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NAME: Miss Phatsalakone Manivong

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Mr. Theerayut Toojinda, Ph.D.)

COMMITTEE MEMBER

(Associate Professor Apichart Vanavichit, Ph.D.)

COMMITTEE MEMBER

(Mr. Somvong Tragoonrung, Ph.D.)

GRADUATE COMMITTEE CHAIRMAN

(Assistant Professor Sudsaisin Kaewrueng, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

MARKER ASSISTED PYRAMIDING OF SUBMERGENCE TOLERANCE, BLAST RESISTANCE AND FRAGRANCE IN GLUTINOUS RICE

PHATSALAKONE MANIVONG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Current local rice cultivars grown by Lao farmers are intolerance to long periods of flooding and susceptible to blast disease. In this study, we develop the new bred glutinous varieties that have fragrance, submergence tolerance and blast resistance characters while have plant type and yield potential similar to the popular Laotian TDK1 variety by using marker-assisted selection (MAS). The three-ways cross (TDK303/IR85264/RGD07529) was made and the progenies were subjected to MAS using six markers, Aromarker, R10783indel, RM212, RM319, RM144 and RM224, were used to select the favored alleles of the *badh2, Sub1, qBL1 and qBL11* loci, respectively. Twenty eight F5 lines were selected and tested for submergence tolerance, blast resistance, fragrance and agronomic characteristics and compared with those of the parents. All of breeding lines exhibited submergence tolerance, blast resistance and fragrance. A wide range of agronomic characteristics was observed in the breeding lines and some breeding lines had shown very good characteristics. This study provides further support that the precision of markers used in MAS can enhance the development of rice varieties in Laos breeding program.

Student's signature Thesis Advisor's signature

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Phatsalakone Manivong

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LIST OF ABBREVIATIONS

AC	=	Amylose content
ANOVA	=	Analysis of variance
2AP	=	2-acetyl-1-pyrroline
badh2	=	Betaine aldehyde dehydrogenase-2
Bp	=	Base pair
Chr	=	Chromosome
Cm	=	Centimeter
cM	=	Centimorgan
^{o}C	=	Degree celsius
DF	=	Days to flowering
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxy-nucleotide triphosphate
F_1	=	First filial
FR	=	Fragrance
GC	=	Gel consistency
GT	=	Gelatinization temperature
ha	=	Hectare
IDs	=	Ideotypes
INDEL	=	Insertion deletion
kb	=	Kilo base pair
KOH	=	Potassium hydroxide
LSD	=	Least significant difference
LP	=	Grain length of polished rice
LU	=	Grain length of unhusked rice
MAS	=	Marker assisted selection
ml	=	Milliliter
mm	=	Millimeter
mM	=	Milli molar

LIST OF ABBREVIATIONS (Continued)

NPB	=	number of plant beginning submerged
NPA	=	number of plant after submerged
nm	=	Nanometer
NEPT	=	number of effective tillers per plant
PCR	=	Polymerase chain reaction
PH	=	Plant hight
PSE	=	Percentage of seedling elongation
PSS	=	Percentage of surviving seedlings
QTL	=	Qualitative trait loci
qBL	=	Blast resistance QTL
RCBD	=	Marker assisted selection
RILs	=	Recombinant inbred lines
SNP	=	Single nucleotide polymorphism
SSR	=	Simple sequence repeat
TP	=	Grain thickness of polished rice
TU	=	Grain thickness of unhusked rice
ul	=	Micro liter
uM	=	Micro molarity
WP	=	Grain width of polished rice
WU	=	Grain width of unhusked rice

MARKER ASSISTED PYRAMIDING OF SUBMERGENCE TOLERANCE, BLAST RESISTANCE AND FRAGRANCE IN GLUTINOUS RICE

INTRODUCTION

Rice (*Oryzae sativa.L*) is one of the world's most important cereals, which collectively provides the largest source of calories for human consumption. It is also considered as one of the most staple food crops in the world especially in Asia because more than half of the world's population consumes rice (Hossain, 1997). Rice (Oryza sativa L.) is the most important food crop in Laos.

In 2010, rice growing areas were approximately 664,425 hectares; in which 2,331,330 tons of rice were produced (an average yield of 3.7 t/ha) (DOA, 2010). The rice production systems in Laos can be classified into three broad ecosystems namely irrigated lowland, rainfed lowland, and rainfed upland (Inthapaya *et al.*, 2006). The rainfed lowland ecosystem accounts for about 78.8% of the total area and 81% of the rice production with an average yield of 3.6 t/ha. The rainfed upland ecosystem accounts for 13.4% of the total area and 8.4% of the rice production with an average yield of 1.8 t/ha. Irrigated environment accounts for 7.8% of the total area and 10.6% of the rice production with an average yield of 4.4 t/ha.

Glutinous rice is Lao PDR's most important cereal. Lao PDR has the highest per capita for the production and consumption of glutinous rice in the world. Lao people also have a particularly strong cultural affinity for glutinous rice (Schiller *et al.*, 2006). In 2005, approximately 83% of the total rice areas in Laos were planted to glutinous rice (Lao-IRRI, 2005). About 91% of glutinous rice is grown in irrigated environment in the dry season. This environment is almost exclusively useful for the improved glutinous varieties released by the Lao National Rice Research Program (NRRP) since 1993. The lowest proportion of total rice area that is growing glutinous

rice (60%) is in the rainfed upland environment in the northern agriculture region (Schiller *et al*., 2006).

In the rainfed lowland areas of Lao PDR, abiotic and biotic stresses are the most limiting factor threatening the rice production. Their effects or damages on rice may range from a minor to a complete loss in yield. Among the important stresses are short terms flooding (submergence) and blast disease is recently the most importance.

Submergence is one of the natural occurrences devastating rice crop in irrigated and rainfed lowland areas in Asia during monsoon season. Transient submergence up to four weeks is common. Twenty two million hectares of rainfed lowland in South and Southeast Asia were reportedly affected by this type of flooding at various stages of growth (Setter *et al.,* 1997; Ram *et al.,* 2002; Jackson and Ram, 2003). Regular flooding of the Mekong River affects 10%–30% of the rice area in the Southern and Central regions (Lao-IRRI, 2006). During 1991 to 1999 significant areas were affected by flooding on five occasions: 1994, 1995, 1996, 1997 and 1999. In 1991, more than 21% (about 70,000 ha) of the total rice area was destroyed by floods. In 1995, almost 30% of the planted area in the central agricultural region was lost. Flooding of the Mekong River in 2000 has also resulted in large crop losses, with preliminary estimates of the area destroyed in the central and southern regions being about 61,000 ha (Schiller *et al*., 2001). Therefore, improvement of rice varieties with submergence tolerance is needed for Lao PDR to sustain the rice production.

Rice blast *(Pyricularia oryzae Cav.)* is the most important disease in Lao PDR. It encountered in lowlands and upland rice crops, which is aggravated by high fertility (mainly N) and dry conditions. It can be reduced yield substantially (Schiller *et al*., 2001; Lao PDR, 2006) and caused a crop loss in different rice ecosystems worldwide, ranging from 40 to 100% in irrigated, 70% in rainfed and 63% in upland rice areas (Phetmanyseng *et al.,* 2011). In 1999, this disease damaged to rice cultivation throughout the central and southern regions in irrigated ecosystem in Lao PDR. In 2006, the outbreak also reported in the central regions causing the yield losses more than 50-70% (Inthapaya *et al.,* 2011). Currently Laos got 92 isolates

collected from all growing areas. They were classified into 12 races called A, B, C, D, E, F, G, H, I, J, K and N. The predominant races A, B, C, D and E are found in both irrigated and rainfed ecosystems in the central and southern regions of Lao PDR. Race F were found in the rainfed ecosystem of the northern and central. Races G, K and N were found in all rice ecosystems in Lao PDR (Inthapaya *et al.*, 2009).

Rice is a staple food in the Lao PDR. Approximately 95 % of population consumes glutinous rice. Majority of the rice varieties growing in Lao PDR (85%) is glutinous. From 1993 to 2006, Lao National Rice Research Program had released 17 improved glutinous varieties including 7 Thadokkham varieties (TDK) from Rice and Cash Crop Research center, 5 Phon Ngam varieties (PNG) from Phon Ngam Rice Research station, 4 Tasano varieties (TSN) from Thasano Rice Research Center and 1 Namtane variety (NTN) from Namtane Rice Research Station. These varieties are widely adopted by farmers (Inthapanya *et al.*, 2006). Of them TDK1 occupied the largest area especially in the central and southern part of the country. TDK1 is a high yield variety with acceptable eating quality; however it lacks of fragrance and cooking quality. It is also susceptible to many diseases and intolerance to submergence. Therefore TDK1 had become the main target of Lao National Rice Research Program to improve for aroma, submergence tolerance and blast resistance.

OBJECTIVES

The objective of this study is:

To breed new glutinous variety which has fragrance, submergence tolerance and blast resistance but have plant type and yield potential similar to the TDK1 rice variety by means of marker-assisted selection (MAS).

LITERATURE REVIEW

The important of rice

Rice (*Oryza sativa* L.) is one of the most important food crops of the world and is the stable food of approximately one-half of the world's population. The organization of *O. sativa* diversity has been of major interest for rice scientists since the early 20th century. *Oryza sativa* contains two major subspecies: the sticky, short grained *japonica* or *sinica* variety and the nonsticky, long grained *indica* variety.*Japonica* varieties are usually cultivated in dry fields, in temperate East Asia, upland areas of Southeast Asia and high elevations in South Asia. The initial and very old distinction of "Hsien" and "Keng" types in China prefigures the main bipolar scheme featuring the current indica and japonica types, well documented in the pioneering work of Kato *et al*., (1928), Matsuo (1952) and Oka (1958).

Indica-type rice provides the staple food for more than half of the world population. Indica rice are grown throughout south and southeast Asia and in most area of the humid tropics have been evolved through selection processes. These varieties are slow growing and are photoperiod sensitive. They have tall, weak, thick culms, long drooping, thin, pale green leaves and relatively large, lax panicles. They are well adapted to conditions of low fertility, adverse weather conditions and poor water control. Their cooking characteristics are preferred by consumers in tropical and subtropical areas (Yoshida S, 1981).

Japonica varieties are widely grown in the temperate zone: the lower Yangtze valley of China, Taiwan, Korea, Japan, part of Australia, California, Europe and Egypt. In comparison with the indicas, japonicas have darker, upright leaves, a shorter, stiffer stalk, more thrifty vegetative growth and earlier maturation. They respond well to the application of fertilizer and are more resistant to lodging. As a result, yield is considerably higher than for the indicas (Yoshida S., 1981)

Indica rice is one of the most economically valuable crops in Laos when compared to other crop that can be planted several seasons in the year. Rice plants can growths in very parts of Laos. In the year 2010, farmers planted rice was 664,425 hectares and an average yield of 3.7 t/ha (DOA, 2010).

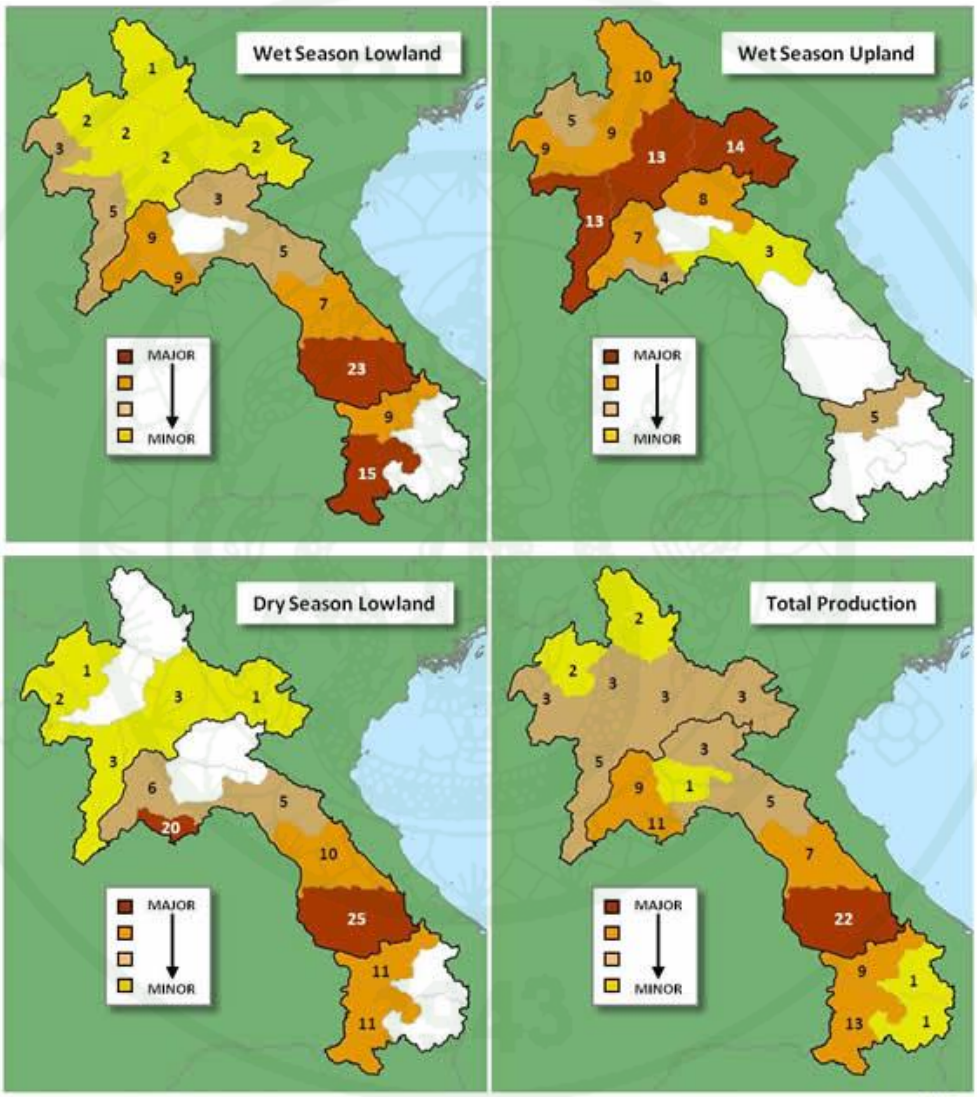


Figure 1 Geography of seasonal rice production in Laos

Source: Ministry of agriculture, Forestry (MAF), Laos 2009

Numbers indicate percent of total national production each province contributed in 2009-2010

http://www.pecad.fas.usda.gov/highlights/2011/12/Laos_13Dec2011/

Submergence tolerance

1. Physiology of submergence tolerance

Submergence stress is a major constraint to rice production during monsoon flooding season in lowlands and rainfed ecosystem. The visible symptoms of injury caused by complete and sustained submergence include phase of faster elongation by leaves accompanied by yellowing of other leaves and slow growth in dry mass of roots and shoots. After water levels fall, the whole shoot, may collapse and later can die (Michael *et al.,* 2003)

The physiological mechanisms provided to the tolerant rice determine plant survival and also recovery under flooding period. Submergence tolerant plant exhibited several morphological adaptations such as decreased chlorosis of tissues and reduced elongation growth in order to save carbohydrates and energy for maintenance processes including the protective antioxidant systems. Submergence stress cause the elongation rate of leaves and stem in some plant species. Under flash flooding, few characters were identified as playing a key role in submergence tolerance in rice, the most critical are: maintenance of high carbohydrate concentration, optimum rates of alcoholic fermentation and energy conservation by maintaining low elongation growth rates during submergence. Protective mechanisms as the up regulation of antioxidant system and low synthesis or sensitivity to ethylene during submergence were also found to be useful (Michael *et al*., 2003 and Sarkar *et al*., 2006).

The main effect of submergence on plant tissues is carbohydrate depletion. As the rice plant is starved of carbohydrates, its leaves turn yellow and die and, ultimately, the plant dies. Photosynthesis decreases because the supply of carbon dioxide is reduced and light intensity is low during submergence; respiration is reduced because oxygen concentrations are low; and ethylene accumulates in the plants (Setter *et al.,* 1988). Factors that increase the damage caused by submergence include (Palada and Vergara, 1972) increased water depth, increased duration of submergence, increased temperature, increased turbidity, increased rate of nitrogen

fertilization, and decreased light intensity submergence also causes mud to be deposited on the leaves of the rice plants, which can inhibit photosynthesis; and when the floodwaters are moving rapidly, rice plants may be uprooted. Prolonged submergence stress can damage plant tissues, set back growth, or even reduce the plant population.

Submergence tolerance is a complex trait; it cannot be attributed just to one or a few physiological or morphological characteristics. Research at IRRI has characterized morphological and physiological differences between submergence-tolerant and -susceptible rice cultivars (Table 1).

