Genetic analysis of resistance to bacterial blight

(*Xanthomonas oryzae* pv. *oryzae*) in rice

(イネにおける白葉枯病菌 (*Xanthomonas oryzae* pv. *oryzae*)に

対する抵抗性の遺伝解析）

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DEDICATIONS

To lord my God, for the life, guidance and protection throughout my life.

To the memories of my Mother Bernadetha Busungu, Grandfather Gabriel Busungu and Aunt Domitila Busungu who inspired me the love of nature and agriculture.

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LIST OF ABBREVIATIONS

acd	: Accelerated cell death
ANOVA	: Analysis of variance
BAC	: Bacterial artificial chromosome
BB	: Bacterial blight
bp	: Base pair
BS	: Brown spots
CAPS	: Cleaved amplified polymorphic sequence
CGSNL	: Committee on gene symbolization, nomenclature and linkage
CSSLs	: Chromosome segment substitution lines
dCAPS	: Derived cleaved amplified polymorphic sequence
DNA	: Deoxyribo nucleic acid
dNTPs	: Deoxy nucleotide triphosphates
EDTA	: Ethylene diamine tetra acetic acid
EMS	: Ethyl methanesulfonate
ER	: Endoplasmic reticulum
EtBr	: Ethidium bromide
GA	: Golgi apparatus
GCA	**:** Genome collections accession
Indel	: Insertion/Deletion
IRGSP	: International rice genome sequencing project
IRRI	: International rice research institute
kb	: Kilo base
LL	: Lesion length
LM	: Lesion mimics
Lsd	: Lesions simulating disease
MAS	: Marker assisted selection
Mb	: Mega base
MNU	: *N*-methyl-*N*-nitrosourea

ORFs	: Open reading frames
PAGE	: Polyacrylamide gel electrophoresis
PCR	: Polymerase chain reaction
QTL	: Quantitative trait loci
RAP-DB	: Rice annotation project database
RGAP	: Rice genome annotation project
RPM	: Rotation per minute
SDS	: Sodium dodecyl sulphate
SNP	: Single Nucleotide Polymorphism
Spl	: Spotted leaf
SSR	: Simple Sequence Repeats
Taq	: Thermus aquaticus
TBE	: Tris Boric acid EDTA
TE	: Tris EDTA
UV	: Ultra-Violet
Xoo	: *Xanthomonas oryzae* pv. *oryzae*

ABSTRACT

Rice (*Oryza sativa*) is one of the most important food crops in the world, feeding 50% of world population daily. Bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases inflicting rice. Developing resistant cultivars is generally regarded as the most effective and economical means of controlling this disease, which is based on study of resistant genes against *Xoo*.

In this thesis, I report the genetic analysis of the *Xoo* resistance of XM14 line. This line was obtained by the mutation induction of an Indica cultivar IR24, which is susceptible to six Philippine *Xoo* races and six Japanese *Xoo* races, using N -methyl-N-nitrosourea. XM14 shows resistance to all the races tested: one Philippine *Xoo* race and six Japanese *Xoo* race. I inoculated Japanese *Xoo* race II to the F_2 population from the cross between XM14 and IR24. The ratio of resistant plants: susceptible plants fitted 1: 3, which suggested that a single recessive gene of XM14 controlled resistance to the *Xoo* race.

Approximate chromosomal location of the resistance gene was determined using F_2 plants from the cross between XM14 and a Japonica cultivar Koshihikari, which is susceptible to Japanese *Xoo* races. Because the distribution of lesion length was continuous, I adopted the analysis using extreme recessive phenotype proposed by Zhang *et al.* (1994). Ten plants with shortest lesion length were genotyped for 116 DNA markers covering the whole rice genome, with the result that the gene was located close to cetromeric region on chromosome 3.

IAS16 line carries IR24 genetic background with a Japonica cultivar Asominori chromosome 3 segment, on which the above resistance gene locus was thought to be located. 194 F_2 plants from the cross between XM14 and IAS16 showed a discrete distribution. The linkage analysis using 6 SSR markers and 12 INDEL markers revealed that the gene is located very close to the centromeric region of chromosome 3. The gene identified in XM14 was new, and named *XANTHOMONAS ORYZAE* PV. *ORYZAE RESISTANCE 42* (*XA42*). *Xa42* is a susceptible wild type allele, whereas *xa42* is a resistant mutated allele.

Then, a high-resolution mapping of *xa42* was performed using 13,000 F_2 plants from the cross between XM14 and IAS16, F_3 lines from selected F_2 recombinants, 2 SSR markers, 12 INDEL markers, 3 CAPS markers and 2 dCAPS markers, with the result that the candidate *xa42* region was narrowed down from 578 kb, which had been obtained in the initial mapping, to 57 kb.

In addition to resistance against multiple *Xoo* races, XM14 line shows brown spots like lesion mimic mutants in its leaves. This line also has a smaller stature than the original cultivar IR24. In *XA42* gene segregating F_2 and F_3 lines from the cross between XM14 and IAS16, homozygotes of *xa42* allele were consistently resistant to six Japanese *Xoo* races used. They also showed brown spots, and significantly short stature compared with the other genotypes, suggesting that *xa42* gene exhibits pleiotropic effect.

Because *xa42* gene is resistant to multiple *Xoo* races, it will be very useful in resistance breeding programs.

CHAPTER ONE

GENERAL INTRODUCTIONS

1.0 INTRODUCTION

Rice (*Oryza sativa* L.) along with maize and wheat are three most important crops in the world. Rice is the most indispensable and principal food crop for more than 50 % of the world's population (Khush 2005, Khush 2013, http://ricepedia.org/). Because of the potential of feeding even more mankind, the Food and Agriculture Organization (FAO) regarded it as a strategic crop for food security in the world (FAO 2017). The rice consumer is increasing and demand for rice is also moving up due to better living standards. A variety of studies have revealed that to meet the increase demand for rice, production has to be increased more than 26 % by 2030 (Khush 2005). Rice is grown in more than a hundred countries, with a total harvested area of approximately 158 million hectares, producing more than 700 million tons annually (470 million tons of milled rice). Nearly 640 million tons of rice is grown in Asia, representing 90% of global production. Sub-Saharan Africa produces about 19 million tons and Latin America some 25 million tons. In Asia and sub-Saharan Africa, almost all rice is grown on small farms of 0.5−3 ha (http://faostat.fao.org/, http://ricepedia.org/).

Although rice yield has more than tripled in most parts of the world from the 1960s to the 2010s (http://faostat.fao.org/, http://ricepedia.org/), hunger and malnutrition still prevail in some regions of the world because of the imbalance between crop production and ever-increasing human demand. The world still faces continuing vulnerability to food shortages. These shortages have resulted from combination of many factors including the ever-increasing world population, plant diseases and drought (Oerke and Dehne 2004).

Rice together with maize and wheat are indispensable crops for world nutrition and livelihoods as they support nearly 60% of global food consumption (Khush 2013). However, crop losses due to pests and pathogens are direct, as well as indirect; they have a number of facets, some with short-, and others with long-term consequences. The phrase "losses between 20 and 40 %" therefore inadequately reflects the true costs of crop losses to consumers, public health, societies, environments, economic fabrics and farmers. Rice production, productivity and quality are threatened by diseases from multiple pathogen species (Khush 2005, Khush 2013, Sharma *et al.* 2012). The use of obnoxious cultivation practices reduced varietal diversity resulting from narrow genetic base, and apparent climatic changes have led to changes in dynamics of rice diseases over time. Hence, the

most important diseases have become more aggressive and spread to new areas. Many diseases which were earlier considered as unimportant have become economically important in many regions. These diseases can cause serious losses in rice production and the income of farmers. They can also threaten food security. Host resistance against multiple pathogen species or the majority of races of a pathogen species is important for sustainable crop production (Wisser *et al.* 2005).

Throughout the world, the rice production is constantly affected by a number of diseases. Among those, rice bacterial blight (BB) caused by *Xanthomonas oryzae pv oryzae* (*Xoo*) constitutes one of major biotic constraint in rice production and productivity (Mew *et al.* 1993, Ou 1985). BB causes annual yield losses ranging from 10 to 20% and up to 50% to 70% in severely infected fields (Mew 1987, Mew *et al.* 1993). This disease also affects grain quality by interfering with the maturation process (Goto 1992).

The main objective of this research was to perform **genetic analysis of resistance to bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) in rice**. The reason for choosing the theme was because BB is one of the most serious bacterial diseases of rice worldwide particularly in Africa and Asia.

Chapter 1 of this thesis, "General Introductions", includes an introductory part and the literature review about the causative agents of *Xoo,* its morphology, how it survives, how it spreads, distribution of the disease, economic impact or losses of the disease, different methods of control, number of identified resistance genes which are resistant to the disease, and molecular mechanisms of BB resistance.

Chapter 2 entitled "Mutation induction of IR24 to breed XM14 line" describes the process of mutation breeding of XM14 line using chemical mutagen "N-methyl-N-nitrosourea" (MNU). Throughout this research, XM14 mutant line was resistant to multiple *Xoo* races and it was crossed to different plant materials in this dissertation. Mutation induction was performed by Prof. Satoru Taura of Kagoshima University.

Chapter 3 entitled "Genetic analysis of *Xoo* resistance gene in XM14 mutant line" describes the experiment to determine type of resistance exhibited and the number of genes involved in the BB resistance in XM14 line.

The Chapter 4 entitled "Chromosomal location of a gene conferring resistance to *Xoo* in XM14 line" describes rough mapping of the resistant gene in XM14 line using the cross between XM14 line and Japanese cultivar Koshihikari. The analysis using plants with extremely short lesion length and published SSR markers facilitated rough mapping of this gene to chromosome 3.

The Chapter 5 entitled “Linkage analysis of resistance gene in XM14 line” presented the experimental linkage analysis using a chromosomal substitution line carrying IR24 genetic background with a Japonica cultivar Asominori chromosomal segment. This experiment determined the location of a gene conferring resistance to BB in XM14 on the centromeric region of chromosome 3, on which no *Xoo* resistance genes have been identified. The resistance gene in XM14 line was assigned a new gene name “*xa42*”.

The Chapter 6 was entitled “Fine mapping of the *xa42* gene”. In this chapter, I confined the location of *xa42* using *xa42*-gene-segregating population composed of 13,000 plants and DNA markers that I designed.

The Chapter 7 was entitled “Resistance of *xa42* against multiple *Xoo* races”. Resistance of *xa42* against multiple *Xoo* races was evaluated using segregating populations and a tightly linked DNA marker of *xa42*.

The Chapter 8 was entitled “Pleiotropic effects of *xa42* gene”. The XM14 line shows brown spots in its leaves similarly to lesion mimic mutants. Relationship between brown spots, stature, and the genotype of *xa42* was evaluated using segregating populations and a tightly linked DNA marker of *xa42*.

Finally, I drew a series of conclusions concerning the investigations performed and the results obtained. In addition, the possible future investigations were also indicated.

Most of the contents in Chapters 2, 3, 4 and 5 were published as a peer-reviewed paper (Busungu C, S Taura, J-I Sakagami, K Ichitani (2016) Identification and linkage analysis of a new rice bacterial blight resistance gene from XM14, a mutant line from IR24. Breeding Science 66: 636-645). Most of the contents in Chapters 6, 7 and 8 will be published as a peer-reviewed paper (Busungu C, S Taura, J-I Sakagami, T. Anai, K Ichitani (2018) High-resolution mapping and characterization of *xa42*, a resistance gene against multiple *Xanthomonas oryzae* pv. *oryzae* races in rice (*Oryza sativa* L.). Breeding Science (in press).)

2.0 LITERATURE REVIEW

2.1Morphology and physiology of Xanthomonas oryzae pv. oryzae

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a member of the family Xanthomonadaceae. The pathogen is a Gram-negative rod-shaped, round-ended species. Individual cells vary in length from approximately 0.7 μm to 2.0 μm and in width from 0.4 μm to 0.7 μm. Cells are motile by means of a single polar flagellum. Colonies on solid media containing glucose are round, convex, mucoid and yellow in colour due to the production of the pigment xanthomonadin, characteristic of the genus (Bradbury 1970a). Bacterial cells produce copious capsular extracellular polysaccharide (EPS). This EPS is important in the formation of droplets or strands of bacterial exudate from infected leaves, providing protection from desiccation and aiding in wind- and rain-borne dispersal (Ou 1972, Swings *et al.* 1990). *Xoo* is obligatory aerobic and does not form spores. Optimal temperature for growth is between 25 and 30°C. Like the genus as a whole, *Xoo* is catalase-positive, unable to reduce nitrate and a weak producer of acids from carbohydrates (Bradbury 1970b). Pathovars *oryzae* and *oryzicola* (*Xoc*) can be differentiated by (a) acetoin production (*Xoo*–, *Xoc*+), (b) growth on L -alanine as sole carbon source (*Xoo*–, *Xo*c+), (c) growth on 0.2% vitamin-free casamino acids (*Xoo*–, *Xoc*+) and (d) resistance to 0.001% $Cu(NO_3)_2$ (*Xoo*+, *Xoc*–) (Swings *et al.* 1990, Vera Cruz *et al.* 1984).

2.2 Disease cycle and epidemiology

The development of bacterial leaf blight depends on many predisposing environmental factors such as presence of rice stubbles and ratoons of infected plants, presence of alternate hosts, presence of bacteria in the rice and irrigation channels, warm temperature, high humidity, typhoon, rain and deep water, over fertilizer handling of seedlings at transplantation. The infected seed and plant debris perpetuate the disease from one season to another season. Potential inoculums sources include volunteer rice plant, infected chaff, weed host and infected seeds in temperate regions (Ou 1972, Swings *et al.* 1990). *Xoo* also penetrate the leaf mainly through hydathodes and wounds. Hydathodes are involved in the specificity of rice cultivar-bacterial strain interaction (Ou 1985). Spread pattern in a rice field has been examined by Huang and De Cleene (1988).

Bacterial cells and exo-polysaccharides fill the xylem and oozes out from the hydathodes forming beads or strands of exudates on the leaf surface, a characteristic sign of the disease and source of secondary inoculums. *Xoo* might as well penetrate to the xylem through wounds or openings caused by emerging roots at the base of the tillers (Mew 1987, Ou 1985, Shen *et*

al. 2002). Inside the xylem, *Xoo* has a tendency to interact with xylem parenchyma cells (Hilaire *et al.* 2001).

The transmission of the pathogen is favored by the environmental factors such as relative humidity, nutrition, temperature, and light, intense wind driven rainfalls which facilitate bacterial entry into plant tissue through wounded leaf edges. The bacteria may also be disseminated in irrigation water as well as by humans, insects and birds (Nino-Liu *et al.* 2006). Cells on the leaf surface may become suspended in guttation fluid as it exudes at night and enters the plant by swimming movement. The bacterium multiplies in the intercellular spaces of the underlying epithem then enters and spread into the plant through xylem (Noda and Kaku 1999, Nino-Liu *et al.* 2006). *Xoo* can survive in rhizosphere of weeds of genera *Leersia* and *Zizania* as well as in the base of the stem and the roots of rice stubble. *Xoo* can also survive in the soil for 1-3 months depending on the soil moisture and acidity. In the tropics, high temperature, humidity and abundance of host plants typically allow *Xoo* to persist throughout the year (Ou 1985).

Severe epidemics often occur following typhoons and the windblown rain, both of which disperse bacteria. Once inside the vascular system, the bacterium multiplies and moves in both directions. Spread takes place in wind and rain, but primarily in flood and irrigation water (Mew 1987, Ou 1985).

The disease is most likely to develop in areas that have weeds and stubbles of infected plants. It can occur in both tropical and temperate environments, particularly in irrigated and rainfed lowland areas. In general, the disease favors temperatures at 25−34°C, with relative humidity above 70%. *Xoo* can live in soil with pH range from 4 to 8.8; optimum pH being 6-6.50. It is commonly observed when strong winds and continuous heavy rains occur, allowing the disease-causing bacteria to easily spread through ooze droplets on lesions of infected plants. Bacterial blight can be severe in susceptible rice varieties under high nitrogen fertilization (Reddy *et al.* 1979).

2.3 Host range

According to a review by Reddy and Yin (1989), the weed hosts of *Xoo* in Japan were first reported under artificial inoculation (Goto *et al.* 1953). Other reported hosts include *Cyperus rotundus L.* and *C. difformis* (Chattopadhyay and Mukherjee 1968), wild rice (Kulkarni and Thombre 1969, Devdath *et al.* 1974), *Leersia hexandra* (Rao and Kauffman 1971) and *Paspalum scrobiculatum* (Reddy and Nayak 1974). The infected weeds were also later found in nature. Gonzalez *et al.* (1991) reported that *Leersia hexandra* was the most important as it served as an aletnative host of *Xanthomonas oryzae* in Texas, America.

2.4 BB symptoms and signs

The most common symptom generally occurs from the maximum tillering stage onward. It begins as water-soaked stripes on the leaf blades. The stripes increase in length and width, become yellow and then white, and may coalesce to cover the entire leaf blade. Drops of bacterial exudates (ooze) may be observed on young lesions (Mew 1987, 1989, Mew *et al.* 1993).

Older infected leaves may appear grayish. Small, circular lesions with water-soaked margins may also form on the glumes with severe infections. Infected plants produce fewer and lighter grains, and the grain is of poor quality. Infected seed may be discolored. The wilt syndrome, known as kresek, is the most destructive manifestation of the disease; it occurs in the tropics from the seedling to the early tillering stage. Leaves of infected plants wilt, roll up, and turn a grayish green color (Ou 1985). The leaves then turn yellow to straw-colored and wither. The entire plant generally dies. Plants that do survive are stunted and yellow in color. Total crop failure is not uncommon with kresek. A third symptom associated with bacterial blight is called yellow leaf or pale yellow leaf. The youngest leaf of the plant becomes uniformly pale yellow or has a broad yellow stripe. With yellow leaf, the bacteria are not present in the leaf itself but can be found in the internodes and crowns of affected stems (Mew 1987, 1989, Ou 1985).

2.5 Distribution and impacts

The bacterial blight is one of the most serious and the oldest recorded rice diseases. The yield losses due to bacterial blight can be as high as 50% when plants are infected at the maximum tillering stage (Gnanamanickam *et al.* 1999; Nino-Liu *et al.* 2006, Tandon and Chaliganjewar 2016). According to historical reviews of BB by Ezuka and Kaku (2000) and Nino-Liu *et al.* (2006), the disease has been known in Japan since 1881 in various localities of southern Japan as ‘white withering disease’ (Nishida 1909). Bacterial leaf blight is said to have been first seen by farmers in the Fukuoka area of Japan in 1884. During 1908-10, it was commonly observed in the southwest of Japan and since 1926, it has also been recorded in the northeast. The disease increased markedly after 1950 and by 1960 it was known to occur in all parts of Japan. Its bacterial nature was established and the causal bacterium was described in 1922 (Ishiyama 1922). Subsequently, it was reported from Japan (Nishida 1909), Korea (Lee 1975), Indonesia (Reitsuma and Schure 1950), Taiwan (Hashioka 1951), China (Bradbury 1986), Mexico (Dickson 1956), India (Dath and Devadath 1983, Srinivasan *et al.* 1959), Sri Lanka (Bradbury 1986), The Philippines (Bradbury 1986), Bangladesh (Alim 1967), Australia

(Buddenhagen *et al.* 1969), Malaysia (Saad and Habibuddin 2010), Latin America (Ou 1977) and United states (Jones *et al.* 1989).

In Africa, BB was first reported in Mali in 1979 (Buddenhagen *et al.* 1979). It was later reported in Senegal, Niger, Nigeria, Gabon, Mauritania, Benin, Cameroon and Tanzania (Afolabi *et al.* 2016, Ashura *et al.* 1999, Jones *et al.* 1991, Manser 1984, Reckhaus 1983, Trinh 1980). Over the last two decades, a significant repetitive appearance and huge yield loss were attributed to this disease in several regions in Africa (Sere *et al.* 2005, Gonzalez *et al.* 2007, Basso *et al.* 2011). BB increase is thought to be associated with the recent introduction of improved varieties from Asia, intensification and expansion of rice cultivation in Africa without effective phytosanitary measures (Verdier *et al.* 2012). BB caused by *Xoo* has been ranked fourth in top ten of most destructive pathogenic bacteria in molecular pathology (Mansfield *et al*. 2012). BB has generally caused havoc and significant yield losses since it discovery. **Table1.1** summarizes some of selected reported losses inflicted by this disease.

Table 1.1. Selective reports of yield losses caused by bacterial blight in representative countries

Country	Yield losses	year	Source
Japan	20-30 %	1969	Mizukami and Wakimoto (1969)
Korea	50%	1975	Lee (1975)
Korea	29%	2002-2005	Noh *et al* . (2007)
China	17%	2008	Qi (2009)
Philippines	50%	1993	Mew *et al.* (1993)
Punjab-India	60-70 %	1981	Raina *et al.* (1981)
Haryana-India	1.9-33.6 %	2004	Sunder *et al.* (2004)
Nepal	26%	1979-1987	Adhikari and Shrestha (1990)
Pakistan	11-80%	2013	Rafi *et al* . (2013)
Pakistan	36-66%	2014	Khan *et al.* (2015)
Togo	50%	2011	Dewa *et al.* (2011)
West Africa	2.7-42 %	1999	Awoderu *et al.* (1991)
West Africa	20-70%	2015	Wonni *et al.* (2016)
Tanzania	40%	2015	Duku *et al.* (2016)

2.6 *Control of Bacterial blight*

2.6.1 Chemical control

Chemical control of bacterial leaf blight in rice field began in the 1950s with the preventive application of Bordeaux mixture. In the 1960s, different kinds of agrochemicals were developed from repeated field trials and made available on a large scale and commercial farming (Ezuka and Kaku 2000, Nino-Liu *et al*. 2006). Some of examples of chemicals that can minimize or control *Xoo* activity include bactericides and zinc thiazole (Chen *et al*. 2015), streptomycins (Zhang *et al.* 2011), Benzylpenicillin, Ampicillin, Kanamycin, Streptomycin, Chloramphenicol and Sinobionic (Khan *et al*. 2012) niclosamide (Kim *et al*. 2016). Despite the fact that some chemicals are effective, they can have residue effect and detrimental effect to the environment (Aktar *et al*. 2009). Also, chemical control might increase cost of production and mostly impractical in bacterial blight disease control (Gnanamanickam *et al.* 1999).

2.6.2 Use of botanicals and Synthetics

Several botanicals and natural synthetics showed active principle for antibacterial activity against BB diseases (Singh *et al.* 2015). These botanicals and natural synthetics were extracted from garlic bulb (*Allium sativum* L.), Datura-white (*Datura stramonium* L.) and Bhoi-ringni (*Solanum incapum* L.) (Grainge *et al.* 1985). Kumar (2006) reported that maximum disease inhibition (58.7%) was obtained with *Lantana camara* (Lantana) followed by *Eucalyptus citridora* (Safeda), *Eupatorium adenophorum* (Kali basuti) and *Agave americana* (Ram ban). Mary (1986) observed that a foliar spray of cowdung extract (20 g/lit) was as effective as Penicillin, Pushamycin and Streptomycin in controlling *Xanthomonas campestris pv. oryzae*. Leaf of *Artabotrys hexapetalus* and seeds of *Moringa oleifera* were found inhibitory to *Xoo*. Safaa (2014) reported that leaf extracts from *Acacia nilotica* was also inhibitory to *Xoo*. Sundin *et al* (2016) discusses the challenges and difficulty of controlling bacterial diseases including the use of botanicals and synthetics. The attainment of most practical and effective management of these diseases can be extremely difficult, and management potential is often affected by grower reliance on highly disease-susceptible cultivars.

2.6.3 Biological control

Biological control using plant growth-promoting rhizobacteria (PGPR) are free-living, root-colonizing bacteria with beneficial effects on crop plants by reducing disease severity and yield enhancement (Murphy 2000, Duong *et al.* 2014). PGPR used as fresh suspensions and powdered formulations have commercial potential in mitigating BB of rice (Chithrashree

2011). Rhizobial inoculants also improve nutrient uptake, growth, seedling vigour and yield of rice (Biswas 2000). Certain strains of *Bacillus* spp. and *Pseudomonas* spp. have also been used as biological control agents to suppress rice BB (Velusamy *et al*. 2006). Biological control appears to be a suitable environment-friendly strategy for disease control and management.

2.6.4 Resistant varieties

Varietal resistance is considered the most practical, environmental friendly and economic way of keeping the disease below the economic injury level (Khan *et al.* 2014, Ogawa and Khush 1989, Tandon and Chaliganjewar 2016). Plant disease resistant cultivars cultivation is not only the central part of plant disease strategies but also most effective, and in addition, it can result in elimination of contamination of the land with chemicals. Furthermore, trends are showing rice consumers worldwide demand foods produced without the use of chemicals. Therefore, disease resistance breeding is the priority of plant disease control. Because bacterial races vary continually, influenced by the artificial and natural selection of resistance genes to bacterial blight, it is critical to explore and identify the new resistant resources to control the continually changing races of *Xoo (*Xia *et al.* 2012).

2.7 Genetic markers in bacterial blight resistance breeding

2.7.1 Genetic markers

A genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. It can be described as a variation (which may arise due to mutation or alteration in the genomic loci) that can be observed. A genetic marker represents a distinct visible character or phenotype, for which alleles at individual loci segregate in a Mendelian manner (Raza *et al.* 2016).

Genetic markers can be used to study the genetics of organisms at the level of single genes. The development of the discipline of genetics would not have been possible without genetic markers such as the visible characters in peas and *Drosophila*. Trees, unfortunately, do not have a large number of visible Mendelian characters and for many years, which was a limitation in forest genetics research. It was not until the early 1970s that biochemical genetic markers such as terpenes and allozymes were developed for trees.

2.7.2 Morphological markers

Morphological markers refer to visually detectable variability in morphology and structure of plants. These characteristics are usually either detectable by eye or by simple visual inspection. This includes plant characteristics that affect agronomic management and production, reaction to biotic and abiotic environmental condition (Bekele and Bekele 2014). An example of morphological marker includes the presence or absence of an awn, leaf sheath

coloration, seed shape, height, grain color and aroma of rice [https://en.wikipedia.org/wiki/Morphology_(biology)]. Morphological mutant traits in a population are mapped and linkage to a desirable or undesirable trait can be determined. There are several undesirable factors that are associated with morphological markers. The first is their high dependency on environmental factors. Often the conditions that a plant is grown in can influence the expression of these markers and lead to false determination. Second, these mutant traits often have undesirable features such as dwarfism or albinism. Lastly, performing breeding experiments with the morphological markers is time consuming, labor intensive, and needs large populations of plants with large plot of land (Lefebvre and Chèvre 1995, Akhtar *et al*. 2010, Barik *et al*. 2015).

2.7.3 Biochemical markers

A biochemical marker includes a protein that can be extracted and observed; for example, isozymes and storage proteins. Before the onset of molecular markers, some of the earlier experiments were carried out using biochemical markers. Certain isozymes (or isoenzymes) have been employed as biochemical markers in various aspects of plant breeding and genetics due to their significance as natural markers. Some of the commonly known biochemical isozyme markers are esterases, peroxidases, dehydrogenases etc. Basically, these markers are gene expression products and are characterized by electrophoresis and staining. By definition, isoenzymes are multiple molecular forms of the same enzyme that execute the same function.

They are the products of the different alleles of one or several genes. In several cases monomer and dimer isozymes are most often employed due to their early segregation process. Biochemical markers are generally co-dominant. Although isoenzymes are potentially reliable markers, their polymorphism is, however, relatively poor within a cultivated species. Polymorphic differences occur on the amino acid level allowing singular peptide polymorphism to be detected and utilized as a polymorphic biochemical marker (Lefebvre and Chèvre 1995, Akhter *et al.* 2010, Barik *et al*. 2015). The only problem with isozymes in MAS is that most cultivars are genetically very similar and isozymes do not produce a great amount of polymorphism, and polymorphism in the protein primary structure may still cause an alteration in protein function or expression (Lefebvre and Chèvre 1995).

2.8 Molecular markers

A molecular marker is a DNA sequence that is readily detected and whose inheritance can be easily monitored. In fact, a piece of DNA or a protein can be used as a marker. However, DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Molecular marker plays a great role to define the distinctiveness of species and their

ranking according to the number of close relative and their phylogenic relationship. A molecular marker very closely linked to the target gene can act as a "tag" which can be used for indirect selection of the gene(s) in a breeding program. Molecular markers are now rapidly adopted and widely used to track loci and genome regions in several crop-breeding programmes, as molecular markers tightly linked with a large number of agronomic and disease resistance traits are available in major crop species (Bueren *et al.* 2010, Jonnah *et al.* 2011).

These molecular markers include: (i) hybridization-based markers such as restriction fragment length polymorphism (RFLP), (ii) PCR-based markers: randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellite or simple sequence repeat (SSR), and (iii) sequence-based markers: single nucleotide polymorphism (SNP) and (iiii) Insertion/deletion (Indel) marker. The majority of these molecular markers have been developed either from genomic DNA libraries (e.g. RFLPs and SSRs) or from random PCR amplification of genomic DNA (e.g. RAPDs and AFLPs). The usefulness, advantages and some disadvantages of these markers are illustrated in **Table 1.2** (Kalia *et al.* 2011, Vieira *et al*. 2016). These DNA markers can be generated in large numbers and can prove to be very useful for a variety of purposes relevant to crop improvement. For instance, these markers have been utilized extensively for the preparation of saturated molecular maps (genetic and physical). Their association with genes/QTLs controlling the traits of economic importance has also been utilized in some cases for indirect marker-assisted selection (MAS) (Kalia *et al.* 2011, Jonnah *et al.* 2011).

Other uses of molecular markers include gene introgression through backcrossing, germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization and phylogenetic analysis (Mishra *et al.* 2014, Collard and Mackill 2008). For plant breeding applications, SSR markers, among different classes of the existing markers, have been proven and recommended as markers of choice (Gupta and Varshney 2000).

Table 1.2. Comparison of most commonly used marker systems

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	High	High	Moderate	Moderate	High
PCR-based	No	Yes	Yes	Yes	Yes
Number of polymorph loci analyzed	1.0-3.0	1.5-50	20-100	1.0-3.0	1
Ease of use	Not easy	Easy	Easy	Easy	Easy
Amenable to automation	Low	Moderate	Moderate	High	High
Reproducibility	High	Unreliable	High	High	High
Development cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

(Source: Kalia *et al.* 2011, Vieira *et al*. 2016).

2.8.1 Molecular markers in plants genome analysis

The concept of genetic markers is not a new one; Mendel used phenotype based genetic markers in his experiment in the nineteenth century. Later, phenotype based genetic markers for *Drosophila* led to the establishment of the theory of genetic linkage. A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. Molecular markers may or may not correlate with phenotypic expression of a trait. Molecular markers offer numerous advantages over conventional phenotype based markers as they are stable and detectable in all tissues regardless of growth, differentiation, development and are not confounded by the environment, pleiotropic and epistatic effects.

The differences that distinguish one plant from another are encoded in the plant's genetic material, the deoxyribonucleic acid (DNA). DNA is packaged in chromosome pairs, one coming from each parent. The genes, which control a plant's characteristics, are located on specific segments of each chromosome. Mendel clearly in his experiment stated that determiner of traits which we now call "genes" are carried within the pollen and egg cells (Westerlund and Fairbanks 2010). Although the whole genome sequence is now available for a few plant species such as *Arabidopsis thaliana* to help identify specific genes located on a particular chromosome, most scientists use an indirect method called genetic markers. Since the markers and the genes are close together at specific DNA segments or locus in

chromosome, they tend to stay together as each generation of plants is produced. Scientists can create a genetic linkage map. Such genetic maps serve several purposes, including detailed analysis of associations between economically important traits and genes or quantitative trait loci (QTLs) and facilitate the introgression of desirable genes or QTLs through marker assisted selection (Semagn *et al.* 2006).

2.8.2 Concept of microsatellite or SSR markers

Microsatellites or simple sequence repeats (SSRs) are extensively employed in plant genetics studies, using both low and high throughput genotyping approaches. They are repeating sequences of 2-6 base pairs of DNA. SSRs are widely distributed throughout the genome, especially in the euchromatin of eukaryotes, and coding and non-coding nuclear and organellar DNA (Gemayel *et al.* 2012).

