tolerance and disease resistance to rice blast and bacterial blight. *Molecular Breeding*, *32*, 709-721.

Malamy, J., Carr, J. P., Klessig, D. F., & Raskin, I. (2014). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science*, *80*(250), 1002-1004.

Mauch-Mani, B., & Slusarenko, A. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonialyasein the resistance of *Arabidopsis* to *Peronospora Parasitica*. *Plant Cell*, *8*, 203-212.

McDonald, B. A., & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology*, *40*, 349-379.

Mew, T.W. (1987). Current status and future prospects of research on bacterial blight of rice. *Annual Review of Phytopathology*, *25*, 359-382.

Mew, T.W., & Vera Cruz, C.M. (1979). Variability of *Xanthomonas oryzae*: specificity in infection of rice differentials. *Phytopathology*, *69*, 152-155.

Muthukrishnan, T., Liang, G. H., Trick, N. H., & Gill, B. S. (2001). Pathogenesis-related proteins and their gene in cereals. *Plant Cell Tissue Organ Culture*, *64*, 93-114.

Ochiai, H., Horino, O., Miyajima, K., & Kaku, H. (2000). Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* strains from Sri Lanka. *Phytopathology*, *90*, 415-421.

Office of Agricultural Economics. (2015). *World Rice Production Export values of Thai rice during 2006-2015.* Retrieved December 22, 2016, from http://www.oae.go.th/oaenew/OAE/index.html

Ogawa, T., Yamamoto, Y., Khush, G. S., & Mew, T. W. (1991). Breeding nearisogenic lines of rice with single gene for resistance to bacterial blight pathogen (*Xanthomonas campestris* pv. oryzae). *Japanese Journal of Breeding*, *41*, 523-529.

Oka, H. I., & Chang, W. T. (1962). Rice varieties intermediate between wild and cultivated forms and the origin of the Japonica type. *Botanical Bulletin-Academia Sinica Taipei Journal*, *3*, 109-131.

Ou, S.H. (1985). *Rice Diseases* (2nd ed.). Amsterdam, Netherlands: Commonwealth Mycological Institute.

Ozeretskovskaya, O. L., & Vasyukova, N. I. (2002). The use of elicitors for protection of cultured plants demands caution. *Applied Biochemistry and Microbiology*, *38*(3), 277-279.

Park, C. J., Lee, S. W., Chern, M., Sharma, R., Canlas, P. E., Song, M. Y., … Ronald, P. C. (2010). Ectopic expression of rice *Xa21* overcomes developmentally controlled resistance to *Xanthomonas oryzae* pv. *oryzae*. *Plant Science*, *179*, 466-471.

Pellegrini, L., Rohfritsch, O., Fritig, B., & Legrand, M. (1994). Phenylalanine ammonia-lyase in tobacco. *Plant Physiology*, *106*, 877-886.

Reiss, E., & Bryngelsson, T. (1996). Pathogenesis-related proteins in barley leaves, induced by infection with *Drechslera teres* (Sacc.) Shhoem. and by treatment with other biotic agents. *Physiological and Molecular Plant Pathology*, *49*, 331-341.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., & van Loon, L .C. (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell*, *10*, 1571-1580.

Piggott, N., Ekramoddoullah, A. K. M., Liu, J.J., & Xu, X. S. (2004). Gene cloning of a thaumatin-like (PR5) protein of western pine (*Pinus monticola* D.Don) and expression studies of the PR-5 group. *Physiological and Molecular Plant Pathology*, *64*, 1-8.

Ponciano, G., Yoshikawa, M., Lee, J. L., Ronald, P. C., & Whalen. M. C. (2006). Pathogenesis-related gene expression in rice is correlated with developmentally controlled *Xa21*-mediated resistance against *Xanthomonas oryzae* pv. oryzae. *Physiological and Molecular Plant Pathology*, *69*(4-6), 131-139.

Ponciano, G., Yoshikawa, M., Lee, J. L., Ronald, P.C., & Whalen, M. C. (2007). Pathogenesis-related gene expression in rice is correlated with developmentally controlled *Xa21*-mediated resistance against *Xanthomonas Oryzae* pv. oryzae. *Physiological and Molecular Plant Pathology*, *69*, 131-139.