Table 1 Relationship between morphological and physiological characteristics and submergence tolerance of rice plants (adapted from Karin *et al.*, 1982 and Mazaredo and Vergara, 1982)

Type	Characteristics	Relationship to submergence tolerance
Morphological	Height	Generally greater in tolerant genotypes
	Culm stiffness	Stiffer in tolerant genotypes
	Culm roundness	Rounder in tolerant genotypes
	Leaf blade length	Longer in tolerant genotypes
	Percentage of lacunae per unit area	Lower in tolerant genotypes
	Air spaces within leaf	Fewer in tolerant genotypes
	Overlapping of first leaf sheath	Greater in tolerant genotypes
	Root length	Longer in tolerant genotypes
Physiological	Carbohydrate content	Decreases more slowly in tolerant genotypes
	Nitrogen content	Higher, decreases more slowly, and is recovered faster in tolerant genotypes
	Silica content	Higher in tolerant genotypes
	Oxidizing power of roots	Stable in tolerant genotypes; decreases in susceptible genotypes
	Photosynthesis and respiration	Higher rates in tolerant genotypes
	Oxygen release	Higher rates in tolerant genotypes
	Potassium and nitrate content	Higher in tolerant genotypes
	Nitrate reductase activity	Higher in tolerant genotypes
	Chlorophyll synthesis	New synthesis in tolerant genotypes

Submergence-tolerant varieties of rice tend to accumulate more starch in their stem sections than do susceptible varieties, and they experience less carbohydrate depletion after submergence (Karin *et al*., 1982; IRRI, 1993; Emes *et al*., 1988). And the decline in photosynthetic ability and chlorophyll content of leaves after submergence progresses more slowly in varieties that are submergence tolerant than

in those that are susceptible (Smith *et al*., 1988). Following submergence, tolerant cultivars accumulate smaller amounts of aldehydes, which possibly are toxic end-products of anaerobic metabolism (IRRI, 1993). The studies that have been done so far indicate that heritability is high and that genre for tolerant are partially to completely dominant (Mohanty *et al.,* 1982; Sinha and Saran, 1988; Haque *et al.,* 1989b). From the results suggest that one major gene is responsible for most of the tolerance of highly tolerant cultivars. Molecular mapping studies have identified a locus on rice chromosome 9 inherited from FR13A that control submergence tolerance (Xu and Mackill, 1995). The submergence tolerance of FR13A is linked to a major quantitative trait locus (QTL), known as submergence 1 (*Sub1*), on chromosome 9 (Xu and Mackill, 1996). This locus explains a large proportion of the variation (35-69 %) in flooding tolerance between indica (tolerance) and japonica (intolerant) rice cultivars (Toojinda *et al*., 2003)

1. Morphological and physiological responses of rice seedlings to complete Submergence

The major morphological and physiological submergence tolerant trait are slow leaf elongation, less chlorosis, high carbohydrate reserve storage during submergence and prompt re-adatation to the aerial environment after de-submergence (Setter *et al.,* 1997; Ito *et al.,* 1999; Ram *et al.,* 2002; Jackson and Ram, 2003).

Shoot elongation during submergence is contalled by hormones such as ethylene, the gas interacting with other hormonese, including abscisic acid (ABA), gibberellins (GA) and auxin (Jaackson, 2008). A cascade model has been proposed based on the study of the stem elongation of deep-water rice stems (Kende *et al.,* 1998). Sequential steps in the proposed chain of reation include (a) accumulation of ethylene, (b) ethylene-induced reduction in ABA concentration, (c) increased responsiveness to GA production, (d) GA-promoted stem elongation. The proposed chain reaction probably applies also to leaf elongation of young rice seedlings during submergence sine ABA is known to decline in submerged rice leaves (Ram *et al.,* 2002), endogenous ethylene has been shown to accumulate to active concentrations

within submerged leaves while submergence tolerant cultivars of *O. sativa* such as FR13A are relatively ethylene insensitive (Jackson *et al.,* 1987). Ethylene also enhanced DMW of intolerant cultivars may be caused by leaf senescence. All submergence tolerant cultivars so far examined have the *Sub1-1*A gene. *Sub1* gene (*Sub-1*A, *Sub-1*B and *Sub-1*C) are thought to contain ethylene response factor domains encoding three ethylene inducible transcription factors (Fukao and bailey-Serres, 2004; Xu *et al.,* 2006). The submergence tolerant rice restricts its growth while in water. Does *Sub1*A stunt its growth via plant hormones? Fukao and Bailey Serres (2008) showed the mechanism by which *Sub1*A regulates plant growth. Slender rice 1(SLR1) and SLR1 like 1 (SLRL1) are repressors of GA signaling. The amount of SLR1 protein is elevated after submergence in submergence tolerant rice (M202 (*Sub1*)), but not in submergence tolerant rice (M202). Additionally, more SLRL1 protein accumulated in submergence tolerant rice (M202). These results suggest that the restriction of growth by submergence tolerant rice is due to the accumulation of SLR1 and SLRL1 through *Sub1*A (Figure 2)

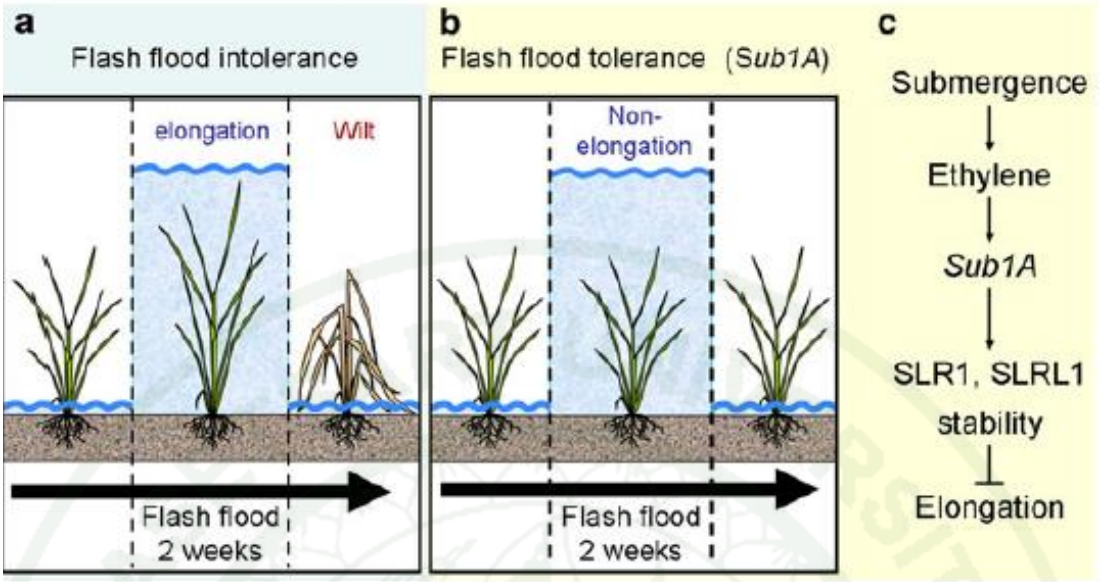


Figure 2 Model of flash flood tolerance and plant hormones in rice. a) The strategy of intolerant rice. b) The strategy of tolerance rice at the seedling stage. c) The scheme of flash flood tolerance. Flash flood tolerant rice carries *Sub1*A, which promotes the accumulation of SLR1 and SLRL1, negative regulators of GA signaling, and inhibits internodes elongation.

Environmental characterization of floodwater

The variations in floodwater characteristics across locations induce different responses in various cultivars marking the interpretation of research data difficult. In particular, conclusions about flooding tolerance at one site cannot be extrapolated elsewhere without information on floodwater characteristics. Several factors contribute to the adverse effects of submergence in rice, and some may have combined effects (Ito *et al.,* 1999). For example, siltation on leaves may cause mechanical damage, and affect light and gas diffusion (Settre *et al.,* 1995). Current experimental evidence demonstrates that limited gas diffusion (setter *et al.,* 1988) and underwater irradiance (Palada and Vergara, 1972; Setter *et al.,* 1995; Ram *et al.,* 1999) are however, the main factors that appear to affect growth, metabolism and survival of rice during submergence.

Breeding and Genetics of submergence tolerance

Submergence tolerant varieties have been developed (Mackill *et al*., 1993), but have not been widely adopted. One reason is that these tolerant varieties lack many of the desirable traits of the widely grown varieties, refered to as "mega varieties" that are population in major rice growing areas of Asia, because of their high yield and grain quality (Mackill *et al*., 2006). The genetic control of submergence tolerance remained ambiguous. Several studies suggested that it was atypical quantitative trait (Suprihatno and Coffman, 1981; Mohanty *et al.,* 1981; Mohanty and Khush, 1985; Haque *et al.,* 1989). The identification of the major QTL *SUBMERGENCE 1* (*Sub1*) on chromosome 9, contributing up to 70% of phenotypic variation in tolerance (Xu and Mackill, 1996), Several independent studies confirmed the major chromosome 9 QTL and identified other minor QTLs that accounted for less than 30% of the phenotypic variation in tolerance (Nandi *et al.,* 1997; Toojinda *et al.,* 2003 and Siangliw *et al.,* 2003). A major QTL for submergence tolerance contributed by FR13A, a submergence tolerant landrace from India, has been identified on chromosome 9 in all mapping studies (Xu *et al.,* 1996; Nandi *et al.,* 1997; Xu *et al.,* 2000; Toojinda *et al.,* 2002). In addition, secondary QTL that influence submergence tolerant has been located in chromosomes 1, 2, 5, 7, 10 and 11 (Toojinda *et al.,* 2002 and Siangliw *et al.,* 2003).

The identification of the *Sub1* QTL enabled its transfer by marker-assisted backcrossing (MABC) into the framer preferred varieties (Xu *et al.,* 2004 and Mackill, 2006). The gene level analyses of the *Sub1* region resolved single nucleotide polymorphisms within *Sub1* region resolved single nucleotide polymorphisms within *Sub1*A and *Sub1*C that could be used for molecular markers and in precision breeding (Neeraja *et al.,* 2007 and Septiningsih *et al.,* 2009). Using MABC, a small genomic region containing *Sub1*A has been introgressed into modern high-yielding varieties, such as Swarna, Samba Mahsuri, IR64, Thadokkam1 (TDK1), CR1009 and BR11 (Septiningsih *et al.,* 2009).

Creating these chromosome linkage maps and locating the submergence tolerance trait on the map have several important implications. First, the markers that map close to *Sub1* should be useful in breeding programmers to select submergence-tolerant offspring and develop new submergence-tolerant lines without the need for as much time consuming and costly outdoor screening (Mackill *et al*., 1999).

Blast disease

The causal agent of rice blast disease is *Pyricularia grisea Secc*. *Pyricularia grisea Secc*has the toleomorph called *Magnaporthe grisea* (Hebert) Barr. The prefect stage of the fungus was first discovered and described as *Ceratopheria grisea* by Hert (1971) which had been changed later to the genus *Magnaporthe* by Barr (1977). As the telemorph was rarely found in the nature but the asexual stage was known. Rosman *et al.,* (1990) stated that it was acceptable to callthis fungus as either *grisea Secc*. *Pyricularia grisea Secc*. Or *Magnaporthe grisea* (Hebert) Barr.

1. Blast symptoms and infection

Rice pathogenic isolate of the blat pathogen produces lesion on all parts of the rice plat., leaves, node, internodes, leaf sheath, collar, neck and panicles. The rice blast disease is characterized by examining these parts of the plant. When lesions appear on leaves, they are often white to gray-green with dark green or brown border. Their shape varies but lesions are characteristically spindle shape. There are several types of lesions based on the resistance and plant age. Leaf reactions vary from pinpoint infection to large elliptical lesions up to 1.5 cm long, 0.3-5.5 cm broad. Large lesions without signs of plant resistance are referred to sometime as acute lesions, while small lesions indicate a degree of resistance.

Blast infection in early plant stages can cause stunting or death, depending on the severity of the attack and on the humidity and temperature during the infection period, neck or panicle usually causes more severe damage to the crop than leaf blast. Neck blast is seen as a gray brown lesion round the upper most nodes just below the

panicle, causing the panicle to fall over (“rotten neck”). Early attack at this point will result in on or very poor gain filling and high harvest losses. A later attack can be less damaging, although the grains will not completely develop.

Figure 3 Blast disease symptoms in rice.

Source: http//www.knowledgebank.irri.Org, http://www.globalplanticlinic.org, http://www.jircas.affrc.go.jp,http://www.ipmcenters.org, http://extension.missouri.edu.

2. Morphology

Conidiophores produced in clusters from each stoma, are rarely solitary and have 2-4 septa. Conidia are pyrifrom with three cells on basal appendage. The average size of conidia was 19-23 x 7-9 μm. Conidia germinate from the apical or basal cell and less frequently from the middle cell. A conidium forms an apressorium at the tip of the germ tube when it germinates on the host plant. At this stage, it is called a resting spore or chlamydospore. It is produced at an carly stage of infection and attaches to the host tissues by secreting a mucilaginous substance. Conidia growth on

media culture at optimum temperature 28^{o}C, and the sporulations were produced rapidly but the production decrease after 9 days. Perithecium of the prefect stage is nonstromatic, with a spherical to subspherical base that is embedded in the host tissue and has a long neck. The asci are eight-spores, cylindrical to clavate and mostly 60-90 x 5-7 μm. Ascospre was fusiform, curved, and rounded at the end. At maturity, the ascospores have extruded from the ostiole in a gelatinous mass (Ou, 1985).

3. Nomenclature and life cycle

The taxonomic definition of the anamorph state of blast fungus (Alexopolos, 1996) is as follows:

Division: Eumycota
Subdivision: Ascomycotina
Class: Pyronomycetes
Order: Diaporthales
Family: Magnaporthaceae
Genus: Magnaporthe

4. The life cycle of blast disease

Infection by the rice blast fungus starts when the three-celled conidium's lands on a host leaf and anchors itself to the leaf cuticle with spore-tip mucilage (Figure 2). Germination proceeds with the extension of a germ tube, which undergoes hooking a swelling at its tip and then differentiates into an infection structure called the aspersorium. During maturation, the appressorium becomes melanized, except for a well-defined pore between the appressorium and the rice leaf (Howard and Valent, 1996). The formation of this infection structure on the host surface marks the onset of the disease. A penetration peg is then driven though the host surface and the infection hypha invades and grows though the rice leaf (Talbot *et al.,* 2003). At this stage, the symptoms become evident and small oval lesions begin to appear, accompanied by

local chlorosis. Eventually, the growing lesions become necrotic and may coalesce. Conidia are carried by air to neighboring plants, spreading the blast disease.

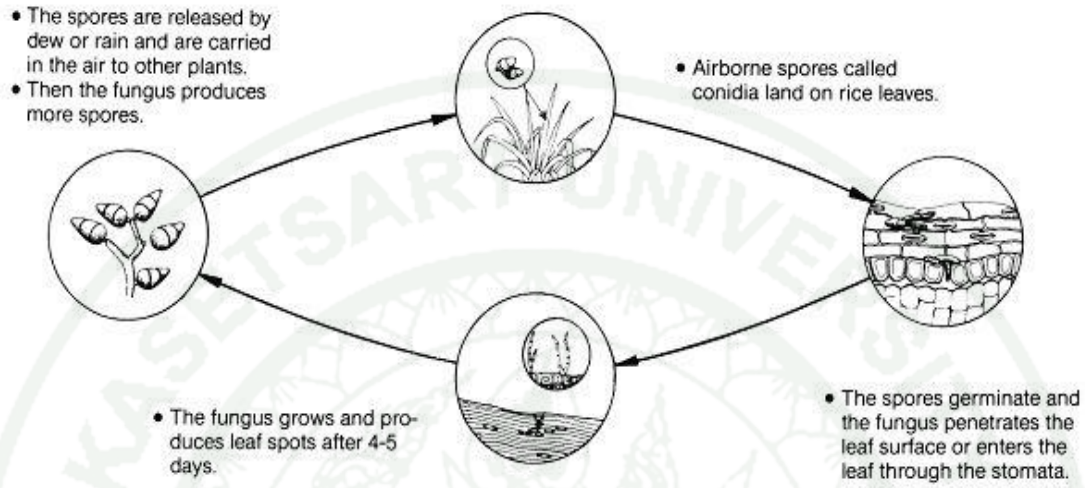


Figure 4 The life cycle infection-related morphogenesis of the rice blast fungus (*M. grisea*) (Wilson and Talbot, 2009)

5. Mechanism of *P. grisea* Infection

Germination of the fungal germ tube is depended upon additional signal; infection signal and vegetative growth signal. Additional signal such as the contact of conidia to a solid surface would help the germination of germ tube (Lee and Dean, 1993). Sensing the infection signal such as hydrophobic leaf surface would cause the swelling of hyphal tip into appressorium which will penetrate into the leaf surface. After penetration, enzymes will take action and life cycle will be completed within 3-4 days.

6. Genetic of blast resistance

Resistant cultivar is one of the solutions to prevent or reduce yield loss due to rice blast epidemics. Blast resistance was classified into two types according to gene expression induced by the attack of the pathogen. One type is called qualitative or

complete resistance while the other is called quantitative or incomplete resistance (Ou, 1979). Qualitative or complete resistance shows reaction indicating the absence of compatible type lesion being controlled by major genes(s) (Ahn, 1994) having race specificity (Marchetti, 1983) and expressing hypersensitivity to the pathogen. The 6 first study on blast resistance gene was reported by Sasaki (1923) who found a single dominant blast resistance gene in Japanese rice variety Tsurugi had initiated a light at the end of the tunnel. Forty-two years later, two resistant genes designated *Pi-1* and *Pi-6* were identified in the United States of America (Atkins and Johnson, 1965). Four dominant genes i.e., *Pi4, Pi13, Pi22* and *Pi25* were also identified (Hsieh *et al*., 1967). The total of 11 major genes designated *Pi-k, Pi-k5, Pi-kh, Pi-ta, Pi-z, Pi-zt, Pia, Pi-b, Pi-f, Pi-i* and *Pi-lm* had been reported fourteen years later (Kiyosawa, 1981). The work on gene mapping had revealed that *Pi5 (t)* and *Pi7 (t)* mapped on chromosome 4 and 11 were linked to marker RG778 and RG103, correspondingly (Wang *et al*., 1994). Based on these information, near isogenic lines (*NILs*) of rice with single resistant gene for each line were developed by backcrossing four donor cultivars to the recurrent parent CO39 (Mackill and Bonman, 1992).

Rice blast *(Pyricularia oryzae Cav.)* the most important of disease in Laos, 200 of blast isolate collected from three part in Laos (north, center and southern regions) can be classified into 92 pathotypes, within 92 pathotypes were classified into 12 races types A, B, C, D, E, F, G, H, I, J, K and N. The predominant race types A, B, C, D and E were observed mostly in rainfed ecosystem of center and southern regions (Inthapanya *et al.*, 2011).