SSRs have been the most widely used markers for genotyping plants over the past 20 years because they are highly informative, codominant, multi-allele genetic markers that are experimentally reproducible and transferable among related species (Vieira *et al.* 2016). In particular, SSRs are useful for wild species (*i*) in studies of diversity measured on the basis of genetic distance; (*ii*) to estimate gene flow and crossing over rates; and (*iii*) in evolutionary studies, above all to infer intraspecific genetic relations. On the other hand, for cultivated plants SSRs are commonly used for (*i*) constructing linkage maps; (*ii*) mapping loci involved in quantitative traits (QTL); (*iii*) estimating the degree of kinship between genotypes; (*iv*) using marker-assisted selection; and (*v*) defining cultivar DNA fingerprints (Kalia *et al.* 2011). SSRs have been particularly useful for generating integrated maps for plant species in which full-sib families are used for constructing linkage maps (Pereira *et al.* 2013), and for combining genetic, physical, and sequence-based maps (Temnykh *et al*. 2001), providing breeders and geneticists with a tool to link phenotypic and genotypic variation (Mammadov *et al.* 2012*)*.

2.8.3 Use of SSR markers in the research of BB resistance rice variety

Pradhan *et al.* (2015) conducted an experiment to pyramid genes conferring resistance to BB to popular deepwater rice variety Jalmagna. This variety is highly susceptible to BB disease. In this study three resistance genes (*xa5* + *xa13* + *Xa21*) were transferred from Swarna BB pyramid line, using a SSR marker-assisted backcrossing (MAB) breeding strategy, into the BB-susceptible elite deepwater cultivar, Jalmagna.

Bhat *et al.* (2015) conducted an experiment using 20 BC_1F_1 cross combinations generated by randomly crossing four elite basmati cultivars with 10 bacterial blight resistant donor lines possessing resistant genes. RG136 and pTA248 closely linked to the BB resistance genes,

xa13 and *Xa21*, respectively, were used to confirm the presence of the BB resistance gene. In this study, five genotypes, namely, Basmati-370 x IRBB-55-4, Basmati-370 x IRBB-55-5, Basmati-370 x IRBB-55-13, Basmati-370 x IRBB-55-17 and Basmati-370 x IRBB-55-18 carrying both *xa13* and *Xa21* along with aroma gene (*fgr*) in homozygous conditions, were identified phenotypically to show complete resistance to BB along with intermediate amylose and high aroma. It is suggested that these genotypes can be effectively used as basmati donors.

A research study was conducted by Mubassir *et al.* (2016) in Bangladesh to investigate genetic diversity of ten rice lines and seventeen rice varieties using four SSR markers RM9, RM296, RM17 and RM324. The results from this study showed significant level of gene diversity value (0.8340) for loci RM9. The PIC values which ranged from 0.588 (RM17) to 0.814 (RM9) were found to be significantly correlated with the results. Most importantly, the UPGMA dendrogram of genetic distance showed that the BB susceptible lines and varieties fall under one cluster whereas the BB resistant lines and varieties falls under the other cluster.

Korinsak *et al.* (2009) used 62 polymorphic markers covering all rice chromosomes to identify the location and linked markers of the resistance gene *xa33* (t). A total number of 139 F_2 progenies generated from a cross between the resistant donor 'Ba7'and 'Pin Kaset' were developed and used in this study. Four SSR markers, viz. RM30, RM7243 and RM400, located on the long arm of the rice chromosome 6, could clearly discriminate between resistant and susceptible phenotypes, and 161 $BC_2F_{2:3}$ individuals carrying *xa33* (t) BB resistance gene were developed through MAS using these SSR markers. This population was inoculated with TXO16 to validate and confirm the location of the gene and linked markers.

Furthermore, many new rice bacterial blight resistance genes have been identified and characterized using SSR markers. Wu *et al.* (2008) found that *xa24* was resistant to the Philippine *Xoo* races 4, 6, 10 and Chinese *Xoo* strains Zhe173, JL691, and KS-1-21, and was mapped on chromosome 2 within a 0.14 cM region, and an approximately 71 kb in length between RM14222 and RM14226. *Xa11*, resistant to Japanese *Xoo* races IB, II, IIIA and V, was mapped on the long arm of chromosome 3 with a genetic distance 2.0 cM and 1.0 cM from the marker RM347 and KUX11 by Goto *et al.* (2009).

2.8.4 Use of RFLP markers in the research of BB resistance rice variety

Tan *et al.* (2004) identified and molecular mapped *Xa29* (t) gene using RFLP markers. *Xa29* (t) was derived from B5, an introgression line carrying chromosomal segment of *O. officinalis,* and BG1222, a variety from Sri Lanka. *Xa29* (t) was located on chromosome 1 within a 1.3 cM region flanked by RFLP markers C904 and R596.

Gu *et al.* (2004) identified a new dominant BB resistance gene located in chromosome 6. The *Xa27* gene was located within a 0.052 cM region flanked by the RFLP markers M964 and M1197, and cosegregated with markers, M631, M1230 and M449. Chen *et al.* (2002) identified and mapped *Xa25* (t) in chromosome 11. *Xa25* (t), which conferred–resistance to PXO339 at the whole period, and had been identified from Minghui63, was located within a 9.5 cM region between NBS109 (a homologous sequence of resistance gene) and RFLP marker G1314.

DNA marker-assisted selection was performed by Huang *et al.* (1997) to pyramid four bacterial blight resistance genes, *Xa4*, *xa5*, *xa13* and *Xa21*. Breeding lines with two, three and four resistance genes were developed by using RFLP markers pTA248, RG136 and RG556 and was tested for resistance to *Xoo*.

2.9 Molecular breeding for BB disease resistance

2.9.1 Conventional breeding

To date, there are more than 40 BB resistance genes have been identified. Most of these genes have been derived from rice cultivars and wild rice (Khan *et al*. 2014). Some of these new genes have already incorporated into the modern rice varieties through conventional breeding. However, the pathogen can easily break down resistance by a single gene. The breakdown of resistance in modern and high-yielding rice varieties after a few years of cultivation was attributed to fast-changing pathogens (Mew 1987, Xia *et al*. 2012, Khan *et al*. 2014). One way to delay the breakdown is to pyramid multiple resistance genes into rice varieties. It is very difficult or nearly impossible with conventional breeding approach.

2.9.2 Marker assisted breeding

Marker assisted selection or marker aided selection (MAS) is an indirect selection process where a trait of interest is selected based on a marker (morphological, biochemical or DNA variation) linked to a trait of interest (e.g. productivity, disease resistance, abiotic stress tolerance, and quality), rather than on the trait itself (Collard and Mackill 2008).

Pradhan *et al.* (2015) used marker-assisted breeding practices to pyramid three resistance genes (*xa5* + *xa13* + *Xa21*) into BB-susceptible elite deepwater cultivar, Jalmagna. In this study, three resistance genes were transferred from Swarna BB pyramid line, using a SSR marker-assisted backcrossing (MAB) breeding strategy.

Suh *et al.* (2013) developed three elite advanced backcross breeding lines (ABL) with three resistance genes by MAS and phenotypic selection in a Japonica genetic background without linkage drag. The background genome recovery of the ABL expressed more than 92.1% using genome-wide SSR marker analysis. Luo *et al* (2012) and Dash *et al*. (2016) also

pointed that the most effective approach to combating BB is the use of broad spectrum resistance varieties obtained through MAS.

2.9.3. Mutational breeding

Mutagenesis is the process whereby sudden heritable changes occur in the genetic information of an organism not caused by genetic segregation or genetic recombination, but induced by chemical, physical or biological agents (Roychowdhury and Tah 2013). Mutation breeding employs three types of mutagenesis. The first type of mutagenesis is mutations which occur as a result of irradiation (gamma rays, X-rays, ion beam, etc.), The second type of mutagenesis is mutations which occur as result of treatment with chemical mutagens such as superoxide, hydroxyl radicals, nitrous acid, ethylnitrosourea, etc). The third type of mutagenesis is site-directed mutagenesis, a molecular biology-based method that is used to make specific and intentional changes to the DNA sequence of a gene and any gene products. Site-directed mutagenesis is one of the most important techniques in laboratory for introducing a mutation into a DNA sequence.

Mutagenesis has contributed a great role in enriching the resistant resources of BB, and the researchers have obtained a series of new genes which were in different resistance levels and resistance spectrums (Xia *et al*. 2012). Until recently, there have been a few identified genes from mutagenesis with BB resistance in rice (Xia *et al*. 2012). Some of BB resistance genes identified from mutagenesis include *xa19* (Taura *et al.* 1991b) and *xa20* (Taura *et al*. 1992a).

2.10 Mechanisms of bacterial blight resistance

2.10.1 Biochemical mechanisms

Plants respond to pathogen infection through an intricate and dynamic defense system that includes structural barriers, toxic chemicals, and attraction of natural enemies of the target pathogens. Biochemical resistance in plants is very complex mechanism. It involves plant-producing defense proteins or compounds which enhance plant resistance to a variety of plant pathogens (Dangl and Jones 2001).

Phenolic compounds are secondary metabolites which are synthesized in plants. They play a role in plant defense against pathogens. Accumulation of these secondary metabolites in plants may play a major role in plant defense response. Khan *et al.* (2014) observed biochemical basis of resistance for BB disease of some rice varieties and suggested resistance of Basmati-385 and Basmati-2000 to BB was due to their high phenolic contents. Correspondingly, Khan *et al.* (2014) pointed out that high phenol production may be due to

loss of virulence in bacterial isolates. Therefore, management of *Xoo* can be achieved through spray of those chemical compounds which trigger phenol production

Taniguchi *et al.* (2014) verified that jasmonic acid (JA) signaling plays an important role in resistance to rice BB caused by *Xoo* in rice. Their study found that many volatile compounds accumulate in response to exogenous application of JA, and the expression of linalool synthase was up-regulated by JA. Vapour application with linalool induced resistance to *Xoo*, and transgenic rice plants over expressing linalool synthase were more resistant to *Xoo*.

2.10.2 Molecular Mechanisms

Rice plant breeding through development of resistant cultivars carrying major resistance genes have been the most effective and economical strategy to control BB disease and to have a beneficial effect on the environment (Huang *et al.* 1997, Jena and Mackill 2008, Singh *et al.* 2001). Qualitative resistance, which confers major gene-specific resistance against some pathogen races, is the easiest to integrate into breeding programs and is usually considered a gene-for-gene type of resistance. For many pathogens and insects, this type of qualitative resistance is not often durable because of rapid changes in the virulence in the pathogen or biotype of the population (Boots *et al*. 2004, Cressler *et al*. 2016)

One useful strategy to prolong the life of major gene resistance is to pyramid several major resistant genes in a resistant cultivar. Cultivars with single major genes for resistance are prone to resistance breakdown due to pathogen variation and evolution. Thus, pyramiding of resistance genes into individual breeding lines is a strategy to provide more durable resistance (Gnanamanickam *et al.* 1999, McDowell and Woffenden 2003). Though pyramiding of known loci is a promising approach for disease management, novel sources of resistance are required to keep the upper hand in the continuous plant-pathogen “arm race”.

2.10.3 Identification of resistance genes to rice bacterial leaf blight

During the last forty years, the interest for developing stable and durable BB resistant cultivars has pioneered discovery and development of more than 40 genes (**Table 1.3**) conferring resistance to various races of *Xoo* (Kim *et al.* 2015, Xia *et al.* 2012, https://shigen.nig.ac.jp/rice/oryzabase/). These genes have been identified in cultivated rice, wild relatives of rice, and some have been derived from artificial mutation induction.

Some of the resistance genes such as *xa24*(t), *Xa39*, *xa19* and *xa20* exhibits broad-spectrum resistance to all *Xoo* races tested (Taura *et al.* 1991b, Taura *et al.* 1992a, Wu *et al.* 2008, Zhang *et al.* 2014) whereas other genes like *Xa1*, *Xa2*, *Xa6*, *xa8*, *Xa10*, *Xa18* and *Xa11*

exhibits *Xoo* race specific (Goto *et al.* 2009, Gu *et al.* 2008, He *et al.* 2006, Liu *et al.* 2004, Sindhu *et al.* 1978, Sindhu and Khush 1978, Yoshimura *et al.* 1998).

These resistance genes are designated from *Xa1*, *Xa2* to *Xa41* according to gene nomenclature system for rice (McCouch and CGSNL 2008). These resistance genes have been mapped on 10 of the twelve rice chromosomes (Khan *et al* .2014). To date, no BB resistance gene have been mapped on chromosome 9 and chromosome 10. Chromosomes 4 and 11 appear to carry most of the known BB resistance genes. Indeed, these chromosomes are known to carry clusters of resistance genes analogs (RGA) (Mago *et al.* 1999; Ghazi *et al.* 2009). Resistance QTLs to rice sheath blight resistance (Zou *et al.* 2000) and resistance genes to blast (Wang *et al.* 1994) also clustered on chromosome 11. Chromosome 2, 8 and 12 appear to carry only one resistance gene: *xa24* on chromosome 2 (Wu *et al.* 2008), *Xa13* on chromosome 8 (Chu *et al.* 2006) and *xa25* on chromosome 12 (Chen *et al.* 2002, Liu *et al.* 2011).

Table 1.3. List of identified genes conferring resistance to bacterial blight in rice

Gene	Chromosome	Gene type	Resistance against	Donor cultivar	Origin	Reference
Xa1	4	D	Japanese race-I	Kogyoku, IRBB1	Japan	Yoshimura *et al.* 1998
Xa2	4	D	Japanese race-	Te-tep, IRBB2	Vietnam	He *et al.* 2006
Xa3/ Xa26	11	D	Philippine, Chinese & Japanese races	WaseAikoku/Minghui 63	Japan	Ezuka *et al.* 1975, Ogawa *et al.* 1986, Sun *et al.* 2004, Xiang *et al.* 2006
Xa4	11	D	Philippine race-I	TKM 6	India	Yoshimura *et al.* 1992, Wang et al. (2001), Sun et al. 2003.
xa5	5	R	Philippine races I, II, III	Aus Boro lines	Bangladesh	Sidhu *et al.* 1978, Iyer-pascuzzi and McCouch 2004
Xa6/ Xa3	11	D	Philippine race 1	Zenith	USA	Sidhu and Khush 1978, Ogawa *et al.* 1990
Xa7	6	D	Philippine races	DV85	Bangladesh	Sidhu *et al.* 1978, Kaji and Ogawa 1995, Lee and Khush 2000
xa8	7	R	Philippine V & VIII	PI231129	USA	Sidhu *et al.* 1978, Singh *et al.* 2002
Xa9/ Xa3	11	R	Philippine races	Khao Lay Nhay and Sateng	Laos	Singh *et al.* 1983, Ogawa *et al.* 1986
Xa10	11	D	Philippine II, V & VIII	Cas209	Senegal	Yoshimura *et al.* 1983, Gu *et al.* 2008, Tian *et al.* 2014
Xa11	3	D	Japanese IB, II, IIIA & V	IR8, IR944	Philippines	Ogawa *et al.* 1986, Goto *et al.* 2009
Xa12	4	D	Indonesian V & Japanese race V	Kogyoku, Java14	Japan	Ogawa *et al.* 1978
xa13	8	R	Philippine race 6	BJ1, IRBB13	India	Ogawa *et al.* 1987, Chu *et al.* 2006,
Xa14	4	D	Philippine race 5	Taichung Native 1(TN1)	Taiwan	Taura *et al.* 1992b
xa15	ND	R	Japanese races	M41, a Harebare	Japan	Nakai *et al.* 1988, Ogawa 1996.
Xa16	ND	D	Japanese H8581, H8584	Tetep	Vietnam	Noda and Ouchi 1989
Xa17	4	D	Japanese H8513 isolates	Asominori	Japan	Ogawa *et al.* 1989
Xa18	ND	D	Burmese BM8427, BM8429 strains	IR24, Toyonishiki, Miyang23	Philippines/ Japan	Yamamoto and Ogawa 1990
xa19	7	R	Japanese races, Philippine I -VI	XM5 (mutant of IR24)	Philippines	Taura *et al.* 1991b , Taura *et al.* unpublished result
xa20	3	R	Japanese races, Philippine I -VI	XM6 (mutant of IR24)	Philippines	Taura *et al.* 1992a, Taura *et al.* unpublished result
Xa21	11	D	Philippine and Japanese races	*O. longistaminata*, IRBB21	Africa, Mali	Song *et al.* 1995, Song *et al.* 1997, Peng *et al.* 2001,
Xa22	11	D	Chinese races	Zhachanglong	China	Li *et al.* 1996, Wang *et al.* 2003
Xa23	11	D	Chinese, Japanese, Philippines races	*O. rufipogon*	China, Cambodia	Zhang *et al.* 1998, Wang *et al.* 2014a, b
xa24 (t)	2	R	Philippine race VI, Chinese	DV86, DV85, Aus 295	Bangladesh	Khush and Angeles, 1999, Wu *et al.* 2008

(Table 1.3. continued)

xa25	12	R	Philippine & Chinese races;	Minghui 63	China	Chen *et al.* 2002, Liu *et al.* 2011
xa26 (t)/ *Xa3*	11	R	Philippine races	Nep Bha Bong	Vietnam	Sun *et al.* 2004, Yang *et al.* 2003
Xa27	6	D	Australian, Chinese, Indian, Indonesian, Japanese, Nepal, Philippines, Korean and Thailand races	*O. minuta*	Philippines	Gu *et al.* 2004, Gu *et al.* 2005
Xa28 (t)	ND	R	Philippine race II	Lota Sail	Bangladesh	Lee *et al.* 2003
xa29 (t)	1	D	Philippine II & V	*O. officinalis*	Philippine	Tan *et al.* 2004
Xa30 (t)	11	D	Indonesian races	*O. rufipogon*	India	Jin *et al.* 2007
xa31 (t)	4	R	Chinese races	Zhachanglong	China	Wang*et al.* 2009
Xa32 (t)	11	D	Philippine races	*O. australiensis*	Australia	Zheng *et al.* 2009
xa33 (t) (re-served)	6	R	Thai races	Ba7	Thailand	Korinsak *et al.* 2009
Xa33 (t)	7	D	Thai races	*O. nivara*	Thailand	Natraj Kumar *et al.* 2012
xa34 (t)	1	R	Chinese, Srilanka races	BG1222	Srilanka	Chen *et al.* 2011
Xa34 (t)	ND	D	Indian races	*O. brachyantha*	Philippine	Ram *et al.* 2010
Xa35 (t)	11	D	Philippine races	*O. minuta* (Acc. No.101133)	Philippines	Guo *et al.* 2010
Xa36 (t)	ND	D	Chinese and Philippine races	C4059 (CSSL from *O. rufipogon*?)	Philippines	Miao*et al.* 2010
Xa38	4	D	Indian Punjab races	*O. nivara* IRGC81825	India	Bhasin *et al.* 2012
Xa39	11	D	Chinese and Philippines races	FF329	China	Zhang *et al.* 2014
*Xa40 (*t)	11	D	Korean *Xoo* races	IR65482-7-216-1-2	Philippines	Kim*et al.* 2015
xa41 (t)/ *11N3*	11	R	Various *Xoo* strains	*O. barthii, O. glaberrima*	Various	Hutin *et al.* 2015

Among these resistance genes, nine resistance genes have been molecularly cloned, including six dominant genes, *Xa21* (Song *et al.* 1995), *Xa1* (Yoshimura *et al.* 1998), *Xa3/Xa26* (Sun *et al.* 2004; Xiang *et al.* 2006), *Xa27* (Gu *et al.* 2005; Bimolata *et al.* 2013), *Xa10* (Tian *et al.* 2014), *Xa23 (*Wang *et al.* 2015*)* and three recessive *xa5* (Iyer and McCouch 2004) and *xa13* (Chu *et al.* 2006), *xa25* (Liu *et al.* 2011). The cloned gene encodes different types of proteins, suggesting multiple mechanisms of resistance gene–mediated *Xoo* resistance (Kim *et al.* 2015, Khan *et al.* 2014, and Zhang *et al.* 2014). **Table 1.3** summarizes the detail of each BB resistance gene.

However, despite of discovery and development of more than 40 new resistance genes, rice BB disease is growing from strength to strength due to *Xoo* pathogen adaptability and variability (Dossa *et al.* 2016). The *Xoo* infecting new areas, new virulent strains emerging and the BB disease are easily breaking down single resistant genes. BB resistance genes breaking down is reported by several scholars in many countries (Verdier *et al.* 2012). The breakdown of resistance in modern and high yield rice varieties after a few years of cultivation was attributed to evolution and recombination of *Xoo* pathogen. Increasing environmental temperatures and climate changes are complicating resistance gene-mediated disease control because high temperatures are promoting new disease development and reducing resistance gene effectiveness (Webb *et al.* 2010). In addition to that, the continuous mono-cropping and deployment of rice cultivars with a narrow genetic base have provided high selection pressure for emerging virulent strains.

2.10.4 Cloning and Characterization of BB genes

Cloning resistant genes at molecular level is the foundation of understanding precisely the molecular mechanism of host resistance to BB. Out of 41 rice BB resistance genes identified, only nine have been cloned and characterized. These include *Xa1* (Yoshimura *et al.* 1998), *Xa3/Xa26* (Sun *et al.* 2004, Xiang *et al.* 2006), *xa5* (Iyer and McCouch 2004), *Xa10* (Tian *et al.* 2014), *xa13* (Chu *et al.* 2006a, b), *Xa21* (Song *et al.* 1995), *Xa23* (Wang *et al.* 2014a, b), *xa25* (Liu *et al.* 2011) and *Xa27* (Gu *et al.* 2005), and these encode various types of proteins (**Table 1.4**).

Based on these studies, the molecular mechanisms of BB resistance in rice seem to be largely different from the mechanisms of resistance to rice blast, although the mechanisms of rice disease resistance remain largely to be elucidated. Most of the characterized BB resistance genes are different from the most common R protein, nucleotide-binding site-leucine-rich repeat (NBS-LRR) protein (Liu *et al.* 2010). However, *Xa21* and *Xa26* encode

similar receptor-like proteins; the products of the other genes are unique and not found in other plant species (Dai *et al.* 2007). Both *Xa21* and *Xa3/Xa26* belong to multigene family; they encode same type of proteins and have 53% sequence similarity (Song *et al.* 1995, Sun *et al.* 2004, Vikal and Bhatia 2017). The only structural difference between two genes is the number of LRR, where *Xa26* encodes 26 LRR, whereas *Xa21* encodes 23 LRR (Song *et al.* 1995, Vikal and Bhatia, 2017). However, the respective LRR domains of Xa3/Xa26 and Xa21 are the important determinants of race-specific recognition during rice-*Xoo* interactions as evidenced from experiment on domain swapping analyses, but a juxtamembrane motif of Xa3/Xa26 also seems to contribute in resistance specificity (Zhao *et al.* 2009). The kinase domain of Xa3/Xa26 can partially replace the function of the kinase domain of Xa21, or vice versa, in *Xoo* resistance, suggesting the partially conserved nature of this domain in defense signaling pathway (Zhao *et al.* 2009).

Table 1.4. The BB resistance genes characterized at molecular level (adopted and modified from Vikal and Bhatia 2017)

S/N	Gene name	Encoded protein	Mechanisms	Reference
1	*Xa1*	NBS-LRR	Induced by stimulus of wounding involved in pathogen infection	Yoshimura *et al.* 1998
2	*Xa3/ Xa26*	LRR receptor-like kinase	The kinase domain is important for complete function of *Xa3/Xa26* for resistance	Sun *et al.* 2004, Xiang *et al.* 2006
3	*xa5*	TFIIA Transcription factor	Positional changes of amino acid from valine to glutamic acid	Iyer and McCouch 2004
4	*Xa10*	Transcription activator–like(TAL)	Programmed cell death	Tian *et al.* 2014
5	*xa13*	MtN3/saliva	Loss-of-function alleles of the susceptibility	Chu *et al.* 2006a, b
6	*xa21*	LRR motif and a serine-threonine kinase-like domain	pathogen recognition and activation of an intracellular defense response	Song *et al.* 1995, Suh et al 2013
7	*Xa23*	Executor R protein, encodes 113 AA, with four potential transmembrane helices	Loss-of-function alleles of the susceptibility	Wang *et al.* 2014a, b
8	*xa25*	MtN3/saliva	Inhibition of *Xoo*	Liu *et al.* 2011
9	*Xa27*	Nuclear localized type-III effector	works as an local defense instead of system defense	Bimolata *et al.* 2013, Gu *et al.* 2004

xa5 consisting of 4 exons and 3 introns, encodes the gamma subunit of eukaryotic transcription factor (TFIIAγ) that contains 106 amino acids. Comparing sequence between resistant and susceptible isolines reveals that an amino acid changes from valine to glutamic acid at position 39, which may result in the resistance of *xa5* and the function of TFIIAγ still keeps. Sequencing TFIIAγ from resistant and susceptible cultivars shows that the amino acid at position 39 highly conserves in resistant varieties and owns two kinds of situations in susceptible varieties: valine and leucine (Iyer and McCouch 2004). *xa13* is a recessive gene which confers high specially resistance to *Xoo* PXO99, containing five exons and encoding a protein of 307 amino acids which targets to the plasma membrane. *xa13* is a promoter-mutation resistant gene, and the expression of *Xa13* is the basis of pathogenic bacteria infecting to rice. The low expression of *Xa13* as a result of promoter-mutation restrains pathogen infection, but causes abnormal development of pollen grains and reduction in setting percentage because of its function involved in pollen development (Chu *et al.* 2006). In contrast to *xa13*, the expression of *Xa27* makes a contribution to restraining invasion of pathogen bacteria. *Xa27* and *avrXa27* are the first cloned pair of resistance gene from rice and a corresponding virulence gene from *Xoo* (Gu *et al.* 2005). *Xa27* is an intron-less gene and encodes a protein composed of 113 amino acids. *Xa27* and its susceptible allele encode the protein with identical sequence. However, expression of only resistant allele occurs when a rice plant is challenged by *Xoo* harboring *avrXa27*, and the expression was confined to the vicinity of tissue infected by *Xoo* harboring *avrXa27*, indicating that *Xa27* works as a local defense instead of system defense. More interestingly, transgenic lines of *Xa27* can mediate resistance to compatible strains of *Xoo.* The experiment of promoter displacement clarified that the diverse expression is attributed to the promoter sequence different from resistant and susceptible plants.

These features suggest that molecular mechanism of rice-*Xoo* system is more complicated and a unique pathosystem to study the interactions between hosts and pathogens. The nine characterized BB resistance genes fall into six different classes of proteins and thus may give a wide scenario of understanding at molecular level.

CHAPTER TWO

MUTATION INDUCTION OF IR24 TO BREED XM14 LINE

1. INTRODUCTION

Mutagenesis is the process whereby sudden heritable changes occur in the genetic information of an organism not caused by genetic segregation or genetic recombination, but induced by chemical, physical or biological agents (Roychowdhury and Tah 2013) Mutation breeding employs three types of mutagenesis. These are induced mutagenesis, in which mutations occur as a result of irradiation (gamma rways, X-rays, ion beam, etc.) or treatment with chemical mutagens; site-directed mutagenesis, which is the process of creating a mutation at a defined site in a DNA molecule; and insertion mutagenesis, which is due to DNA insertions, either through genetic transformation and insertion of T-DNA or activation of transposable elements (Forster and Shu 2012).

Radiation and chemical mutagenesis has been widely used in rice to create useful genetic variations for breeding and rice genome research purposes. From 1930 to 2014 more than 3200 mutagenic plant varieties were released that have been derived either as direct mutants (70%) or as a result of crossing such mutants with other breeding lines /progeny (30%) (https://mvd.iaea.org/, https://www.revolvy.com/main/index.php). During the past fifty years, over 500 rice varieties have been developed worldwide, either directly from induced mutants or as a result of crossing such mutants with other breeding lines (Maluszynsk *et al.* 2000*)*. The schematic representation of the mutation method is presented in **Fig. 2.1**.

Chemical mutagenesis alone has contributed to many useful mutants in rice (Sikora *et al* 2011, Wang *et al.* 2013). Thousands of useful mutants have been produced so far from various chemical mutagens such as sodium azide, ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), hydrogen fluoride(HF), diethyl sulphate, hydroxylamine and N-methyl-N-nitrosourea (MNU) (https://mvd.iaea.org/ Till *et al.* 2003, Till *et al*. 2007, Suzuki *et al.* 2008, Wang *et al.* 2013, Wu *et al.* 2005). Most popular used chemical mutagen in inducing TILLING populations in maize, rice, pea, barley, wheat, soybean and cucumber is ethyl methanesulphonate (EMS) (Martin *et al*. 2009, Kurowska *et al*. 2011). Campbell *et al*. (2012) reported a wheat leaf rust, stem rust, stripe rust, and powdery mildew resistance mutants produced by ethyl methane sulfonate (EMS).

Also, several BB resistance mutants in different resistance level and different spectrums have been produced from different chemical mutants including *N*-methyl-*N*-Nitrosourea

(MNU) (Satoh *et al.* 2010, Taura *et al.* 1991a, 1991b, 1992a, Xia *et al.* 2012). This research study was carried out to create useful mutations using MNU chemical mutagens.

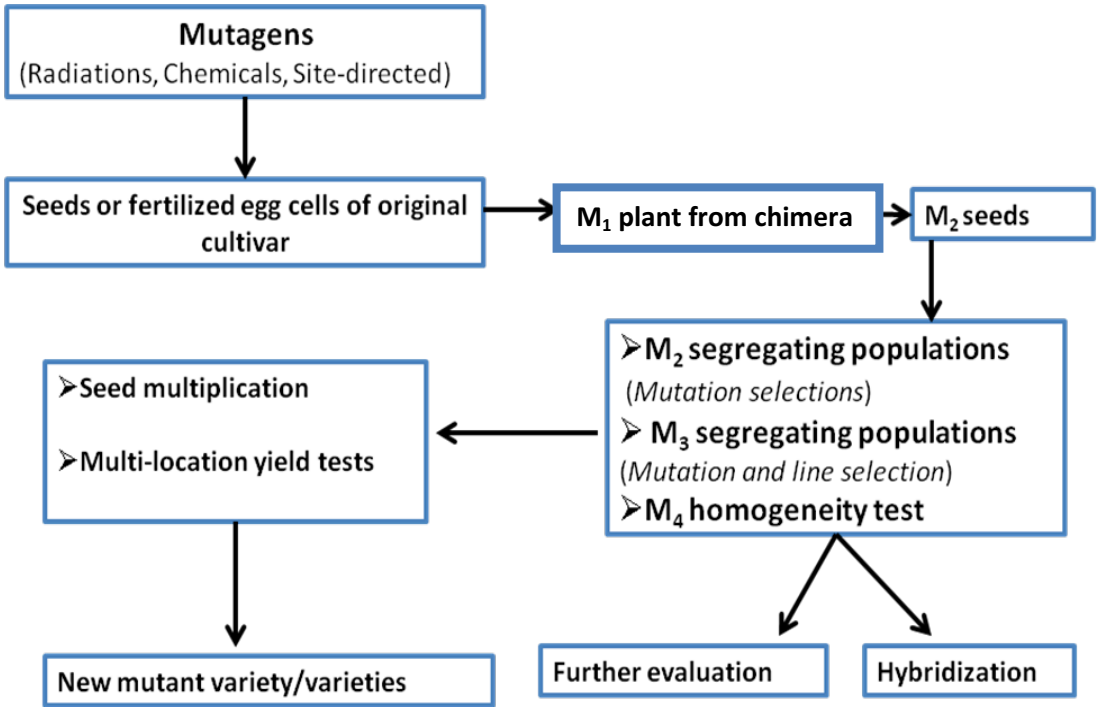

Fig. 2.1. Method of mutation breeding (adopted and modified from Oladosu *et al*. 2016).

2.0 MATERIAL AND METHODS

2.1 Plant material

'IR24' is an elite, semi-dwarf and high-yielding Indica cultivar developed and released in 1972 by International Rice Research Institute (IRRI). This IR24 is known to be susceptible to six Philippine *Xoo* races (Race 1 (PXO 61), Race 2 (PXO 86), Race 3 (PXO 79), Race 4 (PXO 71), Race 5 (PXO 112) and Race 6 (PXO 99) (Taura *et al.* 1991b, 1992a). Moreover, it is susceptible to the six Japanese *Xoo* races (Ogawa and Yamamoto 1987).