Rawal, H. C., Singh, N. K., & Sharma, T. R. (2013). Conservation, divergence, and genome-wide distribution of *PAL* and *POX A* gene families in plants. *International Journal of Genomics*, *2013*, 1-10.

Reichert, J. M., Suzuki, L. E. A. S., Reinert, D. J., Horn, R., & Håkansson, I. (2009). Reference Bulk Density and Critical Degree-of-compactness for No-till Crop Production in Subtropical Highly Weathered Soils. *Soil and Tillage Research*, *102*, 242-254.

Ronald, P. C., Albano, B., Tabien, R., Abenes, L., Wu, K. S., McCouch, S., & Tanksley, S. D. (1992). Genetic and physical analysis of the rice bacterial blight disease resistance locus, *Xa21*. *Mol Gen Genet*, *236*(1), 113-120.

Rossi, M., Goggin, F. L., Milligan, S. B., Kaloshian, I., Ullman, D. E., & Williamson, V. M. (1998). The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proceedings of the National Academy of Sciences of the United States of America*, *95*, 9750-9754.

Roycewicz, P. S., & Malamy, J. E. (2014). Cell wall properties play an important role in the emergence of lateral root primordial from the parent root. *Journal of Experimental Botany*, *65*, 2057-2069.

Talukder, Z. I., Tharreau, D., & Price, A. H. (2004). Quantitative trait loci analysis suggests that partial resistance to rice blast is mostly determined by race–specific interactions. *New Phytologist*, *162*(1), 197-209.

Thipyapong, P., & Steffens, J. C. (1997). Tomato polyphenol oxidase (PPO): differential response of the PPO F promoter to injuries and wound signals. *Plant Physiology*, *115*, 409-418.

Tonnessen, B. W., Manosalva, P., Lang, J. M., Baraoidan, M., Bordeos, A., Mauleon R., Oard, J. (2015). Rice phenylalanine ammonia-lyase gene OsPAL4 is associated with broad spectrum disease resistance. *Plant Molecular Biology.*, *87*, 273-286.

Sanchez-Ballesta, M. T., Lafuente, M. T., Zacarias, L., & Granell, A. (2000). Phenylalanine ammonia-lyase in the response of citrusfruits to cold temperature. *Physiologia Plantarum*, *108*, 382-389.

Santén, K., Marttila, S., Liljeroth, E., & Bryngelsson, T. (2005). Immunocytochemical localization of the pathogenesis-related PR-1 protein in barley leaves after infection by *Bipolaris sorokiniana*. *Physiological and Molecular Plant Pathology*, *66*, 45-54.

Schweizer, P., Buchala, A., Silverman, P., Seskar, M., Raskin, I., & Metraux, J. P. (1997). Jasmonate-inducible genes are activated in rice by pathogen attack without a concomitant increase in Endogenous jasmonic acid levels. *Plant Physiology and Biochemistry*, *114*, 79-88.

Sattari, A., Fakheri, B., Hassan, F. S. C., & Noroozi, M. (2014). Blast resistance in rice: a review of breeding and biotechnology. *International Journal of Agriculture and Crop Science, 7*, 329-333.

Shen, Y., & Ronald, P. (2002). Molecular determinants of disease and resistance in interactions of *Xanthomonas oryzae* pv. oryzae and rice. *Microbes and Infection*, *4*, 1361-1367.

Smetanska, I. (2008). Production of secondary metabolites using plant cell cultures. *Advances in Biochemical Engineering / Biotechnology*, *111*, 187-228.

Stuiver, M. H., & Custers, J. H. (2001). Engineering disease resistance in plants. *Nature*, *411*, 865-868.

Strobel, N. E., Ji, C., Gopalan, S., Kuc, J. A., & He S. Y. (1996). Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZPss protein. *The Plant Journal*, *9*(4), 431-439.

Vallad, G. E., & Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science*, *44*, 1920-1934.

van Loon L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. *European Journal of Plant Pathology*, *119*, 243-254.

van Loon, L. C., Rep, M., & Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology*, *44*, 135-162.

Vaughan, D. A. (1994). *The wild relatives of rice: A genetic resources handbook* (p. 137). Manila: International Rice Research Institute, Manila.

Vaughan, D. A., Lu B. R., & Tomooka, N. (2008). The evolving story of rice evolution. *Plant Science*, *174*(4), 394-408.