The highest virulent frequency was observed in 5 monogenic lines IRBLa-A (*Pia*), IRBL19-A (*Pi19*), IRBLks-S (*Pik-s*), IRBLzt-T (*Piz-t*) and IRBLt-K59 (*Pit*), ranged from 83, 77, 74, 72 and 67%, respectively. In other hand, the lowest frequency was observed in 6 monogenic lines IRBLkh-K3 (*Pik-h*), IRBLta2-Re (*Pita2*), IRBLta2-Pi (*Pita2*), IRBL9-W (*Pi9*), IRBLsh-S (*Pish*) and IRBLz-Fu (*Piz*), ranged from 12, 11, 9, 2, 2 and 0%, respectively (Inthapanya *et al.*, 2011). Monogenic line IRBLz-Fu which is harboring resistant gene *Piz* shows universal resistance to all blast isolates. In addition, monogenic line IRBLsh-S which is harboring resistant gene *Pish*

shows resistance to most of blast isolates collected within the country, except the isolates collected from the upland ecosystem in the southern region (Inthapanya *et al.*, 2011). Therefore, a breeding program should be initiated to transfer these resistance genes from the differential varieties to high quality Lao's rice varieties to control the disease effectively.

Quantitative (Incomplete) resistance has been called field resistance or partial resistance, in general. It is characterized by lesions typically spindle-shaped, fewer in number; reduce in size, slower to develop and shorter-lived (Tabien *et al*., 2002). Partial resistance is more difficult to use than complete resistance due to its quantitative inheritance which usually polygenic and sensitive to environmental factors such as temperature, leaf wetness duration, nitrogen-fertilization, soil type and water stress (Ou, 1985; Roumen, 1994). It has also been stated that quantitative or partial resistance is usually controlled by polygenes that are minor genes (Bonman *et al*., 1992). Examples of partial resistance varieties had been reported as IRAT13, IAC24, IAC27 and Dourado Precose studied by Nottegham (1985). Quantitative resistance could be separated into two components. First, is the efficiency of quantitative resistance in eliminating an avirulent portion of any available inoculum. Second, is the ability to lower the infection efficiency of a virulent portion (Ahn and Koch, 1988).

Rice Diseases

Rice diseases are among the most important limiting factors that affect rice production causing annual yield loss conservative estimated at 5% (Song and Goodman, 2001). Two types of rice diseases are recognized: infectious and noninfectious diseases. The infectious diseases are caused by pathogens or biological vectors, such as fungi, bacteria, viruses, mycoplasmas and nematodes, Noninfectious diseases result from unfavorable environmental of nutritional conditions such as deficiencies or excesses of nutrients, temperature extremes, toxins, etc (Webster, 1992). At every growth stage, the plant is subject to diseases that reduce both yield quality and quantity. The severity of disease depends on the presence of a virulent

pathogen, a disease-conductive growth condition and the susceptibility of the cultivar. The actual number of distinct rice diseases is not clear. Over 80 biotic and abiotic 10 diseases were characterized. However, not all rice diseases are economically important (Ou, 1985).

Control of Rice Blast Disease

Several means to control rice disease have been used such as manipulating the time of planting, fertilizer, water management, the use of fungicides, biological control and cultivation of resistant cultivars.

Manipulating time of planting

In tropical upland rice, crops sown early after the onset of the rainy season are more likely to escape blast infection than the late-sown crops. It was observed in Bangladesh that blast is most severe during seasonal periods of low night temperatures and long dew duration. Sowing early could help to limit the exposure time of the crop to these blast conductive conditions (Bonman, 1992). This technique was also applied in Brazil (Prabhu and Morais, 1986).

Fertilizer application

High doses of nitrogen fertilizer increase the susceptibility of rice to blast disease. The form of N source also influences the severity of blast disease. It is reported that rice plants fertilized with NO^{-3} are more susceptible than those given NH^{+4}. It is also observed that splitting N application can reduce blast disease compared with a single application. Another plant nutrient element, phosphorus, was reported to increase the susceptibility of rice to blast in certain soil (Bonman, 1992).

Water management

This method is based on the observation that drought stress increases blast susceptibility. Providing water to minimize drought stress could help to reduce blast disease (Bonman, 1992).

The use of fungicides

Fungicides currently in use are highly effective. However, less toxic and less expensive chemicals with fewer applications offer important advantages in the current state of heightened environmental and economic awareness. Nowadays, 11 blasticides are registered for blast control in Japan: Blasticidin S, Kasugamycin, Edifenphos, Ferimzone, Fthalide, IBP, Isoprothiolane, Probenazole, Pyroquilon, Tricyclazole, and Carpropamid, These blasticides, excep Blasticidin S, provide systemic resistance in rice against blast disease (Yoshino, 1988; JPPA, 1992). Among these blasticides, Probenazole, which is a resistance inducer, has the most used one, and strong effects with high activity and results in long term control of blast. A rang of microorganisms was screened for promising candidates to be used as biological control agents for rice blast disease (Sy *et al*., 1990). Avirulent isolates of *M.grisea* and the non-rice pathogen Bipolaris sorokiniana were found to reduce blast disease when sprayed on plants. Recently, it was found that a specific pheromone produced by Saccharomyces cerevisiae could minimize infection by inhibiting production of the appressorium. However, not much field application has been done.

Cultivation of resistant rice cultivars

Growing resistant cultivars is the most effective and economical way to control blast disease. The farmers do not have to purchase fungicides and does not contaminate the environment like with the use of fungicides. In some areas where the environment is not highly conductive to blast, the disease can be controlled easily by growing resistant cultivars. However in some tropical area where the environment is favorable for blast disease, resistance can be broken down shortly after a resistant

cultivar is released. Therefore, development of durable resistant cultivars attracted the attention of many breeders.

Integrated pest management

To achieve a satisfactory control in the environment with a high potential for blast epidemics, it is necessary to combine different methods of blast control with cultivation of resistant cultivars and including manipulating time of planting, fertilizer, and water management as well as the use of fungicides.

Broad spectrum of disease resistance

Broad-spectrum resistance and durable resistance to disease are desirable for crop improvement. Broad-spectrum resistance (BSR) refers to resistance against two or more type of pathogen species or the majority of races of the same pathogen species. Durable resistances (DR) refer to resistance that remains effective during its prolonged and widespread use in environments favorable to the pathogen or disease spread.

There are two definitions for broad-spectrum disease resistance. The first one is defined as the resistance to the majority of geographically different isolate of the same pathogen. The second type is the resistance to two or more unrelated pathogens. Whether a broad-spectrum resistance gene is durable or not in multiple locations during a relatively long time is still debatable. To date, several first types of broad-spectrum R gene have been cloned in plant.

Plant disease resistance can be classified into two categories: qualitative resistance conferred by a single resistance (R) gene and quantitative resistance (QR) mediated by multiple genes or quantitative trait loci (QTLs) with each providing a partial increase in resistance. Other terms have also been used for these genetically distinguishable resistances. Compared with qualitative resistance, QR is characterized

by a partial and durable effect of resistance that is generally pathogen species-nonspecific or race-nonspecific but pathogen species-specific.

Gene-for-gene concept

Gene-for-gene resistance has originally been described in the 1940s by Flor who studied the genetics of the interaction between flax and the rust fungus Melampsora lini. He observed that for each dominant resistance gene in the plant, one dominant a virulence gene in the rust fungus was parent (Flor, 1946). The initial definition of pathogen a virulence gene implies that they have the ability to induce resistance in hosts carrying the corresponding resistance gene. At first, the proposed working modelfor these gene-for-gene interactions implied a receptor-ligand model were the R-gene product (receptor) directly binds the avr gene product (elicitor or ligand) to trigger resistance. To date only in a few cases a direct interaction between the elicitor and the R protein has been shown (Deslandes et al., 2003; Jia et al., 2000). Now evidence accumulates that avirulence protein posses virulence functions. Presumably, avirulence protein bind to a plant target different from the R-protein. Resistance gene product might have evolved as guards of the virulence target, sensing its modification followed by initiation of plant defences. This hypothesis, known as the guard-model, has first been described by (Van der Biezen and Jones, 1998).

Compatible Reaction

In the absence of either the resistance gene product or the avirulence gene product, there is no recognition of the pathogen by the plant. This allows the further growth of the pathogen, resulting in a compatible interaction and susceptibility.

Table 2 Gene combinations and disease reaction types in a host pathogen system in which the gene for gene concept for one gene

Virulence or avirulence genes in the pathogen	Resistance or susceptible genes	
	R (resistance dominant)	r (susceptible recessive)
A (avirulent dominant)	AR (-) (incompatible)	Ar (+) (compatible)
a (virulent recessive)	aR (+) (compatible)	ar (+) (compatible)

Mechanisms of R-Avr recognition

In the gene for gene model, also known as receptorligand model, the R protein is proposed to act as a receptor to recognize a corresponding pathogen Avr protein and form an R-Avr complex to activate diverse resistance responses. At present, there are two alternative mechanisms to explain this model: direct and indirect interaction. The direct in-teraction suggests that the pathogen Avr effectors interact with plant R proteins directly to trigger *R* gene-mediated resistance signaling. For example, the rice *R* gene *Pita* was initially shown to directly interact with *AVR-Pita* from *Magnaporthe grisea* but no interaction between *AVR-Pita* and its susceptible allele *Pita* was observed. In addition, a direct interaction recently was observed between the flax *L* alleles and corresponding flax rust *Avr* genes, which provides first evidence for direct, allele-specific inter- action between R proteins and diverse Avr proteins.However, no other clear examples of direct R Avr interaction have been reported. Conversely, most studied data prefer the indirect model also called “guard” hypothesis. In this model, R pro-teins act as “guardee” to monitor the variation/modification of host proteins after coupling with the corresponding Avr effectors.

When plant does not carry an R gene, an Avr protein binds with its virulence target (VT) leading to host susceptibility. When plant carrying an R gene is not attacked by pathogens, the R protein could occur in host cell with inactive self-associated configurations. When an Avr protein is activated through two interaction

models to transfer resistance signals: the Avr protein interacts with the R protein directly to trigger host resistance response or interacts with the R protein indirectly through a host protein (HP) as a molecular chaperon (MC) to from an R-Avr complex to induce resistance. A few cases a direct interaction between the elicitor and the R protein has been shown (Deslandes *et al.*, 2003; Jia *et al.*, 2000). Now evidence accumulates that avirulence proteins bind to a plant target different from the R-protein. Resistance gene products might have evolved as guards of the virulence target, sensing its modification followed by initiation of plant defences. This hypothesis, known as the guard-model, has first been described by (Van der Biezen and Jones, 1998).

Aromatic rice

Rice is consumed mostly as cooked grain with little processing, and grain appearance, cooking quality, and tastes are the factors that determine grain quality. Aromatic or fragrant rice is rice with natural chemical compounds which give it a distinctive scent. It can be used just like conventional rice for cooking, but adds a new dimension of flavor and aroma to meals. The demand for aromatic rice has increased markedly in recent years to the extent that consumers are willing to pay a premium price for aromatic rice. The Jasmine type rice of Thailand and the Basmati rice of India and Pakistan are the aromatic cultivars commonly sold in world trade. This rice is highly valued throughout Asia (Baishya *et al*., 2000) and also have wider acceptance in Europe (Berner and Hoff, 1986), Australia (Reinke *et al*., 1991), USA and the Middle East (Shobha Rani *et al*., 2006). Although most of the trade is from Thailand, India and Pakistan, aromatic rice is cultivated and prized in many other countries of the world.

1. Aroma evaluation methods for different parts of rice

The pleasant aroma associated with aromatic is not only released in cooked rice but is also often emitted by these varieties in the field at the time of flowering (Weber *et al.*, 2000; Widjaja *et al.*, 1996). It was reported that the 2AP aromatic

compound is present in various parts of the rice plant, such as the stems, leaves and grains, but not in the roots (Lorieux *et al*., 1996; Yoshihashi 2002). However, other researches detected 2AP at low levels from rice root and culture media (Vanavichit *et al*., 2005). The following sensory methods have been applied to determine the aroma in rice: chewing several seeds or cooking a sample of seeds from individual plants (Dhulappanavar 1976; Ghose and Butany 1952); heating leaf tissue in water or eluting the aroma from leaf tissue with diluted potassium hydroxide (KOH) (Sood and Siddiq 1978, Hien *et al*., 2006); and heating several half-cut seeds in fresh water (Wanchana *et al*., 2005). However, these sensory evaluation methods are not consistent or reliable because the aroma is subject to human preference. A more reliable method is based on a gas chromatography for quantification of volatile compounds from 100 g cooked rice (Petrov *et al*., 1996). To be more practical to breeders, a method for 1 g of samples was developed (Mahatheeranont *et al*., 2001). From this small volume, 1 ppb of 2-acetyl-1-pyrroline can be detected with repeatability. Recently, a histochemical method was also developed to localize 2AP in plant tissues (Nadaf *et al*., 2006). This method is based on to the structure of 2AP that includes a reactive methyl ketone group, which reacts with 2.4-dinitrophenyl hydrazine to produce an orange-red coloured compound, 2-acetyl-phenyl hydrazone.

Starch Structure of rice

Starch is a carbohydrate polymer that occurs in granular form (starch granule) in the organs of higher plants and is composed almost exclusively of anhydro-α-D-glucose units where it serves as food and energy store. In animals, glucose that is not needed right away to meet energy needs is converted preferentially to glycogen (starch like) that serves as an energy reserve.

Starch is also an industrial product that consists essentially of the starch polysaccharides, minor constituents and moisture and is obtained by industrial wet milling, refining and drying. In general starch contains 20% of water soluble fraction called **amylose** and 80% of a water-insolule fraction called **amylopectine.** These two fractions correspond to different carbohydrates of high molecular weight and formula

$(C_6H_{10}O_5)_n$. The components of strach are hydrolysed progressively to ***cyclodextrins,*** (+)-maltose and finally D-(+)-glucose upon treatment with acid or under the influence of enzymes. Cyclodextrins are polsaccharides of low molecular weight belonging to the general class called *oligosaccharides.* They consist of about six or more D-glucose units joined through 1.4-*alpha* linkages to form rings.

Amylose and Amylopectine in rice

Starch molecules accumulate to form starch grains, which are visible in many plant cells, notably in storage organs such as the potato tuber, and in seeds of cereals and legumes. In rice, Amylose is found inside the microscopic granules, which make up a single rice grain. Amylopectin forms the crystalline or ice-like "skeleton" of these granules. Scientists think the amylose forms into long chain-like arrangements to fill up the spaces inside of the granule. The protein in rice grains is found in pockets between granules of starch.

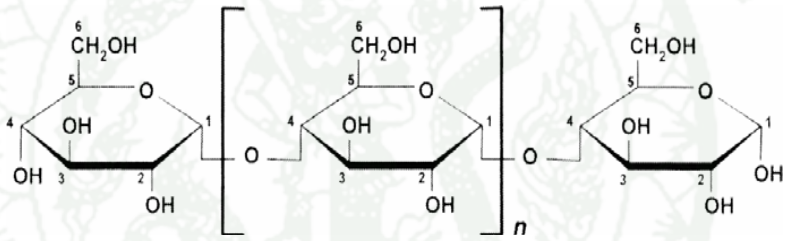
The cooking and eating characteristics of rice are influenced by the amount of amylose found in the grains. This is because the starch granules in the grain expand during cooking, forcing out the chains of amylose in a process scientists call leaching. As the cooked rice cools, the leached amylose chains line up, lock together and form a gel. When rice cools to room temperature or beyond, the chains of amylose crystallise. Generally, the higher the amylose content of rice, the firmer the cooked grain of rice will be. Some types of rice are between 25% and 30% amylose. These high amylose levels tend to make the rice cook firm and dry. Rice with a medium amylose content of between 16% and 22% usually cooks softer and the grains stick together more readily.

Amylose being a component of starch has a straight chain structure consisting of several thousands of D-(+)-glucose units each joined by α glycosidic linkage and C-4 of the next molecule. These bonds can cause the chain to coil helically into a compact shape.

Amylopectin has up to twice as many glucose molecules as amylose and has a highly branched structure consisting of several hundred short chains containing 20-25 D-(+)-glucose units each. One end of each of these chains is joined through C-1 to a C-6 on the next chain giving a more compact structure.

The difference between amylose and amylopectin, amylose, the unbranched type of starch, consists of glucose residues in α-1.4 linkage. Amylopectin, the branched form, has about 1 α-1.6 linkage per 30 α-1.4 linkages, in similar fashion to glycogen except for its lower degree of branching. Amylose also takes up less space than Amylopectin, but is not as easily digested.

a) Amylose



b) Amylopectin

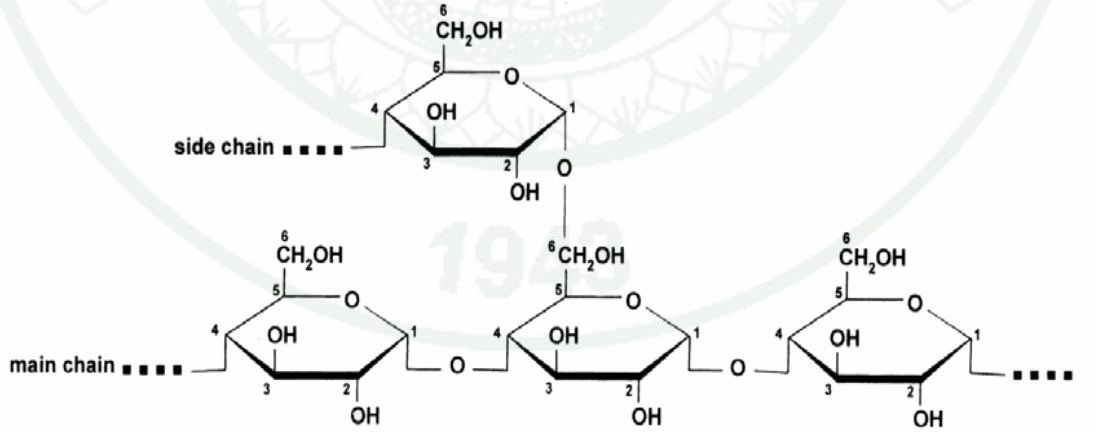


Figure 5 The chemical structure of amylose and amylopectin (Tester *et al.*, 2004)

Quality characteristics of glutinous rice

The glutinous rice starch contains nearly 100% of amylopectin and is insoluble in cold water. The major constituents of starch are very simple substances which are composed of glucose linked residues. Firstly, amylose is amorphous in native starch and is composed of essentially linear chains of α-1.4 linked glucose units. Secondary, amylopectin is a semi-crystalline highly branched polysaccharide with an α-1.4 backbone and α-1.6 branch points. The difference between rice flour and starch is that most of the native proteins and lipids have been removed from the starch. The composition effects the properties of the starch such as the lipid, protein, ash and phosphorus content. The composition of glutinous rice starch such as protein, fat, ash, phosphorus, and moisture content which directly affect to the properties of glutinous rice starch.