2.2 Bacterial races

Philippine Race 5 (PXO 112) was used in the inoculation test during selection of mutants' plants after mutation induction of IR24 with NMU.

2.3 Chemical mutagenic agent

A mutagenic agent, N-methyl-N-Nitrosourea (MNU), belongs to group of cell cycle phase nonspecific, lipid soluble biological alkylating agent (**Fig. 2.2**). It is an alkylating agent because it acts by the process of alkylation to inhibit DNA repair. It has been reported to be

very efficient and effective in inducing mutation to fertilized egg cells at the single-cell stage (Satoh *et al.* 2010).

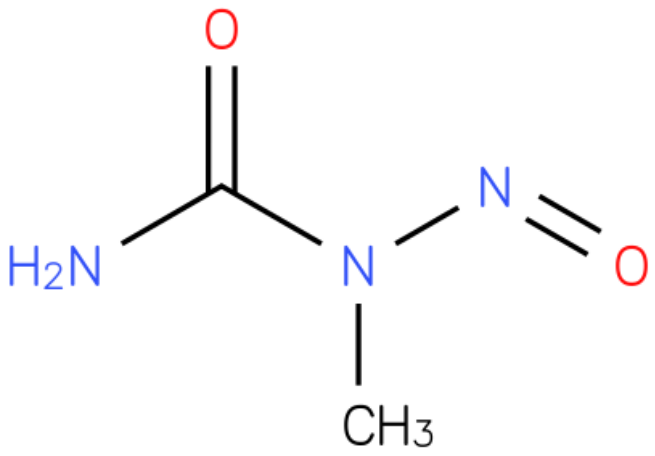

Fig. 2.2. The chemical formula of *N*-methyl-*N*-nitrosoureas. One amino group of urea is substituted by methyl and nitroso groups.

2.4 Mutation induction procedure

The mutation induction producing XM14 line was performed by Prof. Satoru Taura of Kagoshima University when he was in IRRI, Los Baños, Philippine. The method was carried according to Taura *et al.* (1991a, 1991b, 1992a) and illustrated in **Fig. 2.3**. About 1 day before treatment, forty IR24 seedlings at the maximum flowering stage were transplanted from the paddy field to plastic pots, and the pollinated spikelets were cut off with scissors. In order to synchronize the developmental stage of the fertilized egg cells, the plants with unopened spikellets were moved to the dark place just after cutting off the pollinated spikelets. The next day around 11:00 a.m. to 12:00 p.m. they were transferred outside to receive the sunshine. They soon started opening (flowering) and after about one hour the unopened spikelets were cut off. Since many researchers suggest that fertilized egg cells at the single-cell stage in rice achieved a high mutation frequency with MNU (Satoh and Omura 1979, 1986). Spikelets of IR24 were soaked in 1 mM per litre of MNU solutions for 45 minutes at around room temperature in the dark for 10, 12, 14, 16, 18 and 20 hr after flowering. The panicles were rinsed with tap water immediately after MNU treatment. The plants were kept until at maturity where about 10,000 M_1 seeds were harvested.

In the following season, M_1 seeds were sown in the seeding boxes, then transplanted to the paddy field and the segregation of mutants within each line was investigated by inoculation of Philippine *Xoo* race 5 (PXO 112), The M_1 plant were all susceptible to Philippine *Xoo* race 5, which suggested that there was no segregation.

M_1 plants were selfed to produce M_2 generation. M_2 plants resistant to Philippine Race 5 were selected. One M_3 line derived from the resistant M_2 plant was fixed for *Xoo* resistance. The progeny of the M_3 line was named XM14

3.0 RESULTS

3.1 Appearance of XM14 mutant line

The XM14 mutant line looked different from its original wild parent (IR24 cultivar). XM14 has strikingly brown spots (BS) in it leaves (Fig. 2.3), these BS developed impulsively in the absence of infection by pathogens. The BS in XM14 line looks likes lesion mimic mutants and rice spotted leaf mutant (spl) (Zeng *et al*. 2004, Xu *et al*. 2014) and in wheat (Li and Bai 2009).

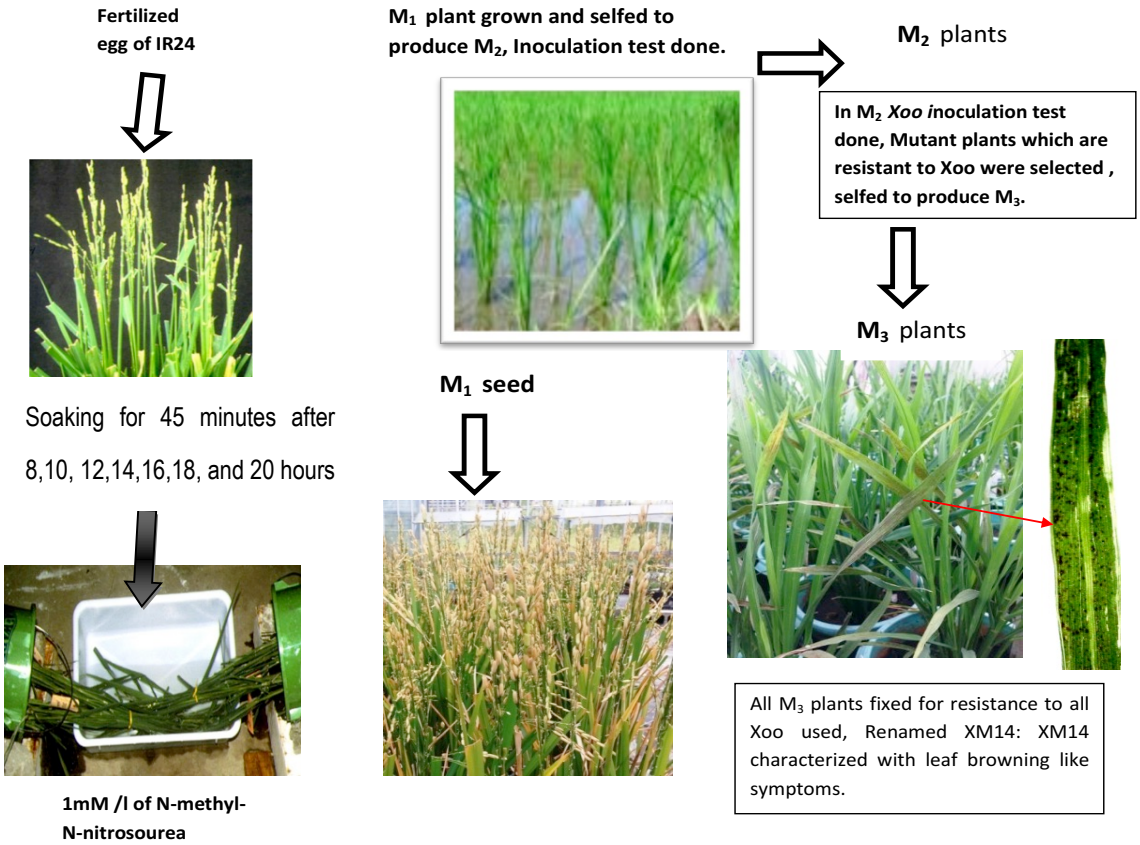

Fig. 2.3. Schematic procedure of IR24 cultivar mutation treatment and production of XM14 line.

4.0 DISCUSSION

The endeavor to produce a long lasting and stable broad spectrum resistance is not only sustainable but also the most effective approach in preventing epidemics and annual yield loss caused by BB. One of the proven ways of creating long lasting and stable broad spectrum resistance is through the use of mutation breeding (Sikora *et al.*, 2011, Wang *et al*. 2013). Thousands of useful mutants have been produced so far from chemical mutagens (https://mvd.iaea.org/, Krishnan *et al*. 2009, Wang *et al*. 2013).

Mutation breeding through using MNU has proved to produce variation and permanent mutations which are useful in disease resistance breeding (Kurata and Yamazaki 2006). One of the examples of usefulness of MNU mutation breeding is identification of *xa19* and *xa20* gene through MNU mutation induction (Taura *et al.* 1991a, b, Taura *et al.* 1992a).

The results reported herein demonstrate that mutation is a good tool for altering the characteristics of rice plants. In this study, it is confirmed how MNU is effective in creating useful mutants for genetic improvements for disease resistance. This chapter reports a mutant line named XM14 produced through MNU mutation induction of IR24 cultivar. In M_1 generation, the plants were heterozygous and no selection was made. In M_2 generation, there was segregation of the population derived from MNU induction. It was therefore possible in this study to start selection and screening a resistant mutant. Extra attention was taken to prevent cross pollination among the M_1 population. Many scholars have reported that cross pollination in M_1 leads to generation of new variation which will be difficult to differentiate from the effects of mutation (Roychowdhury *et al*. 2011, Roychowdhury *et al*. 2012, Roychowdhury and Tah 2013, Olodosu *et al*. 2016).

The XM14 mutant line in this study was tentatively thought to be recessive as there was no segregation in M_1 because recessive mutation can be detected in M_2 and only dominant mutations can be detected and identified in M_1 (Roychowdhury *et al*.2011, Roychowdhury *et al*. 2012, Roychowdhury and Tah 2013). This mutant line is characterized with brown spots (BS) on its leaves which mimic disease symptoms (**Fig. 2.3**). The brown spots in the XM14 mutant line appears about two to three weeks after germination and vanishes before maturity stage. These brown spots very much resemble those that are produced during the hypersensitive resistance response of rice to pathogens. A similar phenotype has been associated with a number of mutants in rice (Kaku and Hiro 1977, Kaku and Ogawa 2001) which have been briefly categorized as disease lesion mimics.

According to Richardson *et al*. (1987), Fix (1993), Glickman *et al*. (1987) and Sato *et al*. (2010) alkylation mutagenic agents such us MNU usually induces a single base substitution. The XM14 line might have resulted from similar base substitution.

5.0 SUMMARY

The present investigation on mutation of IR24 cultivar using N-methyl-N-Nitrosourea to produce XM14 mutant line has yielded new insights about the efficiency of MNU on mutation induction. The XM14 line showed resistance to Philippine race 5 (PXO 112), and brown spot phenotype in absence of any pathogen.

CHAPTER THREE

GENETIC ANALYSIS OF *Xoo* RESISTANCE GENE IN XM14 LINE

1.0 INTRODUCTION

The previous chapter showed that XM14 is resistant to Philippine Race 5 (PXO 112). However, there is no information available as to its resistance against Japanese *Xoo* races. Inheritance pattern of *Xoo* resistance also remains unknown. In this chapter, I evaluated the resistance of XM14 against multiple Japanese *Xoo* races. Then I conducted the genetic analysis of the resistance in XM14, using Japanese *Xoo* race II.

2.0 MATERIAL AND METHODS

2.1 Bacterial races

Japanese *Xoo* races are classified into five groups, I, II, III, IV and V due to their reaction to differential rice varieties (Horino 1978, Horino and Yamada 1979, Yamada *et al.* 1979a). In this study, *Xoo* races used were Japanese *Xoo* race I (T7174), race II (T7147), race III (T7133), race IV (H75373), race V (H75304), and race IIB (H9387).

2.2 Xoo Isolation and Inoculation test

The inoculums were grown on Wakimoto solid medium (Wakimoto 1953) (potato 300 g, sucrose 20 g, $Na_2HPO_4 \cdot 12H_2O$ 2 g, $Ca(NO_3)_2 \cdot 4H_2O$ 0.5 g, agar 25 g, H_2O 1L) at 28℃ for 48 hours (Ou 1985), and then preserved at 4℃ (Hsieh *et al.* 2005). **Fig .3.1** shows the *Xoo* colonies culture at Kagoshima Gene Institute laboratory following Wakimoto solid medium procedure. Single colony was sub-cultured in Wakimoto solid medium with agitation at room temperature for 48 hours. A metal loop was used to streak plate with the mixture of cells over the surface of a semi-solid, agar-based nutrient medium in a Petri dish. The *Xoo* inoculums were then transferred to the test tube and incubated for another 48 hours at 28℃. Finally, inoculum was then diluted with distilled water and the absorbance was adjusted to A= 0.05 (620nm) using a spectrophotomer (**Fig. 3.2**). This value corresponds to the concentration of 10^8 cells/ml, which is optimum concentration required to cause BB disease. *Xoo* was inoculated by clipping method (**Fig. 3.2**) according to procedure described by Kauffman *et al.* (1973). BB severity was scored by measuring lesion length with measuring ruler (**Fig. 3.2**) 18 days after inoculation of *Xoo*.

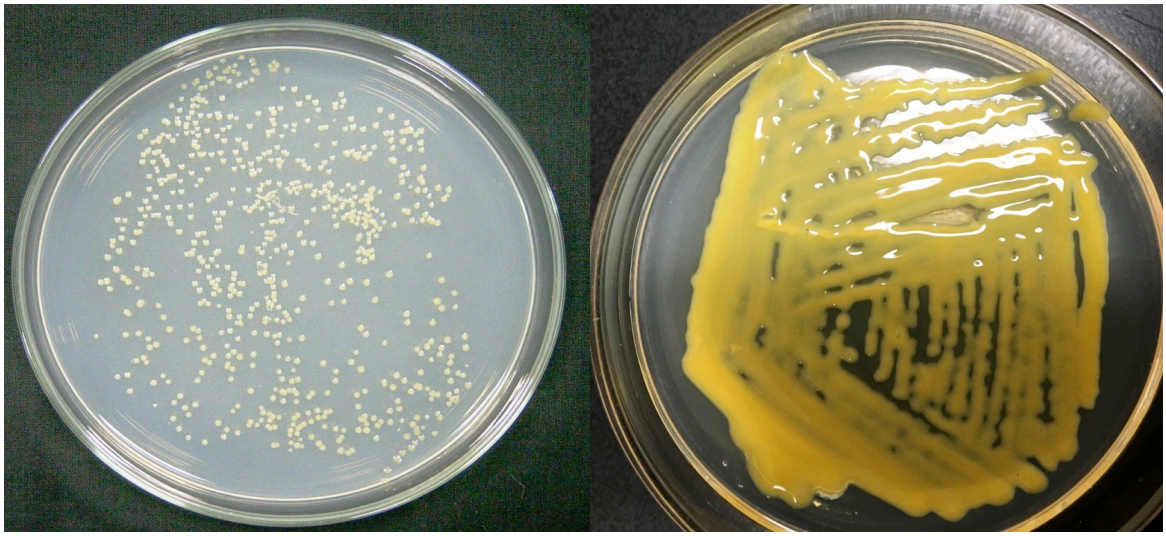

Fig. 3.1. *Xoo* colonies following Wakimoto solid medium preparation protocol.

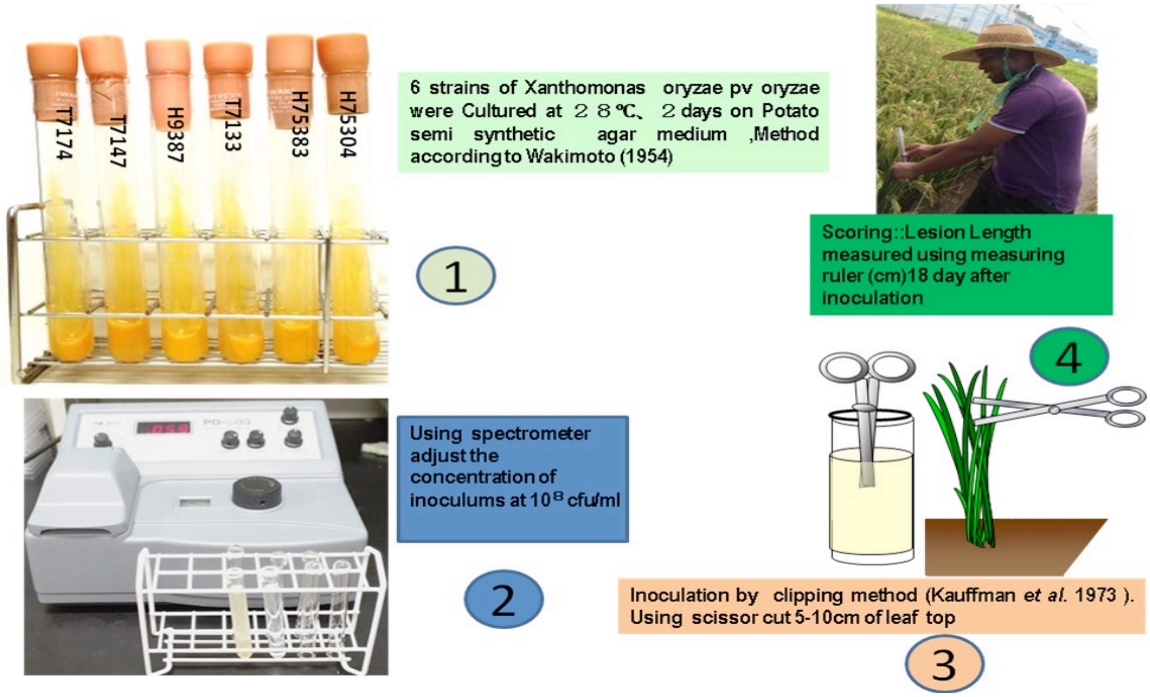

Fig. 3. 2. Illustration showing *Xoo* inoculums preparation, inoculation and scoring.

2.3 Generation of a segregating population

The genetic screens to classify a gene or genes responsible for phenotypic variation such as disease resistance have been a common task in genetics. In crop plant such as rice, identification of genes contributing to the variations in phenotype has great deal of

implications not only in understanding basic processes but also for the improvement of crop attributes (Rafalski 2010). In forward genetics, genetic mechanism of a phenotype is studied by introducing random mutations artificially by means of either chemicals or radiation, and plants showing phenotype of interest are selected to raise a mapping population. The aim of mutagenesis is to introduce maximal genomic variation with minimal reduction in viability to obtain traits that are almost impossible to identify by conventional breeding (Sikora *et al.* 2012). In this study to ascertain the number of gene(s) and inheritance pattern involved in the XM14 line, XM14 was crossed to IR24. The F_1 progeny of this cross was selfed to produce the F_2 generation.

2.4 Preparation of pre-germinated seeds for sowing

The collected seeds were then soaked in tap water, placed in labeled petri dish and then incubated at 29°C for 72 hours in the incubator. After every 24 hours, seeds were washed and rinsed properly with tap water. After 72 hours, almost 90 percentages of seeds germinated and were ready for sowing in the seedling boxes.

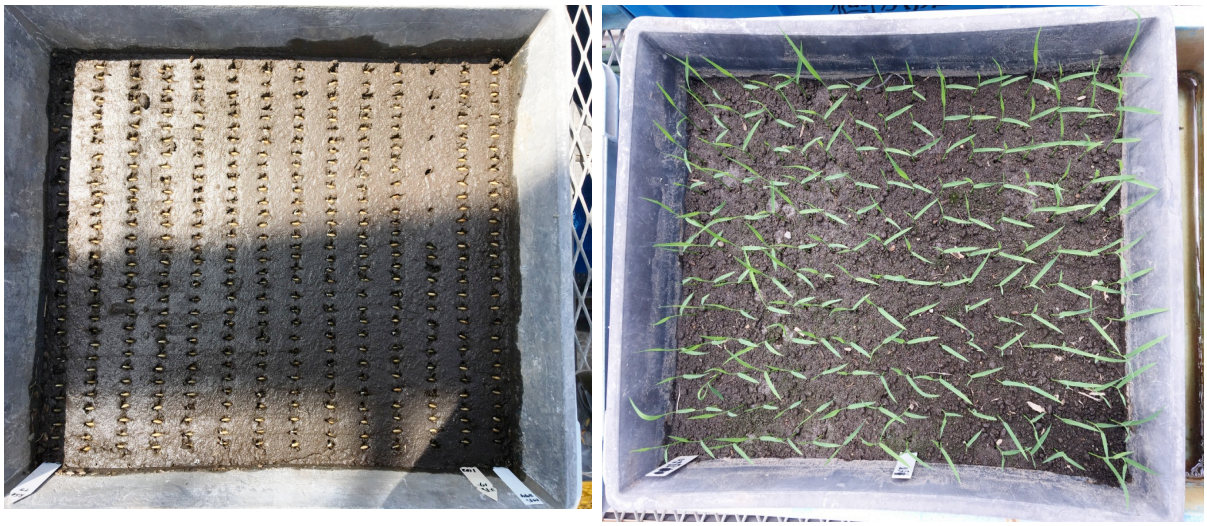

Fig. 3.3. Sowing and growing of seedling in the greenhouse.

2.5 Preparation of seedling boxes and sowing

The soils used in this study were sieved, sterilized with a microwave. A layer of newspaper/magazine was used to cover the bottom of seedling boxes to prevent the soil leakage. The trays were then filled with soil using scoop to about 3-4cm depth. Water was poured gently using watering can. The excess water was drained before sowing. Germinated seeds were then sowed accordingly using forceps (Fig. 3.3).

216 germinated F_2 seedlings were sown in seedling boxes in a greenhouse. About two weeks after sowing, seedlings were transferred out of the greenhouse. Then after about three weeks, seedlings were transplanted to a paddy field in the experimental farm of the Faculty of Agriculture, Kagoshima University, Kagoshima, Japan. Fertilizers were applied one week after transplantation at the rate of 6 g of N, 3 g of K_2O, 3 g of P_2O_5/m^2. Two weeks before *Xoo* inoculation test, N was applied at a rate of 3 g/m^2. The plant spacing was 15 × 30 cm.

3.0 RESULTS

3.1 Reaction of the XM14 line to six races of Japanese Xoo

IR24 was susceptible to the 6 Japanese *Xoo* races used in this study while XM14 was resistant to them (**Table 3.1 and Fig. 3.4**). The average LL of XM 14 reaction for the Japanese *Xoo* races was 0.4 cm whereas that of IR24 reaction was 23.6 cm.

Table 3.1. Reactions of XM14 and IR24 to 6 *Xoo* Japanese races

Xoo races	race I (T7174)		raceIIA(T7147)		race III (T7133)		race IV (H75373)		race V (H75304)		race IIB (H9387)	
Lesion Lengt	Mean [a]	SD [b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
XM14	0.3	0.2	0.2	0.1	1.2	0.6	0.3	0.1	0.3	0.1	0.7	0.2
1R24	30.9	1.5	28.2	2.4	20.6	2.8	28.7	0.5	24.9	3.9	25.6	3.8

[a]Mean (cm)= average lesion length (cm) of three leaves 18 days after *Xoo* inoculation.

[b]SD(cm)=Standard deviation.

The data from four successive years proved that XM14 line was highly resistant to *Xoo* at seedling, tillering and adult stages in all the years I tested (**Table 3.2**). On the contrary, its original parent IR24 was as highly susceptible in all years I tested. All the F_1 plants which were generated by crossing XM14 line with its original parent IR24 were susceptible to *Xoo* after inoculation. F_1 generations resulting from reciprocal crosses between XM14 and its original parent IR24 was phenotypically indistinguishable.

I observed a clear gap and classified the 216 F_2 plants into 53 resistant plants with LL ranging from 0.1 to 2 cm and 163 susceptible plants with LL ranging from 7 to 37 cm (**Fig. 3.5**). The segregation data recorded in F_2 were further subjected to goodness of fit test using the null hypothesis that no significant deviations exist between the observed and expected frequencies. The ratio 53: 163 fitted 1: 3, one-gene segregation (χ^2=0.02, P= 0.88). Thus, this

result suggests that resistance to multiple races of *Xoo* by the XM14 mutant line is controlled by single recessive gene.

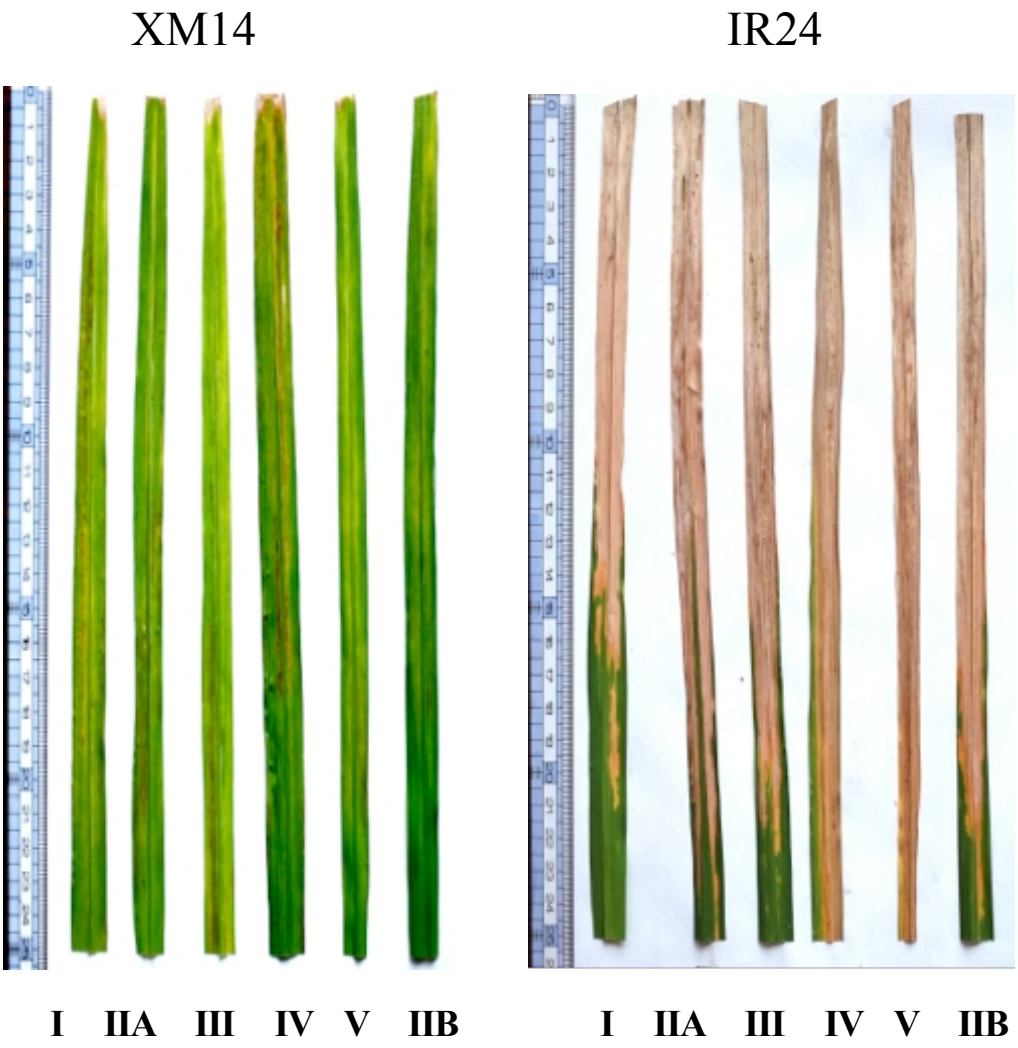

Fig. 3.4. Reactions of XM14 mutant line and its original parent IR24 to six races of Japanese *Xoo* after inoculation test.

Table 3.2. Reactions of XM14 and IR24 to *Xoo* Japanese race IIA (T7147) from 2014-2017

Year	2014		2015		2016		2017	
Lesion length(cm)	Mean [a]	SD [b]	Mean	SD	Mean	SD	Mean	SD
IR24	23.86	3.1	28.2	2.4	20.88	4.65	26.5	3.2
XM14	0.42	0.18	0.3	0.1	0.21	0.2	0.34	0.2

[a]Mean (cm)= average lesion length (cm) of three leaves 18 days after *Xoo* inoculation.

[b]$SD_{(cm)}$=Standard deviation.

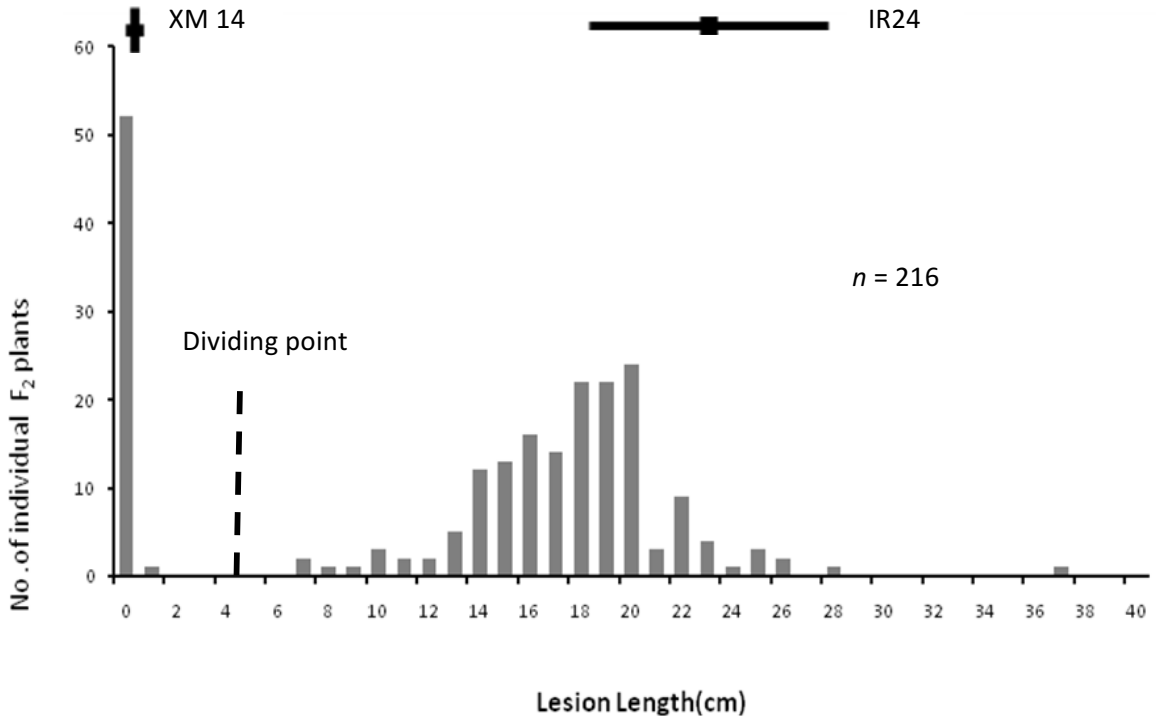

Fig.3.5. Distribution of lesion length in F_2 population from the cross between XM14 (mutant line) and IR24 (original parent) after *Xoo* Japanese race IIA (T7147) inoculation test. Horizontal lines at the top of figure represents range of parental lines. Vertical line crossing the horizontal line represents mean of parental lines

4.0 DISCUSSION

The endeavor to produce a long lasting and stable broad spectrum resistance is not only sustainable but also the most effective approach in preventing epidemics and annual yield loss caused by BB. One of the proven ways of creating long lasting and stable broad spectrum resistance is through the use of mutation breeding (Sikora *et al.* 2011, Wang *et al.* 2013). Thousands of useful mutants have been produced so far from chemical mutagens (https://mvd.iaea.org/, Leung *et al.*,2001, Suzuki *et al.* 2008, Till *et al.* 2003, Till *et al.*, 2008, Wang *et al.* 2013, Wu *et al.* 2005).

Mutation breeding through using MNU has been proved to produce variation and permanent mutations which are useful in disease resistance breeding Examples of usefulness of MNU mutation breeding *xa19* and *xa20* genes (Taura *et al.* 1991b, 1992a).

The XM14 mutant line in this study was tentatively thought to be recessive as there was no segregation in M_1 because recessive mutation can be detected in M_2 and only dominant

mutations can be detected and identified in M_1 (Roychowdhury *et al.* 2011, 2012, Roychowdhury and Tah 2013). This mutant line is characterized with brown spots (BS) on its leaves which mimic disease symptoms (**Fig. 2.3**). The brown spots in the XM14 mutant line appears around two to three weeks after seedling germination and vanishes before maturity stage.

Inoculation tests for over 3 years (**Table 3.1, 4.2, 7.2**) have shown that XM14 is exhibiting broad spectrum resistance to 6 Japanese *Xoo* races. The lesion length (LL) for XM14 ranged from 0.1cm to up 2cm. Using F_2 segregating plants from the cross between XM14 mutant line and its original cultivar IR24 and inoculating Japanese *Xoo* race II (T7147), I observed segregation and separated the plants into resistant (short LL) and susceptible (long LL). Short LL ranged from 0.0cm to 2cm while long LL ranged from more than 4 cm. The F_2 LL distribution showed bimodal distribution and the ratio of resistance: susceptible was 53: 163, fitting 1: 3, one-gene segregation (χ^2=0.02, *P*= 0.88).