Vogt, T. (2010). Phenylpropanoid Biosynthesis. *Molecular Plant*, *3*, 2-20.

Walters, D. R., Newton, A. C., & Lyon, G. D. (2005). Induced Resistance: helping plants to help themselves. *Biologist*, *52*, 28-33.

Wang, X. K., & Li, R. H. (1997). Determination and classification of subspecies of Asian rice and their inter-subspecies hybrids. *Chinese Science Bulletin*, *42*, 2596-2603.

Wagner, E. M. (2013). Monitoring gene expression: quantitative real-time rt-PCR. *Lipoproteins and Cardiovascular Disease*, *1027*, 19-45.

Werner, K., Friedt, W., & Ordon, F. (2005). Strategies for pyramiding resistance genes against the barley yellow mosaic virus complex (BaMMV, BaYMV, BaYMV-2). *Molecular Breeding*, *16*(1), 45-55.

Win, K. M., Korinsak, S., Jantaboon, J., Siangliw, M., Lanceras-Siangliw, J., Sirithunya, P., Vanavichit, A. (2012). Breeding the Thai jasmine rice variety KDML105 for non-age-related broad-spectrum resistance to bacterial blight disease based on combined marker-assisted and phenotypic selection. *Field Crops Research*, *137*, 186-194.

Xiong, L.Z., & Yang, Y. N. (2003). Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid inducible mitogen-activated protein kinase. *Plant Cell*, *15*, 745-759.

Xu, W. H., Wang, Y. S., Liu, G. Z., Chen, X., Tinjuangjun, P., Pi, L. Y., & Song, W. Y. (2006a). The autophosphorylated Ser686, Thr688, and Ser689 residues in the intracellular juxtamembrane domain of XA21 are implicated in stability control of rice receptor-like kinase. *The Plant Journal*, *45*, 740-751.

Yin, Z., Chen, J., Zeng, L., Goh, M., Leung, H., Khush, G. S., & Wang, G. L. (2000). Characterizing rice lesion mimic mutants and identifying a mutant with broad-spectrum resistance to rice blast and bacterial blight. *Molecular Plant-Microbe Interactions*, *13*, 869-876.

Zhang, J., Li, X., Jiang, G., Xu, Y., & He, Y. (2006). Pyramiding of Xa7 and Xa21 for the improvement of disease resistance to bacterial blight in hybrid rice. *Plant Breeding*, *125*(6), 600-605.

Zhang, Y., Luo, L., Xu, C., Zhang, Q., & Xing, Y. (2006). Quantitative trait loci for panicle size, heading date and plant height co-segregating in trait-performance derived near-isogenic lines of rice (*Oryza sativa*). *Theoretical and applied genetics*, *113*(2), 361-368.

Zhang, H., Li, G., Li, W., & Song, F. (2009). Transgenic strategies for improving rice disease resistance. *African Journal of Biotechnology*, *8*, 1750-1757.

Zhao, J., Davis, L. C., & Verporte, R. (2005). Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances*, *23*(4), 283-333.

Zhou, P., Tan, Y., He, Y., Xu, C., & Zhang, Q. (2003). Simultaneous improvement for four quality traits of Zhenshan 97, an elite parent of hybrid rice, by molecular marker-assisted selection. *Theoretical and applied genetics*, *106*, 326-331.

Zhou, L. G., & Wu, J. Y. (2006). Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. *Natural Product Reports*, *23*, 789-810.

APPENDIX

APPENDIX A SCORING SYSTEM FOR EVALUATION OF BB RESISTANCE (IRRI, 1996)

Scoring system used to evaluate breeding lines for BB resistance in the greenhouse and in the field.