Laovachirasuwan *et al.*, 2008, Glutinous rice starch has almost 100% amylopectin content, small particle size (11.06μm), gelatinization temperature in range 58-75ºC, low impurity (0.2% protein, 0.085% lipid, 0.26% ash, 0.1% phosphorus content) and low moisture content (7.96%). The higher amylopectin content had the higher swelling power. This properties lead to trend that the after hydration it develops a highly viscous gelatinous as swelling of the associated matrix.

Cooking and eating characteristics are largely determined by the properties of the starch that makes up 90% of milled rice, with most of these characteristics being influenced by the ratio of two kinds of starch in the rice grain, amylase and amylopectin Amylose is the linear fraction of stach, whereas amylopectin is the branched fraction. Amyloes content strongly affects the cooking and eating quanlities of rice its cohesiveness, tenderness, color, and gloss. The terms usually used to reflect the a amylose content of rice grain are waxy 0-2%, very low 3-9%, low 10-19%, intermediate 20-25% and high more than 25%.

Molecular marker

Marker-assisted selection require identified marker with a high level of accuracy and efficiency, cost effective and easy to use. So, Functional marker is the best for MAS in breeding program because it can predict 100% accuracy. In agriculture, molecular marker is the tool for generating genetic linkage maps and has provided a major contribution to the genetic knowledge of many cultivated plant species useful for crop improvement and increased breeding efficiency. In addition to being of basic importance to genetic and evolutionary studies, molecular marker is useful to localize monogenic and polygenic traits allowing the efficient introgression and selection of individuals with specific characteristics. Basically, any DNA sequence used to distinguish between individuals, lines, varieties or to localize agriculturally important genes and construct genetic linkage map can be considered as a molecular marker. Molecular marker is more specific and accurate than other markers i.e., morphological or biochemical markers. Other advantages are direct measurement on genetic materials, numerous markers in a single population and measurement not subjected to environmental or developmental effect. They could localize any positions on the chromosomes which can be detected and inherited to progenies. Molecular marker or DNA marker can be classified into 2 groups.

Genetic marker

In a broad sense, a genetic marker refers to any heritable character that can be used to distinguish one individual from another in a population. The distinction can be at different levels such as phenotype, protein or DNA. Phenotypic trait can be marker if the variation observed in the population of interest is entirely explained by a single Mendelian factor. At the protein level, allozymes can be used as markers. These are soluble protein with different mobility on an electrophoresis gel. The mobility difference is a result of unequally charged protein due to amino acid substitutions. In current QTL mapping practice, variation at the DNA level is typically used because it is the most abundant and easily scored type of variation due the rapid development of genome technology. Variation in DNA sequence is detected by hybridization and

polymerase chain reaction (PCR) based methods. Commonly used DNA markers include restriction fragment length polymorphism (RFLP). The RFLP may result either from mutation in restriction endonuclease sites or from deletion or insertions of DNA between the sites. Polymorphism detected by PCR result from insertion and deletions between, and mutation in primer binding sites. PCR based markers include sequence tagged sites (STS) random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sample sequenced repeats (SSR or microsatellites), variable number of tandem repeats (VNTR) or minisatellites) and single nucleotide polymorphisms (SNP). Among these marker, RFLP, SSR and SNP are commonly used for mapping QTL.

Marker assisted selection

Plant breeding describes methods for the creation, selection, and fixation of superior plant phenotypes in the development of improved cultivars suited to needs of farmers and consumers. Primary goal of plant breeding with agricultural and horticultural crops have typically aimed at improved yields, nutritional qualities, and other traits of commercial value. Predicted population growth and pressure on the environment, traits relating to yield stability and sustainability should be a major focus of plant breeding efforts. These traits include durable disease resistance, abiotic stress tolerance and nutrient and water-use efficiency (Mackill *et al.*, 1999; Slafer *et al.*, 2005; Trethowan *et al.*, 2005). Despite optimism about continued yield improvement from conventional breeding, new technologies such as biotechnology will be needed to maximize the probability of success (Ortiz, 1998; Ruttan, 1999; Huang *et al.*, 2002). DNA markers can be used to detect the presence of allelic variation in the genes for desired traits. By using DNA markers to assist in plant breeding, efficiency and precision could be greatly increased. The use of DNA markers in plant breeding is called marker-assisted selection (MAS) and is a component of the new discipline of 'molecular breeding'. Molecular markers are especially advantageous for agronomic traits that are otherwise difficult to tag such as resistance to pathogens, insects and nematodes, tolerance to abiotic stresses, quality parameters and quantitative traits (Collard and Mackill, 2008).

In breeding for disease and pest resistance, the segregating populations derived from crosses between the resistant sources and desirable and productive genotypes are selected either at natural disease or pest 'hot-spots' or under artificially created disease and pest nurseries or by infecting individual plants under controlled environments. Screening of plants with several different pathogens and their pathotypes or pests and their biotypes simultaneously or even sequentially is difficult. Availability of tightly linked genetic markers for resistance genes will help in identifying plants carrying these genes simultaneously without subjecting them to the pathogen or insect attack in early generations. Only the materials in the advanced generations would be required to be tested in disease and insect nurseries. Thus, with MAS, it is now possible for the breeder to conduct many rounds of selection in a year without depending on the natural occurrence of the pest or pathogen and theoretically without the pest or pathogen as well. However, the presence of different races or biotypes complicates the development and application of molecular marker assisted selection. Markers developed for one pathotype or biotype may not have application to other locations in which different pathotypes or biotypes occur unless resistance is controlled by the same gene. Pathogens and insects are known to overcome resistance provided by single genes. Durability of resistance has been increased in several crops by incorporating genetic diversity of the major resistance genes. Cultivar diversification, cultivar mixtures, multilines and pyramiding of resistance genes have been successfully used. MAS for resistance genes (R) can be useful in all these approaches. Based on host-pathogen or host-insect interaction alone it is often not possible to discriminate the presence of additional R gene (s). With MAS, new R gene segregation can be followed even in the presence of the existing R gene (s) and hence R genes from diverse sources can be incorporated in a single genotype for durable resistance (Mohan *et al.*, 1997).

Marker-assisted backcrossing

Backcrossing is a plant breeding method most commonly used to incorporate one or a few genes into an adapted or elite variety. In most cases, the parent used for backcrossing has a large number of desirable attributes but is deficient in only a few

characteristics (Allard, 1999). The use of DNA markers in backcrossing greatly increases the efficiency of selection. Three general levels of marker-assisted backcrossing (MAB) were described (Holland, 2004). In the first level, markers can be used in combination with or to replace screening for the target gene or QTL. This is referred to as 'foreground selection' (Hospital and Harcosset, 1997). This may be particularly useful for traits that have laborious or time-consuming phenotypic screening procedures. Furthermore, recessive alleles can be selected, which is difficult to do using conventional methods. The second level involves selecting BC progeny with the target gene and recombination events between the target loci and linked flanking markers referred to as 'recombinant selection'. The purpose of recombinant selection is to reduce the size of the donor chromosome segment containing the target locus (i.e. size of the introgression). This is important because the rate of decrease of this donor fragment is slower than for unlinked regions and many undesirable genes that negatively affect crop performance may be linked to the target gene from the donor parent, referred to as 'linkage drag' (Hospital, 2005). Using conventional breeding methods, the donor segment can remain very large even with many BC generations (Ribaut and Hoisington, 1998; Salina *et al.*, 2003). By using markers that flank a target gene, linkage drag can be minimized. Since double recombination events occurring on both sides of a target locus are extremely rare, loss of vigor of the lines. Recombinant selection is usually performed using at least two BC generations (Frisch *et al.*, 1999b).

The third level of MAB involves selecting BC progeny with the greatest proportion of recurrent parent (RP) genome, using markers that are unlinked to the target locus referred to as 'background selection'. Background selection refers to the use of tightly linked flanking markers for recombinant selection and unlinked markers to select for the RP (Hospital and Charcosset, 1997; Frisch *et al.*, 1999b). Background markers are useful because the RP recovery can be greatly accelerated. With conventional backcrossing, it takes a minimum of six BC generations to recover the RP and there may still be several donor chromosome fragments unlinked to the target gene. Using markers, it can be achieved by BC4, BC3 or even BC2 (Visscher *et al.*, 1996; Hospital and Charcosset, 1997; Frisch *et al.*, 1999 a,b), thus saving two to four

BC generations. MAB can be used in order to trace the introgression of the transgene into elite cultivars during backcrossing.

Marker-assisted pyramiding

Pyramiding is the process of combining several genes together into a single genotype. Pyramiding may be possible through conventional breeding but it is usually not easy to identify the plants containing more than one gene. Using conventional phenotypic selection, individual plants must be evaluated for all traits tested. Therefore, it may be very difficult to assess plants from certain population types (e.g. F_2) or for traits with destructive bioassays. DNA markers can greatly facilitate selection because DNA marker assays are non-destructive and markers for multiple specific genes can be tested using a single DNA sample without phenotyping. The most widespread application for pyramiding has been for combining multiple disease resistance genes (i.e. combining qualitative resistance genes together into a single genotype). The motive for this has been the development of 'durable' or stable disease resistance since pathogens frequently overcome single gene host resistance over time due to the emergence of new plant pathogen races. The combination of multiple genes (effective against specific races of a pathogen) can provide durable (broad spectrum) resistance (Kloppers and Pretorius, 1997; Shanti *et al.*, 2001; Singh *et al.*, 2001). In the past, it was difficult to pyramid multiple resistance genes because they generally show the same phenotype, necessitating a progeny test to determine which plants possess more than one gene. With linked DNA markers, the number of resistance genes in any plant can be easily determined. The incorporation of quantitative resistance controlled by QTLs offers another promising strategy to develop durable disease resistance. Castro *et al.*, (2003) referred to quantitative resistance as an insurance policy in case of the breakdown of qualitative resistance.

Table 3 The DNA marker used for marker assisted selection (Jena and Mackill, 2008)

Traits	Gene/QTL	Markers	Chromosome
Disease resistance			
1. Blast disease	*Pi-1*	RZ536, RG303, NpB181	11
	Pi-2	RG64, XNpb294	6
	Pi-4	RG869, XNpb289, RZ397	12
	Pi-5	RG498, RG788	4
	Pi-7	RG16, RG103A	11
	Pi-10	OPHF6	5
	Pi-11	RG228, RZ617	8
	Pi-b	R2511, C2782B, C379	2
2. Bacterail bight	*Xa-1*	XNpb235, XNpb181	4
	Xa2	Y03700	4
	Xa-3	XNpb186	11
	Xa-4	XNpb181, XNpb78, G181	11
	xa-5	RG556, RZ390, RM122, RM390	5
	Xa-7	G1091	6
	Xa-10	CD0365	11
	xa-13	OPAC05, RG136, R2027	8
	Xa-14	RG620	4
	Xa-21	RG103, PB7-8	11
	Xa-22(t)	R543	11
Insect resistance			
1. Brown planthopper (BPH)	*Bph1*	BpE18-3	12
	bph2	RG463, RG901, CDO344	12
	Bph3	RM190	6
	Bph(t)	RG457	12
2. Whitebacked planthopper (WBPH)	*Wph-l*	RG146B	7

Table 3 (Continued)

Trait	Gene/QTL	Markers	Chromosome
Grain quality and cooking quality			
1. Fragrance	*Badh2*	Aromarker	8
2. Amylose content	*Wx*	Waxy	6
4. Gel consistency	*Wx*	Waxy, RM204	6
3. Gelatinization temperature	*SSIIa*	SNP2340-41, GT11	6
Environment stress tolerance			
1. Submergence tolerance	*Sub1*	R10783Indel	9
2. Drought tolerance	DTQTLchr1	P-3, RM102, RM104	1
	DTQTLchr3	RM81, RM231	3
	DTQTLchr8	RM210	8
3. Salt tolerance	SalTolchr1	RM140, B1.1-1, B1.1-11	1
	salT	CD0548	1
Other trait			
1. Semi dwarfing	*Sd-1*	XNpb363, RG220	3
2. Photosensitivity	*PS*	RG64	6
3. Spikelet width	*wgl(t)*	W168A, Y1060L	5
4. Fertility restorer	Rf-1	XNpbl27, RG134	10
	Rf-4	RG532	1

MATERIALS AND METHODS

Plant Materials

Three breeding lines, TDK303-140-3-93 (fragrance), IR85264-34-141 (submergence tolerance) and RGD07529-1-1-38M-1-0 (blast resistance) were used as parental lines to develop the three ways cross. TDK303-140-3-93 (TDK303) derives from the BC_3F_2 (TDK1/Hom Nangnuan[*4]). It was developed by marker assisted backcrossing (MAB) at NAFRI through the Mekong breeding program. TDK303 has got fragrance phenotype (*badh2*) from Hom Nangnuan. IR85264-34-141 (IR85264) carrying the *Sub1* was developed by MAB at the International Rice Research Institute (IRRI). RGD07529-1-1-38M-1-0 (RGD07529) carrying the qBL1 and qBL11 in the background of RD6 was developed by MAB at Rice Gene Discovery Unit (RGDU).

Table 4 Three line of rice varieties were used in this study.

Breeding lines	Genetic backgrounds	Fragrance	Cooking quality	Submergence	Blast resistance
TDK303	TDK1	Fragrance (*badh2*^{HN})	Glutinous	Intolerance	unknown
IR85264	TDK1	Non-fragrance	Glutinous	Tolerance (*Sub1*)	unknown
RGD07529	RD6	Non-fragrance	Glutinous (RD6 cooking quality profile)	Intolerance	Broad spectrum resistance (*qBL1* and *BL11*)

Development of the three-way population

For combining the genes and QTL, we make the three-ways cross. First, TDK303 (female parent) was cross-pollinated with IR85264 (male parent). The cross was made in the wet season of 2009. Fifty one F_1 plants showing a heterozygous band at Aromarker (fragrance) and R10783 (*Sub1*) were used as female parent to cross with RGDU07529 as showing in Figure 6. The F_1 seeds from the three-way cross were germinated and checked with markers Aromarker, R10783 and RM224 for the

selection of cross-pollination. A hundred plants with positive allele at the Aromarker, R10783 and RM224 markers were genotyped with markers RM144, RM212 and RM319. Out of 100, 10 plants were identified to possess fragrance, submergence tolerance and blast resistance. These F_1 plants were self-pollinated to generate the F_2 population. Step-wise MAS was applied. First, RM212 and RM319 were genotyped 1,040 F_2 plants. The F_2 plants with the positive allele at both markers were then genotyped with RM144, RM224 and R10783. Lastly, the F_2 plants with the positive allele at these markers were genotyped with Aromarker. Finally, 263 F_2 plants were identified to possess the positive alleles at all six marker loci (either heterozygous or homozygous). Of 263 F_2 plants, two plants, RGDU10033-77 and RGDU10046-946 were selected based on plant type. These two plants possess the positive allele in form of homozygous at all six marker loci except for Aromarker and R10783 as heterozygous. They were self-pollinated to develop 636 F_3 plants. Aromarker and R10783 were used to genotype the F_3 population. 136 F_3 plants were found as homozygous at the two markers. Plant type selection was applied to select the F_3 plants with good agronomic characteristics. Ninety F_3 plants were selected and self-pollinated to generate F_4 population. Pedigree selection was applied to select the good plant type within the F_4 families. Fifty four F_4 plants were selected and planted as the plant to row fashion in F_5 generation. Pedigree selection was applied to select the good plant type within the F_5 families. Finally, twenty eight F_5 plants were selected and used for phenotypic evaluation in this study. Phenotypic evaluation included blast resistance, submergence tolerance and grain quality. All evaluations were conducted at Rice Gene Discovery Unit (RGDU), Kasetsart University, Kamphangsean Campus, Thailand.

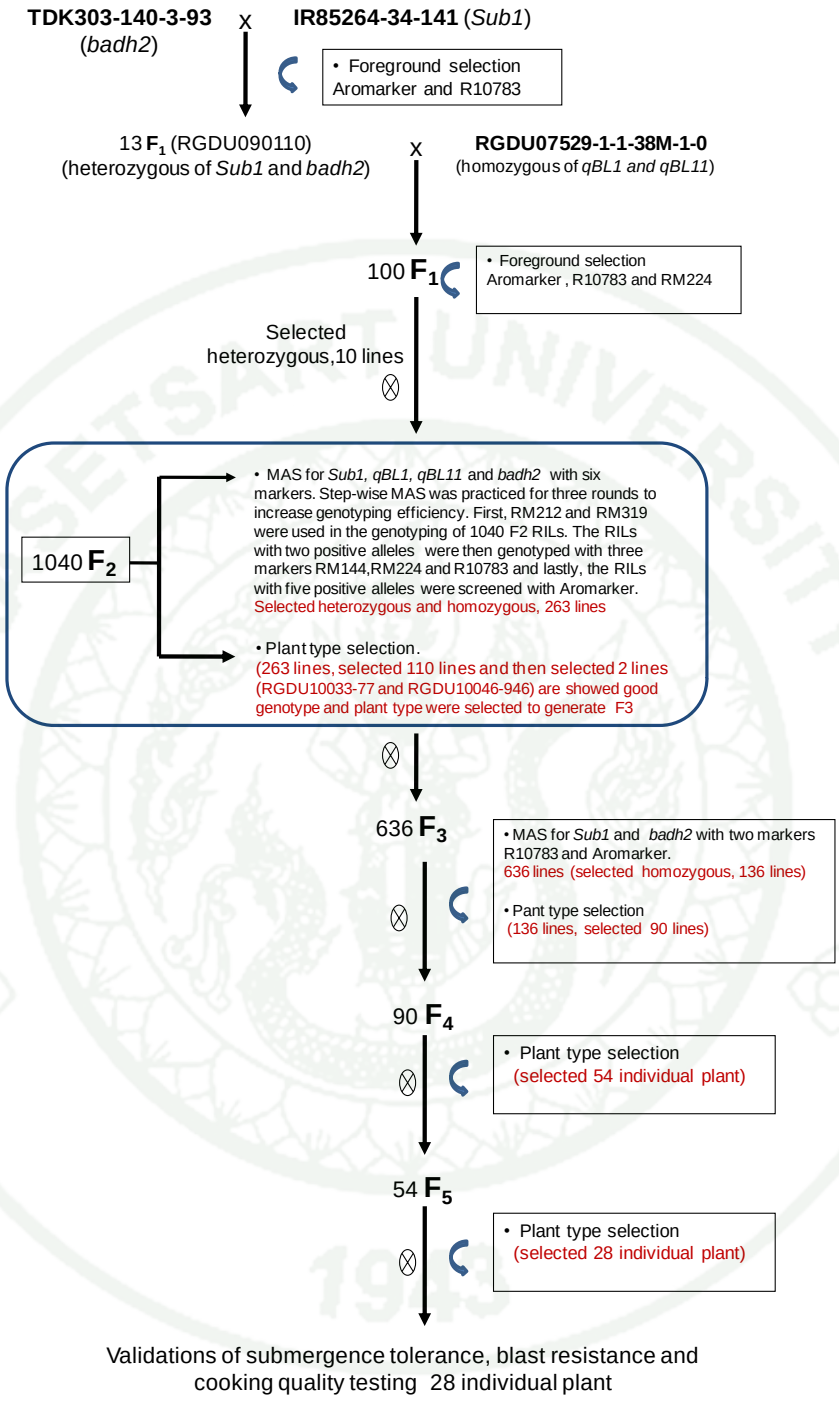


Figure 6 Marker assisted selection scheme showing the development of three way population. Foreground selection with six markers RM144, RM224, RM212, RM319, R10783 and Aromarker was applied in F_1 three-way cross, F_2 and F_3 generation. Plant type selection was applied in F_4 generation to select the F_5 plants used for the QTL validation.