I observed fluctuations of LL after inoculation of *Xoo* Japanese race IIA over years from 2014 to 2017 (**Table 3.2**). These fluctuations might be due to difference in temperature during the growing seasons over the years. However, these changes were not significantly different. According to Webb *et al.* (2010) after monitoring disease severity and virulence of *Xa7* in growth chambers for over 11 years, *Xoo* population size and severity were more in high temperature cropping seasons than in cool seasons

5.0 SUMMARY

XM14 is a mutant line by N-methyl-N-nitrosourea (MNU) treatment to IR24. The XM14 proved to be resistant to six Japanese *Xoo* races, though the original cultivar IR24 was susceptible to them. The F_2 population from the cross between XM14 and IR24 showed that resistance against Japanese *Xoo* race II was controlled by a recessive allele at a single locus.

CHAPTER FOUR

CHROMOSOMAL LOCATION OF *Xoo* RESISTANCE GENE IN XM14 LINE

1.0 INTRODUCTION

The results of Chapter 3 indicate that the resistance of XM14 is controlled by a single recessive gene. However, its chromosomal location remains unknown. It is the basic information as to whether this gene is same as the ones previously identified or newly identified. In this chapter, the approximate gene location was determined by the combination of analysis using extreme recessive phenotype and DNA polymorphism between Indica and Japonica in rice.

2.0 MATERIALS AND METHODS

2.1 Bacterial races

Races used for this chapter were six Japanese races: race I (strain T7174), race IIA (strain T7147), race IIB (strain H9387), race III (strain T7133), race IV (strain H75373), and race V (strain H75304).

2.2 Plant materials

'Koshihikari', a popular Japonica rice cultivar, is cultivated in Japan as well as Australia and the United States. This cultivar is known to be susceptible to all Japanese *Xoo* races (Noda and Ohuchi 1989). Germinated seeds of F_2 segregating populations derived from the cross between XM14 and Koshihikari and parental lines were sown in seedling boxes in a greenhouse in May 2015. Cultivation procedure followed Chapter 3. Along with the respective segregating populations, 10 plants from each parental line were planted.

2.3 Inoculation of Xoo and BB disease scoring

Inoculation of *Xoo* and scoring *Xoo* inoculation followed Chapter 3 section 2.2.

2.4 Preparation for DNA extraction

2.4.1 Leaf sample collection

To extract quality genomic DNA from rice leaves, about 0.1g of a healthy portion of the youngest leave of the tiller was cut apart with sterilized forceps and put into 2mL micro centrifuge tube. In the field, all the centrifuge tubes were contained in racks, upon arriving in the laboratory the racks containing centrifuge tubes were immediately stored in -80C freezer.

2.4.2 Preparations of reagents for DNA extraction

Different reagents were used for DNA extraction and electrophoresis. Stock solutions were prepared as below.

Extraction buffer (1000 ml)

100 ml of 1M Tris (pH 8.0) was taken in measuring cylinder. Then 100 ml 1M NaCl was added to it. 100 ml of 0.5M EDTA (ethylene diamine tetra acetic acid) was mixed with the mixture followed with 10.0g of 1% sodium dodecyl sulphate (SDS). Finally, sufficient sterilized double distilled water was added to make the volume 1000 ml and then sterilized by autoclaving.

1M Tris, PH 8.0 (250 ml)

Trisma base 30.28 gm was added in double distilled water and pH was adjusted to 8.0 with HCl (10.5 ml). After adding double distilled water to 250 ml, the solution was then autoclaved.

1X TE (Tris EDTA) (100 ml)

10 ml 1M Tris (pH 8.0) was taken and 0.2 ml EDTA pH 8.0 was added to it. Then double distilled water was added to make volume 100 ml and the solution was then autoclaved.

RNase A (10 mg/ml) (store at -20°C)

20 % SDS - prepare in hood

In order to make 100ml, a 20 gm sodium dodecyl sulfate (SDS) was dissolved in 80 ml double distilled water, stored at room temperature. SDS emulsifies the plasma and nuclear membrane.

70% (v/v) Ethanol

To make 1000 ml of 70% ethanol, double distilled water was added to 700ml of pure ethanol(99.9(v/v) to make a total volume 1000ml..

5M KOAC (Potassium acetate)

To make 100ml of Potassium acetate stock solution, 49.1 gram KOAC was dissolved in 100ml of double distilled water. Add acetic acid

Isopropanol

2.4.3 DNA extraction

DNA was extracted according to modified Dellaporta method by Dellaporta *et al*. (1983). Two stainless balls were added to 0.1g leaf sample in each micro centrifuge tube. The freeze-dried leaf samples were crushed into powder using Shake Master (Biomedical Science, Tokyo, Japan). 750 µl extraction buffer was added and vortexed well. 250ml of KOAC was added, and the sample was incubated for 20 min on ice. After 20 minutes at 4200rpm centrifugation, DNA was precipitated from the supernatant by adding an equal volume isopropanol, and incubating for 1 h at -20°C. This was followed by another round of 15 minutes at 4200rpm centrifugation; the pellet was washed with 70% ethanol and air-dried. DNA was dissolved in 100mL TE buffer, treated with RNase. Then the samples were vortexed well and kept in -20°C until use.

2.4.4 DNA quantification

Optimum DNA concentration is needed in the microsatellite marker analysis to distinguish the bands clearly. Microsatellite markers, Indel and CAPs markers used in PCR reactions during the course of this study worked better when the DNA concentration was about 10ng/µl -20 ng/µl. On the other hand, excessive or too much DNA concentration produced poor resolution or resulting in a lack of distinct bands in the gel. In this study, DNA guality and quantity was determined by using NanoDrop spectrophotometer (**Fig. 4.1A and 4.1B**). First, The NanoDrop Spectrophotemer is calibrated by measuring 8 samples each with 1.5-2 µl of double distilled water. Then the DNA was measured 8 samples per one time, each sample had 1.5-2 µl DNA. After measuring the average concentration of the entire DNA samples, the concentration DNA samples were diluted by adding sterilized double distilled water to 10ng-20ng/µl and stored at 4 °C ready for polymerase chain reaction.

DNA has a maximal absorbance near 260 nm so UV light of this wavelength is passed through the samples. Higher levels of absorbance are indicative of greater concentrations of DNA present within the samples. This method has the advantage that the quality of the DNA can also be assessed; absorbance at 280 nm is also measured to determine the level of protein contamination. The A260/A280 ratio is indicative of the purity of the DNA samples and values of 1.8 or higher relate to pure DNA samples.

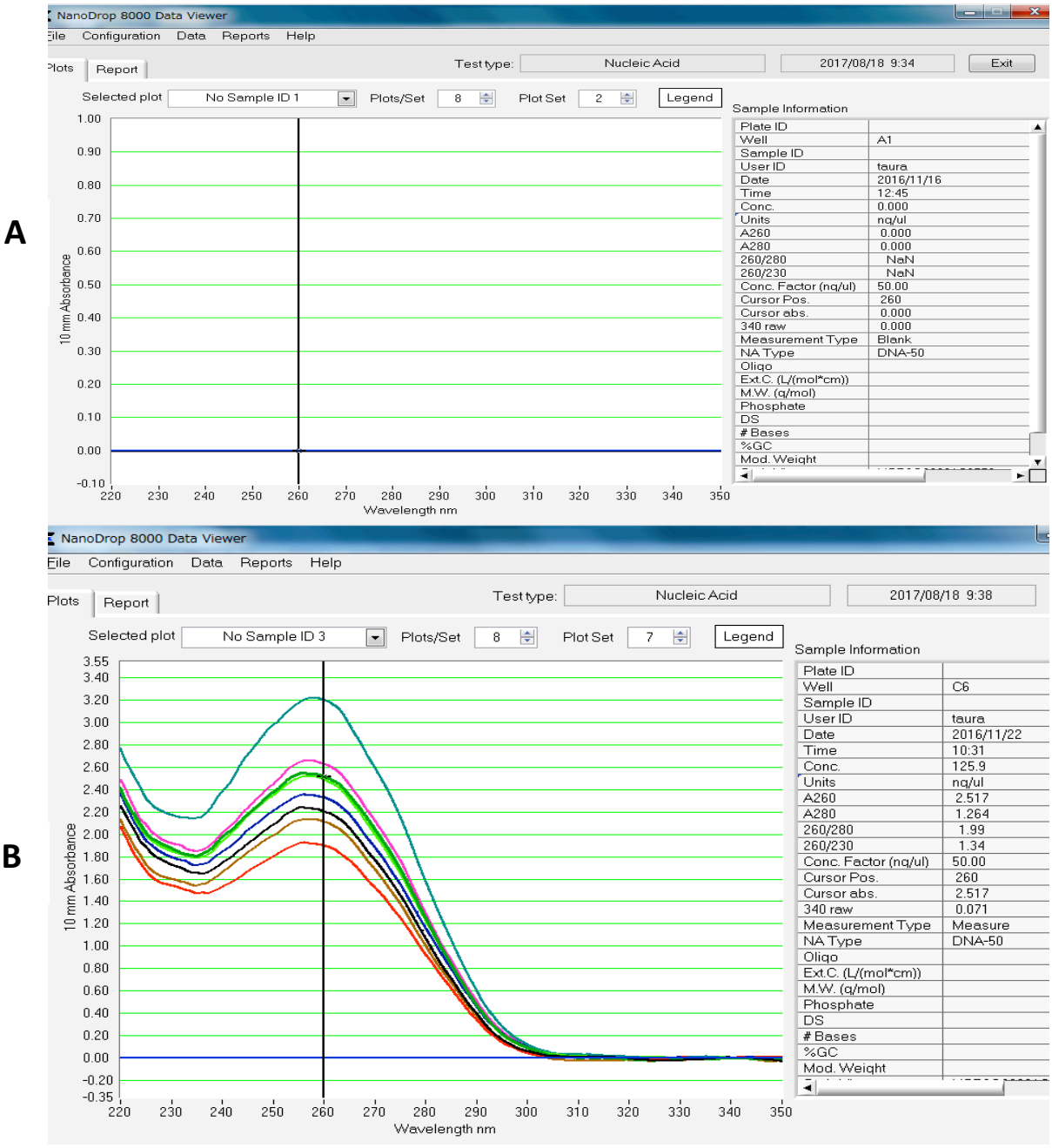

Fig. 4.1. DNA quantification and quality check using Nanodrop 8000 Spectrophotometer after DNA extraction following modified Dellaporta *et al*. (1984) method. A: water blank control measuring with concentration (ng/µl) units. B: DNA quantification curves (absorbance/ wavelength) showing quality and concentration in (ng/µl) units.

2.5 Polymerase chain reaction

Published PCR based SSR DNA markers (Ichitani et al. 2014, IRGSP 2005, McCouch *et al.* 2002, Panaud *et al.* 1996, Rice Genome Research Program (http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html)) were used in this study. Polymerase

chain reaction (PCR) mixture (5 µL) consisted of 10 ng genomic DNA, 200 µM dNTPs, 0.2 µM of each primer, 0.25 U of Taq polymerase (AmpliTaq Gold; Applied BioSystems, CA, USA), and 1 × buffer containing $MgCl_2$. PCR conditions were: 95°C for 5 min, 35 cycles 94°C for 30 s, 55°C for 30 s and 72°C for 30 s with subsequent final extension at 72°C for 7 min.

2.6 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis shows some advantage over agarose gel electrophoresis. Its main advantage over agarose is that acrylamide has smaller pores making it better suited for separating smaller DNA molecules that agarose gel would not be able to separate.

2.6.1 Preparation of reagents

Acrylamide: bisacrylamide (29:1) (30% w/v)

Ammonium persulfate (APS) (10% w/v) is used as a catalyst for the copolymerization of acrylamide and bisacrylamide gels. The polymerization reaction is driven by free radicals that are generated by an oxido-reduction reaction. Tetramethylethylen diamine (TEMED) is a free radical stabilizer, It molecular formula is (N, N, N', N' Tetramethylethylene-1, 2-diamine), TEMED is added at the last in the gel mixture. It reacts with the ammonium persulfate and causes splitting of the persulfate ions into sulfate free radicals. Thus formed sulfate free radicals now initiate the polymerization (free radical reaction) of the acrylamide and cross-linking with bisacrylamide.

5X TBE

5x TBE electrophoresis buffer polyacrylamide gels are poured and run in 0.5x or 1x TBE at low voltage (1-8 V/cm) to prevent denaturation of small fragments of DNA by heating. 5 X Tris/Borate/EDTA (TBE) stock solutions: 54 g of Tris base (Trizma base, Sigma - Aldrich) and 27.5 g of boric acid was dissolved in 900 ml of diluted H_2O. 20 ml of 0.5 M (4.65 g) EDTA was added and the solution was adjusted with diluted H_2O to a final volume of 1 L.

2.6.2 Gel plate assembly

The plates were washed by lab detergent and then rinsed with water. 70 % ethanol was sprayed on the chosen inner surface of the plate and wiped with lint free tissue (three times). The rubber gasket was attached with the short plate. The notches on the gasket should be aligned on the corners; the circular portion of the gasket should be exposed to the inner side of plate. The short plate was laid on the table with the tubing side up and spacers were put

along the inside edges of gasket. The other plate was put on top of the short plate. The clamps were set on both sides of plates and laid on table. The surface of table should be even.

2.6.3 Preparation of the polyacrylamide gel (10 %)

This study used 10 percent polyacrylamide gel (PAGE) as in Ichitani *et al* (2014). For 12.5 ml (**Table 4.1**) of separating gel mix (enough for one gel), 4 ml of Acrylamide: Bisacrylamide 30%(29:1) 2.4 ml of 5x TBE and 5.6 ml of dH2O were mixed. Just before pouring, 200 µl of 10% (w/v) APS and 20 µl TEMED 0.10%) were added to catalyze polymerization. The gel solution was prepared in a beaker with a magnetic stirring bar. The gel was poured smoothly and continuously starting from one corner until it reaches the top portion of the short plate. Then the comb was gently inserted such that half of it is inserted in the gel. The gel was allowed to polymerize for 15 minutes at 28°C in a incubator.

2.6.4 Electrophoresis of the amplified product by PAGE

The steps needed for electrophoresis of the amplified product are given below: The gasket was removed starting from one side of plate assembly. 0.5x TBE buffer was added in the base of the tank. The plate assembly was attached in one side of the tank such that the short plate is facing the inner side.

The other side was followed by the same pattern. It should be confirmed that there is no bubbles at the bottom to avoid short circuit. Same TBE buffer was added on the top of the tank and the comb was removed. 2µl of sample was added in each well. 100 bp DNA ladder was used for size determination. The cover was put on the tank. The electrodes were connected to the power supply and run for about 1hr at 200 volts.

Table 4.1. Ratio of components in 10 % (12.5 ml) PAGE

Reagents	Final volume
Acrylamide: Bisacrylamide 30%(29:1)	4ml
H_20	5.6ml
5× TBE	2.4 ml
APS (10%)	200µl
TEMED	20µL
Total	12.5ml

2.7 Staining and visualization of gel

The power supply was turned off and the plates were removed from the tank. The glass plates were separated by using a plastic wedge. The polyacrylamide gel was removed and soaked into ethidium bromide (10 mg/ml) solution for 10-20 min. The stained gels were put in the exposure cabinet of the gel documentation system. The gels were viewed in the computer monitor by exposing it first to white light. The necessary adjustments were made by moving the gel inside the exposure box. The gel image resolution was adjusted using the camera setting. The gel was exposed to ultraviolet light in GelDoc-It® TS Imaging System (**UVP,** U.S.A). The gel image was saved as jpg format for further scoring and analysis of band patterns.

2.8 Molecular marker data analysis

2.8.1 Scoring of bands

The pattern of bands obtained after amplification with the primers was scored using visual observation (**Fig. 4.2**). First the only DNA ladder and parent bands patterns were observed. Then bands patterns of F_2 segregating plants were scored by comparing the band patterns of parents as control.

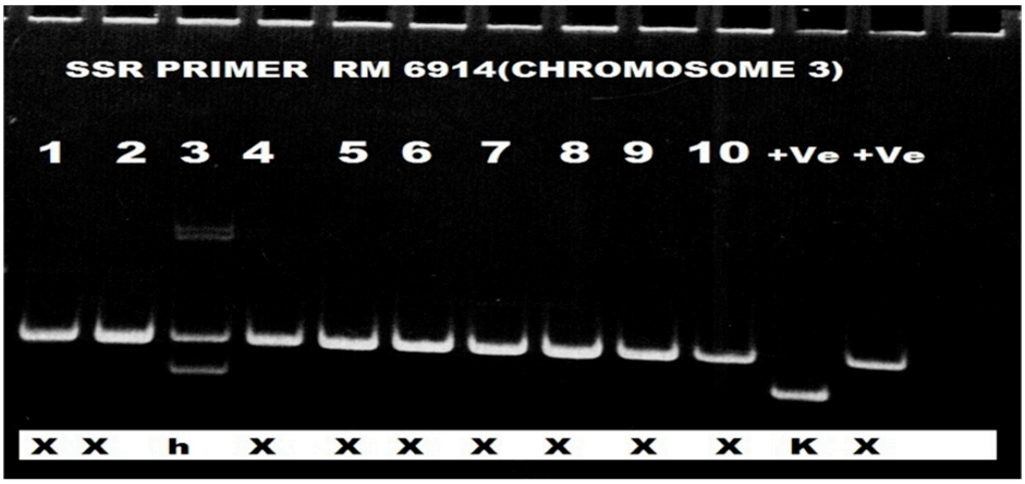

Fig. 4.2. Results of genome chromosome scanning using SSR markers at RM6914 marker locus. Lane 1 to 10 shows genotype scanning of 10 F_2 plants from XM14×Koshihikari with shortest lesion length (cm), nine of which appeared to have XM14 mutant type genotype and 1 had heterozygote genotype. The two lanes at far right are parental genotype and +Ve represents positive controls.

3.0 RESULTS

3.1 Test for resistance to Xoo.

The results obtained from inoculation test confirmed the previous results which showed XM14 mutant line practically exhibits resistance reaction to six Japanese races used. Likewise, it was found that Koshihikari is susceptible to all *Xoo* races used in this study (**Fig. 4.3, Table 4.2)**. The average lesion length of XM 14 reaction for all *Xoo* races was 0.4cm with the range from 0.1 cm to 2cm (**Fig. 4.3, Table 4.2**). The average lesion length of Koshihikari was 13.8cm with the range from 7cm to 23cm (**Fig. 4. 3, Table 4. 2**).

Table 4.2. Reactions in lesion length (cm) of XM14 mutant line, IR24 cultivar, Koshihikari cultivar and IAS16 line after inoculation with six Japanese races of *Xoo*

Xoo races	**race I (T7174)**		**race IIa (T7147)**		**race III (T7133**		**race IV (H75373)**		**race V (H75304)**		**race IIB (H9387)**	
Lesion Length(cm)	**Mean[a]**	**SD[b]**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
XM14	0.3	0.2	0.2	0.1	1.2	0.6	0.3	0.1	0.3	0.1	0.7	0.2
1R24	30.9	1.5	28.2	2.4	20.6	2.8	28.7	0.5	24.9	3.9	25.6	3.8
Koshihikari	15.3	5.6	21.3	2.4	17.8	1.2	11.7	2.4	22.7	2.4	20.6	5.1
IAS16	13.6	3.6	22.3	1.1	20.6	5.3	21.9	2.9	20.5	5.1	24.1	3.1

[a]Mean = average lesion length (cm) of three leaves 18 days after *Xoo* inoculation.

[b]SD=Standard deviation.

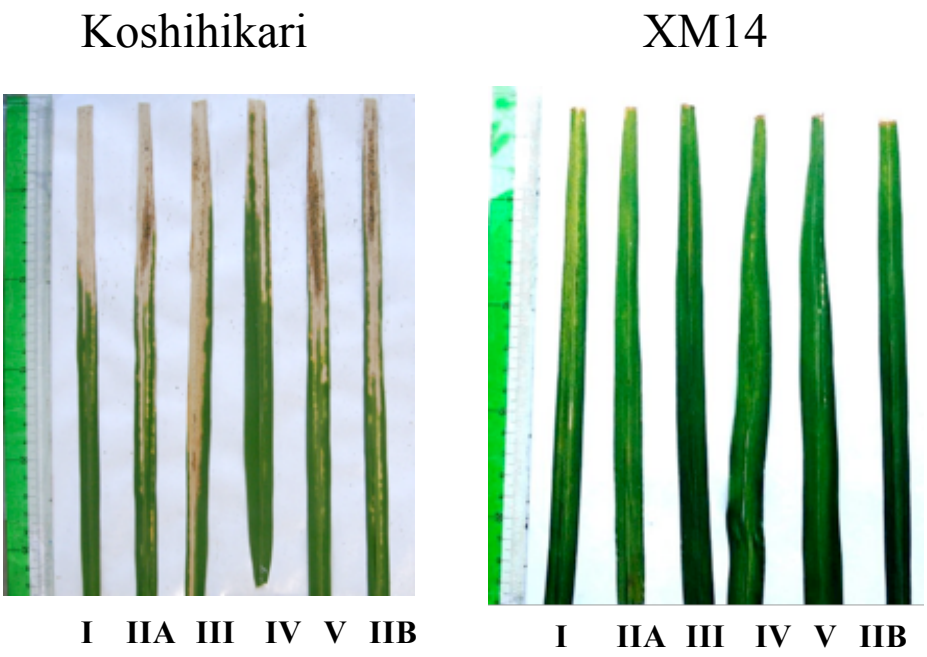

Fig. 4.3. Reaction of parental lines after field inoculation tests with six races of Japanese *Xoo*.

3.2 *Rice genome chromosome scanning*

The F_2 population from the cross between Koshihikari and XM14 showed continuous distribution of LL using *Xoo* race II with no clear gap (**Fig. 4.4)** partly because Koshihikari (Japonica) and XM14 (Indica) have different backgrounds. Large variation in agronomic traits such as tiller number and plant height caused by Indica-Japonica genetic difference might increase LL variation.

In order to determine the location of the gene that confers resistance to BB in XM14, the analysis using extreme recessive phenotype proposed by Zhang *et al.* (1994) was adopted to scan the whole rice genome. Ten F_2 plants with the shortest LL (0.1-4 cm) were selected, and DNA was extracted from each plant. Then genotyping was performed using published 113 DNA markers which covers the whole rice genome (**Table 4.3**).

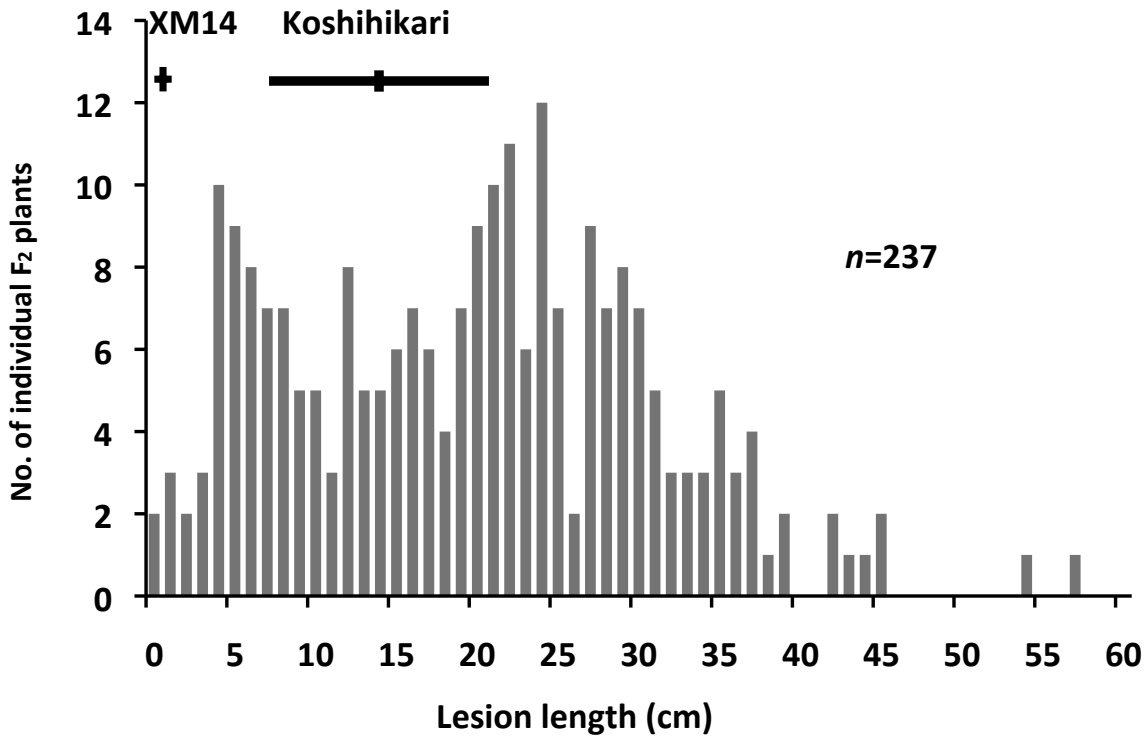

Fig. 4.4. Distribution of lesion length in F_2 population from the cross between XM14 and Koshihikari after *Xoo* Japanese race IIA (T7147) inoculation tests. Horizontal lines at the top of figure represent range of parental lines. Vertical line crossing the horizontal line represents mean of parental lines.

When the DNA marker is linked closely to the resistance gene in XM14, most or all of the ten resistant plants were homozygotes of XM14 allele at the DNA marker locus. Nine plants were homozygotes of XM14 allele at the consecutive four DNA marker loci, RM3400, RM6914, RM1334 and RM5684 on chromosome 3. Further to that, eight plants were homozygotes of XM14 allele at the neighboring DNA marker loci, RM6959, RM3204, RM7642, RM487, RM6832 and RM5488 on chromosome 3. Less than seven plants were homozygotes of XM14 allele at the other loci. These results on whole rice genome scanning for XM14 gene strongly suggest that this gene is located on chromosome 3.

Table 4.3. Genotypes of 113 DNA markers covering the rice genome of ten plants with the shortest lesion length derived from the F_2 population from the cross between Koshihikari and XM14.

Chromo-some	DNA marker[b]	Genotype[a] Individual 1	2	3	4	5	6	7	8	9	10
1	RM1282	H	X	H	K	K	X	H	X	X	H
	RM220	K	X	H	K	K	H	H	X	X	K
	RM259	H	X	H	H	H	H	X	X	X	X
	RM8132	H	X	H	H	H	H	X	H	X	X
	S13623	H	K	K	H	H	H	H	H	X	X
	RM8129	X	K	K	K	X	H	H	H	X	X
	RM246	X	K	K	K	H	H	H	H	X	X
	RM1297	X	H	K	K	H	H	K	H	X	H
	RM212	K	X	K	K	H	H	K	H	X	H
	RM5448	K	X	H	H	H	H	K	H	X	K
	RM8099	K	X	H	H	H	H	H	H	H	H
2	RM211	H	K	K	H	H	H	H	H	H	K
	RM5664	H	K	K	X	K	H	H	K	H	K
	RM6844	H	H	K	H	K	H	H	H	K	K
	RM29	H	H	H	H	X	H	H	H	H	X
	RM1303	H	H	H	H	H	X	H	H	H	H
	RM3525	K	H	K	H	H	X	H	X	X	H
	RM1367	K	X	K	X	H	X	H	X	X	H
	RM240	H	H	K	X	H	X	H	H	X	X
	RM6312	H	H	K	X	H	X	H	H	X	X
3	RM22	H	H	H	X	H	H	K	H	K	H
	E50818	X	H	X	X	X	X	H	X	H	H
	RM6959	X	X	H	X	X	X	X	X	X	H
	RM3204	X	X	H	X	X	X	X	X	X	H
	RM3400	X	X	H	X	X	X	X	X	X	X
	RM6914	X	X	H	X	X	X	X	X	X	X
	RM1334	X	X	H	X	X	X	X	X	X	X
	RM5684	X	X	H	X	X	X	X	X	X	X
	RM7642	H	X	H	X	X	X	X	X	X	X
	RM5488	H	X	H	X	X	X	X	X	X	X
	RM411	H	X	H	X	X	X	X	X	X	X
	RM3698	H	X	H	X	X	X	X	X	X	X
	RM3646	H	X	H	X	X	X	X	X	X	X
	RM487	H	X	H	X	X	X	X	X	X	X
	RM7395	H	X	H	X	X	X	X	X	X	X
	RM6832	H	X	H	X	X	X	X	X	X	X
	RM15451	H	X	H	X	H	X	X	H	H	X
	RM5532	H	X	H	X	H	X	X	H	H	X
	RM6266	H	X	H	X	H	X	X	H	H	X
	RM3513	H	X	H	X	H	X	X	H	H	H
	RM3436	H	X	H	X	H	X	H	H	H	H
	RM3525	H	X	H	X	H	X	H	H	X	H
	RM3346	H	H	H	H	H	H	H	H	X	H
	RM1221	H	H	H	H	H	H	H	H	X	H
4	C61009	X	X	X	X	K	K	H	K	H	K
	RM7279	X	X	X	X	X	K	H	K	H	K
	RM6997	X	X	H	H	H	H	H	H	H	K
	RM303	H	X	H	H	H	H	H	H	H	K
	RM252	H	H	H	H	H	H	H	H	X	K
	RM348	H	K	H	H	K	H	H	H	X	H
	RM8217	H	K	H	H	K	K	H	H	X	H
	RM6246	H	K	H	H	K	K	H	H	X	X
5	RM7373	X	H	H	H	H	H	K	X	H	X
	RM3345	X	H	H	H	H	H	K	H	H	X
	RM7444	X	H	H	K	K	X	K	H	K	X
	RM3777	X	K	K	K	K	X	K	H	K	X
	C50867	K	K	K	K	K	X	K	K	K	X

Chromo-some	DNA marker	Genotype Individual 1	2	3	4	5	6	7	8	9	10
	RM249	H	H	K	K	H	X	H	K	K	X
5	RM7568	H	H	K	K	X	X	X	K	K	X
	E60663	H	H	K	K	X	X	H	H	K	K
	RM6954	H	K	K	K	H	H	H	H	K	K
	RM3476	H	K	H	K	X	K	H	H	K	K
6	E30287	X	X	X	H	X	H	H	X	H	X
	RM253	H	X	X	X	X	X	H	X	H	X
	RM276	H	X	X	X	H	X	H	X	H	X
	RM527	H	X	X	X	H	H	H	X	H	X
	RM3	H	X	X	X	H	H	H	H	K	X
	RM3628	H	H	X	X	X	H	H	H	K	X
	RM6782	H	H	X	X	X	H	H	H	K	K
	RM58114	H	K	H	H	X	K	H	H	H	K
7	RM481	H	K	X	H	H	H	H	H	X	X
	S20268	H	K	X	X	X	H	K	H	H	X
	RM1134	H	K	X	X	H	H	K	H	H	X
	C30372	H	K	X	X	H	H	K	H	H	X
	RM3826	H	K	H	X	H	H	X	X	H	X
	RM234	X	H	H	X	H	X	H	X	X	X
	RM142	X	H	H	X	X	X	X	X	X	X
	RM1306	X	X	K	H	X	X	X	K	H	X
8	RM6369	H	X	H	K	K	X	H	X	X	K
	RM1376	H	K	H	H	X	X	H	X	X	K
	RM6429	H	K	H	H	X	H	H	X	X	K
	RM6215	H	K	H	H	X	H	H	H	X	K
	RM223	H	K	K	H	X	H	H	H	X	X
	RM7556	X	K	K	K	X	H	H	H	X	X
	E4443	X	K	K	K	H	H	H	H	X	X
	RM3120	X	K	K	K	H	H	K	H	X	X
9	RM219	X	H	K	K	H	H	H	H	H	X
	RM7038	X	H	X	K	H	H	X	X	K	X
	RM6771	X	H	X	K	H	H	X	X	K	X
	RM7424	X	H	X	H	H	H	X	X	K	X
	E61552	X	H	X	H	X	H	X	X	K	H
	RM257	X	H	X	H	X	H	X	X	K	H
	RM6971	H	K	H	X	X	K	X	X	K	H
	E21191	H	K	H	X	X	K	X	X	K	H
10	RM216	H	H	K	X	X	K	H	X	X	H
	RM1375	H	H	K	X	H	K	K	X	X	H
	RM258	H	H	K	X	H	H	H	X	X	H
	RM1108	H	H	K	X	H	H	H	X	X	H
	RM5352	H	K	K	H	H	H	H	X	H	H
	RM228	H	K	K	H	X	H	H	H	H	H
11	RM4B	X	H	H	X	H	H	H	H	H	H
	RM5599	X	H	X	X	H	K	H	H	K	X
	S21074	H	K	X	X	X	K	H	H	K	X
	RM5731	H	K	X	X	X	K	H	H	K	X
	RM206	K	K	X	H	H	K	H	H	K	X
	RM6440	K	K	X	H	H	K	H	H	X	X
	RM224	K	K	X	H	H	K	H	X	X	X
12	RM8214	K	X	H	K	X	X	H	K	K	K
	RM6296	K	X	H	K	X	X	H	H	X	H
	RM7102	K	H	X	K	X	H	H	H	X	H
	RM1986	K	H	X	K	H	H	X	H	H	X
	RM1103	X	H	X	X	K	H	X	H	H	X
	L714	X	X	X	X	K	X	H	H	H	X

[a]X, H and K indicate homozygote for XM14, heterozygote and homozygote for Koshihikari, respectively.