Greenhouse Test		Field Test		
Lesion Length (cm)	Description	Scale	Diseased Leaf Area (%)	Description
0-5	R	1	1 - 5	R
>5 – 10	MR	3	6 - 12	MR
>10 – 15	MS	5	13 – 25	MS
>15 cm	S	7	26 – 50	S
		9	>50 %	S

Resistance (R)

Moderately Resistance (MR)

Moderately Susceptible (MS)

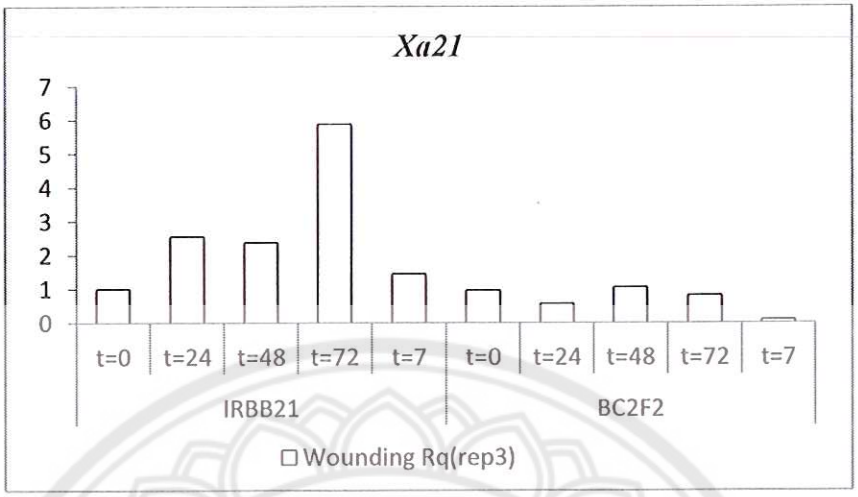
Susceptible (S)

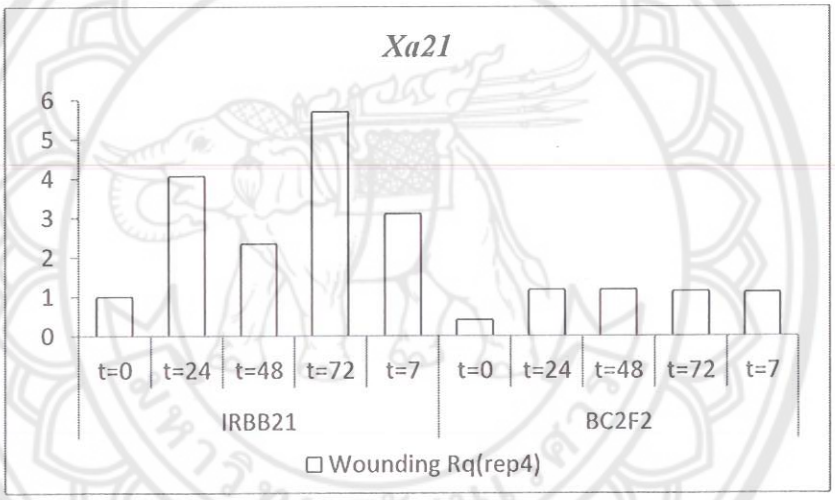
APPENDIX B RELATIVE QUANTIFICATION OF GENE EXPRESSION IN IRBB21 AND BC_2F_2 GIVEN WOUNDING AND *XOO* INOCULATION