DNA markers for a foreground selection

Six makers were used to select for fragrance, submergence tolerance and blast resistance in all breeding generation (Table 6). Indel marker R10783 located in the region of *Sub-1* gene on chromosome 9 was used to select for submergence tolerance (Toojinda *et al.,* 2004). Four microsatellite markers namely RM212, RM319, RM224, RM144, linked to the blast resistance QTL on chromosomes 1 and 11 were used to distinguish resistance and susceptible genotype (Wongsaprom *et al.,* 2009). Aromarker developed based on 8-bps deletion in the seventh exon of the *Os2AP* gene on chromosome 8 was used to distinguish between fragrance and non-fragrance (Wanchana *et al.,* 2003).

DNA markers for cooking quality

Twenty-eight DNA markers located on chromosome 6 were used to screen parental lines (TDK303, IR85264 and RGDU07529) as shown in Table 5. These markers locate in the vicinity of the *Wx* and *SSII*a. Waxy, a microsatellite marker developed by Cornell University, was used to amplify a fragment containing a (CT)n repeat in the putative 5' splice site leader intron of the Waxy gene, which is located on the short arm of chromosome 6 (Tian *et al.,* 2005). GT11, a closely linked microsatellite marker to the *SSIIa* gene developed by the Rice Gene Discovery Unit, Thailand, was used to distinguish high and low GT traits (Lanceras *et al.,* 2000).

Table 5 DNA markers located on chromosome 6 that were used for parental survey.

No.	Chromosome	Marker	No.	Chromosome	Marker
1	6	RM133	15	6	RM225
2	6	RM540	16	6	RM217
3	6	RM435	17	6	RM314
4	6	RM170	18	6	RM111
5	6	RM597	19	6	RM523
6	6	RM589	20	6	RM276
7	6	RM586	21	6	RM50
8	6	RM8114	22	6	GT2209
9	6	RM588	23	6	GT11
10	6	Waxy	24	6	RM136
11	6	RM190	25	6	RM564
12	6	Glu-23	26	6	RM541
13	6	RM587	27	6	RM162
14	6	RM204	28	6	RM3

Table 6 DNA markers and their primer sequences used for MAS in F_1 (three-way), F_2 and F_3

No.	Markers	Traits: qBL1	Chromosome	Direction	Sequence 5' to 3'	Reference
1	RM212	Blast resistance: *qBL1*	1	Forward Reward	CCACTTTCAGCTACTACCAG CACCCAAGGTACCTAGACCACCAC	Wongsaprom *et al.*, 2009
2	RM319	Blast resistance: *qBL1*	1	Forward Reward	ATCAAGGTACCTAGACCACCAC TCCTGGTGCAGCTATGTCTG	Wongsaprom *et al.*, 2009
3	RM144	Blast resistance: *qBL11*	11	Forward Reward	TGCCCTGGCGCAAATTTGATCC GCTAGAGGAGATCAGATCCGCATG	Wongsaprom *et al.*, 2009
4	RM224	Blast resistance: *qBL11*	11	Forward Reward	ATCGATCGATCTTCACGAGG TGCTATAAAAGGCATTCGGG	Wongsaprom *et al.*, 2009
5	Aromarker	Fragrance: *badh2*	8	Forward Reward	TGCTCCTTTGTCATCACACC TTTCCACCAAGTTCCAGTGA	Wanchana *et al.*, 2003
6	R10783	Submergence tolerance: *Sub1*	9	Forward Reward	CTGCTCCGACGACCTGATGG ATTAAATGGAACATTCGAGAAC	Toojinda *et al*., 2004

DNA analysis

1. Genomic DNA

Genomic DNA of each sample was extracted from leaves of the three weeks old seedling. Leaves were collected and cut into small pieces and put in 96 well plastic plates. The samples were stored in a container at -20^{o}C. Genomic DNA was extracted from 30 pieces of the leaf tissue according to the DNA trap method developed by DNA Technology Laboratory, Kasetsart University, Kamphaeng Saen, Thailand. The leaf sample was frozen with liquid nitrogen and ground into a fine powder with tissues striker grinder and the powder was then added to 500 µl of extraction buffer and incubate at 65^{o}C for tow hour. The sample was placed in ice for 5 minutes and added 100 µl of neutralizer and mixed well using vortex genie. The content was spun in a centrifuge at 4000 rpm for 10 min and then aqueous solution was transfer to new plate. DNA was precipitated in 500 µl of trapping buffer and gently mixed and spun at 2,500 rpm for tow min. The supernatant was removed and the pellet was washed twice with each 500 µl of washing buffer I and washing buffer II, spun at 2200-3000 rpm for 2 min, receptively. The sample was dried at 65^{o}C for tow hour, after that DNA was re-hydrated with 100 µl of elution buffer and incubated for 30 min at 65^{o}C. After finished centrifugation of sample for two minutes at 4000 rpm, DNA was transferred into a new 0.2 ml tube to a final concentration of 50-100 ng per µl.

2. DNA Quantification

The concentration of genomic DNA was determined using 1% agarose gel by comparing with standard DNA concentration (50, 100 and 200 ug/ul) Eeletrophoresis was run with 90 volts for 60 minutes in 0.5 % TBE buffer. The gels were strained with 1 ppm of Edthriduim Bromide in distilled water for 20 minutes and restrain with distillated water for 20 minutes. The DNA bands were made visible in UV elluminator and photo of each gel wad recored.

3. Polymerase Chain Reaction (PCR)

Target region of genomic DNA will be amplified by PCR using markers which showed polymorphism between two parents. The PCR reaction was performed in a 10 µl reaction mixture containing 2 µl of template DNA (50 ng), 1 µl of 10x PCR buffer, 0.2 µl of 25 mM $MgCl_2$ (final concentration 2 mM), 0.2 µl of 10 mM dNTP (final concentration 0.2 mM), 0.4 µl of 5 µM forward and reverse primers (final concentration 0.2 µM), 0.05 µl of 1 unit of Taq DNA polymerase (final concentration 0.25 unit). The volume was completed to 10 µl with distilled water. Sample was covered with one drop of mineral oil. PCR reaction was initiated at 95°C denatured temperature for 2 min followed by 40 cycles of 95°C for 30 sec, 55°C for 40 sec, 72°C for 1 min and final 5 min incubation at 72°C was allowed for completion of primer extension. The amplified product was electrophoresed on 4.5% denaturing silver-stained polyacrylamide gel.

4. Polyacrylamide gel electrophoresis and Staining

The polyacrylamide electrophoresis gels were prepared as follows. Wash a chamber plate with absolute ethanol using Kimwipes paper for 3 times, let dry. Wash a glass plate with absolute ethanol using Kimwipes paper for 3 times, let dry. Treat the glass with 1000 µl of Bind Silane solution, and then wash it with absolute ethanol again one time, let dry. Then, prepare a 50 ml of 4.5 % acrylamide gel solution. Apply the gel solution to the assembled gel plates and allow the gel to polymerize during 30 min. The 5 µl of amplification products with sequencing dye solution were denatured for 5 min at 95 °C in the thermo cycler and placed on ice before being applied to the will. The running conditions were, 60 W for 50 °C, after a pre-run of the gels. The electrophoresises were run in 1x TBE solution. The polymorphism of PCR products will be developed with the silver nitrate staining procedure.

Phenotypic evaluation and data recordings

Submergence tolerance (Flash flooding for 17 days)

Twenty eight F_5 lines and six check varieties including FR13A (origin of *Sub1*) and IR85264 (TDK1-*sub1*) as controls for tolerance, original TDK1, RD6, RGDU07529 and TDK303 as an intolerance control, were assessed for their submergence tolerance , two experiments were conducted. The first experiment was conducted under complete submergence in the outdoor lagoon located in Agronomy Field, Kasetsart University, Kamphangsean Campus, Thailand during dry season of 2012-2013. The second experiment was conducted under normal irrigation (control) in the experimental field located near by the outdoor lagoon. Both experiments were arranged in a randomized complete block design with three replications. The tested lines and checks were seeded in three-row plots with 18 plants per plots and 0.25 m between rows. After geminated to twenty-five days old seedlings will be transplanted into pond. Twenty days after transplanting, number of seedlings and plant heights were counted and measured for each plot in both experiment. In the experiment 1, lagoon was filled with water at a level of 1-1.2 m depth. To impose severe submergence stress, Seedlings were subjected to completely submerge for 14-17 days by keeping the water level at 1-1.2 m above leaf tip of the seedlings throughout the experimental period. The lagoon was drained out and seedlings were re-exposed to air for 10 days.

Trait measurements including numbers of surviving seedling and plant height taken 10 days after the water was drained from the submerging ponds, tolerance sore immediately taken after desubmerge using a scale of 1 to 9 (1 = all plants survive and 9 = all plants completely dead) based on Standard Evaluation system (SES)(IRRI, 2002). The percentage of surviving seedlings (PSS) was calculated as total number of surviving seedlings counted 10 days after the water was drained from the submerging ponds, divided by 100.

$$\text{Percentage of surviving seedling (PSS)} = \frac{\text{Number of surviving seedling}}{\text{Total number of seedling}} \times 100$$

The percentage of seedling elongation (PSE) was used as a measure of the increment in shoot height during submergence and calculated as the average different in shoot height be for and after desubmergence. To compare the impact of submergence on shoot elongation, the extension in height in each individual line was set to be 100%.

$$\text{PSE} = \frac{\text{Shoot height after submergence - Shoot height before submergence}}{\text{Shoot height after submergence}} \times 100$$

Figure 7 Submergence screening, during submerge and after submerge. (A) 22 days old seedling after transplanted. (B) submerged uncovered for 17 days by keeping the water level at 60-100 cm above leaf tip of the seedlings throughout the experimental period. (C) The lagoon was drained out and seedlings were re-exposed to air. (D) 10 days after plant re-exposed to air, numbers of survive seedlings were counted and plant heights were recorded.

Blast resistance

Screening for resistance in parental lines:

The parental lines (TDK303, IR85264 and RGDU07529), original parents (TDK1 and RD6) and susceptible check (Sariceltik) were tested for blast resistance against fifty-six blast isolates. Fifty-six blast isolates consisted of fourteen isolates representing the genetic diversity found in the three main rice ecosystems (rainfed

lowland, irrigated lowland and rainfed upland) in Lao PDR (Inthapaya *et al.*, 2011) and forty- two isolates representing the genetic diversity found in the rianfed lowland in Thailand (Hutamekalin, *et al.*, 2001). The list of blast isolates was showed in Table 5 and 6. The blast evaluation was performed by using an artificial inoculation at seedling stage. Single spore isolate was used for Lao strains and mixed isolates were used for Thai strains. The disease assessment was followed a protocol as described by (Roumen, *et al.*, 1997).

Table 7 List of blast isolates number, cluster, pathotype, regions, ecosystems and years of blast isolates collection from various rice growing regions of Laos (Inthapaya *et al.*, 2009; Phetmanyseng *et al.*, 2011).

Isolate no.	Cluster	Pathotype	Regions	Ecosystems	Year
H08-040-1	2	U63-i0-k100-z04-ta403	Central area	Rainfed Lowland	2008
H08-269-1	4	U43-i7-k100-z04-ta003	Southern area	Rainfed Lowland	2008
H08-025-1	5	U23-i0-k100-z00-ta002	Central area	Rainfed Lowland	2008
H08-044-1	8	U63-i6-k100-z00-ta700	Central area	Rainfed Lowland	2008
H08-259-1	9	U03-i7-k127-z04-ta031	-	-	2008
H08-245-1	3	U63-i0-k000-z00-ta031	Northern area	Rainfed Lowland	2008
H08-243-1	10	U41u-i6-k175-z00-ta-ta000	-	-	2008
H08-234-1	11	U53-i5-k107-z04-ta003	-	-	2008
H08-190-1	11	U41-i7-k106-z04-ta021	-	-	2008
H08-184-1	1	63-i0-k100-z14-ta403	Northern area	Rainfed Lowland	2008
H08-171-1	9	U03-i0-k137-z06-ta031	Northern area	Rainfed Upland	2008
H08-158-1	9	U41-i7-k177-z06-ta031	Northern area	Rainfed Upland	2008
H08-027-1	6	U23-i0-k100-z00-ta402	Central area	Rainfed Lowland	2008
X09-42-1	-	-	-	-	2009

Table 8 List of blast isolates by mixed isolate group, isolate code, AFLP group and difference providences of blast isolates collection from various rice growing regions of Thailand (Hutamekalin, *et al.*, 2001).

Mixed isolate group and Isolates code	AFLP Group	Providence	Year
THL832	4	Mae Hong Son	2001
THL710	9	Mae Hong Son	2001
THL282	10	Phrae	2001
THL137	2	Chiang Mai	2001
THL906	12	Yala	2001
THL122	13	Chiang Rai	2001
THL757	14	Mae Hong Son	2001
THL603	16	Surin	2001
THL191	3	Phitsanulok	2001
THL266	3	Lampang	2001
THL456	3	Sakon Nakon	2001
THL653	3	Chiang Mai	2001
THL658	3	Chiang Rai	2001
THL730	3	Mae Hong Son	2001
THL810	3	Ubon Ratchathani	2001
THL838	3	Srisaket	2001
THL967	3	Surin	2001
THL985	3	Nongkhai	2001
THL144	6	Chiang Mai	2001
THL1023	6	Phayao	2001
THL303	-	Lampang	2001
THL906	12	Yala	2001
THL690	6	Lamphun	2001
THL41	7	Phitsanulok	2001
THL855	7	Prachin Buri	2001
THL949	7	Suphan Buri	2001
THL1003	7	Bangkok	2001
THL1009	7	Sra kaew	2001
THL458	3	Surin	2001
THL831	3	Mae Hong Son	2001
THL943	-	Ayutthaya	2001
THL186	UN	Phrae	2001
THL190	UN	Phitsanulok	2001
THL486	UN	Tak	2001
THL634	UN	Lampang	2001
THL868	UN	Sri saket	2001
THL211	1	Chiang Mai	2001
THL244	8	Pathum Thani	2001
THL374	3	Nakorn ratchasima	2001
THL734	3	Mae Hong Son	2001
THL759	4	Mae Hong Son	2001
THL1066	UN	Chiang Mai	2001

Rice seedling preparation

Rice seeds of tested lines were soaked in clean water for 72 hr and then incubated at room temperature. The germinated seeds of each line were planted in plastic trays with 6 x 12 holes (whole size of 5 x 5 x 4 cm) and kept in the greenhouse under high humidity for 14 days old. The plants were watered two times per day, fertilized weekly with 16-00-00 NPK fertilizer. In the final week, the fertilizer was applied three days before inoculation. The experimental unit consisted of 2 trays. Each rice line was planted in one hole of four plants.

Assessment of resistance

Blast isolates were cultured on the agar (Rice bran 20 g, Agar 20 g, yeast extract 2 g and dH_2O 1 liter) in petridish, inocubated at 27ºC for 7 days. The mycelia were scarped with glass rod then plates were transferred to a cabinet with black light for two days to induce sporulation. On the day of inoculation, dH_2O was added into the Petri dish; the mycelial mat was scraped with glass rod. The suspension was transferred to test tube and adjusted a concentration to 5×10^5 conidia per ml in 0.5% gelatin.

Inoculums were sprayed onto 14 day-old rice seedling that planted in plastic tray with soil at the greenhouse by using air brush spray. Inoculated plants were incubated at 25 ºC, 100% humidity for overnight (at least 16 hours). On the next day, the inoculated plants were returned to the greenhouse (Figure 10). Lesion scores were recorded at 7 days after inoculation. Lesion score was scored based on infection type or disease severity index using a scale of 0-6. The classification of resistance and susceptible was followed as described by (Roumen, *et al.*, 1997). Lesion scores are showed in Figure 9 and Table 8.

Table 9 The 7 lesion type scales for the assessment of symptoms induced by the blast pathogen on rice leaves.