4. DISCUSSION

Genetic analysis through chromosome genome scanning is of paramount importance in determining location of candidate genes or candidate region where a gene is located. Information on candidate genes often originates from studies or observation of traits of particular organisms. In the previous chapter on mutation induction and determination of inheritance pattern of a gene conferring BB resistance in XM14 line, it was explained how inoculation and observation of browns spots helped the mutant selection. In this chapter, the rough mapping was performed using extreme recessive phenotype proposed by Zhang *et al.* (1994). 10 plants with shortest LL were selected and used for chromosome scanning. This method allowed us to determine the chromosome location of a recessive gene conferring BB resistance in XM14 line.

Compared with traditional methods in genetic analysis, this method requires identifying only individuals showing contrasting extreme phenotypes in the target population (Zou *et al.* 2016). Further linkage analysis and high resolution mapping will be needed to finally determine the locus of this gene.

5. SUMMARY

The F_2 segregating plants derived from XM14 × Koshihikari were subjected to inoculation of *Xoo* using clipping method. They produced continuous distribution of lesion length. Therefore, analysis using extreme recessive phenotype proposed by Zhang *et al*. (1994) was adopted to determine the approximate chromosomal location of the resistance gene in XM14. Ten F_2 plants with the shortest LL (0.1-4 cm) were selected, and DNA was extracted from each plant. They were genotyped for published 113 SSR markers (Ichitani *et al.* 2014, IRGSP 2005, McCouch *et al.* 2002, Panaud *et al.* 1996, Rice Genome Research Program (http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html)) covering the whole rice genome. I located the resistance gene in XM14 on chromosome 3, probably tightly linked with following DNA markers RM3400, RM6914, RM1334 and RM5684.

CHAPTER FIVE

LINKAGE ANALYSIS OF RESISTANCE GENE IN XM14 LINE

1. INTRODUCTION

Whole rice genome scanning for gene in XM14 using F_2 populations from XM14/Koshihikari crosses with the shortest lesion length (cm) revealed that XM14 resistance gene is located on chromosome 3. However, the locus of the gene conferring resistance to *Xoo* in XM14 has not been confirmed. Thus, this study was conducted to identify chromosomal location of the *Xoo* resistance gene in XM14.

2. MATERIALS AND METHODS

2.1 Bacterial races

Races used for this study were six Japanese races: race I (strain T7174), race IIA (strain T7147), race IIB (strain H9387), race III (strain T7133), race IV (strain H75373), and race V (strain H75304).

2.2 Plant materials

IAS lines are one of the sets of reciprocal chromosome segment substitution lines (CSSLs) between a Japanese Japonica cultivar 'Asominori' and IR24 (Kubo *et al.* 2002). The graphical genotypes of IAS lines are obtainable at http:// www.shigen.nig.ac.jp/rice/oryzabase/strain/recombinant/ genotype IAS. According to Kubo *et al*. (2002), there are 70 IAS lines and among them, the IAS16 line carries IR24 genetic background with Asominori chromosomal segment of chromosome 3, on which resistance gene of XM14 was thought to be located from the initial mapping. I selected IAS16 for the above reason so that I can get polymorphism between Indica and Japonica in recombinants screening. Asominori is resistant to Japanese *Xoo* races I and V while susceptible to races II, III, and IV (Kaku and Kimura 1989). Each *Xoo* race was inoculated to six plants from each line. The previous chapter indicated that population from the cross between XM14 and Koshihikari showed continuous distribution of LL, probably because of diverse genetic background attributable to the Indica–Japonica cross. To minimize the genetic 'noise', the 194 F_2 plants from the cross between XM14 and IAS16 were also subjected to linkage analysis because both lines share the IR24 genetic background. During the following season, F_3 lines from selected F_2 plants from the same cross were grown to confirm the genotypes of the resistance gene in XM14. F_2 plants from the cross between XM14 and IAS16 were planted in 2014. F_3 lines from the same cross were planted in 2015. Germinated seeds of segregating

populations and parental lines were sown in seedling boxes in a greenhouse in May in respective years. Other cultivation procedure followed Chapter 3.

2.3 Inoculation of Xoo and BB disease scoring

Inoculation of *Xoo* and scoring *Xoo* inoculation followed Chapter 3 section 2.2. Resistance of plants to *Xoo* was scored by the mean lesion length (LL) of three leaves from each plant using a ruler 18 days after *Xoo* inoculation. Scoring of LL of F_3 lines was based on visual observation: LL longer than 3 cm judged by visual observation were scored as susceptible, whereas that shorter than 3 cm was scored as resistant.

2.4 Molecular technique

DNA extraction, PCR, electrophoresis, staining and visualization of gels followed Chapter 4.

2.5 DNA markers designing

In Chapter 4, 113 published SSR markers and Insertion/deletion (Indel) markers were used. As the linkage analyses progressed, the target regions of the *Xoo* resistance gene were narrowed. No published DNA markers were present there. Therefore, I developed new PCR-based DNA markers (**Table 5.1**). I used Indel information released by Xu *et al.* (2012) or searched for Indel polymorphism (5–50 bp difference) between a Japonica cultivar 'Nipponbare' (IRGSP 2005, Kawahara *et al.* 2013) and an Indica cultivar '93-11' (Gao *et al.* 2013, Yu *et al.* 2002), and/or an Indica cultivar 'HR12'. Oryza sativa (rice) Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=OGP__4530__ 9512) was used for Indel information. BLAST searching optimized for highly similar sequences was performed using a one thousand to ten thousand base Nipponbare sequence (Os-Nipponbare-Reference-IRGSP-1.0) as the query and 93-11 sequence (GCA_000004655.1) or HR12 sequence (GCA_000725085) as the subject. Uniqueness of the DNA sequences surrounding Indel was confirmed using BLAST with BLASTScope in Oryzabase (Yamazaki *et al.* 2010, http://www.shigen.nig.ac.jp/rice/oryzabaseV4/blast/search). Primers surrounding Indels were designed using Primer 3 (Untergrasser *et al.* 2012). Linkage map involving 16 DNA markers on chromosome 3 was constructed using software (AntMap; Iwata and Ninomiya 2006). The Kosambi function was used to estimate the map distances (Kosambi 1944).

Table 5.1. Primer sequences of DNA markers designed or redesigned for linkage analysis of *xa42* gene .

Marker name	Kind of DNA marker		Primer sequence	Location on IRGSP 1.0 pseudomolecule chromosome 3 From	to	Source
KGC3_15.36	INDEL	F	ATTTCCGATGGATAGATAATTGCTCAA	15369490	15369606	This study
		R	CTCAGTTGGACAGACAGACGTA			
KGC3_15.39	INDEL	F	GCCTGCAAGAATTAACTGCAAAATC	15392101	15392223	This study
		R	TCATATTGGCAGATTAAAGCATGCA			
KGC3_15.57	INDEL	F	TCAAATAGACTGCTGAGAACCGATC	15571005	15571205	This study
		R	GACATGGTGAAGAAATAGCCTCTCC			
KGC3_15.7	INDEL	F	CAACGTCAACATCAATACGACACTA	15729038	15729207	This study
		R	TGAGAACACGATCTTCAGTAAACAG			
KGC3_15.9	INDEL	F	TCGGAGATTGCTATAATAGGGATGA	15966551	15966800	This study
		R	AATTTTACCTCATAACCTGTGCTGT			
KGC3_16.1	INDEL	F	GTTTAGATATCGCTTTCAGGCATGT	16117085	16117235	This study
		R	CGGTTTATAAGGGTAGCCGC			
KGC3_16.3	INDEL	F	ATTAGAGTATCCACCAATAAGCCCG	16323299	16323546	This study
		R	GAGGTAAGATGAGATCGTGTAGGAG			
RM15189	SSR	F	CAGTAAGTGTCTCTGGAAGCTTG	16699297	16699465	IRGSP 2005,
		R	TGCTGAGTAGGTACCTTTCTTAAAAC			redesigned in this study
KGC3_16.7	INDEL	F	TCGGAGATGTGTATTATCATTCAACT	16726679	16726765	This study
		R	GTGGGCGGTTATTCTATATATCAGT			
RM15191	SSR	F	CGTCAATCCATCTTGCCGTTAACC	16747940	16748065	IRGSP 2005,
		R	CTCAGCCCGCCTTGTCGAG			redesigned in this study
RM15206	SSR	F	GAAAGACTCAATAGTAGTACAAAGGAGAG	16965176	16965240	IRGSP 2005,
		R	TCTTCCTGCCAAATATGCAC			redesigned in this study
KGC3_17.02	INDEL	F	CGGAGAAGCTTGATCGGAGG	17022626	17022820	This study
		R	GGAGACCGTATCGACAGTAAATCAA			
KGC3_17.03	INDEL	F	GCCCACCTCCTGCACATT	17034809	17034952	This study
		R	AGTGCCACCCATGACACG			
KGC3_17.1	INDEL	F	ATCATGTCTATCGAGCGTATTTTGG	17120606	17120778	This study
		R	CAATCAGCGTGTCGATTTCTTAGTA			
KGC3_17.2	INDEL	F	GACAGCCCACACCCATATAGAC	17213199	17213308	This study
		R	GAGGATGGCGGAAGGTCG			
RM3400	SSR	F	TCTCTCTCCTCTCTCGCTCG	17266171	17266354	McCouch *et al* . 2002
		R	TAAAACCGAAGTGCTCTCGC			
RM7642	SSR	F	ACGAAATATCAGGGCACCTG	18631946	18632139	McCouch *et al* . 2002
		R	GTTGACTTTGGTCATGAGGG			
RM16	SSR	F	CGCTAGGGCAGCATCTAAAA	23127576	23127743	McCouch *et al* . 2002
		R	AACACAGCAGGTACGCGC			

3.0 RESULTS

4.1 Test for resistance to Xoo.

The results obtained from inoculation test confirmed the previous results which showed XM14 mutant line practically exhibits resistance reaction to six Japanese races used. Likewise, it was found and confirmed that IAS16 line is susceptible to all *Xoo* races used in this study (**Table 4.2).** The average lesion length of XM 14 reaction for all *Xoo* races was 0.4cm with the range from 0.1 cm to 2cm (**Fig. 5.1**). The average lesion length of the IAS16 line was 25 cm with the range from 16cm to 36cm (**Fig. 5.1**). The LL distribution from F_2 segregating population from the cross between XM14 × IAS16 line showed bimodal distribution patterns (**Fig. 5.2 and 5.3)**.

2.2 Linkage analysis

From the above result and subsequent linkage analysis in this chapter, it seems readily obvious that XM14 line carries a novel *Xoo*-resistance gene. Recent *Xoo* studies showed that the latest identified genes conferring resistance to *Xoo* were *Xa40* (t) (Kim *et al.* 2015) and *xa41* (t) (Hutin *et al.* 2015). Therefore, the gene identified in XM14 was named *XANTHOMONAS ORYZAE* PV. *ORYZAE RESISTANCE 42* (*XA42*), according to the gene nomenclature system for rice (McCouch and CGSNL 2008). *Xa42* is a susceptible wild type allele whereas *xa42* is a resistant mutated allele.

Among the eight DNA markers on chromosome 3 used for developing IAS lines, DDBJ accession names of the partial sequence of the six markers, C515, C563, R3156, C1677, R19 and X249, could be obtained in http://rgp.dna.affrc.go.jp/E/publicdata/geneticmap2000/chr03.html. C1677 and R19 are located near the above eleven SSR markers. Among IAS lines, only IAS16 carries Asominori chromosomal segment covering C1677 and R19.

The F_2 population from the cross between IAS16 and XM14 using *Xoo* race II showed clear bimodal distribution of LL. Using the LL of 3 cm as the dividing point, the 194 F_2 plants were classified into 72 resistant plants with LL ranging from 0.1 to 2.8 cm and 122 susceptible plants with LL ranging from 4.5 to 60 cm (**Fig. 5.3**). The ratio was deviated from to 1: 3, one-gene segregation (χ^2=16.084, P<0.001). However, the tight linkage between *xa42* and DNA markers confirmed one-gene segregation (see below). The reason for the deviation was discussed in the next section. Then linkage analysis of *xa42* was performed using 194 F_2 plants and polymorphic DNA markers in **Table 5.1**. KGC3_15.36 and KGC3_15.39 showed polymorphism between Asominori and IR24, but not between XM14 and IAS16.

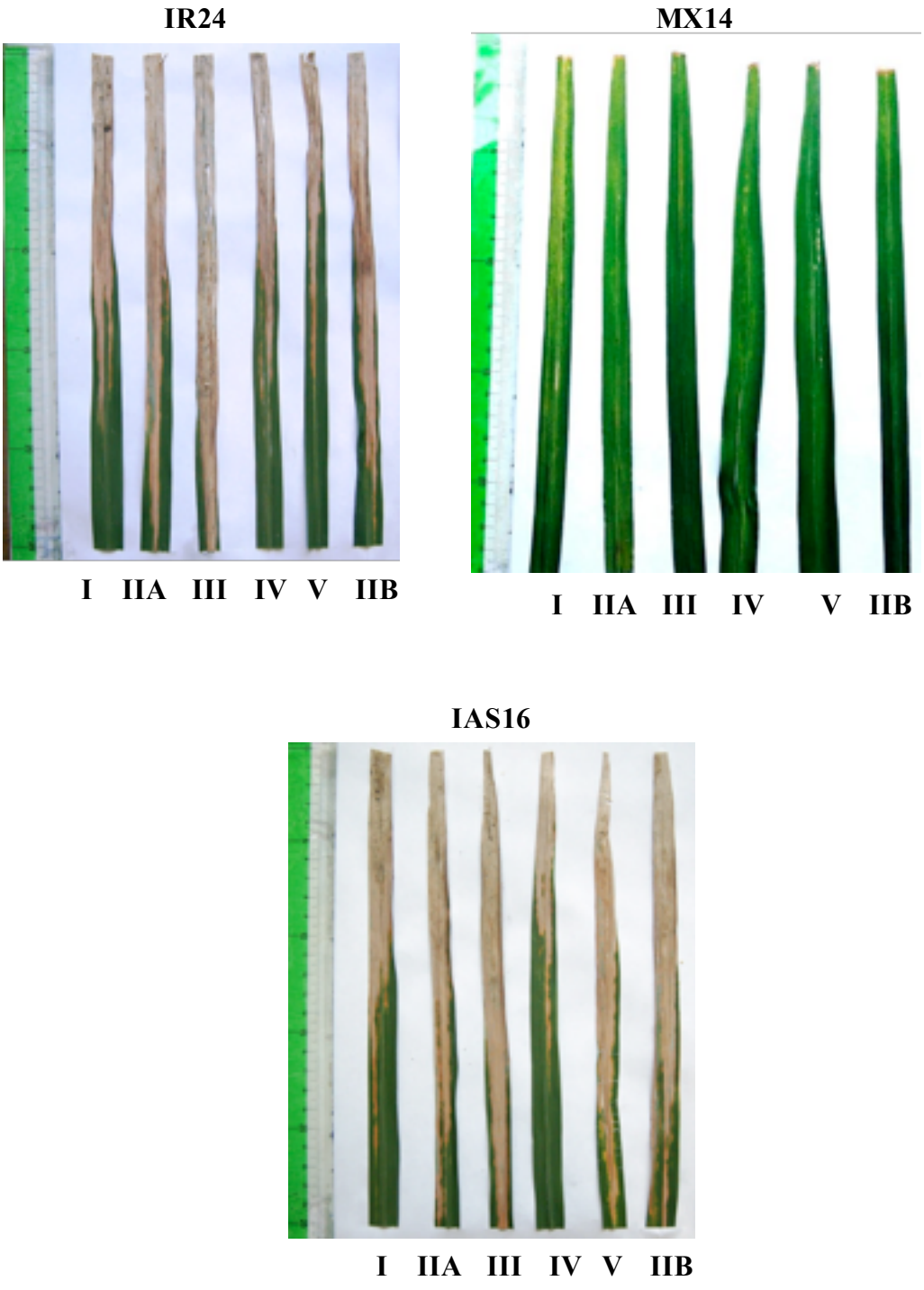

Fig. 5.1. Reaction of parental lines after field inoculation tests with six races of Japanese *Xoo.*

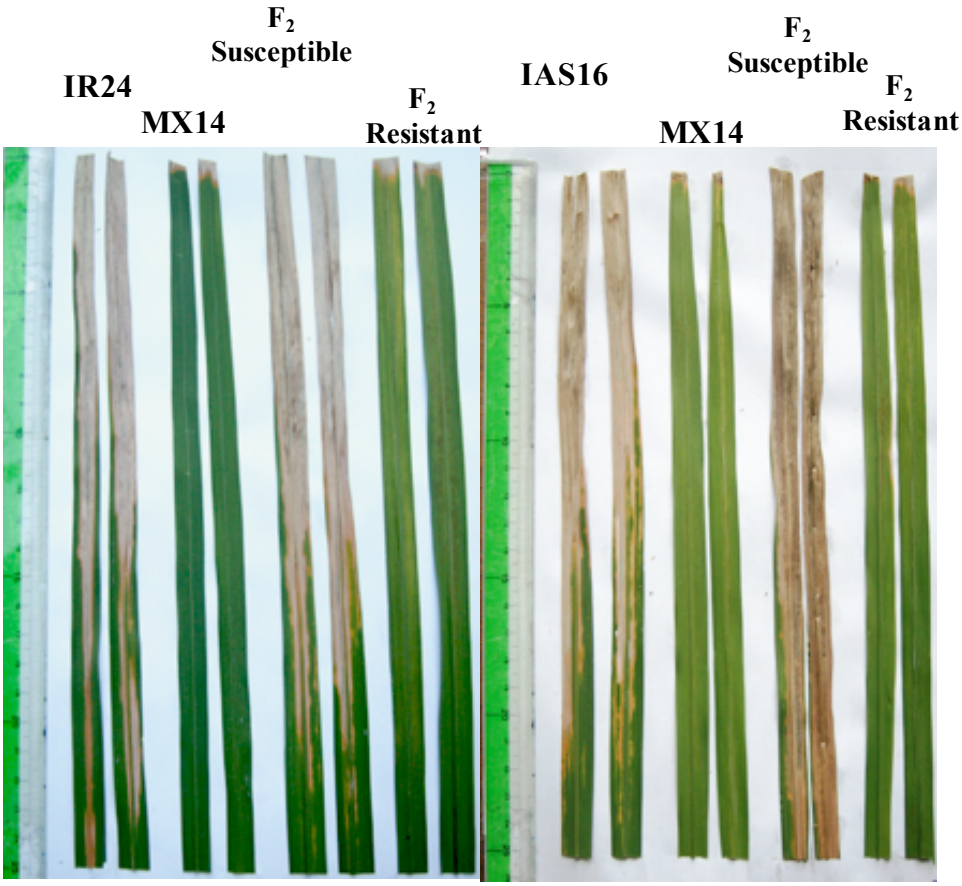

Fig. 5.2. Reaction of parents and F_2 segregating plants after field inoculation test with *Xoo* Race IIA (T7147).

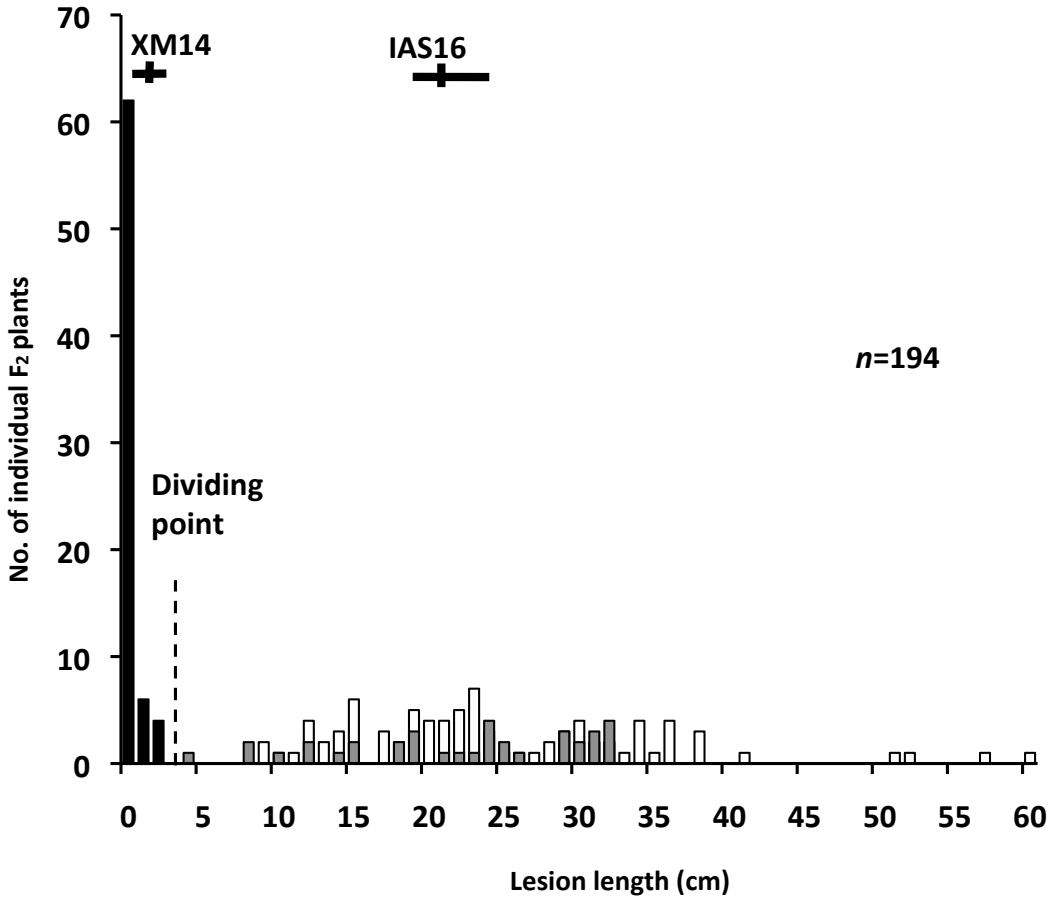

Fig. 5.3. Lesion length distribution in F_2 population from the cross between XM14 (mutant line) and IAS16 line after field inoculation test with *Xoo* Japanese race II (T7147). Three classified genotypes were assessed for RM15189 as indicated: *black*, homozygous for XM14; *white*, heterozygous; and *gray*, homozygous for IAS16. Horizontal lines at the top of figure show the ranges of parental lines. Vertical lines crossing the horizontal line are means of the parental lines.

The other markers in **Table 5.1** showed polymorphism between XM14 and IAS16, in addition to that between Asominori and IR24. These results indicated that the one end of Asominori segment on chromosome 3 in IAS16 is located between KGC3_15.39 and KGC3_15.57.

Fig. 5.3 shows a histogram of LL separated by the genotype of KGC3_16.3. Homozygotes of XM14 were highly skewed toward short LL, and heterozygotes and homozygotes of IAS16 were highly skewed toward long LL. These results showed that *xa42* is linked closely with KGC3_16.3. **Table 5.2** shows LL, genotypes of DNA markers

surrounding *XA42* locus, and results of F_3 generation of informative recombinants and non-recombinants. Plant No. 13 showed LL of 2.8 cm, which was close to the tentative dividing point. It was homozygote of XM14 allele for the all 13 DNA marker loci in **Table 5.2** as was Plant No 12 with LL of 0.1cm, and was fixed for resistant plants in F_3 generation. This result indicates that Plant No. 13 was homozygote of XM14 allele for *XA42* locus. Plant No. 14 showed LL of 4.5 cm, which was also close to the tentative dividing point. It was homozygote of IAS16 allele for the all 11 DNA marker loci in **Table 5.2** as was Plant No 20 with LL of 32.3cm, and was fixed for susceptible plants in F_3 generation. This result indicates that Plant No. 14 was homozygote of IAS16 allele for *XA42* locus.

The two recombinants Plant number 4 and 6 (**Table 5.2**) were homozygotes of XM14 allele because they showed LL shorter than 1.0cm, and were fixed for resistant plants in F_3 generation. In the two plants, recombination events occurred between KGC3_16.1 and RM15189. *XA42* should be located near the loci at which genotypes of the recombinants were homozygotes of XM14 allele.

Therefore, *XA42* is located between KGC3_16.1 and RM15189. The results of the other plants in **Table 5.2** all support this idea. Therefore, the dividing point at 3.0cm clearly classified the F_2 plants into resistant homozygous plants of *xa42* allele and susceptible plants with the other genotypes.

Based on the classification, the linkage map surrounding *XA42* is shown in **Fig. 5.4**. The linkage around *XA42* locus was compared with a restriction fragment length polymorphism (RFLP) marker-based high-density linkage map (Harushima *et al*. 1998), in which some markers have been sequenced. Based on the Nipponbare genome sequence (Os-Nipponbare-Reference-IRGSP-1.0), DNA markers located near each other on Nipponbare pseudomolecules are connected with dotted lines **(Fig. 5.4)**: *XA42* is located around the centromeric region of rice chromosome 3 between DNA markers RM15189 and KGC3_16.1, which have genetic distance of 16,117 kb and 16,699 kb, respectively. *XA42* co-segregated with a DNA marker KGC3_16.3 (16,323 kb).

Table 5.2. F_2 and F_3 tests of selected recombinants to bacterial blight (*Xoo* Japanese race IIA (T7147)) and genotype of recombinants from XM14/IAS16 cross combination using markers on chromosome 3.

F_2	Lesion		Genotypes of the DNA marker loci[a]												No. of F_3 plants	
Indi-	length	Reac-	KGC	KGC	KGC	KGC	RM	KGC	RM	RM	KGC	KGC	KGC	RM	RM	Reaction	
vidual	(cm)	tion[c]	3_15.57	3_15.73	3_16.1	_16.3	15189	3_16.7	15191	15206	3_17.02	3_17.03	3_17.1	7642	16	R	S
1	57.3	S	H	H	H	H	H	H	H	H	X	X	X	X	X	7	23
2	20.6	S	H	H	H	H	H	H	H	H	X	X	X	X	X	9	21
3	19.6	S	H	H	H	H	H	H	H	H	X	X	X	X	X	8	22
4	0.4	R	H	H	H	X	X	X	X	X	X	X	X	X	X	17	0
5	0.6	R	X	X	X	X	X	X	X	X	X	X	X	X	H	NT[c]	NT
6	0.9	R	X	X	X	X	H	H	H	H	H	H	H	H	H	30	0
7	21.2	S	A	A	A	A	H	H	H	H	H	H	H	H	H	5	11
8	24.6	S	A	A	A	A	A	A	H	H	H	H	H	H	H	0	16
9	31.5	S	H	H	H	A	A	A	A	A	A	A	A	A	A	0	11
10	30.1	S	H	H	H	H	H	H	A	A	A	A	A	A	A	5	17
11	22.0	S	H	H	H	H	H	H	H	A	A	A	A	A	A	3	17
12	0.1	R	X	X	X	X	X	X	X	X	X	X	X	X	X	NT	NT
13	2.8	R	X	X	X	X	X	X	X	X	X	X	X	X	X	15	0
14	4.5	S	A	A	A	A	A	A	A	A	A	A	A	A	A	0	15
15	8.6	S	A	A	A	A	A	A	A	A	A	A	A	A	A	NT	NT
16	9.3	S	H	H	H	H	H	H	H	H	H	H	H	H	H	NT	NT
17	21.2	S	H	H	H	H	H	H	H	H	H	H	H	H	H	NT	NT
18	21.4	S	A	A	A	A	A	A	A	A	A	A	A	A	A	NT	NT
19	32.1	S	A	A	A	A	A	A	A	A	A	A	A	A	A	NT	NT
20	32.3	S	A	A	A	A	A	A	A	A	A	A	A	A	A	NT	NT

[a]X, H and A denote homozygote for XM14, heterozygote and homozygote for IAS16, respectively.

[b]R and S denote resistant and susceptible, respectively.

[c]Not tested.

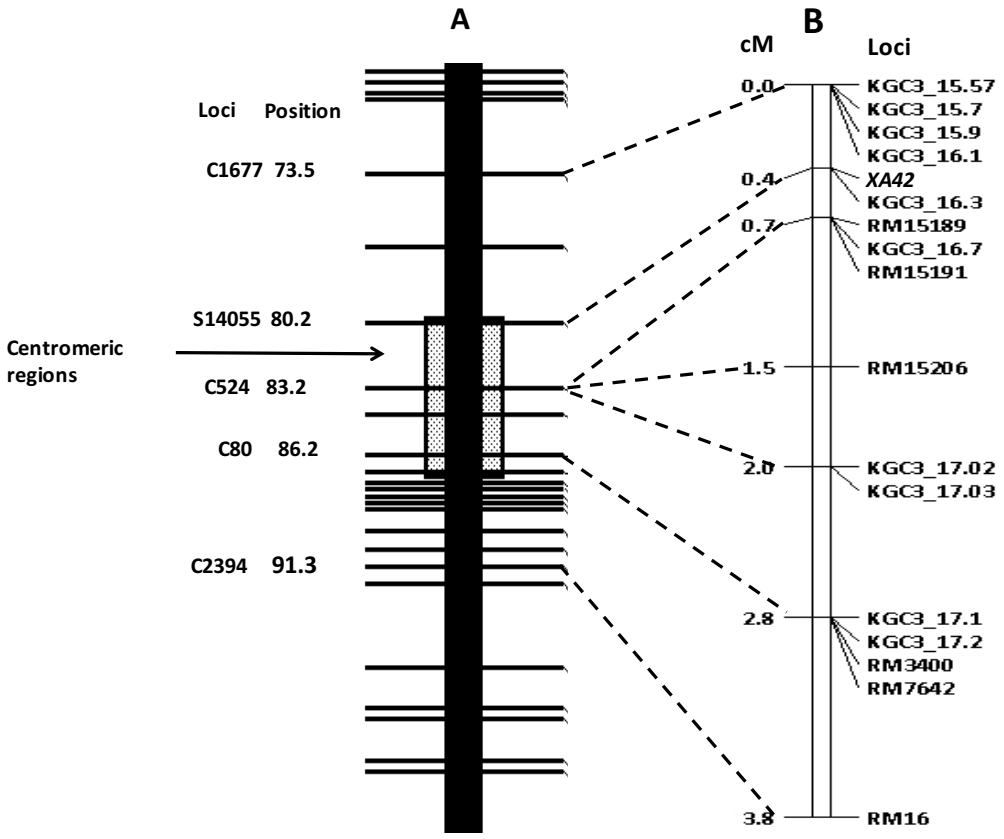

Fig. 5.4. Linkage map showing the location of *XA42* gene on chromosome 3 A, RFLP framework map of chromosome 3 modified from Harushima *et al.* (1998); B, Linkage map of *XA42* gene constructed from F_2 population from XM14 and IAS16 (*n*=194). DNA markers that are located near each other on Nipponbare pseudomolecules are connected with dotted lines.