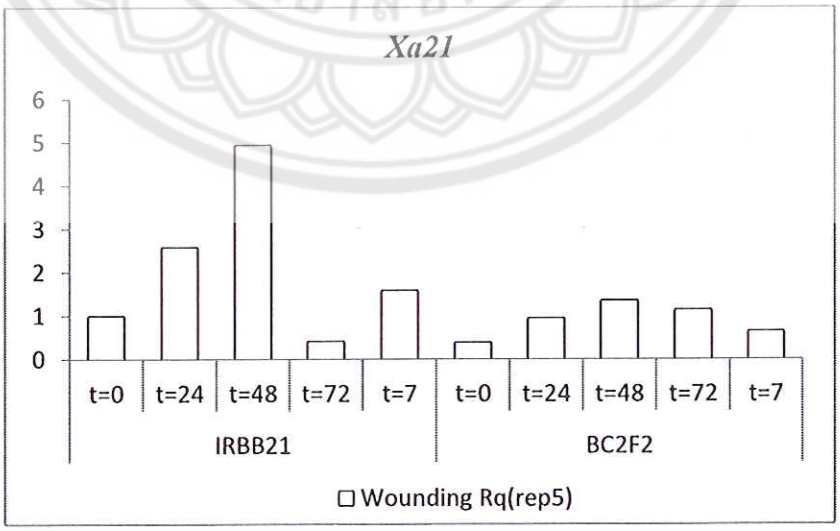
1. The *Xa21* gene

Wounding		Rq(rep1)	Rq(rep2)	Rq(rep3)	Average
IRBB21	t=0	1.00	1.00	1.00	1.00
	t=24	2.56	4.06	2.58	3.07
	t=48	2.37	2.34	4.94	3.22
	t=72	5.88	5.70	0.41	4.00
	t=7	1.45	3.11	1.58	2.05
BC2F2	t=0	0.96	0.41	0.38	0.58
	t=24	0.56	1.18	0.94	0.89
	t=48	1.05	1.17	1.35	1.19
	t=72	0.81	1.14	1.14	1.03
	t=7	0.09	1.11	0.64	0.61
Inoculation		**Rq(rep1)**	**Rq(rep2)**	**Rq(rep3)**	**Average**
IRBB21	t=0	1.00	1.00	1.00	1
	t=24	1.09	1.13	1.00	1.07
	t=48	1.74	2.34	2.20	2.09
	t=72	1.93	1.39	2.66	1.99
	t=7	1.36	0.64	2.38	1.46
BC2F2	t=0	2.16	2.74	2.01	2.30
	t=24	1.23	1.86	1.36	1.48
	t=48	2.40	0.24	0.18	0.93
	t=72	1.42	0.28	0.21	0.6
	t=7	0.61	0.57	0.42	0.53

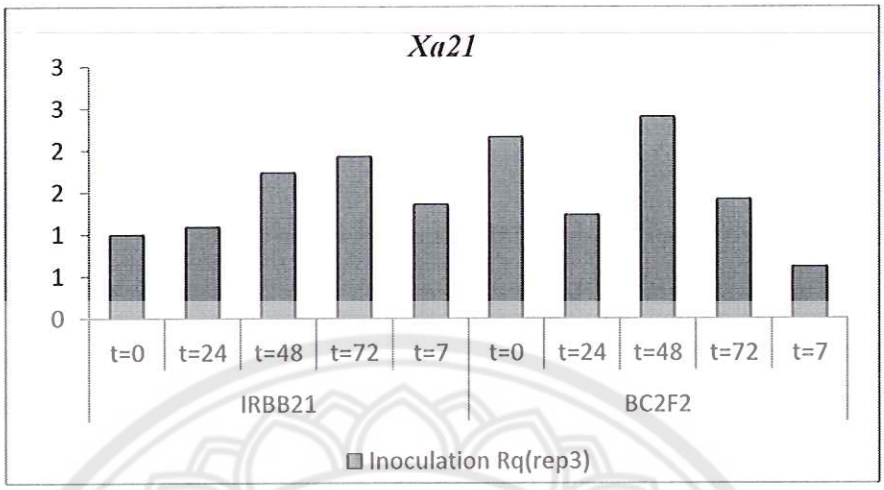
1.1 Relative quantification of *Xa21* after wounding