Lesion type	Symptoms
0	No evidence of infection
1	Brown pinpoint smaller than 0.5 mm., without sporulation
2	Brown pinpoint smaller than 0.5-1 mm., without sporulation
3	Small eyespot about 1-3 mm., lesion capable of sporulation gray center
4	Small eyespot about 3 mm. or more longs, lesion capable of sporulation gray center and dark margin
5	Susceptible sporulation type, coalescence lesion without dark margin
6	Susceptible sporulation type, lesion without dark margin

Figure 8 The 7 lesion type scales for the assessment of symptoms induced by the blast pathogen on rice leaves (Sreewongchai, 2008).

Figure 9 Five steps of methodology of leaf blast inoculation at the seedling stage.

Evaluation of blast resistance in the breeding lines

Unfortunately, all parental lines were highly resistance to broad spectrum of blast isolates both from Lao PDR and Thailand. Four Lao isolates showing a clear differential reaction between parents (H08-040-1, H08-269-1, H08-025-1 and H08-044-1) were used for the validation of the blast resistance.

Assessment of blast resistance

In the greenhouse screening, 28 RILs and 5 original parents (TDK1, RD6, TDK303, IR85264 and RGDU07529) and susceptible check, Saricletik were grown in the plastic tray with 4 plants per line. Nitrogen fertilization was applied twice at 2 g per tray (approximately 184 kg N/ha) at 17 and 21 days after planting. Seedlings were kept in greenhouse for 14 days. The 17-days-old seedlings were then used as plant materials for inoculation with 4 blasts isolates. Inoculums preparation, inoculation method and disease assessment were followed those described above.

Evaluation of grain quality traits

Grain quality including morphological features of the grain and cooking quality was evaluated using grain samples harvested from experimental field planted in 2012. Rice grains of the breeding lines and checks were harvested and sun-dried naturally in a greenhouse. The dried grains were stored at room temperature for one month prior to the evaluation of grain quality traits. Grain samples of 100 g were taken from each replicate and combined. Grain samples were mechanically dehulled and polished by a mini polisher. The evaluations of four cooking quality traits were replicated three times.

Fragrance and non-fragrance were determined by a sensory test as described by Yi *et al*., 2009. The samples were smelled and scored for fragrance by three panelists. Amylose content (AC), gel consistency (GC) and alkali spreading value (ASV) were evaluated following the procedures described by Cagampang *et al.*, (1973), Juliano (1985) and Lanceras *et al.*, (2000). For AC, the absorbance was recorded at 620nm using a spectrophotometer. The AC was estimated using a standard curve developed from known quantities of purified potato amylose from Fluka Thailand. Rice varieties may be classified as high-, intermediate-, low-, very low and no-(glutinous or waxy) amylose classes with >25%, 20-25%, 12-20%, 5-12% and 0-5% of the apparent amylose (Table 7) (Juliano, 1971).

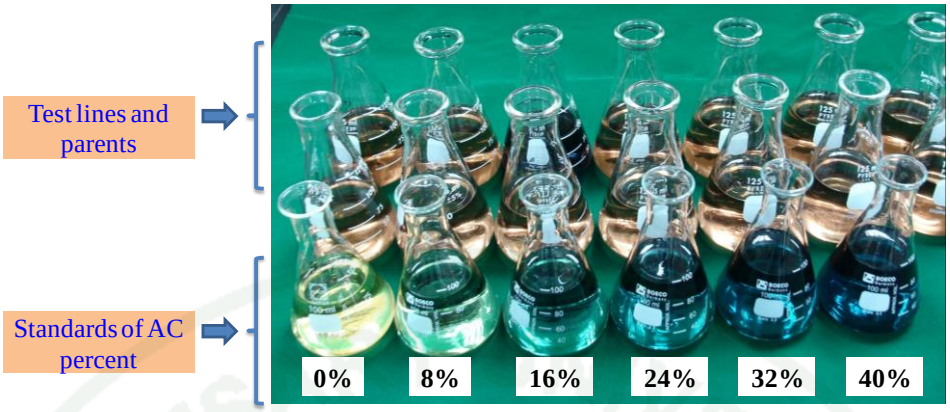


Figure 10 Percentage of AC estimated using a standard curve developed from known quantities of purified potato amylose from Fluka Thailand. The absorbance was recorded at 620nm using a spectrophotometer.

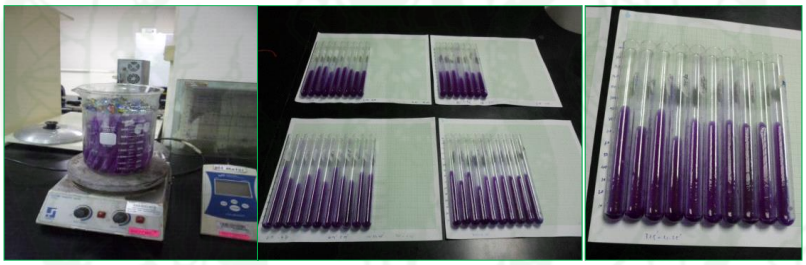
Table 10 Classification of rice varieties based on proportion of amylose content and its related to texture of cooked rice.

Varietal class	% Amylose	Texture
Waxy	0-5%	Sticky, glossy, firm
Very Low	5-12%	Moist, sticky, split
Low	12-20%	Moist, sticky, split
Intermediate	20-25%	Moist, tender, do not hard
High	25-33%	Dry, fluffy and hard

GC was measured by the length in a culture tube of cold gel according to the method of Cagampang *et al.*, (1973). One hundred milligrams of rice powder was put in a 10 mm x 110 mm culture tube and wetted with 0.2 ml of 95% ethanol containing 0.025% thymol blue was added. Two milliliters of 0.2N KOH was added. The sample was mixed using vortex Genie mixer. The test tube was covered with glass marble. The sample was cooked in a boiling water bath for 10 minutes, making sure that the tube content reach 2/3 the height of the tube. The test tube was removed from the water bath and let stand at room temperature for 5 minutes. The tube was cooled in an ice-water bath for 20 minutes and laid horizontally on a laboratory table lined with

millimeter graphing paper. The total length of the gel was measured in millimeter one hour later as distance from the bottom of the tube to the front of the gel migration. The gel length thus obtained provides a measurement of the GC: the longer the distance, the softer the gel. The gel consistency value was evaluated by hard (26-40mm), medium (41-60mm) and soft (61-100mm), short gel indicates hard GC and long gel represents soft GC.

Comical and step of gel consistency test



Score of gel consistency (GC)

Figure 11 Schematic of gel consistency was measured by the length in a culture tube of cold gel according to the method of Cagampang *et al.*, (1973).

Table 11 Gel consistency is classified as given below.

Category	Consistency (mm)
Soft	61-100
Medium	41-60
Medium hard	36-40
Hard	26-35

Gelatinization temperature was indirectly measured by evaluating the ASV using the method of Little *et al.,* (1958). Each sample was tested three times. Each time, six intact milled grains were put in a petridish, to which 10 ml of 1.7% KOH was added. The grains were carefully separated from each other using a forceps and incubated at 30˚C for 23 hours to allow spreading of the grains. The spreading value of the grains was scored on a numerical scale of 1 to 7 by visual assessment. 1, grain unaffected; 2, grain swollen; 3, grain swollen, collar incomplete and narrow; 4, grain swollen, collar complete and wide; 5, grain split , collar complete and wide; 6, grain dispersed, merging with collar and 7, grain dispersed and disappeared completely. Alkali spreading value (ASV) corresponds to GT as follows; 1-2 high (74-79^{o}C), 3 high- intermediate, 4-5 intermediate (70-74^{o}C) and 6-7 low (55-69^{o}C). A larger ASV represents more spreading in alkali, indicating a lower GT and a smaller ASV indicates a higher GT.

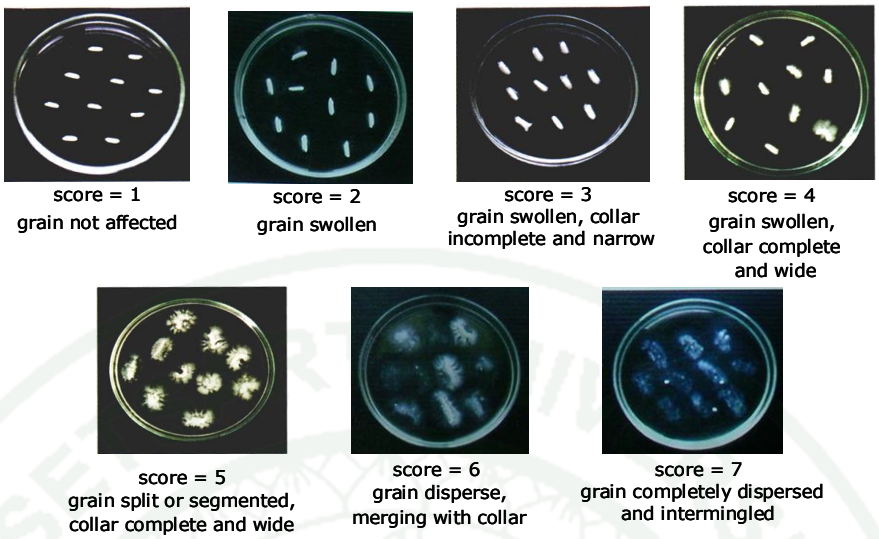
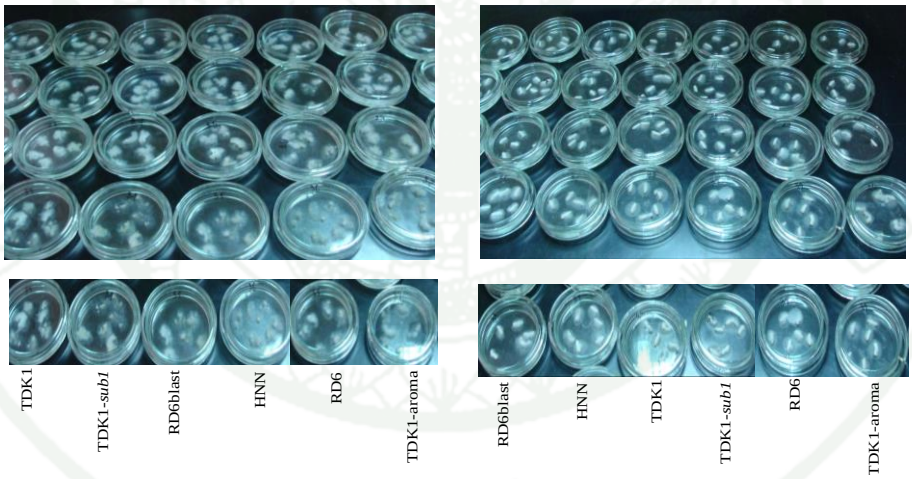


Figure 12 Alkali spreading scores for measuring gelatinization temperature by used 1.7 % and 1.3% KOH (Little *et al.*, 1958)



Alkali condition of 1.7% KOH Alkali condition of 1.3% KOH

Figure 13 Gelatinization temperature (GT) of RILs tested with two conditions of alkali digestibility values used 1.7% KOH and 1.3% KOH, at 23 hour.

Table 12 Alkali spreading scores for measuring gelatinization temperature (GT)

Gelatinization Temperature (^oC)	Range	Range of scores	Range of cooked time (minute)
< 65	Low	6 - 7	12 - 16
70-74	Intermediate	4 - 5	16 - 24
>75	High	1- 3	>24

Morphological features of rice grains include the appearance of the endosperm and the size and shape of the kernel. Ten seeds of milled rice kernel were used for the measurements of length and breadth using vernier calipers and the length/breadth ratios (L/B) were calculated. The appearance of the rice endosperms was determined by a visual assessment according to the procedure described by Tan *et al.* (2000).

Evaluation for Agronomic characteristics

Agronomic characteristics of the breeding lines and parents were tested in non-replicated trial conducted at the Kamphaeng Saen campus (KPS), Kasetsart University Thailand during the month of November to February, 2012 - 2013. The seeds of tested lines and parents were sown in a seed bed nursery. Three-week-old seedlings were then manually transplanted into the rice field with one seedling per hill. Plot size was 2m x 3m and each plot consisted of eight rows with twelve plants per row and had a planting density of 25 cm between plants (within a row), and 25 cm between rows. The field was fertilized twice by hand broadcasting with 120 kg/ha of urea, 75 kg/ha of P2O5 and 75 kg/ha of K2O. Weed control was performed using chemical herbicide and hand weeding. Field water was maintained during the tillering stage at approximately 10cm until 15 days before harvesting. Data collections of morphological traits were taken as shown in the Table 12.

Table 13 Description and unit of traits taken from the field experiment in 2012-2013

Traits	Unit	Descriptions
Days to 100% flowering (DF100)	days	Number of days from planting to 100% Of the plants within a plot with flowers
Plant height (PH)	cm	Height of plant from soil level to the tip of leaf
1000 grain weight (GW)	gram	Weight of 1000 seeds
Grain length of polished rice (LP)	mm	Seed length of polished rice
Grain length of unhusked rice (LP)	mm	Seed length of paddy rice
Grain width of polished rice (WP)	mm	Seed width of polished rice
Grain thickness of polished rice (TP)	mm	Seed thickness of polished rice
Grain thickness of unhusked rice (TU)	mm	Seed thickness of paddy rice
Grain width of unhusked rice (WU)	mm	Seed width of paddy rice

RESULTS AND DISCUSSION

Results

Marker assisted selection (MAS)

MAS were conducted in all breeding generation. Number of lines being tested and the foreground markers were presented in Table 15. In this study, we combined target genes and QTL by developing the three-ways cross. First, TDK303 (as female parent) was cross-pollinated with IR85264 (as male parent) to develop 54 F_1 seeds. Thirteen F_1 plants were used as female parent to cross with RGDU07529 to produce 100 F1 three-ways seeds. The 100 F_1 plants were genotypes with markers for a presence of the *badh2* (Aromarker), *Sub1* (R10783) and *qBL11* (RM224) alleles. Ten F1 three-ways plants were selected and self-pollinated to produce 1040 F_2 seeds in which they were genotyped with six markers (RM144, RM224, RM212, RM319, R10783 and Aromarker). Of 1040 plants, 263 possessing the positive allele at all six marker loci (either heterozygous or homozygous) were identified. Of 263 F_2 plants, two plants, RGDU10033-77 and RGDU10046-946 were selected based on the expression of excellent plant type. These plants carried all positive marker alleles except for the Aromarker and R10783 markers (as heterozygous). They were self-pollinated to develop 636 F_3 plants in which they were subjected to screen by Aromarker and R10783 markers. 136 F_3 plants were found to possess the homozygous positive allele at the two markers. Plant type selection was applied to select the F_3 plants with good agronomic characteristics. Ninety F_3 plants were selected and self-pollinated to generate F_4 population. Pedigree selection was performed in F4 and F5 generations. Finally, twenty eight F_5 plants were selected.

Table 14 Population development and Marker assisted selection for aroma, submergence tolerance and blast resistance.

Generations	Cross	Foreground markers	Additional markers	Tested samples	Selected samples	Plant type selection
F_1	TDK303/IR85264	Aromarker and R10783	*badh2 and Sub1*	54	13	
F_1	(TDK303/IR85264)/RGD07529	Aromarker, R10783, RM224	*badh2, Sub1 and qBL11*	100	10	
F_2	(TDK303/IR85264)/RGD07529	Aromaker, R10783, RM212, RM319, RM144, RM224	*badh2, Sub1, qBL1and qBL11*	1040	263	
F_3	(TDK303/IR85264)/RGD07529	Aromaker, R10783	*badh2, Sub1*	636	163	90
F_4	(TDK303/IR85264)/RGD07529			90		54
F_5	(TDK303/IR85264)/ GD07529			54		28

Submergence tolerance

Twenty eight breeding lines (F_5) and 6 standard checks, including FR13A and IR85264 (submergence tolerance) and TDK1, RD6, RGD07529 and TDK303 (intolerance) were assessed for their responses to submergence stress for 17 days. The result indicated that submergence stress was clearly limited the plant growth as seen in the significant difference of the NEPT (69 days) between control and submerged experiments (Figures 16 and 17). Average NEPT (69 days) was 21 and 2 (tillers) in control and submerged respectively while average PH (69 days) between control and submerged was not significantly differed (62.6 cm). Ten days after the water was drained from the submerging ponds. Intolerance checks (TDK1, RD6, TDK303 and RGD07529) showed typical symptoms such as high PSE and low PSS: while tolerant checks (FR13A and IR85264) showed opposite phenotypes (low PSE and high PSS).

Complete submergence decreased PSS in both tolerance and intolerance varieties but the decrease was significantly less in the tolerance one. The PSS was 95 and 78% for FR13A and IR85264 respectively. The PSS of the breeding lines carrying the *Sub1* ranged from 39.72% to 92.92% (average of 66.3%) which were slightly lower than that of the tolerant check, FR13A (PSS=95%). Ten breeding lines consisted of RGDU10033-MAS-77-438-51(PSS = 79%), RGDU10033-MAS-77-149-14 (PSS = 80%), RGDU10033-MAS-77-149-16 (PSS = 81%), RGDU10033-MAS-77-327-45, RGDU10033-MAS-77-438-48, RGDU10033-MAS-77-438-47 and RGDU10033-MAS-77-303-36 (PSS = 83%) and RGDU10033-MAS-77-524-75 (PSS = 87%), RGDU10033-MAS-77-438-46 (PSS = 89%) and RGDU10033-MAS-77-149-17 (PSS = 90%) showed higher PSS than the donor parent IR85264-34-141 (PSS = 78%). The rest of the tested lines showed PSS equal to or slightly below that of the IR85264. However, the PSS of all breeding lines was significantly higher than that of intolerant checks, RD6 (PSS = 6%), TDK1 (PSS = 0%) and intolerance plants, RGD07529 (PSS = 4%) and TDK303 (PSS = 16%).

Shoot elongating (SE) under water when rice plant experiences submergence stress is one of the key traits determining submergence tolerance. The SE of some

intolerant varieties increased rapidly underwater (Figure 17). PSE was high in RD6 (66.7%) and RGD07529 (46.9%) but low in TDK1 (18.1%) and TDK303 (29.9%). All breeding lines showed low PSE which was not significant differed from tolerant parent IR85264 but significantly differed from intolerant parents (Figures 18, Table 15).

Total 15 Comparisons of submergence tolerance performance among parents and RILs calculated by combined analysis.