4.0 DISCUSSION

Linkage analysis showed that this gene was located on centromeric region of chromosome 3. About 40 resistance genes conferring host resistances to *Xoo* have been identified (Xia *et al.* 2012, Khan *et al.* 2014, Kim *et al.* 2015). Among all resistance genes, only *Xa11* has been reported to be located on chromosome 3, not on centromeric region but on the long arm (Goto *et al.* 2009). Located on the centromeric region of chromosome 3, a new gene name *xa42* was assigned to this resistant gene in XM14, according to the gene nomenclature system for rice (McCouch and CGSNL 2008). Many resistance genes 'breakdown' when they have been widely used for numerous years in a large population. Exploitation of new resistance genes is urgent. The new resistant gene *xa42* in this study would be useful in resistance breeding programs and genetic analysis of *Xoo* resistance.

It remains unknown that this gene is resistant to *Xoo* races other than Japanese race II (T7147), because it is difficult to inoculate plants in segregating populations with many races. Because the probability of identifying *Xoo* resistant mutant is small (Taura *et al.* 1991a), simultaneous plural resistance mutations on one M_2 line seem improbable. Therefore, it is plausible that *xa42* conferred resistance to the all races tested so far. The fact that many of resistant genes reported so far has showed resistance to plural races supports this idea. Using the progeny of resistant plants derived from the F_2 population from the cross between XM14 and IR24, I will clarify whether the multi-resistance of XM14 is conditioned by *xa42* gene only or combination of plural genes.

XA42 segregation was deviated from the expected ratio 1:3 in the cross XM14 and IAS16: *xa42* allele located on Indica XM14 chromosome was transmitted more than *Xa42* allele on Japonica Asominori chromosome. This pattern is same as that reported by Fukuta *et al.* (2000): segregation was skewed in favour of Indica over Japonica alleles in chromosome 3 around the centromeric region. Such segregation distortions might have been caused by reproductive barrier such as gametophyte genes.

Centromeric region is recombinationally suppressed (for a review, Hall *et al.* 2004). Frequently cited rice molecular linkage map by Harushima *et al.* (1998) showed the location of centromeric region on chromosome 3 is encompassed by two RFLP markers, S14055 and R606A, linked to each other with a genetic distance of 6.8 cM. *xa42* gene is located on the centromeric region of chromosome 3 (**Fig. 5.4**). The ratio of the physical distance/ the genetic distance between the closest encompassing DNA markers of *XA42*, KGC3_16.1 and RM15189, is 582kb/0.7cM=831, whereas that of the whole rice genome is 38,900kb (IRGSP 2005) /152,16 cM (Harushima *et al.* 1998)=256. At the centromeric sub-region apart from *XA42* by 3.0 cM, physical and genetic distance between KGC3_17.1 and RM7642 is 1,511kb and 0 cM, respectively, suggesting that recombination is highly suppressed in the sub-region. Since *XA42* is apart from that sub-region and recombination was not suppressed so much, I could narrow the chromosomal region of *XA42* by map-based strategy.

Of all 41 identified BB resistance genes, only nine genes have been isolated and characterized (Kim *at al*. 2015). Among them, *xa5*, *xa13* and *xa25* are recessive resistance genes. *xa5* gene encodes small subunit of transcription factor IIA (TFIIAγ) (Iyer and McCouch, 2004). *xa13* and *xa25* genes belong to the MtN3/saliva gene family (Yang *et al.* 2006, Liu *et al.* 2011). Using the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/), I searched for candidate genes of *xa42* in the chromosomal region encompassed by KGC3_16.1 and RM15189. We found 15 genes coding known

proteins (**Table 5.3**) and 13 predicted genes coding hypothetical proteins or those for non-protein coding transcript. No genes in **Table 5.3** encode transcription factor or similar proteins or belong to the MtN3/saliva gene family. Spectra of isolated recessive resistance genes to *Xoo* race are also different: The homozygote of *xa5* is resistant to Japanese races IA, IB, II, IIIA, IIIB and IV, Philippine races 1-5, susceptible to Philippine race 6 (Ogawa *et al.* 1991). The homozygotes of *xa13* is susceptible to Philippine races 1-5 (Singh *et al.* 2001), and resistant to Philippine race 6 (Chu *et al.* 2006), which is virulent to most resistant genes (Ogawa *et al.* 1991). The homozygote of *xa25* is susceptible to Philippine races 1-8, Japanese races II and IIIA, resistant to a Philippine race 9 (PXO339) (Chen *et al.* 2002).

These facts suggest that the cloning of *xa42* gene could lead to a new resistance mechanism against *Xoo*. Once this gene is cloned, its recessive inheritance could be converted into a dominant trait by knocking down the corresponding dominant allele in transgenic rice using artificial microRNA technology (Li *et al.* 2012), which could contribute to hybrid rice breeding program.

Xoo symptoms could be affected by environmental condition and rice developmental stage (Mew 1987). The genetic background of IR24 in XM14 has a large advantage studying *Xoo* resistance in rice: Many kinds of experimental lines for *Xoo* resistance have been constructed, near-isogenic lines carrying single *Xoo* resistance gene (Ogawa *et al.* 1991), pyramid lines carrying multiple resistance genes (Yoshimura *et al.* 1996, Huang *et al.* 1997), artificially induced mutant lines (Taura *et al.* 1991a). Therefore, the effect of newly identified genes such as *xa42* on resistance to *Xoo* could be compared with other previously-published genes easily. The effect of pyramiding of *xa42* with other genes could be evaluated easily, too. Iyer-Pascuzzi and McCouch (2007) reviewed the genetic and molecular resistance mechanism of *xa5* and *xa13* with special reference to their recessive inheritance. As for pyramiding, when used in combination with other resistance genes, both *xa5* and *xa13* provide stronger and broader levels of resistance than when used alone. The pyramiding of *xa42* with other resistance genes is worth consideration.

In this study, the combination of rough linkage analysis using extreme recessive phenotype using Indica-Japonica cross and precise linkage analysis using CSSLs effectively mapped recessive mutant resistance gene induced in IR24. The same mapping strategy could be applied to other previously identified *Xoo* resistant mutants with IR24 background such as XM5 (Taura *et al.* 1991b) and XM6 (Taura *et al.* 1992a), which will also contribute to the study of *Xoo* resistance in rice.

Selection of *Xoo* resistant recessive mutant plants in paddy field contains transplanting large number of M_2 generation seedlings, *Xoo* inoculation, LL measuring, which are very time-consuming. Marker assisted transfer of this gene to other genetic background needs several times of backcrossing. If this gene is cloned and loss of function mutation is the mechanism of resistance, application of TILLING to the selection of resistant gene carriers in seedling stage of M_2 generation induced by chemicals such as MNU and EMS (Suzuki *et al.* 2008, Till *et al.* 2007) will enhance the development of the resistant gene carrier under diverse genetic backgrounds.

Table 5.3 Annotation data by rice annotation project-database(RAP-DB) of putative ORFs presumably coding known proteins in the candidate chromosomal region of *XA42* (Chromosome 3 Spanning from 16301696bp to16700996 bp)

ORFin RAP-DB	ɔcation on IRGSP 1.0 pseudomolecule of chromosome 3	Description in RAP-DB
Os03g0401333	16301696-16306081 (+ strand)	Hypothetical protein. (Os03t0401333-00)
Os03g0401951	16358834-16359470 (+ strand)	Hypothetical gene. (Os03t0401951-01)
Os03g0402000	16359486-16362281 (- strand)	TRAPP I complex, Bet3 domain containing protein
Os03g0402400	16384695-16386794 (- strand)	Similar to Ribosome-associated protein p40-like
Os03g0402800	16407695-16410007 (- strand)	Tify domain containing protein ;Similar to ZIM motif family protein, expressed.
Os03g0403100	16421056-16421056(+ strand)	Similar to DNA-directed RNA polymerase subunit
Os03g0403400	16449407-16451763 (+ strand)	Six-bladed beta-propeller, TolB-like domain containing protein
Os03g0403600	16457908-16459449 (+ strand)	von Willebrand factor, type A domain containing protein
Os03g0404800	16551354-16561430(+ strand)	NAD(P)-binding domain containing protein
Os03g0405000	16571992-16577673(+ strand)	NAD(P)-binding domain containing protein
Os03g0405100	16580159-16586153 (+ strand)	Ubiquinone biosynthesis protein COQ9 domain containing protein
Os03g0405500	16590192-16595911 (+ strand)	Similar to PDI-like protein
Os03g0406100	16645952-16646434 (+ strand)	Similar to ids4-like protein
Os03g0406200	16654335-16658094 (+ strand)	Elongation factor 1 beta
Os03g0407000	16696295-16700996 (- strand)	von Willebrand factor, type A domain containing protein. (Os03t0407000-01)

Source (*rapdb.dna.affrc.go.jp/)*

5.0 SUMMARY

IAS16 line carries IR24 genetic background with a Japonica cultivar Asominori segment of chromosome 3, on which the resistance gene locus was thought to be located. The F_2 population from IAS16 ×XM14 showed a discrete distribution of lesion length when inoculated with Japanese *Xoo* race IIa. The linkage analysis indicated that the gene is located on the centromeric region. The resistance gene in XM14 was a new gene, and named *XA42*. This gene would be useful in resistance breeding programs and genetic analysis of *Xoo* resistance.

CHAPTER SIX

FINE MAPPING OF *XA42* GENE

1.0 INTRODUCTION

Chapter 5 described the identification of a new recessive R gene from the 'XM14' line. This gene was named and registered as *xa42* at the *XA42* locus according to the gene nomenclature system for rice (McCouch and CGSNL 2008). Linkage analysis showed that *XA42* is located around centromeric regions of chromosome 3 between DNA markers KGC3_16.1 and RM15189. To identify this gene at molecular level, one must narrow down the candidate chromosomal region. Thus, the aim of this study was to fine map the *XA42* gene.

2.0 MATERIAL AND METHODS

2.1 Bacterial Races, Inoculation and Scoring

The present study used Japanese race IIA (strain T7147) only. Inoculation of *Xoo* and scoring *Xoo* inoculation followed Chapter 3 section 2.2. BB severity was scored by measuring the lesion length (LL) of the inoculated leaves of rice plants with measurement using a ruler 18 days after *Xoo* inoculation. The LL of one representative leaf from each plant was measured after careful visual observation.

2.2 Plant materials

Progeny of the cross from the cross between XM14 and IAS16 was used for this study. All plant materials were grown and analyzed following Chapter 3, with some exceptional cases described in the next subsections.

2.3 Fine mapping of XA42

In 2015, about 10,000 F_2 germinated seed were sown. 982 F_2 plants were transplanted to a paddy field. The remaining 9000 F_2 plants were kept in nursery trays just for screening for recombinants between DNA markers encompassing *XA42* gene. The potentially useful recombinants were transferred to pots and later on the seeds were harvested for further F_3 tests of *xa42* gene. In 2016, a total of 2950 F_2 segregating plants and 30 plants from each parental line were subjected to fine mapping in the same manner as 2015. In addition to F_2 plants, 24 F_3 lines (30 plants per line with some exceptional cases) from recombinants obtained in 2015 were subjected to the progeny test for the genotype of *XA42* locus.

2.4 Molecular techniques

DNA extraction, PCR, electrophoresis, staining and visualization of gels followed chapter 4.

2.5 DNA Markers/Restriction enzyme

In this chapter, I used 19 DNA markers (**Table 6.1**), 3 of which have already been described by Chapter 5. The other 16 markers, that is, 1 SSR, 2 dCAPS, 2 CAPS markers and 11 InDel, were designed according to procedure elaborated below. In Chapter 5, *XA42* gene was located between DNA markers KGC3_16.1 and RM15189. Apparently, a single sequence repeat (SSR) located between them was available. I checked for uniqueness of the DNA sequences surrounding the SSR using BLAST with BLASTScope in Oryzabase (Yamazaki *et al.* 2010). Primer pairs were designed automatically using software (Primer 3 ver. 4; http://bioinfo.ut.ee/primer3-0.4.0/primer3/, Untergasser *et al.* 2012). We adopted the following primer design parameters: Primers were 20–35 nucleotides long, with optimum set at 25. Primer Tm was 55–65°C with optimum set at 60°C. The maximum Tm difference was 2°C. Primer GC% contents of 20–80.

For Indel markers, we used Indel information released by Xu *et al.* (2012) or looked for Indel polymorphism (5–50 bp difference) between a Japonica cultivar 'Nipponbare' and an Indica cultivar '93-11' and/or an Indica cultivar HR12. *Oryza sativa* (rice) Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=OGP__4530__9512) was used for Indel information. Blastn search optimized for highly similar sequences was applied using one thousand to ten thousand base Nipponbare sequence (Os-Nipponbare-Reference-IRGSP-1.0) as query and 93-11 sequence (GCA_0000046551) or HR12 sequence (GCA_000725085) as subject. Uniqueness of the DNA sequences surrounding Indel was confirmed using BLAST with BLASTScope in Oryzabase (Yamazaki *et al.* 2010).

As for CAPS and dCAPS markers, I used SNP information from Huang *et al.* (2012) and the Rice SNP seeker database (Alexandrov *et al.* 2015). Using various SNP datasets available in a single interface (Mansueto *et al.* 2016), I was able to detect SNP which can distinguish Indica (IR24) and Japonica (Asominori). I confirmed the SNP information using the genome information of five rice cultivars: Japonica cultivar Nipponbare, Indica cultivar 93-11, Indica cultivar HR12, Japonica Hitomebore (GCA_000321445.1), and Japonica Koshihikari (GCA_000164945.1) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP__

4530__9512). The DNA sequences surrounding the SNP were checked for uniqueness using BLAST with BLASTScope in Oryzabase (Yamazaki *et al.* 2010). DNASIS Pro software (ver. 2.10; Hitachi Ltd., Yokohama, Japan) was used to detect restriction enzymes that can recognize SNP.

Table 6.1. Primer sequences and information of DNA markers designed or used in fine mapping of *xa42* gene.

	Marker name	Kind of DNA marker	Restriction Enzyme		Primer sequence	Location on IRGSP 1.0 pseudomolecule chromosome 3 From	to	Source
				F	GTTTAGATATCGCTTTCAGGCATGT	16117085	16117109	
1	KGC3_16.1[a]	INDEL		R	CGGTTTATAAGGGTAGCCGC	16117216	16117235	This study
				F	TGTTTCACATCGGGGACTTGAATTT	16180443	16180467	
2	KGC3_16.180	INDEL		R	GACAAACGGGACAAGGCTAAATTAT	16180526	16180550	This study
				F	ACATAACGTGGCACCAAAACTAG	16208945	16208967	
3	KGC3_16.209	INDEL		R	GCTCGATTTATAGGGTGCAAAATCT	16209086	16209110	This study
				F	TTCTAGGTCGATCGGTGCATTTATG	16255712	16255736	
4	KGC3_16.255	INDEL		R	CGTCTGGTAATTGTGAAACATGACT	16255856	16255880	This study
				F	TAATTGGCGCTGAGATATGTCCGAT	16278421	16278445	
5	KGC3_16.278	INDEL		R	GAATCGAACCTGGACCTTTTACTTG	16278578	16278602	This study
				F	ATTAGAGTATCCACCAATAAGCCCG	16323299	16323323	
6	KGC3_16.3	INDEL		R	GAGGTAAGATGAGATCGTGTAGGAG	16323522	16323546	This study
				F	GGTTATTTTCTTAAACCCGCTATTGG	16342875	16342900	
7	KGC3_16.342	SNP	HsP92	R	ATAAATAGTGTATAGCAGCGGGTTG	16342934	16342958	This study
				F	GATGCCATCCTTCTTCACCCTT	16370161	16370182	
8	KGC3_16.370	INDEL		R	GGTAGAAGTAGAGCCATGGAACTTG	16370289	16370313	This study
				F	GATAATGCGAAAGAGAATCAAGGGG	16371045	16371069	
9	KGC3_16.371	INDEL		R	AGAAGCACGAGTTTAATAAGGCCTA	16371279	16371303	This study
				F	ATTTTACCAATTATAGTTTTCGTCCGgAT	16399678	16399703	
10	KGC3_16.399	dCAPs	BseG I	R	GCTTTACCTGGCATATTCTAGACCT	16399803	16399827	This study
				F	CTCGTCTCCATCAAGTTTTCAGGTC	16407423	16407447	
11	KGC3_16.407	SSR		R	TAGTGTACCATGATTTGCAAGCCTT	16407536	16407560	This study
				F	CCCTCCGTACAAGTACAATACATaGC	16421768	16421790	
12	KGC3_16.421	dCAPs	AluI	R	CCCTCTCCAAGTAAATCCATGTCTT	16421914	16421938	This study
				F	AGTGCATATTCTTTCGTCCAACTTT	16422886	16422910	
13	KGC3_16.422	SNP	BssSI	R	TTAACCTTCCTTTACTATCGGTGTC	16423007	16423031	This study
				F	GCTTTATATCTGTCAACTGGATTAGAATTC	16514854	16514883	
14	KGC3_16.514	INDEL		R	AGGTGAGTATAATAAGCAAGTTGAGT	16514952	16514977	This study
				F	GGTACAATATAACAGTTCCACCAAGA	16552916	16552941	
15	KGC3_16.552	SNP	HaeIII	R	GTTTGGAATTGACTGATTAGCCACA	16553069	16553093	This study
				F	GTTTTGATAGAGCGCAATTTGTCAT	16594790	16594814	
16	KGC3_16.594	INDEL		R	ATCCCAAGCTGCCAGTATAAATTAA	16594863	16594887	This study
				F	TGCCTGTAAAAGTTCTTGATGGAAT	16613174	16613198	
17	KGC3_16.613	INDEL		R	TTTAGCTTCACAGTGTAAAAGTTAG	16613383	16613407	This study
				F	CTTATTGGTTGGCGTGGTGTTT	16636613	16636634	
18	KGC3_16.636	INDEL		R	AGAAGCACGAGTTTAATAAGGCCTA	16636819	16636845	This study
				F	CAGTAAGTGTCTCTGGAAGCTTG	16699297	16699319	IRGSP 2005, Redisigned
19	RM15189	SSR		R	TGCTGAGTAGGTACCTTTCTTAAAAC	16699440	16699465	This study

[a]KG, C3, and numerals denote Kagoshima University, chromosome 3 and nucleic bases in mega bases, respectively.

Primers surrounding Indel and SNP were designed using Primer 3 (Untergrasser *et al.* 2012) with the parameters above. When SNPs were not detected by restriction enzymes, dCAPS markers were designed using dCAPS finder software (Neff *et al.* 2002).

2.6 Mapping and Gene annotation

F_2 plant recombinants were used to determine the exact position of the nearest recombination event to the target region on chromosome 3 centromeric regions. The Fine map of the target *XA42* gene was constructed according to the physical distance of the DNA markers used in the study (**Table 6.1**). In addition to F_2 plants, the progeny test of all recombinants obtained in 2015 was applied to confirm the recombination events, segregation or non-segregation reaction against *Xoo*. Candidate genes of *XA42* were sought within the candidate chromosomal region using 'the rice annotation project database (http://rapdb.dna.affrc.go.jp/)' (Kawahara *et al.* 2013, Sakai *et al.* 2013) and 'rice genome annotation project (*rice.plantbiology.msu.edu/)'* (Kawahara *et al.* 2013).

3.0 RESULTS

3.1 Fine mapping of xa42 gene

In 2015, 982 F_2 plants from the cross between IAS16 and XM14 were evaluated for LL inoculated with *Xoo* race IIA (strain T7147). The distribution of LL showed a bimodal distribution (**Fig. 6.1**). The dividing point was set at 5 cm LL because of the clear gap of LL at 5 cm. LL shorter than 5 cm was regarded as resistant, whereas LL longer than 5 cm was regarded as susceptible to BB. Based on that dividing point, the resistant and susceptible plants were, respectively, 288 and 694. In 2016, the 2950 F_2 plants from the same cross combination were evaluated for LL inoculated with *Xoo* race IIA (strain T7147) (**Fig. 6.2**). The ratio in 2015 and 2016 both deviated from 1 resistant: 3 susceptible, one-gene segregation (2015: χ^2=9.810, P<0.01, 2016: χ^2=39.333, P<0.001). However, the tight linkage between *XA42* and DNA markers KGC3_16.370 (see below) confirmed one-gene segregation. The result in previous chapters showed that *XA42* was located between DNA markers KGC3_16.1 and RM15189. A total of 126 recombinants between the two markers were selected in 2015. Non-recombinants between them were treated as non-recombinants between all inside markers, because the genetic distance between the two markers were only 0.7 centi Morgan.

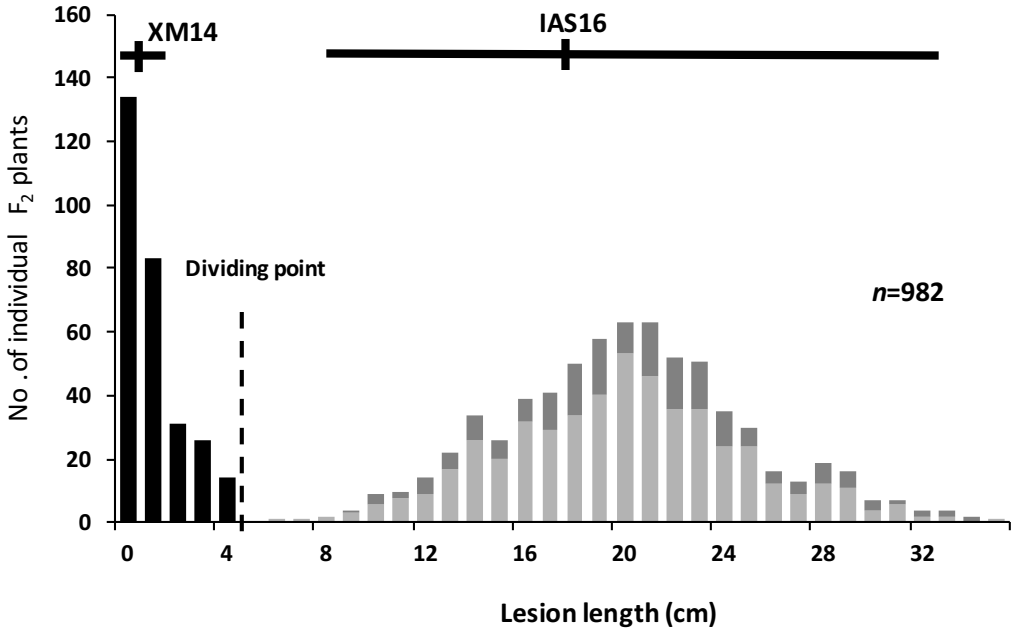

Fig.6.1. Lesion length distribution of 982 F_2 plants (planted in 2015) from the cross between XM14 and IAS16 after *Xoo* Japanese race IIA (strain T7147) inoculation. Three classified genotypes were assessed for KGC3_16.370 as indicated: *black*, homozygotes for XM14; *gray* heterozygotes; *dark gray*, *homozygotes* for IAS16. Horizontal lines at the top of the figure show the ranges of parental lines. Vertical lines crossing the horizontal line show means of the parental lines.

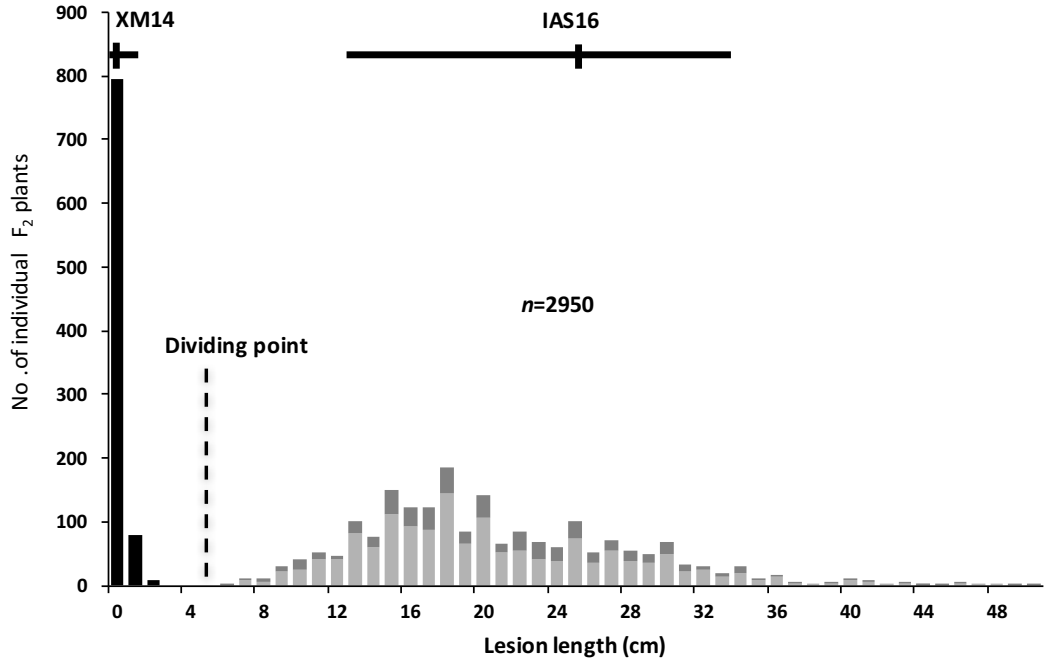

Fig. 6.2. Lesion length distribution of 2950 F_2 plants (planted in 2016) from the cross between XM14 and IAS16 line after *Xoo* Japanese race IIA (strain T7147) inoculation. Three classified genotypes were assessed for KGC3_16.370 as indicated: *black*, homozygotes for XM14; *gray*, heterozygotes; *dark gray*, *homozygotes* for IAS16. Horizontal lines at the top of figure show ranges of parental lines. Vertical lines crossing the horizontal line show means of the parental lines.

To draw a fine map of *XA42* gene, 19 new polymorphic DNA markers were developed (**Table 6.1, Fig. 6.3**). Reaction to *Xoo* and DNA marker genotypes of Plants 7–17 in Table 6.2 all suggested that *XA42* was located between KGC3 16.1 and KGC3_16.594. Therefore, in 2016, the DNA marker combination of KGC3_16.1 and KGC3_16.594 was used for recombinant screening. In all, 29 recombinants between them were selected. Then they were subjected to genotyping of internal DNA markers (**Table 6.1**). In addition to F_2 tests, we performed F_3 test of 55 recombinants. The results of genotyping of informative F_2 plants and their F_3 tests facilitated detailed mapping of the *XA42* gene (**Table 6.2**).

Genetic information from each informative recombinant was mutually consistent (**Table 6.2**). The discontinuous LL distribution like **Fig. 3.5** was observed in F_3 lines in **Table 6.2** in 2016: LL shorter than 3.0 cm were scored as resistant (R), whereas those longer than 5.0 cm were scored as susceptible (S). Important recombination events occurred between DNA markers KGC3_16.3 and KGC3_16.407. Plant Nos. 12 and 13 both show that the *xa42* gene is located on the left side of DNA marker KGC3_16.399 because the F_3 lines of the two plants were fixed for susceptible plants with no brown spots. Plant No. 17 showed that the gene was located on the right side of DNA marker KGC3_16.342 because the F_3 line of the plant is composed of 5 resistant plants with brown spots and 24 susceptible plants with no brown spots. The result for Plant No. 22 was consistent with that. Therefore, *XA42* gene was located in the 57 kb-chromosomal region between DNA markers KGC3_16.342 and KGC3_16.399. Results for other plants in **Table 6.2** all support this idea. A linkage map comparing results obtained from our previous chapters and this chapter is portrayed in **Fig. 6.4.** The search for candidate gene using RAP-DB (http://rapdb.dna.affrc.go.jp/) found six ORFs in the candidate region of *XA42* (**Table 6.3**). The rice genome annotation project (rice.plantbiology.msu.edu/) found eight ORFs, four of which might correspond to those found in RAP-DB, and two of which encoded retrotransposon proteins. The other two are LOC_Os03g28430 described as encoding hypothetical protein and LOC_Os03g28420 described as 3-oxoacyl-synthase. Its first exon corresponds to Os03g402200.

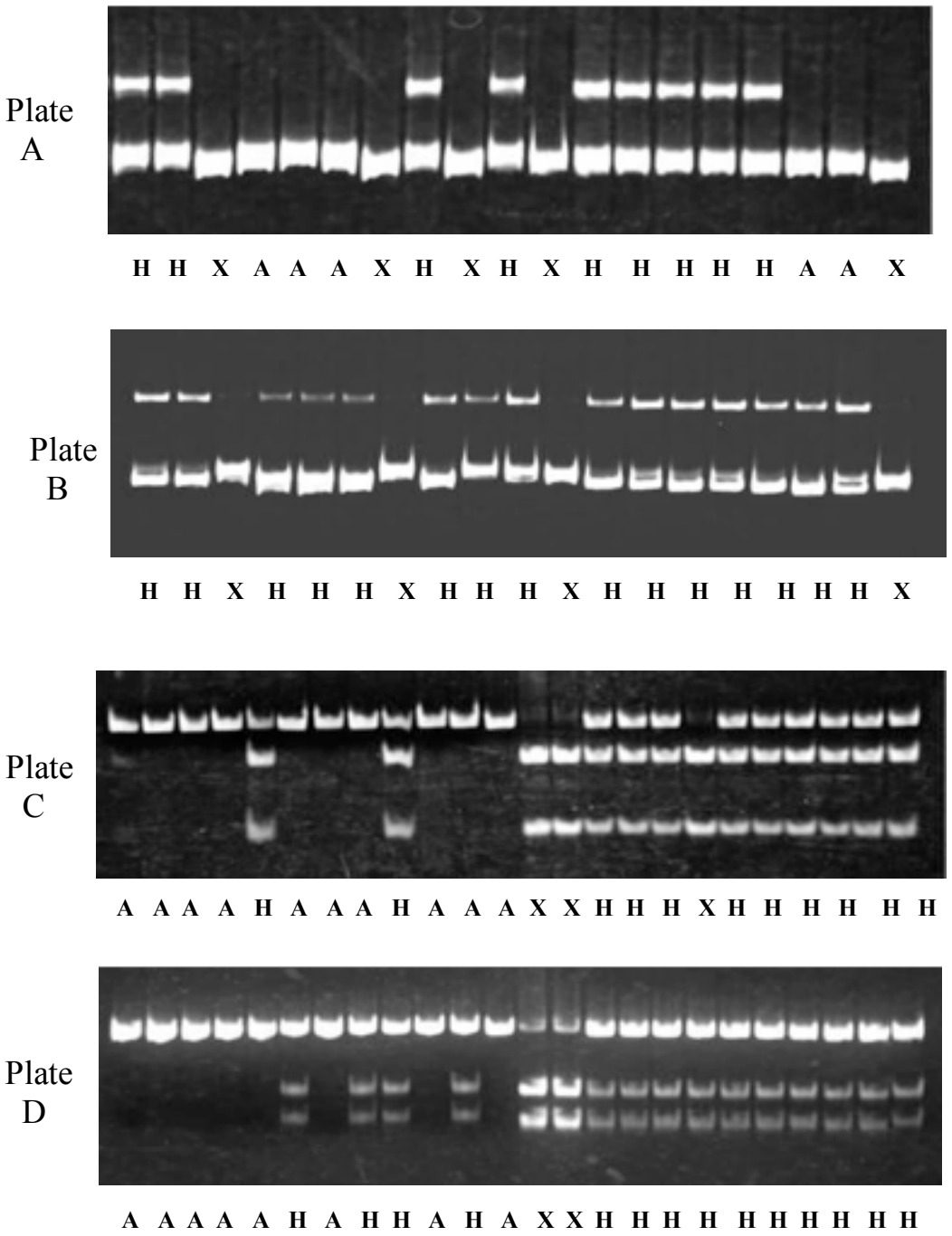

Fig. 6.3. Plates showing recombinant screening using indel and CAPs markers. Plate A and B show PCR results after running 10% PAGE, Plate A and B are KGC3_16.27 and KGC3_16.3 indel markers, respectively. Plate C and D show restriction enzyme digestion after PCR and 10% PAGE running. Plate C and D are KGC3_16.365 and KGC3_16.368 CAPS markers, respectively. X, H and A denote homozygote for XM14, heterozygote and homozygote for IAS16, respectively.

Table 6.2. Genotypes of informative recombinants for the DNA marker loci linked with *XA42* in the F_2 population (XM14 ×IAS16), brown spots, and reaction against X*oo* Japanese race II (T7147) inoculation in the F_2 and F_3 generations.