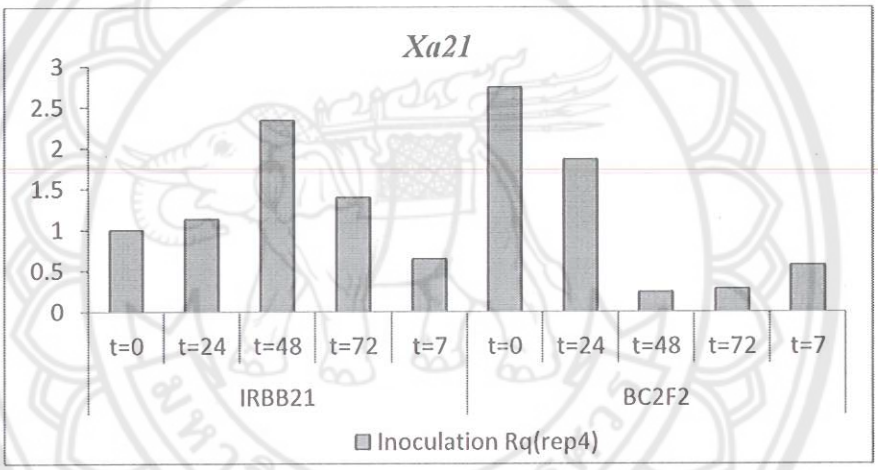


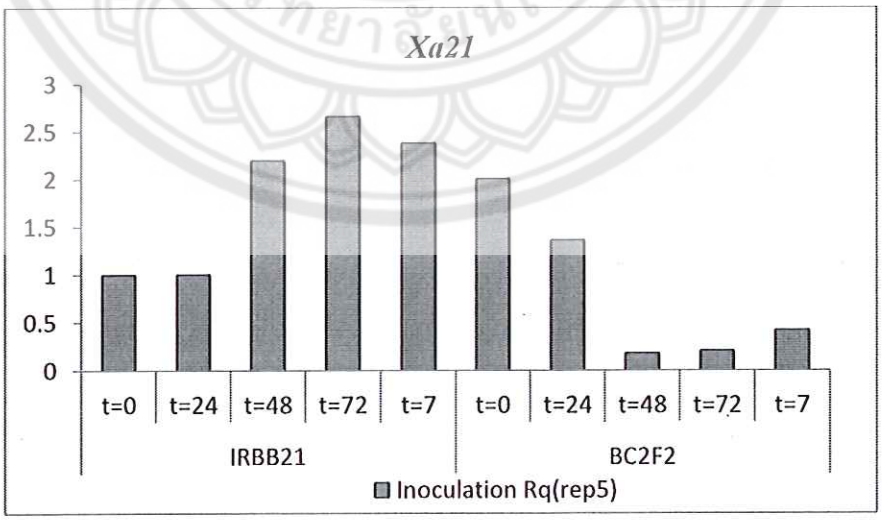




1.2 Relative quantification of *Xa21* after inoculation



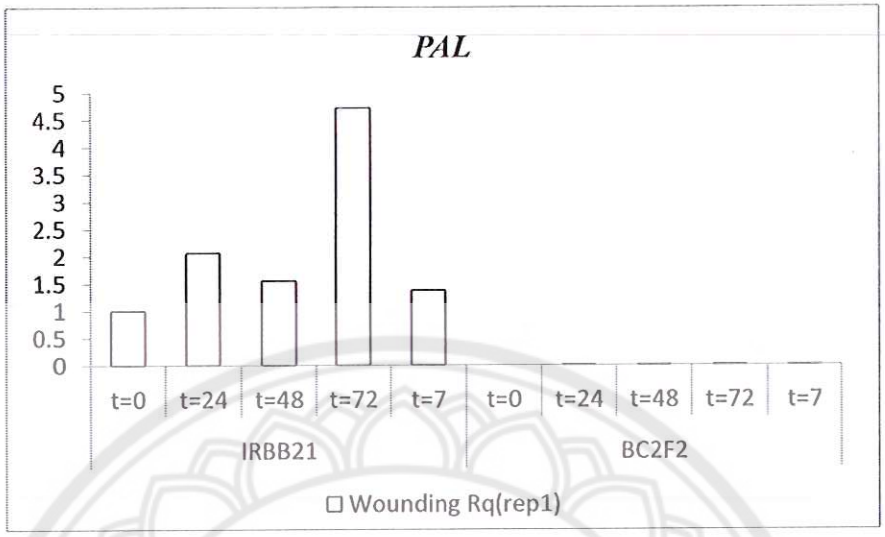


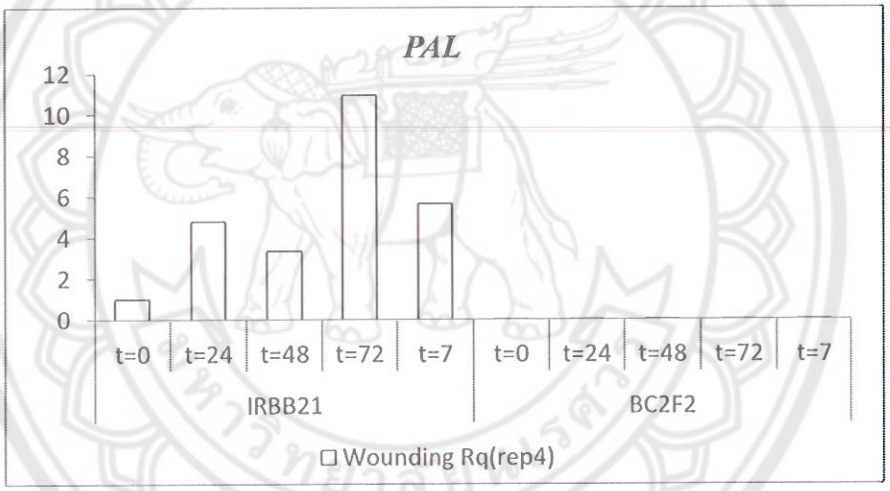


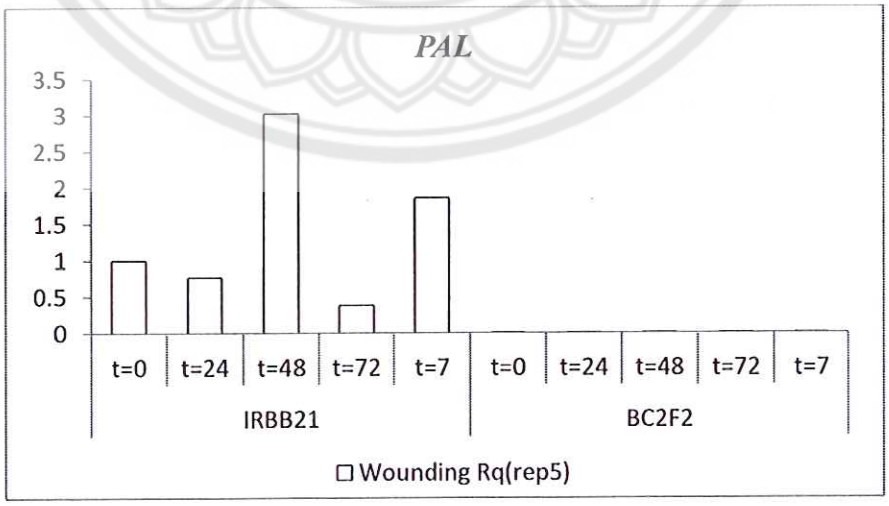
2. The *Phenylalanine ammonia-lyase* (*PAL*) gene

Wounding		Rq(rep1)	Rq(rep2)	Rq(rep3)	Average
IRBB21	t=0	1.00	1.00	1.00	1.00
	t=24	2.06	4.79	0.77	2.54
	t=48	1.55	3.36	3.02	2.64
	t=72	4.72	10.95	0.38	5.35
	t=7	1.37	5.65	1.86	2.96
BC2F2	t=0	0.00	0.00	0.00	0.00
	t=24	0.00	0.00	0.00	0.00
	t=48	0.00	0.00	0.00	0.00
	t=72	0.00	0.00	0.00	0.00
	t=7	0.00	0.00	0.00	0.00
Inoculation		**Rq(rep1)**	**Rq(rep2)**	**Rq(rep3)**	**Average**
IRBB21	t=0	1.00	1.00	1.00	1.00
	t=24	2.04	0.61	0.82	1.16
	t=48	2.34	0.65	0.80	1.26
	t=72	3.94	0.82	1.24	2.00
	t=7	3.02	0.64	1.03	1.56
BC2F2	t=0	0.00	0.00	0.00	0.00
	t=24	0.00	0.00	0.00	0.00
	t=48	0.00	0.00	0.00	0.00
	t=72	0.00	0.00	0.00	0.00
	t=7	0.00	0.00	0.00	0.00

2.1 Relative quantification of *PAL* after wounding







APPENDIX C Real-Time qPCR data analysis (Bio-Rad, 2006)

1. Amplification efficiency

E is calculated from the slope of the standard curve using the following formula:

$$E = 10^{-1/slope}$$

2. Relative quantification normalized to a reference gene

First, normalize the CT of the target gene to that of the reference (ref) gene, for both the test sample and the calibrator sample:

$$\Delta CT_{(test)} = CT_{(target,\ test)} - CT_{(ref,\ test)}$$

$$\Delta CT_{(calibrator)} = CT_{(target,\ calibrator)} - CT_{(ref,\ calibrator)}$$

Second, normalize the ΔCT of the test sample to the ΔCT of the calibrator:

$$\Delta\Delta CT = \Delta CT_{(test)} - \Delta CT_{(calibrator)}$$

Finally, calculate the expression ratio:

$$2^{-\Delta\Delta CT} = \text{Normalized expression ratio}$$