Traits	Parents												RILs mean		LSD (0.05)		F-test		CV (%)	
	FR13A		IR85264		TDK303		RGDU0529		TDK1		RD6									
	NC	SC	NC	SC	NC	SC	NC	SC	NC	SC	NC	SC	NC	SC	NC	SC	NC	SC	NC	SC
NETP (52d)	3	6	3	4	3	3	4	3	4	5	4	3	4	4	1	3	**	ns	22.3	42.5
NETP (69d)	27	8	26	2	23	0	20	0	33	0	21	0	21	2	5	2	**	**	20.9	61.0
PH (52 d)	57.8	66.1	46.7	53.3	54.2	60.1	58.8	56.8	47.1	53.9	53.3	57.3	49.8	53.7	4.9	4.9	**	**	7.8	8.9
PH (69d)	65.1	75.1	59.7	55.7	66.9	78.1	77.1	83.2	57.7	70.4	66.9	95.1	62.6	62.6	7.8	10.1	**	**	6.2	9.9
PES	12.8	13.6	27.7	8.2	23.6	29.9	31.3	46.9	22.5	18.1	31.0	66.7	26.4	17.5		15.2		**		53.2
NP (52d)		13.7		13.3		11.0		8.7		5.7		10.0		13.8		5.1		**		22.6
NP (69d)		13.0		10.3		2.0		0.7		0.0		0.7		9.3		4.6		**		30.2
PSS		95.0		78.0		16.3		4.2		0.0		6.1		66.3		26.6		**		24.6

Notes:

NC = normal condition, SC = submerged condition, NETP (52 days) = number of effective tillers per plant beginning; NETP (69 days) = number of effective tillers per plant after submerged; PH (52 days) = plant height before submerged; PH (69 days) = plant height after submerged; PES= percentage of seedling elongation; NPB=number of plant beginning submerged; NPA=number of plant after submerged; PSS=percentage of surviving seedlings, **=significant at $P < 0.01$; ^{ns}=not significant

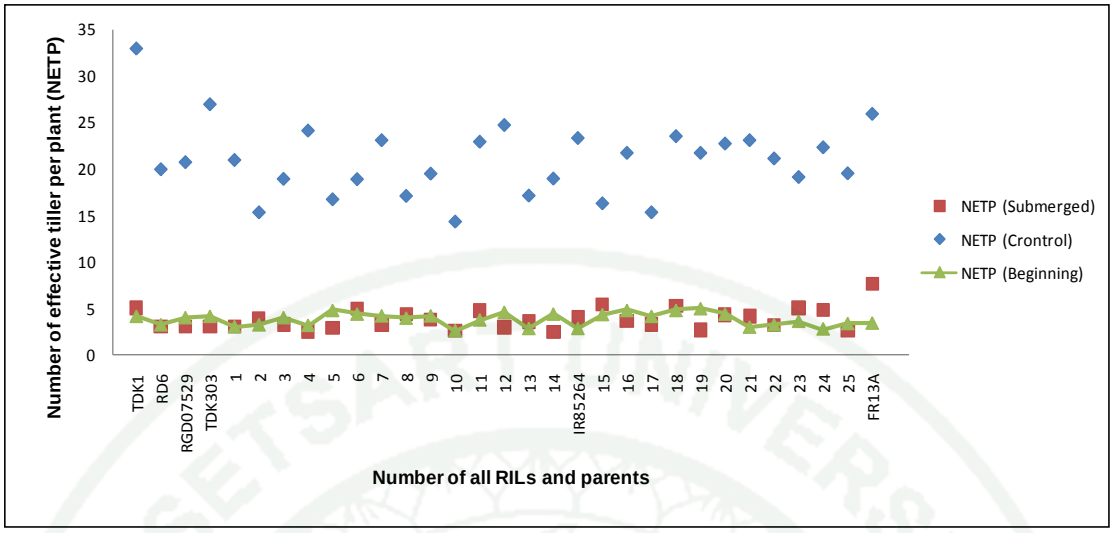


Figure 14 Comparison of the plant growth of the tested lines and checks based on numbers of tiller (NETP) before (52 days) and after (69 days) submergence.

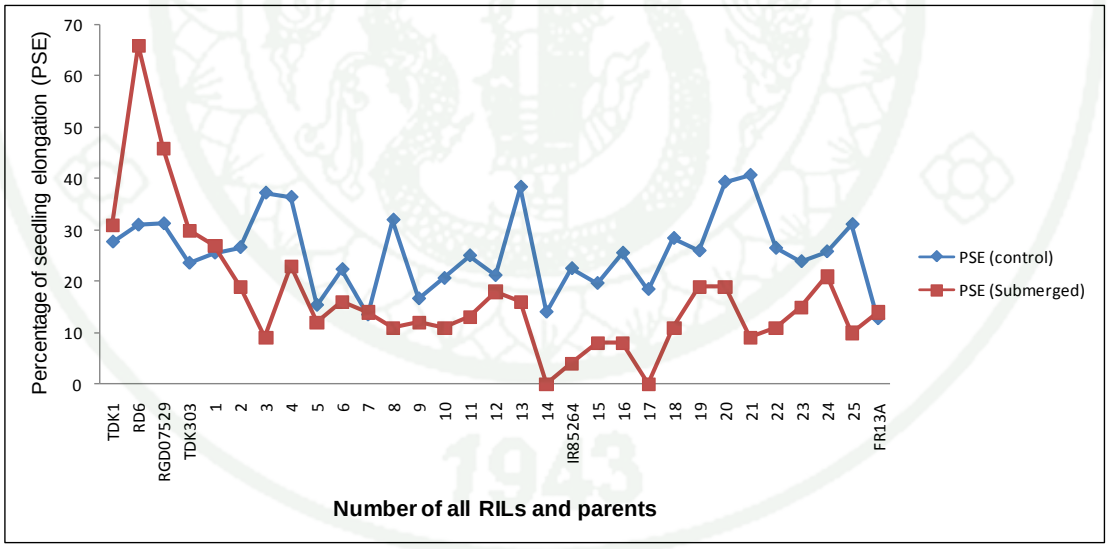


Figure 15 Comparisons of the plant growth of the tested lines and checks based on the plant height (PH) before (52 days) and after (69 days) submergence.

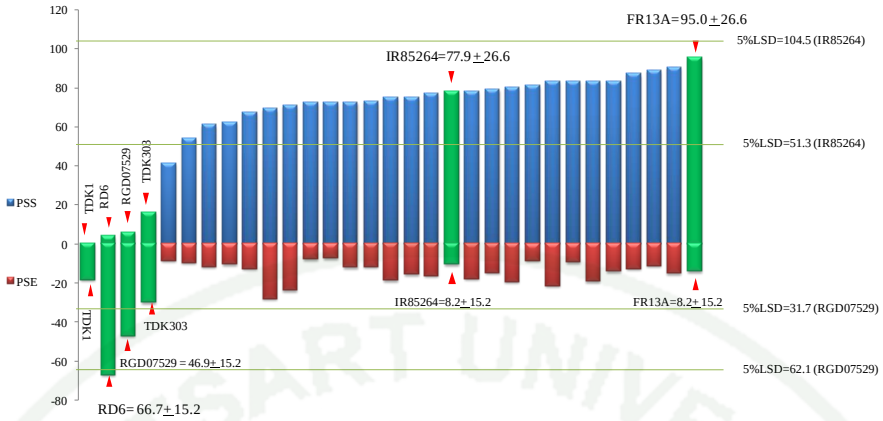


Figure 16 PSS and PSE of 25 breeding lines derived from the three-way cross (TDK303-140-3-93/IR85620-34-141/RGD090110-4/RGD07529-1-1-8M-1-0) and checks.

Blast resistance

1. Evaluation of blast resistance in parental lines with Laos and Thai isolates.

Three check varieties, TDK1, RD6 and Sariceltik and three parental lines, IR85264, TDK303 and RGD07529 were screened for blast resistance at seedling stage against 14 Laos isolates and 42 Thai isolates (9 mixed isolates) (Table 18 & 19). We classified resistance reaction based on lesion scores into three types as resistance (R: lesion score 0, 1 and 2), moderate resistance (MR: lesion score 3 and 4) and susceptible (S: lesion score 5 and 6). Sariceltik was very susceptible to all Laos and Thai isolates. TDK1 showed high level of resistance to all isolates from Laos except for H08-044-1 and to all mixed isolates from Thailand. RD6 showed high level of resistance to all isolates from Laos except for H08-269-1 and to most mixed isolates from Thailand except for mixed 3, 4, 8, and 9. All parental lines (IR85264, TDK303 and RGD07529) were resistance to all tested isolates from Thailand and Laos. This is an unfortunated result because NAFRI had reported that TDK1 is very susceptible to blast disease in Laos PDR. However, we selected four isolates from Laos (H08-025-1, H08-040-1, H08-044-1 and H08-269-1) in which some parental lines showing MR to evaluate the breeding lines for blast resistance.

Table 16 Pathogenicity of the four recombinant strains and their parents based a differential reaction on the eleven rice varieties inoculated with 14 isolates from Laos. Disease scores were rated at seedling stage following the 0-6 scales described by International Rice Research Institute (IRRI).

Varieties/Pedigree	Blast isolates													
	H08-190-1	H08-184-1	H08-27-1	H08-040-1	H08-245-1	H08-259-1	X09-042-1	H08-158-1	H08-171-1	H08-025-1	H08-044-1	H08-269-1	H08-243-1	H08-234-1
IR85264-34-141	1(R)	0(R)	1(R)	0(R)	1(R)	1(R)	1(R)	1(R)	2(R)	1(R)	4(MR)	0(R)	0(R)	0(R)
TDK303-140-3-93-4-27	0(R)	1(R)	0(R)	0(R)	0(R)	1(R)	1(R)	1(R)	2(R)	2(R)	1(R)	0(R)	1(R)	0(R)
RGD07529-1-1-38M-1-0	2(R)	1(R)	0(R)	0(R)	0(R)	3(MR)	3(MR)	2(R)	2(R)	1(R)	1(R)	0(R)	1(R)	3(MR)
TDK1	0(R)	0(R)	1(R)	0(R)	0(R)	1(R)	1(R)	1(R)	1(R)	0(R)	5(S)	0(R)	1(R)	0(R)
RD6	3(MR)	3(MR)	2(R)	6(S)	0(R)	3(MR)	2(R)	2(R)	2(R)	2(R)	1(R)	S(6)	2(R)	3(MR)
Sariceltic	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	4(MR)	6(S)	3(MR)	6(S)

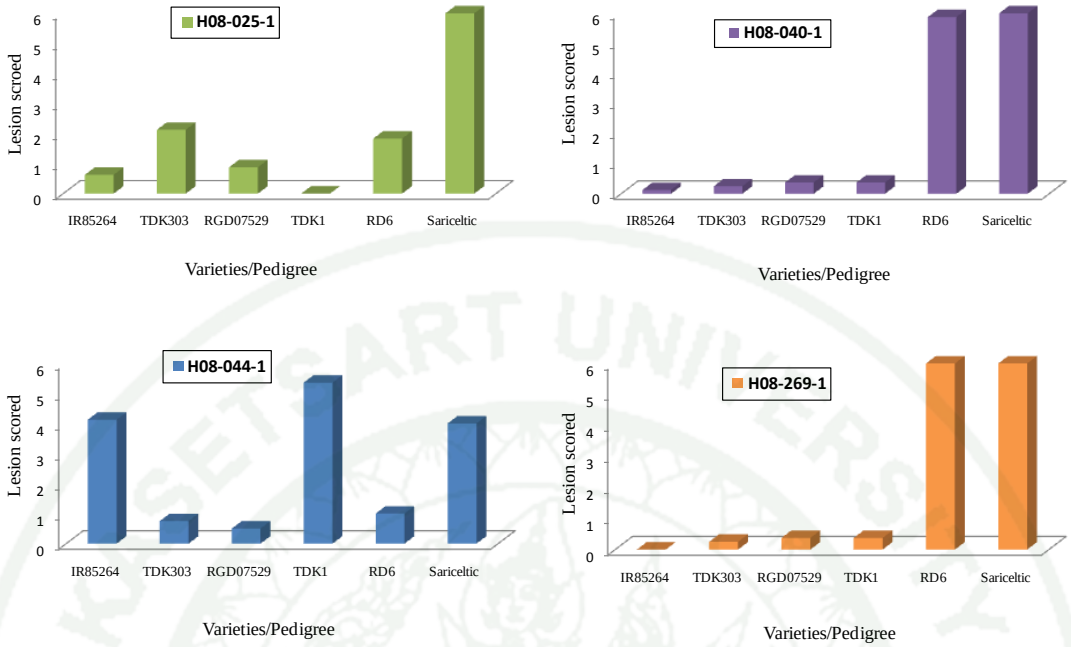


Figure 17 Distribution freguency of the lesion score obtained on the Prarent inoculated with 4 Laos isolates. lesion types were scored 0 (resistant) to 6 (susceptible) according to a standard reference scale.

Table 17 Pathogenicity of the three recombinant strains and their parents based a differential reaction on the seven rice varieties inoculated with 9 mixed isolates from Thai. Disease scores were rated at seedling stage following the 0-6 scales described by International Rice Research Institute (IRRI).

Varieties/Pedigree	Mixed isolate1	Mixed isolate2	Mixed isolate3	Mixed isolate4	Mixed isolate5	Mixed isolate6	Mixed isolate7	Mixed isolate8	Mixed isolate9
IR85264-34-141	0(R)	0(R)	0(R)	1(R)	0(R)	0(R)	0(R)	0(R)	0(R)
TDK303-140-3-93-4-27	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)
RGD07529-1-1-38M-1-0	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)	0(R)
TDK1	0(R)	0(R)	0(R)	1(R)	0(R)	0(R)	0(R)	0(R)	0(R)
RD6	0(R)	4(MR)	5(S)	5(S)	0(R)	4(MR)	0(R)	5(S)	5(S)
Sariceltik	5(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)	6(S)

2. Evaluation of blast resistance in breeding lines

Twenty eight breeding lines, 3 parental lines and four checks (TDK1, RD6, Sariceltik and US2) were inoculated with four blast isolates from Laos, H08-025-1, H08-040-1, H08-044-1 and H08-269. All breeding lines showed high level of resistance (Score 0-1) against all tested isolates. RD6, Sariceltik and US2 were susceptible to H08-025-1, H08-040-1, H08-044-1 and H08-269-1 except for RD6 that was resistant to H08-025-1(Table 20).

Table 18 Pathogenicity of the F_5 RILs strains and their parents inoculation with 4 isolates from Laos. Disease scores were rated at seedling stage following the 0-6 scales described by International Rice Research Institute (IRRI).

Variety/Pedigree	Blast resistance gene	Blast isolates			
		H08-025-1	H08-040-1	H08-044-1	H08-269-1
RGD10033-MAS-77-43-2	*qBl1*,*qBl11*	0(R)	1(R)	0(R)	0(R)
RGD10033-MAS-77-43-3	*qBl1*,*qBl11*	0(R)	1(R)	0(R)	0(R)
RGD10033-MAS-77-149-14	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-149-16	*qBl1*,*qBl11*	0(R)	0(R)	1(R)	0(R)
RGD10033-MAS-77-149-17	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-149-18	*qBl1*,*qBl11*	0(R)	1(R)	1(R)	0(R)
RGD10033-MAS-77-291-20	*qBl1*,*qBl11*	0(R)	1(R)	0(R)	0(R)
RGD10033-MAS-77-291-21	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-291-22	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-291-23	*qBl1*,*qBl11*	0(R)	0(R)	1(R)	0(R)
RGD10033-MAS-77-291-24	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-291-25	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-298-26	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-298-27	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-298-31	*qBl1*,*qBl11*	0(R)	0(R)	1(R)	0(R)
RGD10033-MAS-77-303-35	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-303-36	*qBl1*,*qBl11*	0(R)	1(R)	0(R)	0(R)
RGD10033-MAS-77-327-42	*qBl1*,*qBl11*	0(R)	1(R)	0(R)	0(R)
RGD10033-MAS-77-327-43	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-327-44	*qBl1*,*qBl11*	0(R)	1(R)	0(R)	0(R)
RGD10033-MAS-77-327-45	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-438-46	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-438-47	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-438-49	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-438-50	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-438-51	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-524-76	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10033-MAS-77-524-77	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10046-MAS-592-13	*qBl1*	0(R)	0(R)	4(MR)	1(R)
RGD10046-MAS-609-2	*qBl11*	0(R)	0(R)	0(R)	0(R)
RGD10046-MAS-576-40	-	1(R)	0(R)	2(R)	0(R)
RGD10046-MAS-592-5	-	0(R)	0(R)	0(R)	0(R)
RD6	-	0(R)	6(S)	0(R)	6(S)
TDK1	-	0(R)	0(R)	4(MR)	0(R)
IR85264-34-141	-	0(R)	1(R)	4(MR)	0(R)
TDK303-140-3-93-4-27	-	0(R)	0(R)	0(R)	0(R)
RGD07529-1-1-38M-1-0	*qBl1*,*qBl11*	0(R)	0(R)	0(R)	0(R)
Sariceltik	-	6(S)	6(S)	0(R)	6(S)
US2	-	6(S)	6(S)	2(R)	6(S)

Cooking quality

The chemical grain quality traits were measured using seeds harvested from paddy field in 2012. Table 22 showed the summary of measured traits. Amylose content (AC) of the breeding lines were ranged from 5.8-6.9 % that was not significant differed from that of all parental lines.

Gel consistency (GC) of the breeding lines ranged from 102-118 mm, in which they indicated as soft gel quality. Gelatinization temperature (GT) of the breeding lines tested with two condition of alkali digestibility. The alkali digestibility values using 1.7% KOH of the breeding lines were ranged score of 5.4 - 6.8. Gelatinization temperature of the breeding lines were approximately 55-69^{o}C, indicated as low gelatinization temperature (<70^{o}C). Considering alkali digestibility values 1.3% KOH of the breeding lines ranged score of 2.3 - 3.9. Gelatinization temperature of the breeding lines was approximately 70-74^{o}C, in which they indicated as intermediate gelatinization temperature. In case of aroma, all breeding lines are aromatic.