F_2						Genotypes of the DNA marker loci[a]																	No.of F_3 plants
Indi-			Lesion	React-	Brown	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	KGC3	RM	Reaction
vidual[b]	Year	place	Length	tion[c]	spots[d]	16.1	16.180	16.209	16.255	16.278	16.3	16.341	16.371	16.37	16.399	16.407	16.421	16.514	16.594	16.613	16.636	15189	R : S[e]
1	2015	Field	18.8	S	N	A	A	A	A	A	H	H	H	H	H	H	H	H	H	H	H	H	7: 22
2	2015	Field	14.6	S	N	A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	0: 30
3	2015	Field	19.4	S	N	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A	A	A	0: 30
4	2015	Field	31.5	S	N	H	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A	A	0: 30
5	2015	Field	18.3	S	N	H	H	H	H	H	H	A	A	A	A	A	A	A	A	A	A	A	0: 30
6	2015	Field	21.6	S	N	H	H	H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	6: 24
7	2015	Field	0.1	R	B	H	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	30: 0
8	2015	Field	2.8	R	B	H	H	H	H	X	X	X	X	X	X	X	X	X	X	X	X	X	30: 0
9	2015	Field	12.7	S	N	H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	31: 128
10	2015	Field	14.5	S	N	X	X	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	31: 109
11	2015	Field	22.1	S	N	H	H	H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	42: 117
12	2015	Field	1.1	R	B	X	X	X	X	X	X	X	X	X	H	H	H	H	H	H	H	H	50: 0
13	2015	Field	0.3	R	B	X	X	X	X	X	X	X	X	X	H	H	H	H	H	H	H	H	30: 0
14	2015	Field	23.3	S	N	X	X	X	H	H	H	H	H	H	H	H	H	H	H	H	H	H	43:110
15	2015	Field	18.5	S	N	H	H	H	H	H	H	H	H	H	H	H	H	H	X	X	X	X	42: 117
16	2015	Field	23.4	S	N	X	X	X	X	X	H	H	H	H	H	H	H	H	H	H	H	H	34: 116
17	2015	Field	18.2	S	N	X	X	X	X	X	X	X	H	H	H	H	H	H	H	H	H	H	5: 24
18	2016	Field	27.3	S	N	H	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	0: 30
19	2016	Field	18.9	S	N	H	H	H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	10: 20
20	2016	Field	27.4	S	N	H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	A	A	6: 24
21	2016	Field	20	S	N	H	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	0: 30
22	2016	Field	44.5	S	N	H	H	H	H	H	H	H	H	A	A	A	A	A	A	A	A	A	7: 23
23	2016	Field	6.5	S	N	H	H	H	H	H	H	H	H	H	H	H	A	A	A	A	A	A	2: 28
24	2016	Field	0.1	R	B	H	H	H	H	H	H	X	X	X	X	X	X	X	X	X	X	X	57: 0
25	2016	Field	0.1	R	B	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	X	X	60: 0
26	2016	Field	22.8	S	N	H	H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	6: 24
27	2016	Field	0.1	R	B	X	X	X	X	X	X	X	X	X	H	H	H	H	H	H	H	H	30: 0
28	2016	Field	0.2	R	B	X	X	X	X	X	X	X	X	X	X	X	H	H	H	H	H	H	28: 0
29	2015	pot[b]				A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	0:28
30	2015	pot[b]				A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	0:22
31	2015	pot[b]				A	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	0:30
32	2015	pot[b]				H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	4:14
33	2015	pot[b]				H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	2:27
34	2015	pot[b]				H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	4:15
35	2015	pot[b]				H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	6:24

[a] X, H, and A respectively denote homozygotes for XM14, heterozygotes, and homozygotes for IAS16.

[b] F_2 plants from plant number 29, 30,31,32,33,34 and 35 were sown and pots and its progeny (F_3) were subjected to test for resistance reaction against X*oo* Japanese race II in the field.

[c] R and S respectively denote resistance and susceptible.

[d] B and N respectively denote plants showing brown spots on their leaves and those showing normal leaves.

[e] LL shorter than 3.0 cm were scored as R, whereas those longer than 5.0 cm were scored as S.

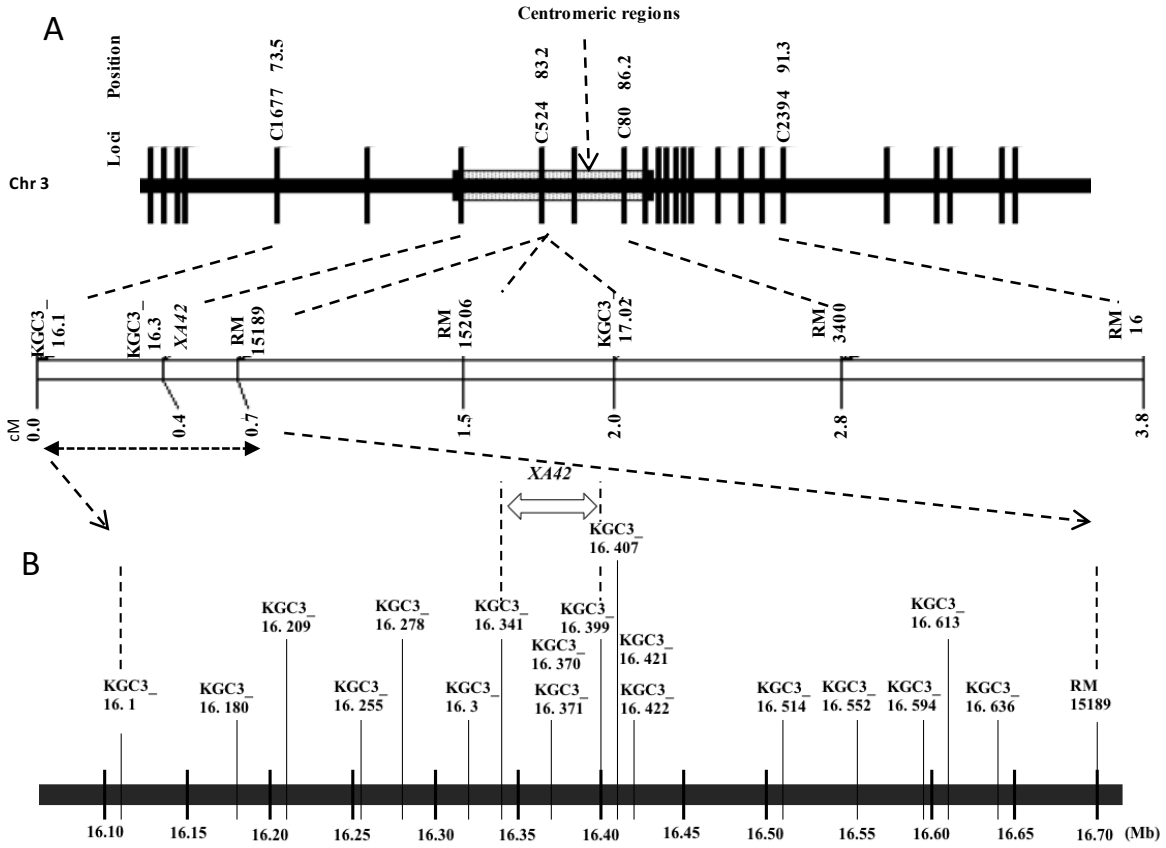

Fig. 6.4. Fine mapping of *XA42*. A, comparative linkage map of *XA42*. Modified from Fig. 5.3 in Chapter 5. B, Candidate chromosomal region of *XA42* on Os-Nipponbare-Reference-IRGSP-1.0.

Table 6.3. Comparative annotation data by RGAP and RAP-DB of putative ORFs presumably coding known proteins in the fine mapped candidate chromosomal region of *XA42.*

Predicted gene locus in RGAP	Location (from to)	orientation	Description	Prediced protein length (amino acid)	Predicted gene locus in RAP-DB	Location (from to)	orientation	Description	Prediced protein length (amino acid)
Loc_Os03g28370	16346430 16351718	+	retrotransposon protein, putative, unclassified, expressed	1275	None				
Loc_Os03g28380	16355479 16353107	-	retrotransposon protein, putative, unclassified, expressed	705	None				
Loc_Os03g28389	16358668 16359485	+	expressed protein	171	Os03g0401951	16358834 16359470	+	Hypothetical gene	169
					Os03g0401900	16358768 16359256	-	irgsp1_predicted_locus	163
Loc_Os03g28400	16362293 16358668	-	transport protein particle component, Bet3, domain containing protein, expressed	188	Os03g0402000	16362182 16362281	-	TRAPP I complex, Bet3 domain containing protein.	188
Loc_Os03g28430	16369610 16370585	+	hypothetical protein	111	None				
Loc_Os03g28420	16379063 16372118	-	3-oxoacyl-synthase, putative,	499	Os03g0402200	16378724 16379079	-	Hypothetical protein	90
Loc_Os03g28410	16386431 16383886	-	ribosomal protein S2, putative, expressed	274	Os03g0402400	16384695 16386794	-	Similar to Ribosome-associated protein p40-like	187
Loc_Os03g28910	16398112 16397246	-	expressed protein	289	Os03g0402600	16397246 16397680	-	irgsp1_predicted_locus	145

Source (*rapdb.dna.affrc.go.jp/ rice.plantbiology.msu.edu/)*

4. DISCUSSION

In this chapter, I performed fine mapping of *XA42*, narrowing the area of interest to 57 kb located between the two DNA markers, KGC3_16.342 and KGC3_16.399. This gene cosegregated with two DNA markers, KGC3_16.370 and KGC3_16.371.

The *XA42* gene candidate region was narrowed down from 582 kb (between KGC3_16.1 and RM15189) to 57 kb (KGC3_16.342 and KGC3_16.399) (**Table 6.1).** The fine map depicted in this study **(Fig. 6.4**) established the foundation for *XA42* gene map-based cloning.

The analysis of ORFs coding known proteins using the rice annotation project database *(http://rapdb.dna.affrc.go.jp/*) and rice genome annotation project *rice.plantbiology.msu.edu/*of the 56kb candidate region were summarized in Table 6.3. Some interesting proteins which can be related to disease resistance were found. One of them is LOC_Os03g28420 described as encoding 3-oxoacyl-synthase. Although Acyl-carrier-protein synthase I precursor has been reported to be associated in control of bacterial disease in tomato and coffee (Alvarenga *et al*. 2010, Bent 1996, Medeiros *et al*. 2010), microarray analysis showed changes that occurred in the abundance of protein transcripts which don't show specific protein in triggering plant disease resistance.

In addition, Bet3 domain containing protein (Loc_Os03g28400) or TRAPP I complex (Os03g0402000) was found, which could be involved in transport protein particle component and trafficking in endoplasmic reticulum (ER) to Golgi apparatus (GA). TRAPP I is located at very conserve area in chromosome and several researches have associated it influencing disease resistance in animals but no reports of TRAPP I involvement in plant disease resistance (Bruggeman *et al.* 2015, Brunet and Sacher 2014, Kim and Jones 1997, McDowell and Woffenden 2003, Sacher *et al.* 2008, Vukašinovic and Žárský 2016). Genes tentatively coding ribosome-associated protein p40-like (Os03g0402400), two retrotransposon proteins (LOC_Os03g28370 and LOC_Os03g28370), expressed protein (LOC_Os03g28910) and hypothetical gene (Os03g0401951) were also found. Ribosomal proteins have played part in conferring disease resistance in plants (Kim and Jones 1997, Nagaraj *et al.* 2016). Retro transposon has also been reported to be associated with some plant disease resistance (Hayashi and Yoshida 2009). However, to date no isolated plant disease resistance gene encodes 3-oxoacyl-synthase or TRAPP I complex, Bet3 domain containing protein, or ribosome-associated protein (p40-like) (**Table 6.3**). These data suggest that *xa42* is a new kind of resistance gene.

We downloaded the chromosome 3 sequences of nine cultivars (**Table 6.4**) ('Nipponbare' (IRGSP 1.0, NC_029258.1), 'Nipponbare' (assembled by Beijing Genomics Institute, CM000140.1), 'HEG4' (CM003066.1), 'A123' (CM003078.1), 'Hitomemore' (DG000055.1), 'Koshihikari' (DG000027.1), 'RP Bio-226' (CPO12611.1), '93-11' (CM000128.1), 'IR8' (CM007598.1), 'Kasalath' (no accession name, downloaded from http://rapdb.dna.affrc.go.jp/download/irgsp1.html) and found that the DNA length surrounded by the two DNA markers KGC3_16.342 and KGC3_16.399 ranged from 57 kbp (NC_029258.1) to 143 kbp (CM003066.1). Both NC_029258.1 and CM000140.1 are Nipponbare chromosome 3 sequences, but the *XA42* candidate region length of CM000140.1 is 132 kbp. My preliminary analysis of these genome alignments suggests that most of the sequences not covered by IRGSP 1.0 (NC_029258.1) are repetitive sequences scattered in rice genome (data not shown). However, some of these sequences could be unique, and contain genes not found in IRGSP 1.0 (data not shown). These data suggest that there might have been other candidate genes for *XA42* than listed in Table 6.3 To identify *XA42* gene, DNA sequencing of the candidate *XA42* region of both XM14 and IR24 is necessary.

Results of this study might be important both for elucidating the mechanisms of BB resistance genes in triggering resistance response, and for advancing the genetic engineering of BB resistance genes for broad and durable resistance. To support these goals, however, expanding the mapping populations to close the gap from KGC3_16.342 and KGC3_16.399 is necessary.

5.0 SUMMARY

This chapter describes fine mapping of *xa42* at *XA42* locus, a new rice BB resistance (R) gene in XM14, a mutant line originating from IR24. The candidate gene region was narrowed down from 582 kb, which had been obtained in our previous study, to 57 kb.

A total of 9 genes were predicted in the final mapped interval, according to the rice annotation project database (*http://rapdb.dna.affrc.go.jp/*) and rice genome annotation project *(http://rice.plantbiology.msu.edu/index.shtml*).

Findings in this study provide a solid base for map-based cloning of the *xa42* resistance gene underlying in rice. The high-density markers developed around *XA42* will be useful in MAS for BB resistant breeding.

Table 6.4 Comparable rice genomes showing base composition and length in which *XA42* gene candidate region is located.

Rice accession	Varietal group	Assembly	Chr 3 accession	Base composition in the *XA42* candidate region					
				A	C	G	T	Unidentified	Sum
IRGSP 1.0 Nipponbare	Japonica	GCA_001433935.1	NC_029258.1	15737	12498	11873	14845	2000	56953
Nipponbare genome assembled by Beijing Genomics Institute	Japonica	GCA_000149285.1	CM000140.1	35298	25332	25361	35156	11350	132497
HEG4	Japonica	GCA_000817615	CM003066.1	37715	28030	28452	36982	11911	143090
A123	Japonica	CA_000817635.1	CM003078.1	39180	29236	29381	38090	1832	137719
Hitomebore	Japonica	GCA_000321445.1	DG000055.1	8318	7579	7105	7999	110955	141956
Koshihikari	Japonica	GCA_00016945.1	DG000027.1	6472	5568	5377	5899	118640	141956
RP Bio-226	Indica	GCA_001305255.1	CPO12611.1	33726	25176	24921	32882	6850	123555
Kasalath	Indica (aus)	no name (downloaded from http://rapdb.dna.affrc.go.jp/download/irgsp1.html)	chr03.fasta	17119	12451	11946	15634	14621	71771
9311	Indica	GCA_000004655.2	CM000128.1	39026	28645	28208	38217	701	134797
IR8	Indica	GCA_001889745.1	CM007598.1	38797	28739	28637	37921	0	134094

CHAPTER SEVEN

RESISTANCE OF *xa42* AGAINST MULTIPLE *Xoo* RACES

1. INTRODUCTION

In the previous chapters, I identified a new resistance gene from 'XM14' line. This gene was named and registered as *XA42* according to the gene nomenclature system for rice (McCouch and CGSNL 2008). The previous chapters also revealed that XM14 line showed resistance to six Japanese races, in additional to being resistant to Philippines race 5 (strain PXO 112) of BB. The purpose of this study is to determine if *xa42* in XM14 is resistant against multiple *Xoo* races.

2. MATERIALS AND METHODS

2.1 Bacterial races

In this chapter, six Japanese *Xoo* bacterial races were used: Japanese *Xoo* Race I (T7174), Race II (T7147), Race III (T7133), Race IV (H75373), Race V (H75304), and Race IIB (H9387).

2.2 Plant materials

The F_3 progenies derived from the cross between XM14 and IAS16 was used for this study. Cultivation method followed Chapter 3, with some exceptional cases described in the next subsections. Germinated seeds were sown in seedling boxes in a greenhouse in May in 2016. About two weeks after sowing, seedlings were transferred out of the greenhouse in 2016. Then, after about three weeks, seedlings were transplanted to a paddy field in the experimental farm of the Faculty of Agriculture, Kagoshima University, Kagoshima, Japan.

2.3 Test for resistance of xa42 against multiple Xoo races

In 2016, six F_3 lines were selected so that *XA42* gene segregation was expected and for which recombination had occurred very close to *XA42* gene (**Table 7.1**). About 150 plants per F_3 line were transplanted to the paddy field. At the booting stage, the tillers of each plant from each line were separated into two halves (right and left) using polypropylene ropes. Different *Xoo* races were inoculated to each half of tillers. Each F_3 line was divided into three sublines of approximately fifty plants each. One subline was inoculated with race I (strain T7174) and race IIA (strain T7147). Another subline was inoculated with race III (strain T7133) and race IV (strain H75373). The other subline was inoculated with race V (strain H75304) and race

IIB (strain H9387). The LL of one representative leaf from bundle of half tillers of each plant was measured after careful visual observation.

2.4 Molecular technique

DNA extraction, PCR, electrophoresis, staining and visualization of gels followed Chapter 3. Only one DNA marker KGC3_16.370 was used in this chapter.

Table 7.1. Genotypes of Segregating F_3 lines used in F_3 test with multiple *Xoo* races inoculation

F_3 line	F_2 plant in Table 6.2	Le-sion length	Genotypes of the DNA marker loci [a]																	No.of F_3 plants Reac-tion R : S[b]
			KGC3 16.1	KGC3 16.180	KGC3 16.209	KGC3 16.255	KGC3 16.278	KGC3 16.3	KGC3 16.341	KGC3 16.370	KGC3 16.371	KGC3 16.399	KGC3 16.407	KGC3 16.421	KGC3 16.514	KGC3 16.594	KGC3 16.613	KGC3 16.636	RM 15 189	
BUF$_3$ 1	11	22.1	H	H	H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	42: 117
BUF$_3$ 2	9	12.7	H	H	H	H	H	H	H	H	H	X	X	X	X	X	X	X	X	31: 128
BUF$_3$ 3	15	18.5	H	H	H	H	H	H	H	H	H	H	H	H	H	X	X	X	X	42: 117
BUF$_3$ 4	10	14.5	X	X	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	31: 109
BUF$_3$ 5	14	23.3	X	X	X	H	H	H	H	H	H	H	H	H	H	H	H	H	H	43:110
BUF$_3$ 6	16	23.4	X	X	X	X	X	H	H	H	H	H	H	H	H	H	H	H	H	34: 116

[a] H and X respectively denote heterozygotes and homozygotes for XM14.

[b] R and S respectively denote resistant and susceptible. Plants with lesion length of 0.1-3cm were regarded as R. Those with lesion length longer than 5.0 cm were regarded as S (see Table 6.2).

3.0 RESULTS

3.1 Resistance of xa42 to multiple Xoo races

Inoculation of *Xoo* to parental lines showed that IAS16 line was susceptible, whereas the XM14 line was resistant to the six Japanese *Xoo* races used for this study. All F_3 lines showed segregation in the reaction against the six races (**Fig 7.1A and 7.1B, Fig. 7.2**, **Table 7.2**). For this study, I inoculated two races to one plant. The reaction against one race was almost identical to that against the other race. When a plant showed short LL against one race, it showed short LL against the other race. A plant showing long LL against one race showed long LL against the other race. The parental F_2 plants of F_3 lines used in this experiment were all heterozygous at KGC3_16.370 locus, which proved to co-segregate with *XA42* in the previous subsection. Homozygotes of XM14 allele at KGC3_16.370 locus showed very short

LL (shorter than 1 cm in most cases), indicating that they were resistant against inoculated *Xoo* races. Heterozygotes and homozygotes of IAS16 allele at the locus showed long LL (longer than 10 cm in most cases), indicating that they were susceptible to inoculated *Xoo* races (**Fig. 7.1A and Fig. 7.1B, Table 7.2**). The segregating F_3 plants showed a clear bimodal distribution pattern like that was shown by F_2 after inoculation with inoculums from six *Xoo* races (**Fig. 7.2**).

When the six F_3 lines were summed up, the segregating chromosomal region around *XA42* gene was limited to 121 kb (**Table 7.1**). When reactions to multiple strains were summed up for each F_3 line, the ratio of resistant: susceptible all fitted to 1: 3 ($0.10<p<0.70$ for $\chi^2(1: 3)$), as expected from one-gene segregation, but different from the result obtained in F_2 generation (see previous chapters). The discrepancy is discussed in the next section.

Table 7.2. Reactions in means and standard errors lesion length (cm) of parental lines (XM14, IR24, and IAS16) and F_3 plants at KGC_16.37 DNA marker locus after inoculation of six Japanese races of *Xanthomonas oryzae* pv. *oryzae.*

	T7174	T7147	H9387	T7133	H75373	H75304
Parents						
XM 14	0.30±0.06	0.21±0.04	0.20±0.04	0.22±0.04	0.21±0.03	0.23±0.03
IAS16	14.90±0.80	22.40±1.00	25.80±1.00	17.80±0.70	22.30±0.90	19.40±0.90
IR24	15.80±0.90	20.90±0.08	28.80±0.90	21.60±0.90	25.10±0.80	19.80±0.70
F_3						
X genotype ^{R}	0.34±0.02	0.42±0.03	0.37±0.04	0.32±0.03	0.32±0.02	0.39±0.03
A genotype ^{S}	13.40±0.40	19.70±0.50	24.20±0.80	17.10±0.80	16.84±0.60	20.30±0.50
H genotype ^{S}	13.80±0.30	19.30±0.50	24.20±0.40	15.90±0.40	16.20±0.40	19.50±0.40

R and S denotes plants resistance and susceptibility to *Xoo*, respectively.
X denotes F_3 plants with XM14 genotype.
A denotes F_3 plants with IAS16 genotype.
H denotes F_3 plants with heterozygous genotype

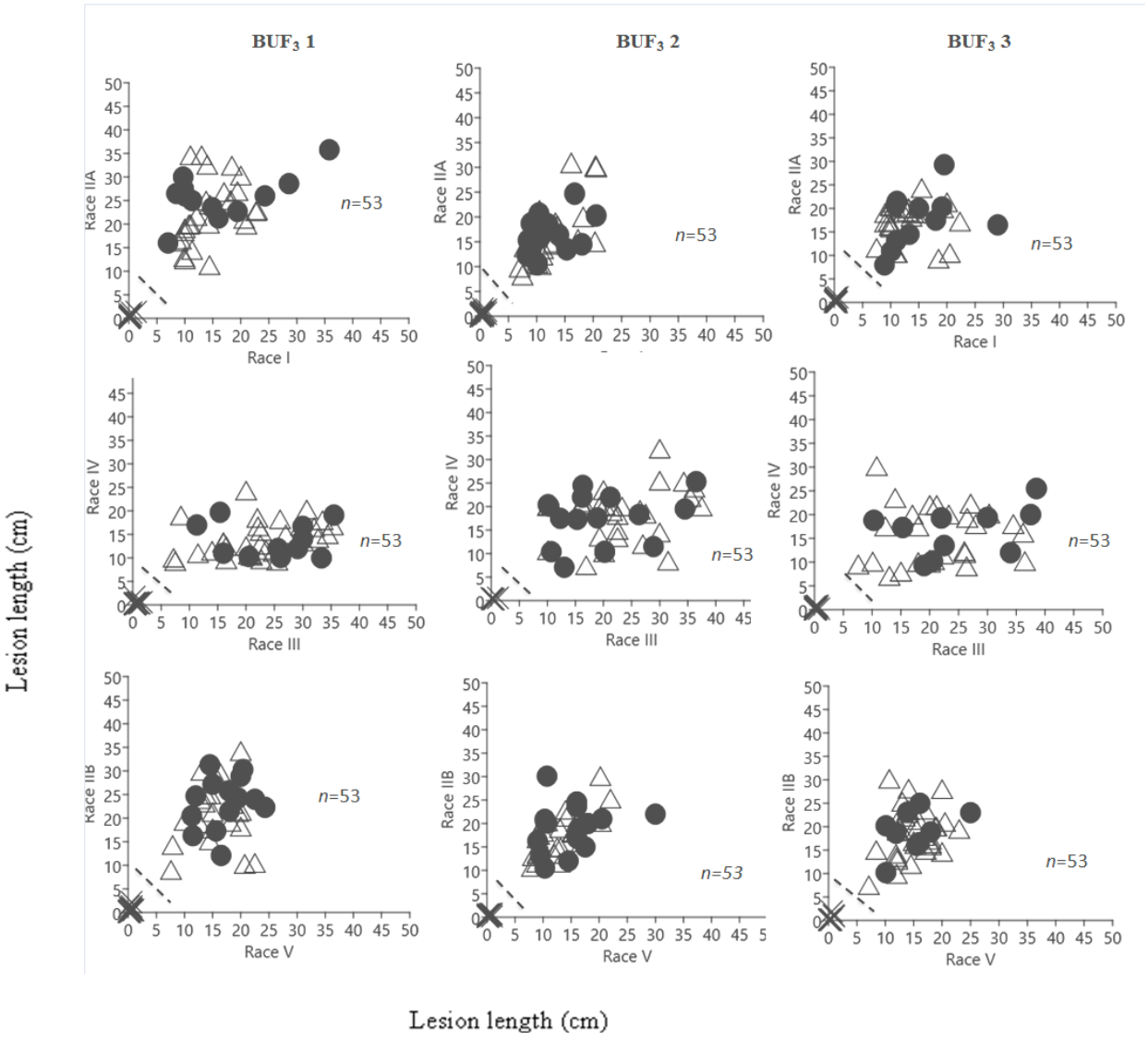

Fig. 7.1A. Lesion length (cm) distribution inoculated with six Japanese *Xoo* races shown along each axis of subfigures of F_3 plants (BUF_3 1, BUF_3 2, BUF_3 3) from the cross between XM14 and IAS16. X, solid circle and open triangle respectively denote homozygotes for XM14, heterozygotes and homozygotes of IAS16 at the KGC3_16.370 locus. Dotted lines denote the dividing point between resistant and susceptible plants.

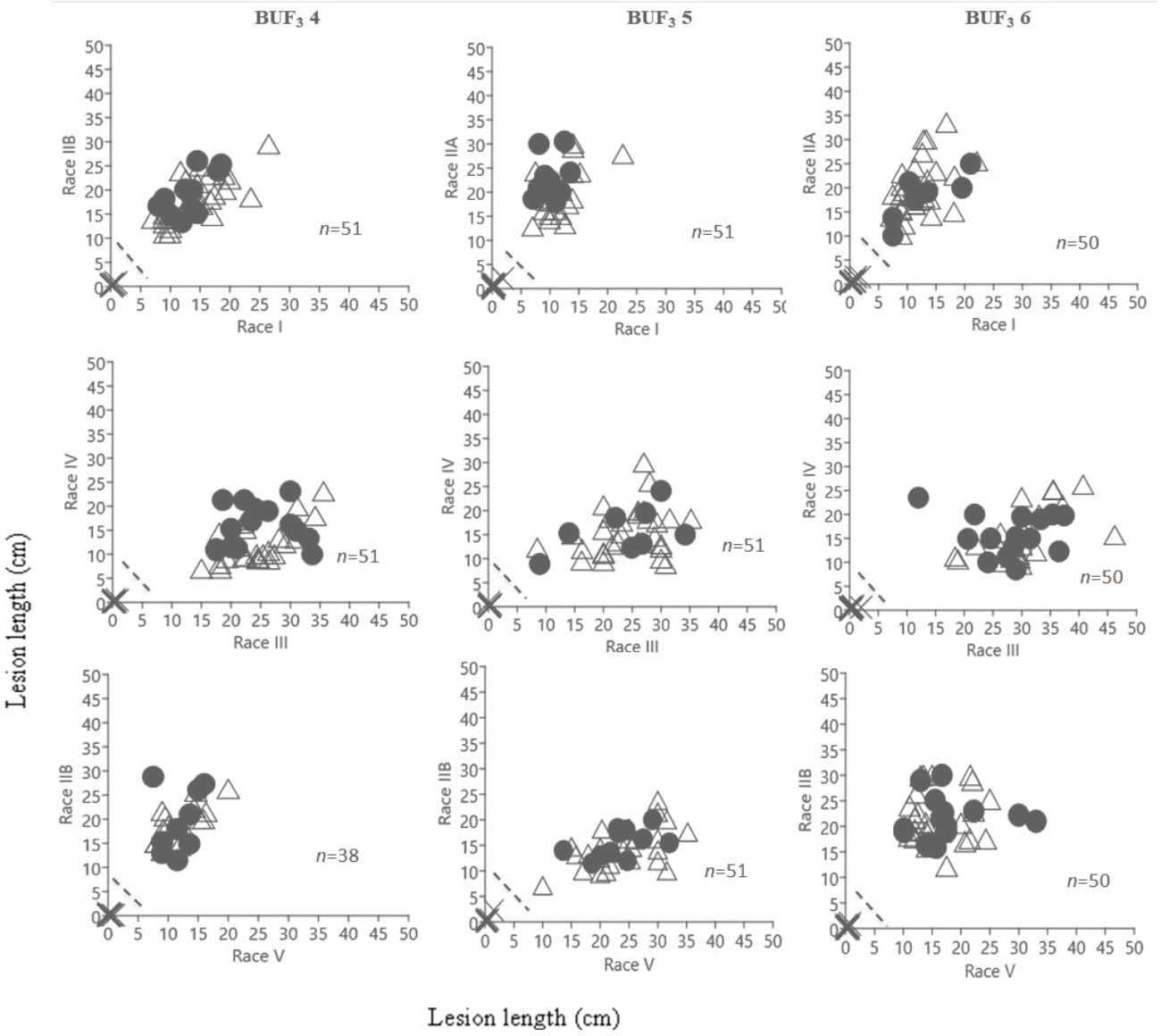

Fig. 7.1B. Lesion length (cm) distribution inoculated with six Japanese Xoo races shown along each axis of subfigures of F_3 plants ($BUF_3$4, $BUF_3$5, $BUF_3$6) from the cross between XM14 and IAS16. X, solid circle and open triangle respectively denote homozygotes for XM14, heterozygotes and homozygotes of IAS16 at the KGC3_16.370 locus. Dotted lines denote the dividing point between resistant and susceptible plants.

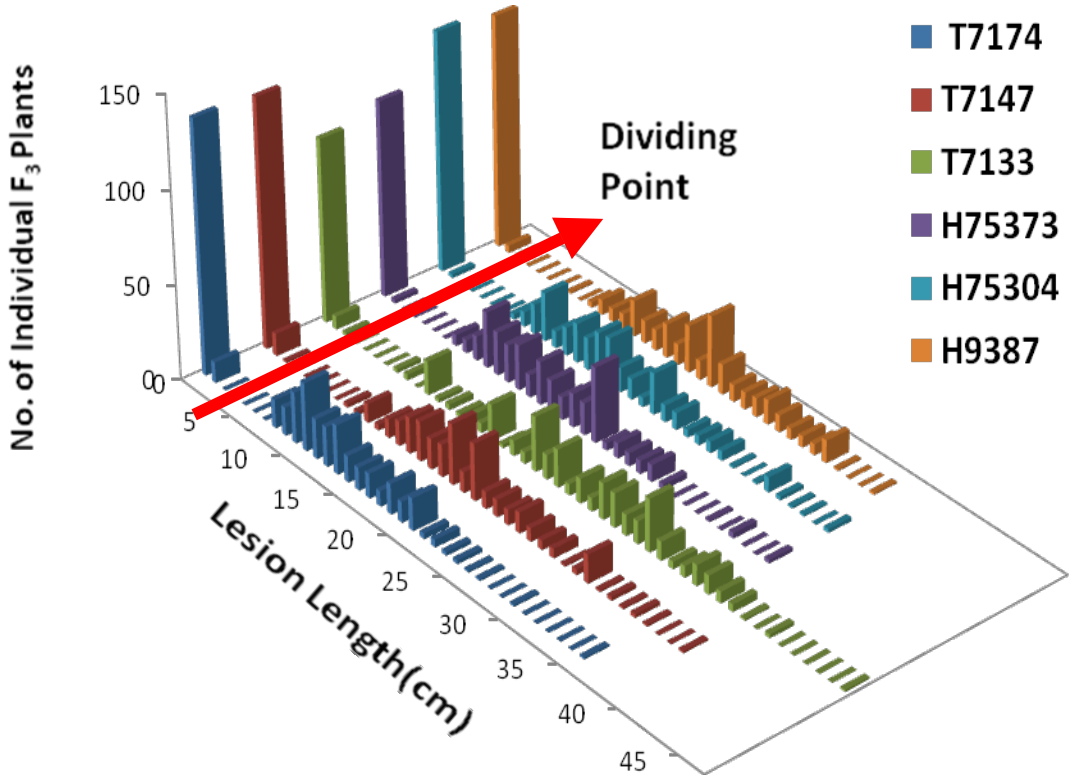

Fig.7.2. A distribution of lesion length in F_3 population from the cross between XM14 and IAS16 after X*oo* race I (T7174), X*oo* race II (T7147), X*oo* race III (H9387), X*oo* race IV(T7133), X*oo* race V(H75373) and X*oo* race IIB (H75304) inoculation test.

4. DISCUSSION

Because the probability of identifying *Xoo* resistant mutant is small (Taura *et al*. 1991a), the existence of simultaneous plural resistance mutations on one mutant line seems improbable. Therefore, these results strongly support the idea that *xa42* is resistant to the six Japanese *Xoo* races. Suzuki *et al.* (2008) induced mutation in a Japonica cultivar Taichung 65 using 1mM of MNU, the same method of obtaining XM14 (Chapter 2), and estimated the mutation frequency as 3.2×10^3 nucleotide changes in a 4.3×10^8 rice genome corresponding to one mutation in every 135 kb. If the estimate is applied to our experimental results and the above rice genome information, there is high possibility that only one mutation occurred in the *XA42* candidate chromosomal region. Therefore, these facts strongly support the idea that one mutation in *XA42* locus induced resistance to the six Japanese *Xoo* races.

XA42 gene segregation in F_2 generation from the cross between XM14 and IAS16 was distorted. However, that in selected F_3 lines was not distorted. According to Fukuta *et al.* (2000), segregation of chromosomal regions of both the short arm and long arm on chromosome 3 were skewed in favor of Indica allele. Some possibility exists that genes on one arm are insufficient to distort segregation, and that the F_3 lines were fixed for one arm, not expressing segregation distortion.

Repeated R gene failure and breakdown upon pressure from new strains and favorable environment to *Xoo* have been reported many times, as reviewed by Khan *et al.* (2014). Planting of rice varieties with broad-spectrum disease resistance is the most sustainable strategy to protect rice from diseases and to ensure stable rice production. Results from this study suggest strongly that XM14 line, which has *xa42* gene, falls in the category of broad-spectrum resistance. To prove that *xa42* is truly a broad spectrum R gene, it should be tested with international *Xoo* races, especially those from south Asian and African countries where putative new *Xoo* races have been reported (Gonzalez *et al.* 2007, Mishra *et al.* 2013, Verdier *et al.* 2012). If proven, the *xa42* gene will be very useful in resistance breeding programs.

5. SUMMARY

Resistance of *xa42* against multiple *Xoo* races was evaluated using segregating populations and tightly linked DNA markers of *xa42*. The experimental results strongly suggest that *xa42* is consistently resistant to the six Japanese *Xoo* races. If *xa42* is truly a broad spectrum gene, this gene will be very useful in rice resistance breeding programs.

CHAPTER EIGHT

PLEIOTROPIC EFFECT OF *XA42* GENE

1. INTRODUCTION

Pleiotropy occurs when one gene influences two or more seemingly unrelated phenotypic traits (https://en.wikipedia.org/wiki/Pleiotropy). Therefore, a mutation in a pleiotropic gene may have an effect on several traits simultaneously due to the gene coding for a product used by a myriad of cells or different targets that have the same signaling function.

Pleiotropy can arise from several distinct but potentially overlapping mechanisms, such as molecular gene pleiotropy, developmental pleiotropy, and selectional pleiotropy. Molecular gene pleiotropy occurs when the gene product interacts with multiple other proteins or catalyzes multiple reactions. Developmental pleiotropy occurs when mutations have multiple effects on the resulting phenotype. Selectional pleiotropy occurs when the resulting phenotype has multiple effects on fitness (Paaby and Rockman 2012).

In previous chapters, I reported the identification of a new resistance gene in XM14 produced by chemical mutation induction to IR24. In additional to being resistant to six races of Japanese *Xoo,* XM14 line exhibits smaller stature than its original cultivar, and brown spots like lesion mimic (LM) mutants in its leaves (**Fig. 2.3** and **Fig. 8.1**). This suggests that *xa42* gene is exhibiting pleiotropic effect. Li and Bai (2009) found LM that resembles plant disease symptoms in wheat which shows pleiotropic effects and were related to broad-spectrum resistance to wheat rust *(Puccinia triticina*). Similar observations of lesion mimics with broad-spectrum resistance to pathogen rice have been reported (Yin *et al.* 2000). According to Campbell and Ronald (2005), genes that regulate expression of LM symptoms in plants may play an important role in signal pathways of plant defense. This study was conducted to determine if *xa42* gene is exhibiting pleiotropic effects.

2.0 MATERIALS AND METHODS

2.1 Bacterial races

Japanese *Xoo* bacterial races that were used in this study were Japanese *Xoo* race I (T7174), race IIa (T7147), race III (T7133), race IV (H75373), race V (H75304), and race IIB (H9387).

2.2 Plant materials

IR24, XM14, IAS16, F_2 and F_3 progenies (**Table 7.1**) derived from the cross between XM14 and IAS16 were used for this study. All plant materials were grown and analyzed, following Chapter 3 with exceptional cases described in the next subsections. Germinated seeds were sown in seedling boxes in a greenhouse in May, 2016. About two weeks after sowing, seedlings were transferred out of the greenhouse. Then, after about three weeks, seedlings were transplanted to a paddy field in the experimental farm of the Faculty of Agriculture, Kagoshima University, Kagoshima, Japan.

2.3 Evaluation of pleiotropic effects of xa42 gene on browning spots and agronomic traits(Culm length, plant height and number of tillers).

The XM14 line exhibits brown spots in its leaves (**Fig. 8.1, Fig. 2.3**). In all, 982 F_2 plants for *xa42* mapping in 2015, 2950 F_2 plants for *xa42* mapping and the six F_3 lines for test for resistance of *xa42* against multiple *Xoo* races in 2016 were evaluated for their brown spots immediately before *Xoo* inoculation test. The 'B' score was given to plants with brown spots on their leaves; an 'N' score was given to those with normal leaves. At the maturity stage in 2016, three agronomic traits, culm length, plant height and number of tillers, were evaluated in F_2 generation (250 plants), F_3 generation (297 plants), parental lines (20 plants per line), and IR24 (20 plants per line). The F_2 plants in this test were selected randomly in 2950 F_2 plants. The F_3 came from the progeny of recombinants as illustrated in recombinants table in the previous chapters, two of the six F_3 lines used for multiple *Xoo* races test of *xa42* gene (BUF_3 4 and BUF_3 5). Statistical analyses were conducted using software (SPSS statistics 23; IBM Inc. New York, USA).

2.4 Molecular technique

DNA extraction, PCR, electrophoresis, staining and visualization of gels followed Chapter 4. Only one DNA marker KGC3_16.370 was used in this chapter.

3.0 RESULTS

3.1Relation between resistance to Xoo and brown spots

In 2015, 982 F_2 plants were examined for the presence of brown spots on their leaves. All 288 resistant plants were found to exhibit brown spots, although the 694 susceptible plants showed no signs of brown spots. In 2016, I used 2950 F_2 and 920 F_3 plants for the presence of brown spots (**Tables 8.1** and **8.2**). Results showed that all the F_2 and F_3 plants which had brown spots consistently exhibited a resistant reaction to *Xoo* races used for this study. Genetic analysis at DNA marker KGC3_16.370 locus showed that these plants were homozygotes of XM14 allele. Therefore, they proved to be homozygotes of *xa42* allele.

Fig. 8.1. Leaf appearance of parental lines (XM14 and IAS16) and F_2, F_3 plants from the cross between them. XM14 showed brown spots on its leaves. IAS16 showed normal leaves. In the F_2 and F_3 populations, Segregation occurred and both plants with normal leaves and those with brown spots on their leaves appeared.

In contrast, all the F_2 and F_3 with normal leaves exhibited susceptible reactions to the *Xoo* races used for this study and were either homozygotes of IAS16 allele or heterozygote at KGC3_16.370 locus. These results suggest strongly that brown spots are caused by *xa42* gene.

3.1 Pleiotropic effect of xa42 gene on agronomic traits

Table 8.3 presents the culm length, plant height and number of tillers of parental lines (XM14, IR24, and IAS16), the F_2 population and sum of the two F_3 lines derived from the cross between XM14 and IAS16. Analysis of variance (ANOVA) revealed significant difference among parental lines and among genotypes at KGC3_16.370 locus in the segregating populations for the entire three traits. According to the multiple mean comparisons, XM14 showed significantly smaller value than IAS16 and IR24 for culm length and plant height. In the segregating population, homozygotes of XM14 showed a much smaller value than heterozygotes and homozygotes of IAS16 allele for culm length and plant height. I compared homozygotes of XM14 allele and the combination of the other genotypes, which corresponded respectively to resistant plants and susceptible plants in F_2 and F_3 populations, by applying *t*-tests: resistant plants showed significantly smaller values than susceptible plants for all three traits.

4. DISCUSSION

Results show a strong and significant correlation between BB resistance and brown spots (BS), which suggests that *xa42* gene exhibits a pleiotropic effect. The BS in XM14 line might be attributed by chlorophyll mutation. Several chlorophyll mutants such as rice spotted leaf (spl) and lesion mimics (LM) have reported to exhibit shorter culm length, shorter plant height, and few tillers even some sterility (Takahashi *et al.* 2003, Yin *et al.* 2000, Bruggeman *et al*. 2015, Chen *et al*. 2016).

Brown spot mutants are generally called spotted leaf (*spl*) in rice. They are regarded as lesion mimic mutants. Yin *et al.* (2000) reported that four mutants (*spl1*, s*pl5*, *spl9*, and s*pl11*) show enhanced resistance to blast, and that *spl11* shows resistance to four Philippine *Xoo* races. Lesion mimic mutants have been studied extensively in light of programmed cell death leading to resistance to pathogens in many plants such as wheat (Li and Bai 2009) and *Arabidopsis*, as reviewed by Lorain *et al.* (2003). Therefore, *xa42* might be a kind of lesion mimic mutant. However, the brown spots on its leaves are not as dense and thick as those of typical *spl* mutants (**Fig. 8.1**).

Table 8.1. Relationships between genotypes at KGC_16.370 locus, brown spots and reaction against *Xoo* race IIA (T7147) inoculation in the F_2 population from the cross between IAS16 and XM14.

Year	Genotype at KGC3_16.370 locus	Reaction against *Xoo* race IIA		Browning symptom	
		Resistant	Susceptible	B[a]	N
2015	Homozygote for XM14 allele	288	0	288	0
	Heterozygote	0	506	0	506
	Homozygote for IAS14 allele	0	188	0	188
2016	Homozygote for XM14 allele	885	0	885	0
	Heterozygote	0	2065	0	2065
	Homozygote for IAS14 allele	0	536	0	536

[a]B and N respectively denote plants showing brown spots on their leaves and those showing normal leaves.

Table 8. 2. Relationships between browning symptoms and genotypes at KGC_16.370 locus) in the F_3 lines used for the resistance against multiple *Xoo* races.

Genotype at KGC3_16.370	Brown spots in F_3 lines[a]											
	BUF_3 1		BUF_3 2		BUF_3 3		BUF_3 4		BUF_3 5		BUF_3 6	
	B	N	B	N	B	N	B	N	B	N	B	N
Homozygote for XM14 allele	42		31		42		31		43		34	
Heterozygote		80		80		30		73		80		79
Homozygote for IAS16 allele		37		48		87		36		30		37
Total	159		159		159		140		153		150	

[a]B and N respectively denote plants showing brown spots on their leaves and those showing normal leaves.

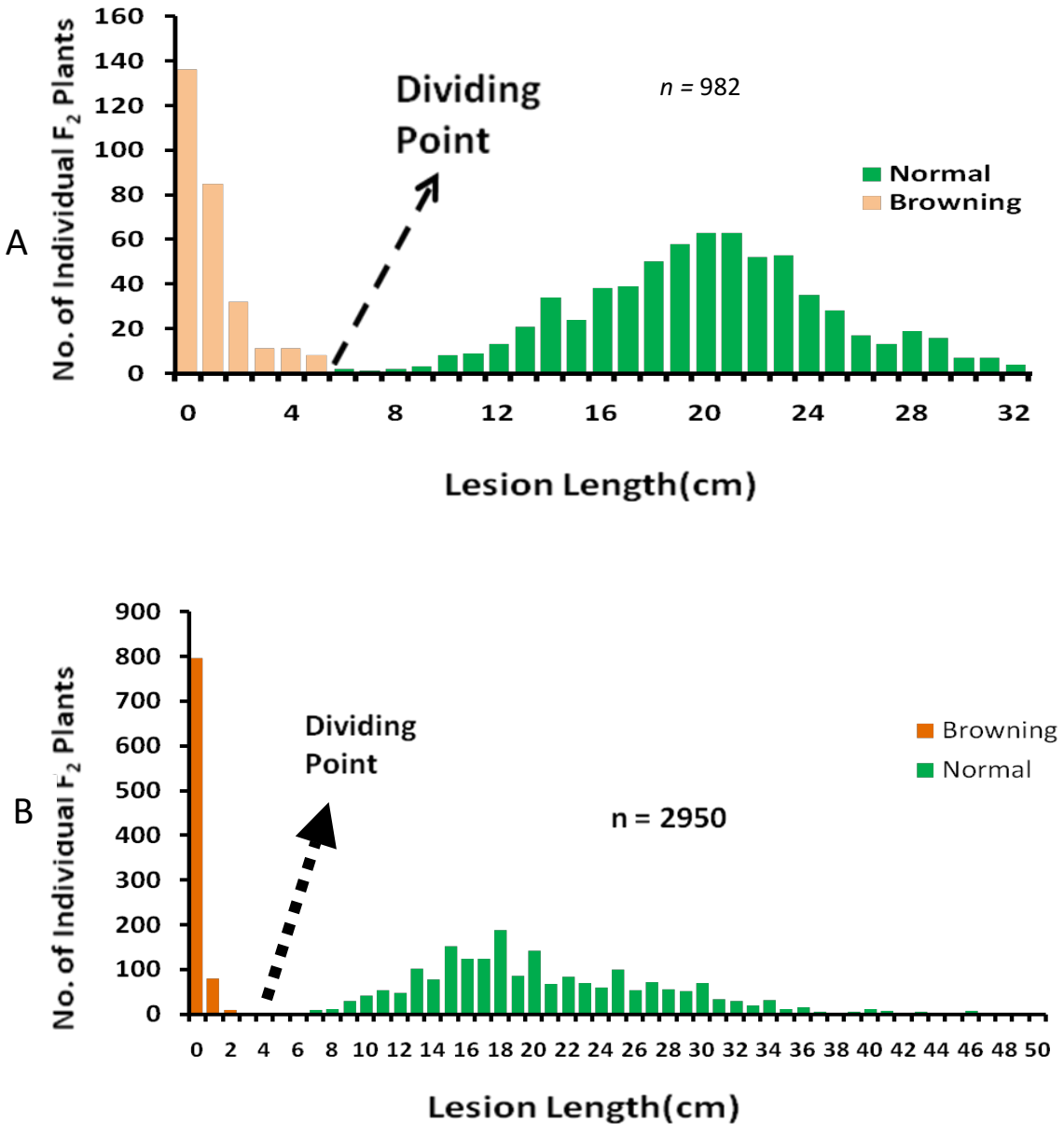

Fig. 8.2.A; graph showing distribution graph of F_2 lesion length in relation to brown spots (browning) after *Xoo* strain IIA (T7147) field inoculation test in 2015, 8.2.B; graph showing distribution graph of F_2 lesion length in relation to brown spots after *Xoo* strain IIA (T7147) field inoculation test in 2016.

Table 8.3 .Tukey HSD mean comparisons of culm length(cm), plant height(cm) and number of tillers of parental lines(XM14, IR24, and IAS16) , and F_2 and segregating F_3 lines at KGC3_16.37 locus derived from the cross between XM14 and IAS16.

Group	Culm length (cm)	Plant height (cm)	Number of tillers
Parents (*n* =60)			
XM14	60.5 a [a]	79.1 a	9.5 a
IR24	63.1 b	84.0 b	10.6 ab
IAS16	64.2 b	86.2 b	11.6 b
F-values	1.06*	1.90*	2.15*
F_2 (*n* = 250)			
Homozygote for XM14 allele	59.7 a	81.3 a	10.1 a
Homozygote for IAS16 allele	67.5 b	91.0 b	12.3 ab
Heterozygote	69.5 b	92.6 b	14.0 b
F-values	41.14*	40.61*	39.61*
F_3 (*n* =297)			
Homozygote for XM14 allele	60.7 a	81.2 a	10.0 a
Homozygote for IAS16 allele	65.5 b	86.0 b	11.6 ab
Heterozygote	67.2 b	88.0 b	13.1 b
F- values	13.60*	13.92*	22.37*

[a]Values followed by the same letter in each trait in the same group are not significantly different at *p*=0.05 according to Tukey's HSD test.

* denotes significant different at the 0.05 level by ANOVA.

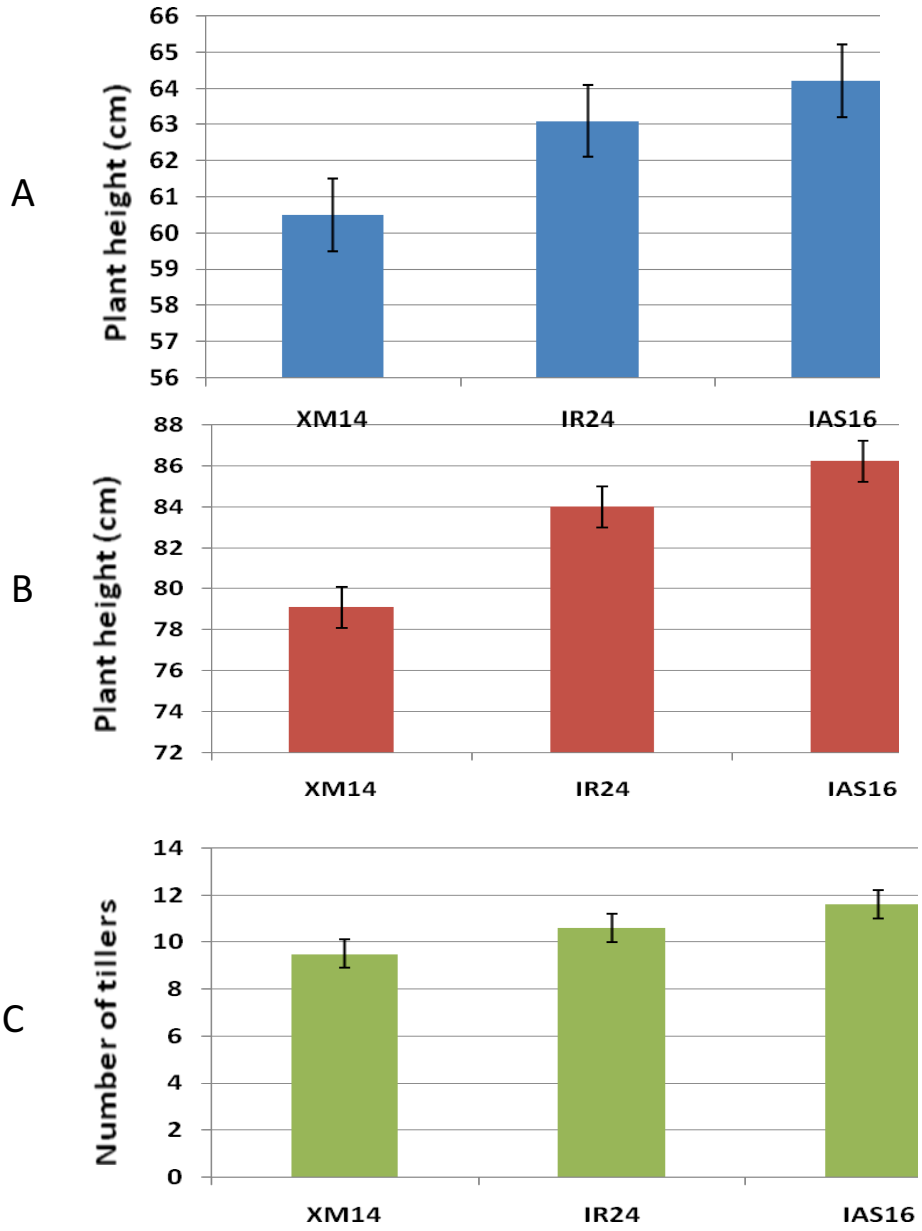

Fig. 8.3. Graph showing; A. Culm length; B, Plant height and C, Number of tillers of the XM14 line, IR24 Cultivar and IAS16 line. Error bars represent standard deviation from the mean culm length (cm), mean plant height (cm) and mean number of tillers.

Table 8.4. The data shows agronomic traits presented in mean (cm) and standard deviation (cm) of culm length, plant height and number of tillers of parental line, F_2 and F_3 populations (both derived from XM14/IAS16 cross combination)

	Parental line		t-value
	XM14 ^{R}	**IR24^S**	
Culm length(cm)	60.50±2.10	64.30±3.50	0.32*
Plant height(cm)	79.22±1.80	84.10±3.40	0.53*
Number of tillers	9.45±2.00	11.50±1.81	0.73*
	XM14 ^{R}	**IAS16 ^{S}**	
Culm length(cm)	60.50±1.90	64.65±2.72	0.54*
Plant height(cm)	79.22±1.32	86.80±3.75	0.20*
Number of tillers	9.45±0.92	12.00±1.20	0.81*
	F_2 (From XM14/IAS16 combination)		
	XM14 genotype^R	**IAS16 /vheterozygote genotype^S**	
Culm length(cm)	60.73±3.12	68.56±4.16	0.50*
Plant height(cm)	82.37±1.95	91.52±2.21	0.66*
Number of tillers	9.36±2.20	13.04±3.06	0.01*
	F_3 (From XM14/IAS16 combination)		
	XM14 genotype^R	**IAS16 /vheterozygote genotype^S**	
Culm length(cm)	61.37±3.07	66.01±3.26	0.80*
Plant height(cm)	81.58±2.53	86.92±3.61	0.86*
Number of tillers	9.32±1.09	12.07±1.06	7.79*

R and S denotes for resistance and susceptibility to *Xoo*, respectively.
*denotes for significant different at the 0.05 level.

According to Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/), two *spl* genes, *SPL3* and *SPL30*, were registered as located on chromosome 3. A comparative linkage map of chromosome 3 based on findings reported by Harushima *et al.* (1998), Tsunematsu *et al.* (1996), and Yoshimura *et al.* (1997) shows that *SPL3* is located on the short arm, not close to the centromeric region. Therefore, *SPL3* and *XA42* differ. *SPL30* was found to cosegregate with a DNA marker RM15380 in the segregating population comprising 2890 plants (Huang *et al.* 2011). RM15380 is located in 18632 kb region in Rice_IRGSP_Ver1_0, which is more than 2,000 kb from the *XA42* candidate region (**Fig. 6.4**). Therefore, *XA42* differs from two reported *spl* genes on chromosome 3. Of about 40 reported rice BB resistance genes (Khan *et al.* 2014), only *Xa3* gene was reported to exhibit brown spots resembling disease symptoms (Kaku and Hori 1977). Cultivars carrying the *Xa3* resistance gene develop brown spots as a necrotic resistant response after inoculating the plant with *Xoo* inoculums (Kaku and Hiro

1977, Kaku and Ogawa 2001). The positive relation between brown spots and BB resistance reported in *Xa3* seems similar to our observation in *xa42*. However, the brown spots in *Xa3* appear after inoculation in contrast to those of *xa42* with brown spots appearing even without inoculation of *Xoo*. Although the mechanisms of brown spots in *Xa3* are not fully elucidated, the map-based cloning of *Xa3* gene revealed that it encodes leucine- rich repeats (LRR) receptor kinase-like protein (Sun *et al.* 2004, Xiang *et al.* 2006). Annotation data of *XA42* candidate gene (**Table 5.3**) confirm that LRR receptor kinase-like protein is not associated with resistance in *xa42*. The mechanism of brown spots in association with resistance in *xa42* must be explored further to improve rice resistance using this gene.

The resistance to multiple *Xoo* race and short stature of XM14 line might be associated with chlorophyll mutation. Chen *et al.* (2016) reported the identification of a rice pale green leaf mutant, *lc7*, which is defective in chlorophyll synthesis and photosynthesis but which confers resistance to multiple strains of *Xoo*. The leaves with brown spots caused by *xa42* looked paler than normal leaves (**Fig. 8.1**). The chlorophyll content and tone of the leaf from homozygous carrier of *xa42* gene and their parental lines were not examined in this study. Such characterization will be necessary to elucidate the resistance mechanisms of the *xa42* gene. Similar to homozygotes of *xa42*, homozygote of *lc7* showed significantly fewer tillers and significantly shorter stature than wild type (Chen *et al.* 2016). The pleiotropic effect of *xa42* on agronomic traits can sometimes be negative in rice production when *xa42* gene is used for breeding rice cultivars with multiple *Xoo* resistance. Therefore, the combination of *xa42* and genes masking the negative effect of *xa42*, for example, allele conditioning tall stature on QTL controlling plant height, represents a possible solution.

5. SUMMARY

Many plant mutants develop spontaneous lesions that resemble disease symptoms in the absence of pathogen attack. In additional to being resistant to six races of Japanese *Xoo*, XM14 line exhibits smaller stature than its original cultivar, and brown spots like lesion mimic mutants in its leaves. The experiment in this chapter was conducted to determine if *xa42* gene is exhibiting pleiotropic effects. The result showed that brown spots on leaves and short stature XM14 is caused by pleiotropic effect of *xa42*. This pleiotropic effect of *xa42* might be negative to practical rice production. The possible solution to that was discussed.

CONCLUSIONS

Bacterial blight (BB) caused by *Xanthomonas. oryzae pv. oryzae* (*Xoo*) is one of the most extremely devastating diseases of rice causing huge yield losses in almost all the rice growing regions in the world. It is particularly more prevalent in Africa and South Asia. Bacterial leaf bight is one of the oldest recorded rice diseases and has been problematic for over a century. *Xoo* spreads rapidly from diseased plant to healthy plant and from field to field in water droplets. Identification and consequently deployment of resistant varieties to BB is the most pragmatically sustainable and environmentally safe way of controlling this bacterial disease.

The major objective of this research work was to genetic analysis of resistance gene bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) in rice. Among many *Xoo* resistant genes, I studied the one in XM14 of which breeding process was described in Chapter 2. The research study investigated and analyzed a number of research topics which includes DNA markers design, determination of inherent pattern of a mutant gene, the analysis using extreme recessive phenotype proposed by Zhang *et al.* (1994), genome wide scanning, linkage analysis, fine mapping of gene, investigation of a gene with multiple resistance to *Xoo,* and study on pleiotropic effects of a gene.

This research study uncovered new findings, confirmed findings from other researchers and added valuable knowledge to the plant breeders, pathologist and all researcher which are interested in resistance genes against *Xoo.* The followings are the some of the findings that this study revealed.

Chapter 1 of this dissertation describes the literature review of BB caused by *Xoo.* This chapter reviewed the past and recent literature about the causative agents of *Xoo***,** its morphology, how it survives, how it spreads, distribution of the disease, economic impact or losses of the disease, different methods of control, identified genes which are resistant to the disease and molecular mechanisms of BB resistance.

Chapter 2 with the title “Mutation induction of IR24 to breed XM14 line” describes the process of breed XM14 line. An mutagenic agent, N-methyl-N-nitrosourea (MNU),was applied to *Xoo* susceptible IR24 to obtain a *Xoo* resistant mutant line, subsequenty named XM14.

Chapter 3 was entitled with “Genetic analysis of *Xoo* resistance gene in XM14 line”. This experiment was performed in order to determine the number of genes involved in the BB resistance in XM14 line. Similarly, this experiment was undertaken to understand inheritance

patterns. The F_2 population from the cross between XM14 and IR24 showed that resistance against Japanese *Xoo* race II was controlled by a recessive allele at a single locus.

Chapter 4 focused on "Chromosomal location of *Xoo* resistance gene in XM14 line". The XM14 mutant line was crossed with a Japanese popular variety Koshihikari. The F_2 segregating plants derived from XM14 × Koshihikari upon inoculation using clipping method. They produced continuous distribution of lesion length. Then the analysis using extreme recessive phenotype proposed by Zhang *et al*. (1994) was adopted determining the location of the gene which confers resistance in XM14 mutant line. The gene was located on chromosome 3, around DNA markers RM6914 and RM6959.

In chapter 5, to minimize the genetic 'noise' caused by Indica-Japonica cross such as XM14 and Koshihikari and to maximize the usefulness of Indica-Japonica DNA polymorphism, I adopted IAS lines, which carry Asominori chromosomal segments under IR24 background (Kubo *et al.* 2002). IAS16 line carries IR24 genetic background with a Japonica cultivar Asominori segment of chromosome 3, on which the resistance gene locus was thought to be located. The F_2 population from IAS16 ×XM14 showed a discrete distribution of lesion length when inoculated with Japanese *Xoo* race IIa. The linkage analysis indicated that the gene is located on the centromeric region. The resistance gene in XM14 was a new gene, and named *XA42*, according to the gene nomenclature system for rice (McCouch and CGSNL 2008).

Chapter 6 focused on fine mapping of *xa42* gene. In order to fine map, DNA markers, mostly Indels, and few CAPS, dCAPSs and SSR markers, were designed. For higher chance of getting recombination events, the mapping populations was expanded to 13,000 F_2 segregating plants. Eventually the candidate gene region was narrowed down from 582 kb, which had been obtained in Chapter 5, to 57 kb, and the *Xa42* was flanked between DNA markers KGC3_16.342 and KGC3_16.399.

In chapter 7, resistance of *xa42* against multiple *Xoo* races was examined. According to Wisser *et al*. (2005), host resistance against multiple pathogen species or the majority of races of a pathogen species is important for sustainable and long term control of plant disease. In this chapter, 1229 F_3 segregating plants from the XM14 × IAS16 cross combination were inoculated with six Japanese races of *Xoo.* The LL distribution graph for each race showed a bimodal clear cut distribution pattern. This result with DNA marker genotypes co-segregating with *XA42* confirms that *xa42* confers resistance to multiple *Xoo* races.

Chapter 8 focused on pleiotropic effect of the *xa42* gene. XM14 exhibits brown spot in absence of pathogen inoculation, short stature compared to its original parent IR24, and fewer

number of tillers than its original cultivar. In this chapter, F_2 and F_3 segregating plants from the XM14×IAS16 cross combination were tested for plant height, culm length, brown spots and number of tillers. The results confirm that *xa42* exhibits pleiotropic effect.

In conclusion of this dissertation, I would recommend possible areas for further research in regard to *xa42* and bacterial blight(BB) caused by *Xanthomonas oryzae pv. Oryzae*.

(1) In order to prove that *xa42* is really durable and a broad spectrum R gene, it should be tested with international *Xoo* races, especially those from south Asian and African countries where putative new *Xoo* races have been reported.

(2) Some lesion mimic mutants (LMM) like *lsd1* is known to confer enhanced resistance to both fungal and bacterial pathogens (Dietrich *et al.* 1994). It is postulated that *LSD1* may negatively regulate a broad set of defense response components against more than one species of pathogen. *xa42* also has chance to have resistance to other bacterial species like blast disease or other fungal diseases.

(3) More research should be performed to isolate and clone *xa42* gene so that some investigation on transformation can be possible. Last but not least is elucidation of the mechanisms for resistance and pleiotropic effects in *xa42* gene

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