Table 19 Comparison of the grain-quality performance of RILs and parental lines calculated by the combined analysis of variance data in all of trait.

Trait	Parents					RILs		LSD (0.05)	F-Test	CV (%)
	TDK303	IR85264	RGD0529	TDK1	RD6	Mean	Range			
AC (%)	6.7	6.2	6.3	6.3	5.7	6.3	5.8-6.9	0.6	ns	5.8
GC (mm)	103.9	109.5	111.5	110.3	110.1	110.4	102.4-118.4	8.0	*	5.8
GT (1.3% KOH)	3.4	3.8	3.8	4.3	3.8	3.1	2.3-3.9	0.8	**	12.9
GT (1.7% KOH)	6.6	6.8	6.7	6.7	6.8	6.1	5.4-6.8	0.7	**	5.6
Fragrance	2	0	0	0	1	1	1-2	1	**	43

Notes: **=significant at $P < 0.01$

^{ns}=not significant different with 95%

Agronomic characteristics of the breeding lines

All measured traits of breeding lines were statistically significant difference from parents except PH, GW, SF, LU, TU and HR. Significant genotypic variations were observed for DH, PH, GW, SF, LU, WU, TU and HR. The mean values of the breeding lines ranged from 95.0 to 127.0 days (DH), 78.9 to 92.9 cm (PH), 23.2 to 39.4 g (GW), 15.2 to 55.2 % (SF), 9.9 to 10.6 mm (LU), 2.44 to 3.11mm (WU), 1.7 to 2.46 mm (TU) and 66.0-77.3 % (HR). TDK1 were 128 days, 113.0 cm, 40.7 g, 10.7 %, 10.8 mm, 2.72 mm, 2.13 mm and 70.9 %.

Table 20 Comparison of the agronomic characters trait of RILs and the parental lines calculated by obtained analysis of variance data in all of trait.

Trait	Parents					RILs		LSD (0.05)	F-Test	CV (%)
	TDK303	IR85264	RGD0529	TDK1	RD6	Mean	Range			
DF	115	128	-	128	-	111	95.0-127.0	16	ns	8.8
PH	98.6	109	-	113	-	85.9	78.9-92.9	7	**	5
GW	39.9	45	-	40.7	-	31.3	23.2-39.4	8.1	**	12.6
SF	10.3	12.7	-	10.7	-	35.2	15.2-55.2	20	**	27.7
LP	7.0	7.3	7.0	7.3	6.9	6.97	6.68-7.25	0.3	**	2.0
LU	10.4	10.3	2.61	10.8	9.98	10.3	9.9 - 10.6	0.3	**	2.3
WP	2.3	2.4	2.1	2.4	2.1	2.29	1.40-3.18	0.9	**	1.9
WU	2.52	2.72	2.01	2.72	3.02	2.7	2.44-3.11	0.3	ns	7. 4
TP	1.7	1.8	1.7	1.8	1.7	1.81	1.46-2.16	0.4	ns	9.5
TU	2.04	2.13	2.01	2.13	1.98	2.0	1.7 - 2.46	0.3	**	1.1
HR	68.5	70.8	-	70.9	71.6	71.4	66.0-77.3	-	-	-

Notes:

DF=Days to flowering (days); PH=lant height (cm); GW=1000 grain weight (g); SF=Spikelets fertility (%); LU=Grain length of unhusked rice (mm); WU=Grain width of unhusked rice; TU= Grain thickness of unhusked rice (mm); HR=head rice, precentage of head rice (%)

[a-]ndicates a

**Significant at P < 0.01; [ns]not significant different at 95% confidence level

Discussion

Breeding superior rice varieties can be achieved through the precision of molecular marker-assisted selection. In this study, gene-specific (Aromarker and R10783indel) and microsatellite (RM212, RM319, RM224 and RM144) markers were employed in all generation. We selected 28 (F_5) breeding lines that combined all favorable alleles of *Sub1, badh2, qBL1* and *qBL11* loci. All selected lines are submergence tolerance as seen by the evidence that PSS and PSE of the breeding lines were not significantly differed from those of IR85264-34-141 and FR13A (Table 15 and 18), All breeding lines showed high level of resistance (Score 0-1) against all tested isolates and were not significance differed from RGD07529, a tolerance parent (Table 20. All breeding lines showed aromatic characteristic. These results indicated a high accuracy of MAS.

In the current study, QTLs for submergence tolerance, the *Sub1* locus (Nandi *et al.,* 1997; Sripongpangkul *et al.,* 2000; Kamolsukyunyong *et al.,* 2001., Siangliw *et al.,* 2003; Toojinda *et al*., 2003) and broad spectrum resistance QTL loci (Wang *et al.,* 1994; Wang *et al.,* 2001; Prashanth *et al.,* 2002; Lin *et al.,* 2007; Wongsaprom *et al.,* 2009) were confirmed for the use of MAS (Zhou *et al.,* 2003; Wan *et al.,* 2005; Neeraja *et al.,* 2007). The result indicated the following: 1) the *Sub1* locus is the major gene contributing to a high plant survival through the reduction of plant growth to store carbohydrates and energy when rice plants experience a compete submergence (Xu and Mackill, 1996); 2) the *badh2* locus is major gene determining the presence of 2AP in rice grains (Wanchana *et al.,* 2003; Bradbury *et al.,* 2005); 3) the *qBl1* and *qBL11* QTL locus is major QTL for broad spectrum resistance to blast disease (Wongsaprom *et al.,* 2009; Korinsak, 2010). MAS can be integrated into an ongoing rice breeding program, which can accelerate the development of ideal genotypes within four years however, MAS can cause a distorted segregation of markers favoring the responsive alleles along various segments of chromosomes where the selective markers are located (Xu *et al.,* 1997). These selections affect the frequencies of alleles at linked marker loci, causing the distorted segregation of markers to extend over some distance along carrier chromosomes (Xu *et al*., 1997;

Septiningsih *et al*., 2003; Thomson *et al*., 2003). However, the linkage drag was not observed in this exoeriment.

The significant variations among the individual breeding lines were observed for traits related to submergence tolerance such as PSS and PSE. These indicated a quantitative nature of such trait. To date, there have been a number of reports on QTLs associated with submergence tolerance (Xu and Mackill, 1996; Nandi *et al.,* 1997; Sripongpankul *et al.,* 2000; Siangliw *et al.,* 2003; Toojinda *et al.,* 2003; Angaji, 2008). The *Sub1* tolerance gene inherited from FR13A has been known to control ethylene- and gibberellin-mediated changes in gene expression that include a regulation of genes controlling carbohydrate consumption and cell elongation (Jackson and Ram, 2003; Fukao *et al.,* 2006; Xu *et al.,* 2006). The effect of *Sub1* on carbohydrate consumption and cell elongation was confirmed by this study. A significantly improved submergence tolerance through the reduction of the PSE was observed (the PSE of most breeding lines was significantly different from RD6 and TDK1), indicating that the presence of *sub1* inherited from IR85264 clearly reduced the elongation of plant under the submergence event. The PSS and PSE showed a significant negative correlation, and the different genomic locations of the QTLs for the PSS and PES were detected. These results indicated that there might be other mechanisms, in addition to the reduction of the PSE that leads to a high PSS when rice plants are subjected to complete submergence. The result also clearly showed that the genetic background played a critical role in determining the level of submergence tolerance in rice plants that carry the *Sub1* locus, a major QTL for submergence tolerance.

Resistance to the blast pathogen has been shown to follow the classic gene-for-gene system, where a major resistance gene is effective against *M. grisea* strains containing the corresponding avirulence gene (Silue *et al.,* 1992). In the present study, the genetic analyses confirmed that there was a based on resistance reaction to the blast isolates. The flanking marker RM319-RM212 and RM224-RM144 were useful for rice breeders to accelerate the improvement of blast resistance through marker assisted selection strategy. The result were present to confirmed the gene for broad

spectrum blast resistance in the breeding lines derived from the three way cross were located to RM319-RM212 and RM224-RM144 interval. The use of DNA markers, which permit the genetic dissection of the progeny at each generation, increases the speed of selection process (Tanksley *et al.,* 1989). Blast resistance of all breeding lines were not significant different from the parents. showed very high level of resistance (Score 0-1) against all tested isolates (Table 18). Our validation experiments indicated that the breeding lines are resistant to blast disease.

The mean of values of the AC, GC, GT and FR of breeding lines were not significantly different from the parents. But the AC, GC, GT and FR among individual were slightly different from each other (Table 4). The amylose to amylopectin ratio was the most important factor, followed by the protein content, which indicated cooking and eating qualities of cooked rice. In this study, all breeding lines have excellent cooking and eating qualities. Most of agronomic characteristics of the breeding lines were similar to those of the TDK1. This result confirms that molecular markers can rapidly assist the development of new varieties that possess submergence tolerance, broad spectrum blast resistance and cooking quality characters with considerable saving in time. The new breeding lines will help Lao farmers to prevent their yield losses due to submergence and blast disease in the future.

CONCLUSION

1) MAS can accelerate rice breeding program in developing glutinous rice with fragrance, submergence tolerance, blast resistance and cooking quality. Glutinous rice lines were successfully developed through integration of MAS and a conventional breeding method.

2) Breeding lines with $Sub1^{IR}$ were tolerant to complete submergence and the breeding lines with two QTL (*qBL1* and *qBL11*) were resistance to broad spectrum of blast isolates.

3) Breeding lines with $badh2^{TDK303}$ showed similar cooking quality to TDK303.

4) MAS has been proven as an effective breeding method for the development of superior genotype in the self-pollinating crop with less production cost and more rapid compared to pedigree (Fahim *et al.*, 1998).

5) MAS, can be routinely used to develop new rice genotype in Laos rice breeding program. The new breeding lines will help Lao farmers to prevent their yield losses due to submergence and blast disease.

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APPENDIX

Appendix Table1 Number of plants at the beginning of and 10 days after submergence of RILs population

Entry	Varieties/Pedigree	Number of plant		Death tiller	% of Survival
		Beginning	After		
1	RGDU10033-MAS-77-291-20	14	6	8	41
2	RGDU10033-MAS-77-298-27	12	6	6	54
3	RGDU10033-MAS-77-43-3	16	10	6	61
4	RGDU10033-MAS-77-291-21	13	8	5	62
5	RGDU10033-MAS-77-291-24	16	15	1	67
6	RGDU10033-MAS-77-303-35	19	11	8	69
7	RGDU10033-MAS-77-327-43	17	12	5	71
8	RGDU10033-MAS-77-524-74	20	11	9	72
9	RGDU10033-MAS-77-149-18	18	13	5	72
10	RGDU10033-MAS-77-298-26	15	7	8	72
11	RGDU10033-MAS-77-438-50	11	6	5	73
12	RGDU10033-MAS-77-327-42	18	13	5	75
13	RGDU10033-MAS-77-298-31	14	11	3	75
14	RGDU10033-MAS-77-291-23	16	12	4	77
15	RGDU10033-MAS-77-291-22	11	9	2	78
16	RGDU10033-MAS-77-438-51	14	9	5	79
17	RGDU10033-MAS-77-149-14	15	14	1	80
18	RGDU10033-MAS-77-149-16	15	12	3	81
19	RGDU10033-MAS-77-327-45	15	13	2	83
20	RGDU10033-MAS-77-438-48	17	14	3	83
21	RGDU10033-MAS-77-438-47	16	12	4	83
22	RGDU10033-MAS-77-303-36	18	14	4	83
23	RGDU10033-MAS-77-524-75	15	13	2	87
24	RGDU10033-MAS-77-438-46	16	14	2	89
25	RGDU10033-MAS-77-149-17	17	14	3	90
	FR13A	14	13	1	95
	IR85264-34-141	13	10	3	78
	RGDU07529-1-1-38M-1-0	9	1	8	4
	TDK303-140-3-93-6-8	11	2	9	16
	RD6	10	1	9	6
	TDK1	6	0	6	0
	Mean	15	10		66
	LSD (0.05)	2	5		27
	% CV	21	29		25

Appendix Table 2 Plant height at the beginning of and 10 days after submergence of RILs lines.

Entry.	Varieties/ Pedigree	Plant height		Increase in height during test (cm)	Increase in height during test (%)
		Before (cm)	After (cm)		
1	RGDU10033-MAS-77-43-3	54	58	5	9
2	RGDU10033-MAS-77-149-14	61	62	0	0
3	RGDU10033-MAS-77-149-16	58	65	7	11
4	RGDU10033-MAS-77-149-17	55	61	6	10
5	RGDU10033-MAS-77-149-18	53	59	7	12
6	RGDU10033-MAS-77-291-20	55	69	15	27
7	RGDU10033-MAS-77-291-21	48	59	11	23
8	RGDU10033-MAS-77-291-22	55	60	4	8
9	RGDU10033-MAS-77-291-23	55	55	0	0
10	RGDU10033-MAS-77-291-24	48	53	6	12
11	RGDU10033-MAS-77-298-26	50	55	6	11
12	RGDU10033-MAS-77-298-27	54	64	10	19
13	RGDU10033-MAS-77-298-31	51	59	8	16
14	RGDU10033-MAS-77-303-35	52	60	8	16
15	RGDU10033-MAS-77-303-36	58	65	6	11
16	RGDU10033-MAS-77-327-42	53	63	10	18
17	RGDU10033-MAS-77-327-43	56	64	8	14
18	RGDU10033-MAS-77-327-45	54	64	10	19
19	RGDU10033-MAS-77-438-46	56	68	12	21
20	RGDU10033-MAS-77-438-47	52	56	5	9
21	RGDU10033-MAS-77-438-48	50	59	9	19
22	RGDU10033-MAS-77-438-50	50	57	7	13
23	RGDU10033-MAS-77-438-51	54	58	5	8
24	RGDU10033-MAS-77-524-74	53	59	6	11
25	RGDU10033-MAS-77-524-75	54	62	8	15
	FR13A	66	75	9	14
	IR85264-34-141	54	56	2	4
	RGDU07529-1-1-38M-1-0	57	95	38	66
	RD6	57	83	26	46
	TDK1	54	70	16	31
	TDK303-140-3-93-6-8	60	78	18	30
	Mean	54	63		
	LSD (0.05)	8	10		
	% CV	9	10		

Appendix Table 3 characteristics of the cooking quality of RILs and parents lines.

Variety/Pedigree	Alkalai digestibility		Amylose	Gel Consistency	Fragrance
	1.7% KOH	1.3% KOH	content (% db)	(mm)	(score)
RGD10033-MAS-77-43-2	5.6	3.1	6.5	110.7	1
RGD10033-MAS-77-43-3	5.8	2.8	6.8	113.7	1
RGD10033-MAS-77-149-14	6.1	3.4	6.5	111.5	2
RGD10033-MAS-77-149-16	6.1	3.3	6.8	111.1	2
RGD10033-MAS-77-149-17	5.3	3.6	6.2	108.5	2
RGD10033-MAS-77-149-18	5.6	3.5	6.7	112.7	2
RGD10033-MAS-77-291-20	5.9	3.1	6.5	109.7	1
RGD10033-MAS-77-291-21	6.1	3.0	6.4	109.7	2
RGD10033-MAS-77-291-22	5.9	2.6	6.7	113.5	2
RGD10033-MAS-77-291-23	5.8	2.8	6.5	121.7	1
RGD10033-MAS-77-291-24	5.7	2.3	6.5	109.1	1
RGD10033-MAS-77-291-25	5.6	2.9	6.2	108.5	1
RGD10033-MAS-77-298-26	5.4	3.1	6.1	117.1	1
RGD10033-MAS-77-298-27	6.2	3.2	6.5	106.5	2
RGD10033-MAS-77-298-31	5.9	2.7	6.0	111.1	2
RGD10033-MAS-77-303-35	6.0	2.8	6.3	111.1	2
RGD10033-MAS-77-303-36	5.8	2.8	6.3	115.3	2
RGD10033-MAS-77-327-42	6.0	2.5	6.3	108.3	1
RGD10033-MAS-77-327-43	5.8	2.6	5.9	107.3	2
RGD10033-MAS-77-327-44	5.6	3.1	6.5	105.9	2
RGD10033-MAS-77-327-45	5.7	2.7	6.3	111.7	1
RGD10033-MAS-77-438-46	5.7	2.7	6.3	109.1	2
RGD10033-MAS-77-438-47	5.8	2.5	6.5	102.3	1
RGD10033-MAS-77-438-49	5.5	2.3	6.1	107.3	2
RGD10033-MAS-77-438-50	6.2	2.6	6.2	109.3	1
RGD10033-MAS-77-438-51	6.4	2.6	5.8	107.1	1
RGD10033-MAS-77-524-76	5.9	2.4	6.4	111.7	2
RGD10033-MAS-77-524-77	5.7	3.0	6.2	113.3	2
TDK1	6.7	4.3	6.3	110.3	0
RD6	6.8	3.8	5.7	110.1	-
Homnangnuan	6.9	3.8	6.2	116.9	2
IR85264-3-141	6.8	3.8	6.2	109.5	0
TDK303-140-3-93-4-27	6.6	3.4	6.7	103.9	2
RGD07529-1-1-38M-1-0	6.7	3.8	6.3	111.5	-
Mean	6.1	3.1	6.3	110.4	1
min	5.4	2.3	5.8	102.4	1
max	6.8	3.9	6.9	118.4	2
F-test	**	**	ns	*	**
LSD (0.05)	0.7	0.8	0.6	8.0	1
% C.V	5.6	12.9	5.5	5.8	43

CIRRICULUM VITAE

NAME : Miss. Phatsalakone Manivong

BIRTH DATE : Aril 25, 1987

BIRTH PLACE : Vientiane, Laos

EDUCATION :

YEAR	INSTITUTE	DEGREE/DIPLOMA
2007	National University of Laos	H.Agr.D.
2009	National University of Laos	B.Agr.Sc

POSITION/TITLE : Senior Researcher

WORK PLACE : Rice and Chas Crop Research Center
National Agriculture and Forestry Research Institute (NAFRI), Laos

SCHOLARSHIP/AWARDS : National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand