FINE MAPPING OF BACTERIAL BLIGHT RESISTANT GENE FROM *Oryza glaberrima* Steud. AND EXPRESSION ANALYSIS OF PUTATIVE CANDIDATE GENES IN THE REGION

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in
BIOTECHNOLOGY
(Minor Subject: Plant Breeding and Genetics)

By

Ankita Babbar
(L-2018-A-166-M)

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CERTIFICATE I

This is to certify that the thesis entitled. **"FINE MAPPING OF BACTERIAL BLIGHT RESISTANT GENE FROM *Oryza glaberrima* Steud. AND EXPRESSION ANALYSIS OF PUTATIVE CANDIDATE GENES IN THE REGION"** submitted for the degree of **Master of Science** in **Biotechnology** (Minor subject: **Plant Breeding and Genetics**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Ms. Ankita Babbar (L-2018-A-166-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the thesis entitled, "**FINE MAPPING OF BACTERIAL BLIGHT RESISTANT GENE FROM *Oryza glaberrima* Steud. AND EXPRESSION ANALYSIS OF PUTATIVE CANDIDATE GENES IN THE REGION**" submitted by **Ms. Ankita Babbar (L-2018-A-166-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of **Biotechnology** (Minor subject: **Plant Breeding and Genetics**) has been approved by the Student's Advisory Committee along with External Examiner after an oral examination on the same.

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ABSTRACT

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most devastating disease of rice leading to huge yield losses in Southeast Asia. A bacterial blight recessive gene *xa-45(t)* was identified from the *O. glaberrima* accession IRGC102600B and a QTL was mapped to the rice chromosome 8. This QTL limits an 80 kb region on Nipponbare reference genome IRGSP-1.0 harboring 9 candidate genes. Fine mapping of this region was carried out using a RIL population developed from the cross of rice cultivar Pusa 44 and an Introgression line IL274. The F_6 and F_7 RIL population were phenotypically evaluated by leaf clipping method against *Xoo* pathotype-VII. The phenotypic assessment confirms 1:1 segregation ratio of the gene in the mapping population indicating single gene control of BB resistance by *xa-45(t)*. For narrowing down the 80 kb region, the sequencing of all the nine candidate genes were performed using the two parents. The presence of 18 SNPs and Indels were observed between the parents of which 7 were used for the marker's development. The analysis of the genotypic data confirms that among the 9 loci, the LOC_Os08g42410 could be considered as the putative candidate for the bacterial blight resistance gene *xa-45(t)*. Further, relative expression analysis of the candidate genes at different time intervals of BB infection showed over expression of LOC_Os08g42410 specific transcripts and thus strongly supports its candidacy towards the bacterial blight gene *xa-45(t)*.

Keywords: Bacterial blight, *Oryza glaberrima*, Bacterial blight recessive genes, Quantitative trait loci, Single Nucleotide polymorphism and Insertion-Deletions.

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ਸਾਰ ਅੰਸ਼

Xanthomonas oryzae pv. *oryzae* (*Xoo*) ਕਾਰਨ ਹੋਣ ਵਾਲਾ ਝੁਲਸ ਰੋਗ ਝੋਨੇ ਦੀ ਇੱਕ ਬਹੁਤ ਹੀ ਵਿਨਾਸ਼ਕਾਰੀ ਬਿਮਾਰੀ ਹੈ ਜਿਸ ਕਾਰਨ ਦੱਖਣ-ਪੂਰਬੀ ਏਸ਼ੀਆ ਵਿੱਚ ਝੋਨੇ ਦੀ ਫ਼ਸਲ ਦਾ ਬਹੁਤ ਵਧੇਰੇ ਨੁਕਸਾਨ ਹੁੰਦਾ ਹੈ। ਮੌਜੂਦਾ ਅਧਿਐਨ ਦੌਰਾਨ *O. glaberrima* ਦੇ ਅਕਸੈਸ਼ਨ IRGC102600B ਤੋਂ ਝੁਲਸ ਰੋਗ ਦੇ *xa-45(t)* ਨਾਮਕ ਇੱਕ ਪ੍ਰਤੀਸਾਰੀ ਜੀਨ ਦੀ ਪਹਿਚਾਣ ਕੀਤੀ ਗਈ ਅਤੇ ਝੋਨੇ ਦੇ ਗੁਣਸੂਤਰ 8 ਉਪਰ QTL ਇੱਕ ਦੀ ਮੈਪਿੰਗ ਕੀਤੀ ਗਈ। ਇਹ QTL ਨੇ 9 ਕੈਂਡੀਡੇਟ ਜੀਨਾਂ ਨੂੰ ਸੰਭਾਲ ਕੇ ਰੱਖਣ ਵਾਲੇ ਨੀਪੋਨਬੇਅਰ ਰੈਫਰੇਂਸ ਜੀਨੋਮ IRGSP-1.0 ਉਪਰ 80 kb ਖੇਤਰ ਦਰਸਾਇਆ। ਝੋਨੇ ਦੀ ਕਿਸਮ ਪੂਸਾ 44 ਅਤੇ ਅਨੁਕ੍ਰਮਤ ਲਾਈਨ IL274ਦੀ ਕਰਾਸਿੰਗ ਤੋਂ ਵਿਕਸਤ RIL ਜੰਨਸੰਖਿਆ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਇਸ ਖੇਤਰ ਦੀ ਫਾਈਨ ਮੈਪਿੰਗ ਕੀਤੀ ਗਈ। ਪੱਤਾ ਕੱਟਣ ਦੀ ਵਿਧੀ ਦੀ ਵਰਤੋਂ ਕਰਕੇ *Xoo* ਪੈਥੋਟਾਈਪ-VII ਵਿਰੁੱਧ F_6 ਅਤੇ F_7 RIL ਜੰਨਸੰਖਿਆ ਦੀ ਫਿਨੋਟਿਪਿਕ ਪੜਤਾਲ ਕੀਤਾ ਗਈ। ਫਿਨੋਟਿਪਿਕ ਮੁਲਾਂਕਣ ਨੇ ਮੈਪਿੰਗ ਜੰਨਸੰਖਿਆ ਵਿੱਚ ਜੀਨ ਦੇ 1:1 ਸੈਗਰੀਗੇਸ਼ਨ ਅਨੁਪਾਤ ਦੀ ਪੁਸ਼ਟੀ ਕੀਤੀ ਜਿਸ ਤੋਂ *xa-45(t)* ਦੁਆਰਾ ਝੁਲਸ ਰੋਗ ਪ੍ਰਤੀਰੋਧਕਤਾ ਦੇ ਇੱਕਹਿਰੇ ਜੀਨ ਨਿਯੰਤਰਨ ਦਾ ਪਤਾ ਚੱਲਿਆ। 80 kb ਖੇਤਰ ਨੂੰ ਸੰਕੀਰਨ ਲਈ, ਦੋ ਮਾਪਿਆਂ ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਸਾਰੇ ਦੇ ਸਾਰੇ ਨੌਂ ਕੈਂਡੀਡੇਟ ਜੀਨਾਂ ਦਾ ਅਨੁਕ੍ਰਮਣ ਕੀਤਾ ਗਿਆ। ਮਾਪਿਆਂ ਵਿੱਚ 18 SNPs ਅਤੇ ਇੰਨਡੇਲਸ ਦੀ ਹੋਂਦ ਦਾ ਪਤਾ ਚੱਲਿਆ ਜਿਹਨਾਂ ਵਿੱਚੋਂ 7 ਨੂੰ ਮਾਰਕਰ ਵਿਕਸਤ ਕਰਨ ਲਈ ਵਰਤਿਆ ਗਿਆ। ਜੀਨੋਟਿਪਿਕ ਆਂਕੜਿਆਂ ਦੇ ਵਿਸ਼ਲੇਸ਼ਣ ਤੋਂ ਇਸ ਗੱਲ ਦੀ ਪੁਸ਼ਟੀ ਹੋਈ ਕਿ 9 ਲੋਕਾਇ ਵਿੱਚੋਂ, LOC_Os08g42410 ਨੂੰ ਝੋਨੇ ਵਿੱਚ ਝੁਲਸ ਰੋਗ ਦੇ ਪ੍ਰਤੀਰੋਧਕ ਜੀਨ *xa-45(t)* ਲਈ ਪੁਟੇਟਿਵ ਕੈਂਡੀਡੇਟ ਮੰਨਿਆ ਜਾ ਸਕਦਾ ਹੈ। ਝੋਨੇ ਦੇ ਝੁਲਸ ਰੋਗ ਦੇ ਸੰਕ੍ਰਮਣ ਦੇ ਵੱਖੋ-ਵੱਖਰੇ ਅੰਤਰਾਲਾਂ ਉਪਰ ਕੈਂਡੀਡੇਟ ਜੀਨਾਂ ਦੇ ਰੇਲੀਟਿਵ ਐਕਸ਼ਪ੍ਰੇਸਨ ਨੇ LOC_Os08g42410 ਵਿਲੱਖਣ ਟ੍ਰਾਂਸਕ੍ਰਿਪਟਸ ਦਾ ਓਵਰ ਐਕਸਪ੍ਰੇਸ਼ਨ ਦਰਸਾਇਆ ਅਤੇ ਸਿੱਟੇ ਵਜੋਂ ਝੁਲਸ ਰੋਗ ਦੇ ਪ੍ਰਤੀਰੋਧਕਤ ਜੀਨ *xa-45(t)* ਪ੍ਰਤੀ ਉਮੀਦਵਾਰੀ ਦਾ ਜੋਰਦਾਰ ਸਮਰਥਨ ਕੀਤਾ।

ਮੁੱਖ ਸ਼ਬਦ: ਝੁਲਸ ਰੋਗ, *Oryza glaberrima*, ਝੁਲਸ ਰੋਗ ਦਾ ਪ੍ਰਤੀਸਾਰੀ ਜੀਨ, ਮਾਤਰਾਤਮਕ ਗੁਣ ਲੋਕਾਇ, ਇੱਕਹਿਰੀ ਨਿਉਕਲੀਓਟਾਇਡ ਬਹੁਰੂਪਕਤਾ, ਇਨਸ਼ਰਸ਼ਨ-ਡਲੀਸ਼ਨਸ

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CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal food crops of Asia which is consumed by more than half of the world's population. It is cultivated throughout the year in different regions of India and diverse ecologies by covering more than 40 Mha of the area and providing 21% of global human per capita energy (www.knowledgebank.irri.org). Rice production stood at 101.98 MT in the kharif (summer sown) season of the 2019-20 crop year (July-June) (www.fao.org). The total rice produce of India is not satisfactory to meet the actual demand of the country due to very high population density. The major constraint to rice productivity is the pressure of various abiotic and biotic stresses. These stresses represent an utmost threat to global food security. The produce is highly undermined due to the adverse conditions involving temperature, drought as a part of environmental factors and biotic factors includes a huge number of pathogens. Among the biotic and abiotic stresses, more than 40% of rice yield is lost every year due to biotic stresses only. It emphasizes the need for further increase in productivity of rice for the fulfilment of demand.

Out of various rice infecting diseases, bacterial blight is an ancient and an acute disease attributable to bacteria, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Mew 1987; Vikal and Bhatia, 2017). This bacterium is responsible for causing 20-30% yield loss annually specifically in irrigated and rainfed lowland rice growing areas throughout Asia (Mew 1987, Bhasin *et al* 2012; Chen *et al* 2011). The first outbreak of this disease occurred in 1975 in Bihar, followed by the spread of disease in the Palakkad district of Kerala (Rangaswami, 1975; Venkatesan and Gnanamanickam, 1999). Consequently, the disease occurrence has been observed in Andhra Pradesh, Haryana, Kerala, Orissa, Punjab and Uttar Pradesh. The most crucial stage for disease occurrence is the invasion of *Xoo*. *Xoo* invades host via natural openings, and colonize the xylem vessels. Following the invasions, the bacteria proliferate leading to the occurrence of disease. The disease once established envisage as tiny water-soaked lesions on the edges of leaf blades, leading to yellowing of leaves followed by wilting of plant. Heavy infestation of bacterial blight leads to a condition called 'Kresek'.

A varied number of disease control measures are followed which includes excessive use of pesticides and insecticides that poses high risk to the environment and mankind. Among the disease control measures, the best possible way is environment-friendly agricultural practices. These practices involve restricted use of pesticides by development, improvement and utilization of the resistance mechanisms already present in the nature. The utilization of host plant resistance specifically of the wild germplasm represents an ecofriendly and cost-effective approach to achieve the desirable yield in the diseased state

(Kumar *et al*, 2012). The genus *Oryza* encompass various wild species that substantiate as virtually untapped repository of genetic diversity which can be used to perk up the rice crop. The elite rice species cultivated includes *O. sativa* and *O. glaberrima*. *O. sativa* (2n=24, AA) is a high yielding Asian rice variety grown worldwide while *O. glaberrima* (2n=24, AA) constitutes a low yielding African rice variety which is limited to some parts of West Africa.

Diversifying the genetic pool for the discovery and relocation of resistance genes from wild relatives of rice is an important strategy, extensively used in the breeding programs all across the world (Kumar *et al* 2012). The two options available for breeders include, stacking of the existing *Xa/xa* genes through the use of marker-assisted selection or discovery of new resistance genes (Vikal *et al* 2007). Till date, 46 genes providing resistance against bacterial blight, called *Xa /xa* genes, have been identified through genetic mapping studies (Kim *et al* 2015; Busungu *et al* 2016; Kim *et al* 2018 Kim *et al* 2019). It is to be noted that, the major apprehension for breeding of BB resistance varieties is the shorter duration of resistance due to rapid evolvement of pathogenic variation in *Xoo* (Vera Cruz *et al* 2000; Suh *et al* 2013). Thus, the life span of a single resistance gene is always at stake, therefore, the research for discovering new BB resistance is always active.

Among all the 46 genes, 18 BB resistance genes *viz. xa5* (Blair *et al* 2003), *xa8* (Sidhu *et al* 1978), *xa9* (Yoshimura *et al* 1983; Sun *et al* 2004; Xiang *et al* 2006), *xa13* (Yoshimura *et al* 1995), Zhang *et al* 1996; Chu *et al* 2006), *xa15* (Noda and Ohuchi 1989), *xa19* (Taura *et al* 1991), *xa20* (Taura *et al* 1992), *xa24* (Mir & Khush 1990; Khush & Angeles 1999), *xa25* (Gao *et al* 2001,2005), *xa26* (Lee *et al* 2003; Sun *et al* 2004), *xa28* (Lee *et al* 2003), *xa31* (Wang *et al* 2009), *xa33* (Korinsak *et al* 2009), *xa34* (Chen *et al* 2011), *xa41* (Hutin *et al* 2015), *xa42* (Busungu *et al* 2016), *xa44* (Kim 2018) and *xa45* (Neelam *et al* 2019) are found recessive, rest all are dominant type of genes. In addition to this, from the 46 BB resistance genes discovered till now, *Xa21* (Khush *et al* 1990; Ronald *et al* 1992), *Xa23* (Wang *et al* 2006), *Xa27* (Amnte-Bordeos *et al* 1992; Lee *et al* 2003), *Xa29(t)* (Tan *et al* 2004), *Xa30(t)* (Jin *et al* 2007), *Xa32(t)* (Zheng *et al* 2009), *Xa33(t)* (Natarajkumar *et al* 2010), *Xa34(t)* (Ram *et al* 2010), *Xa35(t)* (Guo *et al* 2010), *Xa38* (Bhasin *et al* 2012), *xa41(t)* (Hutin *et al* 2015), *xa45* (Neelam *et al* 2019) have been discovered and successfully introgressed from wild rice *viz.O. longistaminata, O. rufipogon, O. minuta, O. officinalis, O. rufipogon, O. australiensis, O. nivara, O. brachyantha, O. minuta, O. nivara, O. barthii* and *O. glaberrima* respectively.

The School of Agricultural Biotechnology, (PAU), is involved in preserving an active collection of 1600 wild species, secured from International Rice Research Institute (IRRI), Los Baños, Philippines, and ICAR-National Rice Research Institute (ICAR-NRRI), Cuttack,

India, (formerly CRRI, Central Rice Research Institute). These accessions were screened against *Xanthomonas* pathotypes prevalent in Punjab, constitutively from many years since 2001 (Vikal *et al* 2007, Neelam *et al* 2016). The accession IRGC 102600B belonging to *Oryza glaberrima*, was found resistant against *Xoo* pathotype VII. This accession was explored for the conferring resistance, for the mapping and transfer of the responsible QTL/gene to elite cultivar. From the mapping population developed from wild parent crossed with Pusa 44 cultivar, a QTL, *xa-45* was mapped on long arm of chromosome 8 with LOD score of 33.22 bracted by the flanking C8.26737175 and C8.26818765 SNP markers. The SNP marker, C8.26810477, stood as the peak marker, explaining 49.8 % of total phenotypic variance and was located at 202.90 cM on linkage map. The region spanned 80 Kb on Nipponbare reference genome IRGSP-1.0, harbouring 9 candidate genes. The present study focuses on the enrichment of this 80 kb region with a greater number of markers to decipher the putative gene responsible for resistance. Also, the resulting putative gene function would be validated through differential gene expression analysis. The present work was implemented under the following objectives:

1. To fine map novel bacterial blight resistance gene from *O. glaberrima* and development of linked marker.
2. To study expression analysis of putative candidate BB resistance genes in the identified QTL region.

CHAPTER II

REVIEW OF LITERATURE

Rice is one of the most prominent crops serving as the staple food for half of the population worldwide. It is a beneficial source of livelihood for many rural households and plays a vital role in India's food security. It is the most important grain with regard to human nutrition and caloric intake as it provides more than one-fifth of the calories consumed worldwide by humans (Smith and Bruce 1998). Among the major rice production worldwide, China and India produce half of the global rice. According to the USDA 2020 statistics, 160 million hectares (Mha) of global land is been solely used for rice production which is about 500 million tonnes (Mt). In order to sustain ever increasing food grain requirement, the production rate of rice needs to be elevated. This production estimate is severely scaled down by a variety of biotic and abiotic stress factors. Amongst these stresses, biotic agents are highly responsible for hampering rice production leading to huge economic losses. Engaging the resistant crop have revolutionized the agricultural scenario for combating the biotic stress.

2.1 Causative agent of Bacterial Blight

2.2 *Xoo* infection

2.3 Disease control

2.4 Artificial screening technique

2.5.1 BB resistance mechanisms

2.5.2 Breeding approaches

2.6 Wild species of rice

2.7 High resolution genetic mapping of BB resistance genes

2.8 Expression analysis of BB resistance gene

2.1 Causative agent of BB

Bacterial blight is caused by a gram-negative bacterium *Xanthomonas oryzae pv. oryzae* is widely prevalent and destructive disease of rice. It is one of the seed borne diseases (Srivastva and Rao 1963) and vascular bundle disease, can be leaf blight type, wilting type and withering type. It has been a reason for huge loss of agricultural produce worldwide. The disease is not only involved in grain yield reduction, rather, also responsible for declining the quality of paddy straw which is actually used as fodder. The disease was first observed in 1884 by Japan farmers. It has been observed, that the disease is prevalent in both temperate and tropical areas but poses major threat to rice production in irrigated, rainfed lowland areas.

The first outbreak of BB in India was observed in year 1975 in Bihar and its neighboring state (Rangaswami 1975). The compatible reaction of *Xoo* with host is responsible for occurrence of disease. Also, significant heavy infestation of bacteria leads to a severe condition called 'Kresek' owing to huge losses in the field. The yield loss generally caused by the disease is 20-30%, but can even reach up to 80% in severe infections (Mew *et al* 1993, Pradhan *et al* 2015).

2.2 Symptoms of *Xoo*

The bacterium was categorized as *Bacillus oryzae* (Mizukami and Wakimoto 1969). But then renamed to *Pseudomonas oryzae* and later *Xanthomonas oryzae* (Ishiyama 1922) also reclassified as *X. campestris pv. oryzae* in 1978 (Dye 1978). It is an obligate aerobic bacterium with 25-30 °C optimal temperature range (Sharma *et al* 2017). It exists with single flagella in singular form or in chains. (Swings *et al* 1990). The bacterium belongs to the family of γ-proteobacteria that are responsible for type III secretion system for pathogenicity (Zhu *et al* 2001). *Xoo* is unable to reduce nitrate and it is a weak producer of acids from carbohydrates (Bradbury 1984).

The disease is observed at seedling and adult stage but superlatively during flowering stage of rice. It enters the leaf through hydathodes or wounds at leaf tip or margin (Ou 1985). Further, it multiplies in the intercellular spaces of underlying epithelial cells, and spreads in plant by gaining access to parenchymatous cells of xylem vessels, resulting in yellow lesions with wavy margins along the veins that extends to the sheath (Noda and Kaku 1999; Hilaire *et al* 2001). The Initial stage of disease is recognized as lesions at leaf tip, but with expansion of infection, the lesions turn yellow with initiation of leaf curling, slowly lesion starts covering whole leaf blade turning into saprophytic growth. In severe circumstances, the plant completely dries and there is no panicle emergence, if emerges, produces sterile grains with poor quality.

In Punjab, currently ten distinct *Xoo* pathotypes (PbXo-1 to PbXo-10) were reported (Lore *et al* 2011, 2013). Before the release of high-yielding but susceptible varieties, the disease was found only in East Asia. But, with the introduction of high yielding varieties bacterial blight has become one of the major constraints to rice production. The pathogen may survive on infected rice plants or other hosts (wild rice and gramineous weeds) over winter (Ou 1985). During winters, the pathogen survives in soil, weeds, straw, stubbles or other wild hosts and again enters the plant through natural openings in next kharif season. A yellow soluble pigment called xanthomonadin and an extracellular polysaccharide (EPS) is produced for protecting the bacteria from dessication and facilitating rain-borne dispersal.

2.3 Disease control

The disease can be managed by chemical means, biological control and host plant resistance. Among the various management strategies, Cook (1993) has stated that biological measures depend on conditions governing plant growth. Studies reveal the bacteria responds distinctly to different chemicals. In 1979 Chand *et al* reported reduction in lesions by disinfecting rice seeds with bleaching powder and 30% chlorine. The disease was suppressed by foliar spray of cow dung (20g/l) (Mary *et al* 1986). Khan *et al* (2012) tested six different broad-spectrum antibiotics but these could temporarily control the disease. But race development possesses serious problems in devising an effective control. Also, extensive use of chemicals has detrimental effect on the environment, farmers and consumers health (Schantz *et al* 2001). Therefore, the best strategy to combat such a disease is development of resistance among the cultivated varieties. The host plant resistance is the most ecological friendly and cost-effective approach to achieve the desirable yield in diseased state (P N Kumar *et al* 2012) and various wild accessions of rice can be exploited for the same. Wild species actually serve as a untapped reservoir of genetic diversity which can be used to perk up the rice crop. Broadening the genetic base for identification and transfer of resistance genes from wild relatives of rice is an important strategy in breeding programs across the world (P N Kumar *et al* 2012) to control the disease. Transfer of novel resistance genes from wild species to cultivated rice to widen its gene pool and their deployment in breeding programs, provide sustainable bacterial blight resistance to *Xoo* can help in the purpose (Suh *et al* 2009; P N Kumar *et al* 2012).

2.4 Phenotyping and Bioassay

Screening techniques have been devised and standardized by many researchers accordingly. The most common methodology for bioassay is the leaf-clipping method devised by Kauffman *et al* 1973. *Xoo* strains are firstly isolated and streaked on Wakimoto media followed by incubation at 27-30°C. The colonies so obtained are used to prepare the inoculum. To evaluate the disease reaction, population is inoculated with leaf-clipping method specifically at maximum tillering stage of plants (Kauffman *et al* 1973). The disease severity for the inoculated plants is usually recorded 10-14 days post inoculations. The mean lesion length or SES qualitative score is generally used for phenotyping of population. The data is recorded on the scale of 0-9 following the standard values stated by IRRI 2002.

Table 2.1: Disease Reaction assessment

Score	Lesion length	Disease reaction
0	< 0.5 cm	Immune
1	< 1 cm	Highly Resistant
3	< 3 cm	Resistant
5	3-5 cm	Moderate
7	> 5 cm	Susceptible
9	> 10-12 cm	Highly Susceptible

2.5.1 BB resistance mechanism

2.5.1a Biochemical basis

The biochemical mechanism involved in resistant reaction of host is referred as bacteriostasis. This process is an incompatible reaction leading to accumultation of fluorescent yellow green compound and ultimately death of host cell. During these incompatible reactions, lignin-like polymerase are rapidly formed that facilitate deposition of peroxidase, toxic phenols and free radicals (Reimers and Leach 1991). These compounds contributed well towards the defense reaction of resistant hosts.

2.5.1b Molecular basis

Gene for gene interactions have been described for various plant-pathogen combinations (Martin *et al* 1993, Song *et al* 1995). Studies prove that plants have developed innate mechanisms for survival under pathogen attack. Two instrinsic line of defense are Pattern-Trigerred immunity (PTI) and Effector-Trigerred immunity (ETI) response. The former response in rice involves production of reactive oxygen species (ROS), callose deposition in cell wall, increase in calcium concentration, activation of MAP kinases and phytoalexins production (Leach *et al* 2014). While the later response, ETI is mainly governed by the resistance genes inherent to the host. From the studies of such interactions, it has been concluded that a single resistance gene (R) is responsible for imparting disease resistance, which corresponds to a single avirulence (*avr*) gene of pathogen. On the infestation of pathogen, the R genes allows the host to recognize the pathogen entering through different opening of the plant and initiates a cascade of signals that is responsible for production of different molecules. The recognition event allows to stimulate the host defence response and ultimately results in resistance (Kunnel 1996). With years of studies and research made, it can

be stated that BB resistance exhibits, both quantitative and qualitative inheritance. Different BB R genes produce different R proteins in defense to the *Xoo* attack.

2.5.2 Breeding approaches for BB resistance

Various breeding strategies are deployed worldwide for the exploitation of already available host resistance mechanism. It serves as an economically and eco-friendly strategy for disease management. Flor (1971) elaborated the concept of gene-for-gene interaction which is efficiently applied for interacting among the rice and a specific race of *Xoo*. The host plant resistance gene is involved in sensing the elicitor molecules encoded by an avirulence gene (*avr*) released by the pathogen following infection. This recognition steps initiates a series of defence response in the host and prevent the further invasion of pathogen. This interactive mechanism is generally exploited by the scientists all around the world for combating the biotic stress pertaining to bacterial blight.

Table 2.2: Gene interactions among host and *Xoo*

Pathogen Avirulence/ Virulence genes	**Plant Resistance/ Susceptibility genes**	
	R (Resistant)	**S (Susceptible)**
A(Avirulent)	AR (Resistance)	Ar (Susceptible)
A (Virulent)	aR (Susceptible)	ar (Susceptible)

2.6 Wild species of rice

The wild relatives of genus *Oryza* are the unparalleled reservoir of genetic diversity, highly exploited for expanding the crop improvement strategies worldwide. The wild species exhibits 50-60% higher genetic diversity as compared to cultivated species. These wild species harbor inferior agronomic traits like poor plant type, poor grain type and yield along with many diversified morphological characters for adaptation to different habitats. The *Oryza* genus incorporates two domesticated species (*O. sativa* and *O. glaberrima*) and 22 wild species. The genus comprises of nine genome sets, AA, BB, CC, BBCC, CCDD, EE, FF, GG, and HHJJ representing an enormous gene pool for genetic improvement of cultivars.

Various breeding strategies are employed for the transfer of genes from distinct genome sets of wild species into the cultivars. These strategies are dependent on relatedness among the species, possible incompatibility barrier and ultimately the nature of target trait. Successful examples for identification and introgression of BB resistance gene from wild relatives of genus *Oryza* are described below:

Table 2.3: BB resistance genes identified and introgressed from wild rice species

R-genes	Wild species	Reference
Xa21	*O. longistaminata*	Khush *et al* 1990; Ronald *et al* 1992
Xa23	*O. rufipogon*	Wang *et al* 2006
Xa27	*O. minuta*	Amnte-Bordeos *et al* 1992; Lee *et al* 2003
Xa29(t)	*O. officinalis*	Tan *et al* 2004
Xa30(t)	*O. rufipogon*	Jin *et al* 2007
Xa32(t)	*O. australiensis*	Zheng *et al* 2009
Xa33(t)	*O. nivara*	P N Kumar *et al* 2010
Xa34(t)	*O. brachyantha*	Ram *et al* 2010
Xa25(t)	*O. minuta*	Guo *et al* 2010
Xa38(t)	*O. nivara*	Bhasin *et al* 2012
xa41(t)	*O. barthii*	Hutin *et al* 2015
xa45(t)	*O. glaberrima*	Neelam *et al* 2019

2.7 High resolution genetic mapping of BB resistance genes

The studies on genetics of BB resistance genes initiated in Japan with the identification of *Xa1* dominant gene spanning 6.6 kb region, owing resistance to Japanese race-1 bacterium. The gene was mapped with the aid of RFLP marker (Sakaguchi 1967; Yoshimura *et al* 1995). Since then, mapping of BB resistant gene attracted scientist's attention. Further, He *et al* (2006) refined the already identified *Xa2* locus to a 190 kb region by designing 120 new SSR markers in the region. Among these, 12 SSR markers were found polymorphic for the parental lines ZZA and IRBB2 and their respective population. Also, many studies have been reported for *Xa4*, initiating with its identification by Petipisit *et al* (1977), mapping to chromosome 11 by Yoshimura *et al* (1995), followed by Li *et al* (1999) and Wang *et al* (2001) to refine the locus. Following this, Sun *et al* (2003) reported fine mapping of *Xa4* locus to a 47 kb region that stood very useful for cloning purpose.

Apart from RFLPs and SSR markers, SNP markers could also be used for fine mapping a loci. The first report for refining the locus using SNP markers was stated by Blair *et al* (2003). The report claims fine mapping of *xa5* locus to 4.5-6.1 cM.

In Punjab state, 7 *Xoo* pathotypes exists. Vikal *et al* (2015) reported fine mapping of already identified *xa8* gene conferring complete resistance against 5 out 7 prevalent pathotype in Punjab (Singh *et al* 2002). *xa8* has been mapped on chromosome 7 at 19.9 cM genetic

distance from SSR marker, RM214. The gene has been fine mapped to 9.5 kb region with the aid of new SSR markers within the region. The interval so obtained, harbours three candidate putative genes LOC_Os07g07400, LOC_Os07g07410, LOC_Os07g07420. Gu *et al* (2008) reported fine mapping of *Xa10* between marker M491 and M419 at genetic distance of 0.28 cM, harbouring six putative candidate genes on the basis of annotation results.

Chu *et al* (2006) stated fine mapping of *xa13* gene which lies on the long arm of chromosome 8 to a fragment of 14.8 kb. The region was narrowed on the basis of collective results of map-based cloning and sequence-specific molecular markers revealing two intact candidate gene in the region. The findings facilitated marker-assisted transfer of *xa13*. Similarly, *Xa23* already mapped on long arm of chromosome 11 to a 1.7 cM region has been refined to a 0.4 cM region pertaining to 6 candidate gene between the STS markers Ljl38 and A83B4 STS with Lj74 as the co-segregating marker (Wang *et al* 2014).

Wu *et al* 2008 reported fine mapping of *xa24* to a 71 kb fragment on chromosome 2L by filtering recombinants in accordance to the applied SSR markers result. This gene confers resistance at seedling and adult stage against Philippine *Xoo* races 4,6,10 and Chinese *Xoo* KS-1-21, Zhe 173 and JL 691.

Xa25 conferring resistance to chinese strain JL691 and *Xoo* strain PXO339 was mapped to centromeric region of chromosome 12. It spaced 2.5 cM from a disease resistance gene homologous sequence, NBS109, and 7.3 cM from a RFLP marker G1314 (Chen *et al* 2002). Against the same Chinese *Xoo* strain JL691, Yang *et al* (2003) stated high resolution mapping of *Xa26* identified in Minghui 63. The gene was mapped from F_2 population on chromosome 11 between the markers RM224 and Y6955RA to a 1.68 cM. The region so identified was delineated to 20 kb fragment with the analysis od recombinantion events achoring the BAC clones involved. Also, this locus was found tighlty linked to *Xa4*.

Another gene *Xa27*(t) has been found to confer resistance to 30 strains of bacterial blight, the gene has been fine mapped to 0.052 cM within the markers M964 and M1197. The genetic interval was cosegregating with M631, M1230, M449 (Gu *et al* 2004). In year 2008 Cheema *et al* reported the identification of *Xa30(t)* from *O. nivara* (IRGC 81825) which showed resistance against 7 *Xoo* pathotypes of nothern India. The experiment followed genotyping with bacterial artificial chromosome (BAC) clone, leading to a 38.4 kb region on chromosome 4L. Till then, none of the *Xa* genes were mappend on chromosome 4L. Similarly, *Xa31(t)* was localised to 0.2 cM bracketed by G235 and C600 RFLP markers on chromosome 4 which was further limited to a 100 Kb region using BAC library for genotyping (Wang *et al* 2009).

Zheng *et al* (2009) identified a novel BB resistance gene from wild rice (*Oryza australiensis*) introgression line, C4064 on chromosome 11 designated as *Xa32(t)*. Another gene *Xa33* was identified from the developed BC_1F_2 of *O. nivara* IRGC 105710 and TN1 with the aid of SSR marker. Around 72 polymorphic SSRs were devised to develop a coarse map for *Xa33* on chromosome 7 within RM5711 and RM6728. *In silico* analysis revealed 8 candidate genes among which, a gene coding for serine-threonine kinase was considered to be the putative gene (P N Kumar *et al* 2012). Chen *et al* (2011) reported a novel recessive gene *xa34(t)* belonging to chromosome 1 in a 1.42 cM region between RM10927 and RM10591. Further the fine mapping was conducted by the sequencing analysis, which lead to designing of new 43 Indel markers and found BGID25 marker at 0.4 cM distance. Confining the locus to 204 kb region braceted by RM10929 and BGID25.

Guo *et al* (2010) reported a novel dominant BB resistance gene *Xa35(t)* showing resistance to PXO61, PXO112 and PXO339, originated from *O. minuta* (accession 101133). The locus pertained to a 1.80 cM region on chromosome 11, co-segregating with marker RM144. On the same chromosome, another gene *Xa36(t)* was mapped at a 4.5 cM region within SSR markers RM224 and RM2136 (Miao *et al* 2010). At PAU, study was conducted for designing a linked marker in close proximity to *Xa38*. The gene annotation analysis revealed the presence of 3 candidate genes in the region which were proceeded for target sequencing. One loci, LOC_Os4g53050 harbor 48 bp deletion in *O. nivara* acc. IRGC 81825, around which the new primers were designed which can be efficiently used for marker-assisted selection (Bhasin *et al* 2012).

Another BB resistance gene *Xa40* was identified from the population derived from the two crosses 11325/Anmi and 11325/Ilpum. The gene was found involved in providing resistance to all Korean *Xoo* races (Kim *et al* 2015). Another resistant locus identified against Korean *Xoo* races was *Xa43(t)*. The locus was fine-mapped to 119 kb interval flanked by marker IBb27os11_14 and S_BB11.ssr_9 harboring 9 target ORFs (Kim and Reinke 2019). Neelam *et al* (2019) reported a study aiming the identification of a recessive BB resistance locus *xa-45(t)* from *O. glaberrima* acc. IRGC 102600B. The locus was identified following ddRAD sequencing approach that delimited it to a 80 kb region on Nipponbare reference genome IRGSP-1.0.

Table 2.4: BB resistance genes

R-gene	Donor	Marker type	cM position	Reference
Xa1 Cloned	*Oryza sativa* ssp. japonica	RFLP	108.2	Sakaguchi (1967); Yoshimura *et al* (1995)
Xa2	*O. sativa* ssp. indica	SSR	464	Sakaguchi (1967); He *et a* (2006)
*Xa3/Xa26*Cloned	*O. sativa* ssp. japonica	RFLP	114.4	Ezuka *et al* (1975); Sun *et al* (2004)
Xa4	*O. sativa* var. indica	RFLP	110.4 -113.8	Petpisit *et al* (1977); Sidhu *et al* (1978); Yoshimura *et al* (1995)
xa5 Cloned	*O. sativa* ssp. Indica	CAPS	4.5- 6.1	Blair *et al* (2003)
Xa6/Xa3	Zenith	RFLP	109.8-114.4	Sidhu *et al* (1978)
Xa7	*O. sativa* ssp. Indica	RFLP, STS, SSR	107.5	Sidhu *et al* (1978); Porter *et al* (2003)
xa8	PI231129	SSR	14.8-54.6	Sidhu *et al* (1978)
xa9	Khao Lay Nhay and Sateng,	RFLP	109.8-114.4	Yoshimura *et al* (1983); Sun *et al* (2004); Xiang *et al* (2006)
Xa10 Cloned	Senegal	RFLP/CAPS	96.5-106.5	Gu *et al* (2008); Yoshimura *et al* (1983)
Xa11	IR8, RBB11	SSR	1-2	Ogawa and Yamamoto (1986)
Xa12	*O. sativa* ssp. japonica	-	66	Ogawa *et al* (1978)
xa13 Cloned	*O. sativa* ssp. indica	STS	109.3- 111.2	Yoshimura *et al* (1995); Zhang *et al* (1996); Chu *et al* (2006)
Xa14	*O. sativa* ssp. indica	RFLP	40- 78.2	Taura *et al* (1987)
xa15	*O. sativa* ssp. japonica	-	-	Noda and Ohuchi (1989)
Xa16	*O. sativa* ssp. indica	-	-	Noda and Ohuchi (1989)
Xa17	*O. sativa* ssp. japonica	-	-	Ogawa *et al* (1989)

Table 2.4: Contd..

R-gene	Donor	Marker type	cM position	Reference
Xa18	*O. sativa* ssp. japonica	-	-	Ogawa and Yamamoto (1986)
xa19	*O. sativa* ssp. indica	-	-	Taura *et al* (1991)
Xa20	*O. sativa* ssp. indica	-	-	Taura *et al* (1992)
Xa21, Cloned	*O. longistaminata*	PTA248 STS	95.4	Khush *et al* (1990); Ronald *et al* (1992)
Xa22	*O. sativa* ssp. japonica	RFLP	112.8-119.5	Lin *et al* (1996)
Xa23, Cloned	*O. rufipogon*	RFLP/ SSR	92.4–125.6	Wang *et al* (2006)
Xa24	*O. sativa* ssp. indica	SSR	187.5-196.8	Mir & Khush (1990); Khush & Angeles (1999)
Xa25	*O. sativa* ssp. indica	SSR	102.7-113.2	Gao *et al* (2001,2005)
xa25, Cloned	*O. sativa* ssp. indica	STS	0-140	Chen *et al* (2002); Liu *et al* (2011)
xa26(t)	*O. sativa* ssp. indica	RFLP	112.9-117	Lee *et al* (2003); Sun *et al* (2004)
Xa27 Cloned	*O. minuta*	RFLP	0-139	Amnte- Bordeos *et al* (1992); Lee *et al* (2003)
xa28	*O. sativa* ssp. indica	Mutagenesis	95.4	Lee *et al* (2003)
Xa29	*O. officinalis*	RFLP	71.2-73.1	Tan *et al* (2004)
Xa30	*O. rufipogon*	SSR	68.8	Jin *et al* (2007)
xa31	Zhachanglong	RFLP	106.5-108.2	Wang *et al* (2009)
xa34(t)	BG1222 (aus rice cultivar)	SSR	78.4-79.7	Chen *et al* (2011)
Xa34(t)	*O. brachyantha*	SSR	-	Ram *et al* (2010)
Xa35(t)	*O. minuta*	SSR	117.3-123.2	Guo *et al* (2010)
Xa36	C4059	SSR	117.9-120.1	Miao *et al* (2010)
Xa38	*O. nivara*	STS/ SSR	126.5-128.5	Bhasin *et al* (2012)

Table 2.4: Contd..

R-gene	Donor	Marker type	cM position	Reference
Xa39	FF329	SSR	102.9-85.7	Zhang *et al* (2015)
Xa40(t)	*O. sativa* ssp. indica	SSR	27.65	Kim *et al* (2015)
xa41(t)	*O. barthii and O. glaberrima*	TF	18.17	Hutin *et al* (2015)
xa42	XM14, a mutant of IR24	SSR	80.2	Busungu *et al* (2016)
Xa43(t)	P8 (Columbia XXI)	SNP	126	Kim & Reinke (2019)
xa44(t)	P6 (IR73571-3B-11-3- K3 /Ilpum)	SNP	160	Kim (2018)
xa45(t)	*O. glaberrima*	SNP, STS	202.9	Neelam *et al* (2019)

2.8 Expression analysis of BB resistance genes

The first BB resistant gene, *Xa1* conferring resistance to Japanese race 1 of *Xoo*, been isolated by map-based cloning. The amino-acid sequence of the gene revealed that it belongs to NBS-LRR family of R proteins. The RT-PCR analysis for *Xa1* revealed the induction of gene by wounding and pathogen inoculation in IRBB1, which was absent in T7 133 and IR24 (Yoshimura *et al* 1998). Iyer *et al* 2004 reported the use of RT-PCR analysis for the *Q94HL4* and *TFIIAγ* candidate genes for *xa5*. Both the genes were detected at all time points and in wounded controls. With no difference in the coding sequence of *Q94HL4* and similar expression for susceptible and resistant, this gene was demarcated for not a putative gene. *TFIIAγ* gene is considered putative for *xa5*.

Another study for the quantitative expression analysis of *xa7* analysed the three candidate genes using real-time fluorescent quantitative PCR technique confirming the expression of these gene in defence responses against bacterial blight (Jingluan *et al* 2012). The findings of Yang *et al* 2006, indicated that *Os8N3* is a host susceptibility gene for bacterial blight targeted by type III effector PthXo1. In year 2018, *xa44(t)* was identified as 120 kb segment harbouring 9 ORFs. Among the 9 ORFs, two ORFs revealed significant differential expression levels of the candidate genes (Os11g0690066 and Os11g0690466) (Kim *et al* 2018).

CHAPTER III

MATERIAL AND METHODS

The present study entitled, "Fine mapping of Bacterial Blight resistant gene from *Oryza glaberrima* Steud. and expression analysis of putative candidate genes in the region", was carried out at the School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, Punjab, India. A RIL population of F_6 and F_7 derived from the cross of *O. sativa* cv. Pusa 44 with an introgression line IL274 selected from the backcross population was applied for the fine mapping of *xa-45(t)* resistance gene following expression studies of putative candidate gene(s). The detailed information on the protocols followed, material, and tools used in the study, to meet the objectives of the research, are discussed under the following headings:

3.1 Site of experiment

All the experiments were performed in the laboratories and field of the School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana, India.

3.2 Plant material

In the study conducted so far, *O. glaberrima* accession IRGC102600B was observed to exhibit higher resistance against 7 *Xoo* pathotypes prevalent in Punjab (Vikal *et al*. 2007). Following the strategy, a stable and agronomically BC_3F_8 introgression line IL274 was developed that was used further for crossing with Pusa 44 for generating mapping populations from F_2 onwards (Figure 3.1).

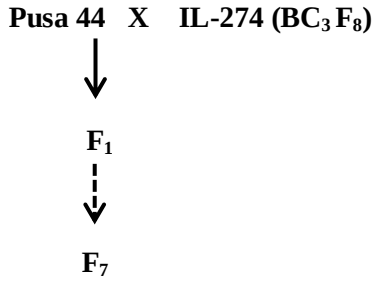


Figure 3.1: Crossing scheme for development of mapping population

3.3 Phenotypic evaluation

Screening of the population was practiced sequentially at experimental field area of School of Agricultural Biotechnology. The bacterial inoculations have proceeded following Kauffman's leaf-clipping method to evaluate the disease reaction of F_6 and F_7 populations during kharif 2019-2020 season.

3.3.1 Bacterial Inoculations

The isolates of *Xoo* strain PbXo-7 was utilized for the experiment. The strains were isolated from the ooze coming out of cut leaves that are reservoirs of specific bacterial strains. The ooze so obtained was streaked on Wakimoto media plates at 27-30°C. The colonies so obtained were further stored as stock at 4°C. *Xoo* isolates were revived on the same media at 30°C and growth was observed after 3 days. The bacterial colonies so obtained were suspended in sterile distilled water to a concentration of about 10^8 cell/ml to prepare the inoculum. The inoculum of *Xoo* was used immediately after preparation, to inoculate the population grown in the field. The inoculations were carried at the maximum tillering stage with the help of scissors following the leaf-clipping method (Kauffman *et al* 1973).

3.3.2 BB disease evaluation

Disease reactions of the population were evaluated 14 days post inoculations (Figure 3.2). The mean lesion length or SES qualitative score was used for phenotyping of the population. The data was recorded on the scale of 0-9 as in table (IRRI 2002) (Table 3.1)

Table 3.1: Disease scoring IRRI 2002

Score	Lesion area (%)	Reaction recorded
0	0	Highly Resistant (HR)
1	1-5	Resistant (R)
3	6-12	Moderately Resistant (MR)
5	13-25	Moderately Susceptible (MS)
7	26-50	Susceptible (S)
9	75	Highly Susceptible (HS)

A A B B C C D D E E F F

Figure 3.2: Disease score; A - 0, B - 1, C - 3, D - 5, E - 7, F - 9.

3.4 Molecular studies

The various protocols and procedures used for narrowing down the 80 kb region and expression analysis of the F_7 population are described below

3.4.1 DNA extraction

DNA of Pusa 44 (susceptible parent), IL274 (resistant parent), and F_6 population was extracted following the standard CTAB method (Saghai-Maroof *et al* 1984). Following the procedure, fresh young leaves of described plants were collected from field before inoculation of plants with *Xoo*. With the aid of liquid nitrogen, leaves were finely grounded using pestle-mortar. The finely crushed powder so formed was transferred to 2ml microcentrifuge tubes, to which 800 µl of CTAB was added. Followed by incubation of these tubes at 65^0C for 45 minutes in water bath. The components of CTAB buffer are mentioned in the table below.

Table 3.2: Components for extraction buffer (CTAB)

Component	Final concentration
CTAB	2.0%
Tris HCl (pH 8.0)	100mM
NaCl	1.4M
EDTA	20mM
Mercaptoethanol*	0.2%

*Mercaptoethanol was just added before using CTAB.

During the incubation period, the homogenate was slightly shaken in 15 minutes intervals to ensure complete mixing. After incubation of 45 minutes of, 800 µl of chloroform: isoamyl alcohol (24:1) solution was added to the homogenate. This was followed by shaking provided by a rotary shaker for 30 minutes until a dark green emulsion of organic phase was formed. The tubes were then placed in a fixed angle rotor centrifuge and were allowed to centrifuge at a minimum of 10,000 rpm for 15 minutes at room temperature. Following centrifugation, two phases are formed, an organic and another aqueous phase. The aqueous phase (supernatant) was then transferred to a 1.5ml microcentrifuge tube following RNase treatment with 5µl of RNase (10mg/ml) with incubation at 37ºC in water bath for half an hour. After the treatment, approximately half the volume of refrigerated isopropanol was added to the tubes, and tubes were gently inverted 2-3 times. The good quality DNA precipitate as a white cotton thread. To ensure appropriate precipitation of DNA, tubes were stored in -20 ºC refrigerator for 30-45 minutes. Then centrifugation was performed for the purpose of pelleting

down the precipitate which was then washed with 70% ethanol twice and air-dried for an hour by keeping the tubes in angular position on blotting paper. The DNA was finally dissolved in 100-200µl of 1X TE (Tris-EDTA buffer, 1mM EDTA, 10mM Tris HCl pH 8.0) buffer and kept at room temperature overnight and then stored at -20ºC after thorough mixing for further use.

3.4.2 Estimation of quantity and quality of DNA

Following DNA extraction, genomic DNA needs to be quantified to verify its intactness and purity. The crude genomic DNA was assessed using 0.8% Agarose gel electrophoresis and visualized in a gel documentation system (Biorad).

DNA quantity and quality was estimated by two methods:

a) Gel Electrophoresis

b) Nanodrop method

3.4.2 a) Gel electrophoresis method

To assess the quality, quantity, and integrity, the DNA was analysed on 0.8% agarose gel. The preparation of 0.8% Agarose gel involves mixing of 4 gm of Agarose powder in 0.5X TBE buffer (500ml) in a flask. The gel solution was heated in microwave oven for 8-10 minutes till a clear gel is obtained. The gel was allowed to cool down by gentle stirring and 25µl ethidium bromide (5µl/100ml) was added to agarose gel solution. The agarose gel so formed was then poured in the gel casting mould having combs and stoppers. The gel was then allowed to solidify for 45-60 minutes at room temperature.

For quantitation purpose, 2µl of each DNA sample was mixed with 8µl of 1X loading dye (Bromophenol Blue prepared by adding 500µl loading dye of 6X consisting of 0.4% w/v sucrose or 30% glycerol and 0.25% w/v bromophenol blue in 100µl of sterile water). Using a micropipette, the samples were loaded into the wells of gel and were allowed to run for about 1-2 hours at a constant voltage of 5V/cm. The visualization of the gel was done under a UV transilluminator using photo gel documentation system and photograph was captured. The concentration of each DNA sample was determined by comparison of width and intensity of each band with a known concentration band (λ DNA) loaded in the gel. The DNA sample quality was estimated based on whether the DNA formed a single high molecular weight band (good quality) or a smear (degraded/ poor quality).

Based upon both the methods of quantification, normalization of the DNA sample was done to the concentration of 400ng/µl by the addition of an adequate quantity of 1X TE buffer. The stock DNA was further diluted to a concentration of 25ng/µl with sterile ddH_2O for further dilution.

3.4.2 b) Nanodrop method

For assessing the DNA purity, nanodrop method involves the determination of the absorbance ratio at 260/280 nm. A ratio of ~1.8 pertains to pure DNA sample. In case the ratio is lower in either case, it indicates the protein, phenol, or other contaminants that absorb strongly at or near 280 nm. The DNA quantity in different samples varied from 100-2000 ng/µl. Further, dilutions to 50 ng/µl DNA were made that were used for PCR analysis.

3.4.3 Designing gene-specific overlapping primers

The experiments were initialized by designing locus-specific primers for the 9 candidate genes using Primer 3 software keeping the parameters to default. Before primer designing the genomic sequences of candidate genes were evaluated and downloaded from the Nipponbare reference genome available at the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/). The primers were designed for full-length gene amplification using overlapping primers specifically producing 1 kb amplicons (Table 3.3).

Table 3.3: Gene-specific overlapping primers

Candidate genes	Genomic sequence size (bp)	Number of overlapping primers
LOC_Os08g42360	1547	2
LOC_Os08g42370	3692	4
LOC_Os08g42380	1078	1
LOC_Os08g42390	3195	3
LOC_Os08g42400	3530	3
LOC_Os08g42410	5318	5
LOC_Os08g42420	5607	4
LOC_Os08g42430	685	2
LOC_Os08g42440	4358	5

3.4.4 PCR analysis

The overlapping primers so designed were used for PCR amplification of Pusa 44 (susceptible parent) and IL274 (resistant parent). A PCR reaction of a higher volume of 20-50 µl was carried out for sequencing purposes (Table 3.4). Also, the PCR reaction involved use of a hi-fidelity *Taq polymerase* Extaq (Takara) which is feasible for providing better sequencing results (Figure 3.3). The reactions involved duplicates for obtaining stringent sequencing data.

Table 3.4: PCR reaction for overlapping primers

Components of PCR reaction	Volume
Parental DNA	10 µl
Forward primer	5 µl
Reverse primer	5 µl
$MgCl_2$	0.5 µl
PCR Buffer	6 µl
dNTPs	6 µl
Extaq	0.5 µl
Nuclease-free H_2O	17 µl

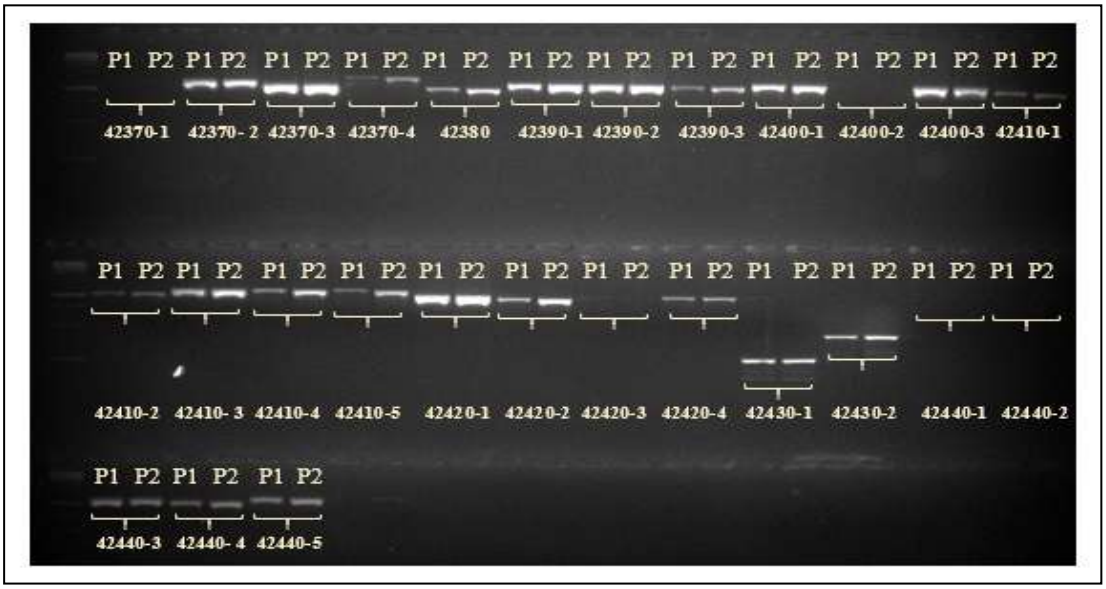


Figure 3.3: Representative picture of standardization of PCR conditions of all gene-specific overlapping primers; P1-Pusa 44 P2- IL 274

3.4.5 Sequencing analysis

The amplicons so obtained from overlapping primers were used for outsourcing sequencing analysis from Agrigenome. The results were obtained as AB1 files and chromatograms. The chromatograms were used to manually check the quality of peaks and the accuracy of data. While the ABI files included the sequences, these files were first used for aligning each overlapping primer of a single gene to fetch a continuous stretch of sequence. From these full-length gene sequences, the overlapping regions in between different primers were removed.

These alignments were carried out using DNA Baser Assembler (Figure 3.4). First of all, the forward and reverse sequences of a single primer were assembled to generate one

sequence for each primer. It was followed by combining such sequences for all the primers of a gene. This way contigs were generated for all the amplicon sequences. All these contigs of candidate genes were used for finding the variations among Pusa 44 and IL274. This comparison was carried out using CLUSTAL X offline tool to identify the putative SNPs and Indels for all the loci.

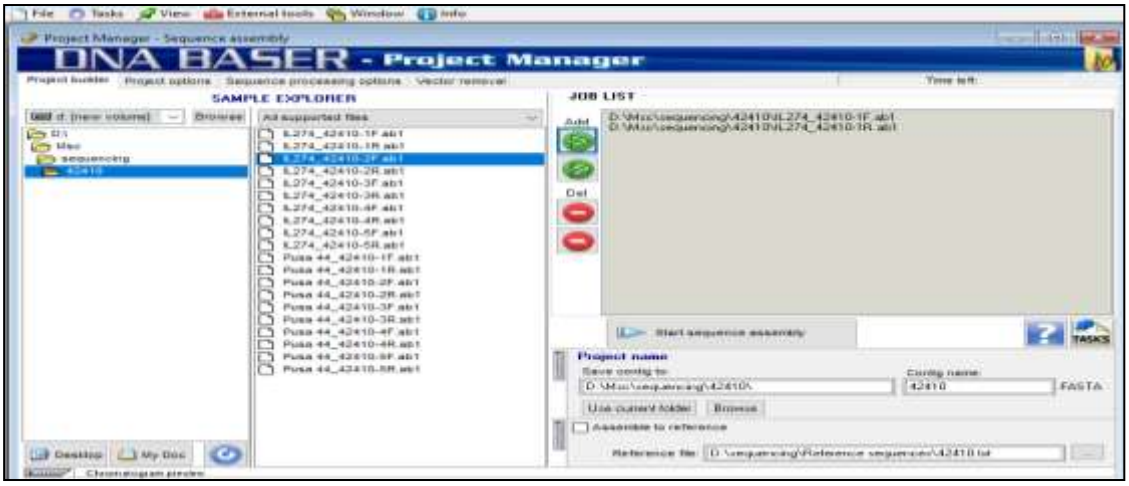


Figure 3.4: Window of DNA baser tool and the input command

3.4.6 dCAPs primer designing

The candidate SNPs identified, were further used for designing dCAPs markers using an online tool dCAPs finder 2.0. The genomic sequence of two haplotypes which was identical except for the SNP was used for designing the primers. The input required here was, the SNP in between the sequence with approximately 25 nucleotides on each side (Figure 3.5). And this sequence was filled in the boxes provided for adding wild and mutant sequences, where Pusa 44 was considered as wild haplotype while IL274 as mutant.

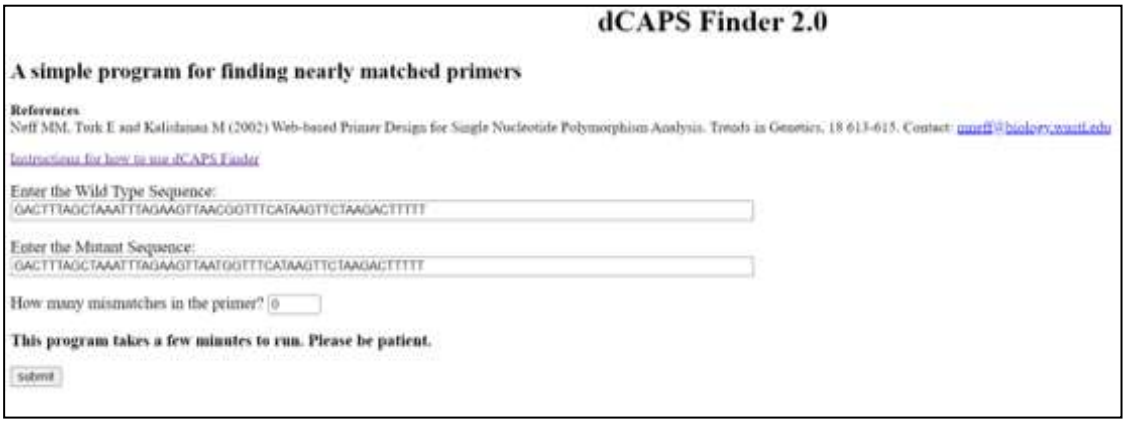


Figure 3.5: Input window of dCAPs Finder 2.0 online tool

For designing the primers, initially, the output from zero mismatches shows whether a CAPS marker is present or not. If CAPS marker was not obtained, mismatch was set to 1 to

search for a dCAPs marker. Further, the number of mismatches was increased in each run until a potential dCAPs marker has been identified. The figure represents an output window, where the sequences of forward and reverse primers are displayed. Also, the existing restriction sites and their respective enzymes are stated in the result window of the software (Figure 3.6).

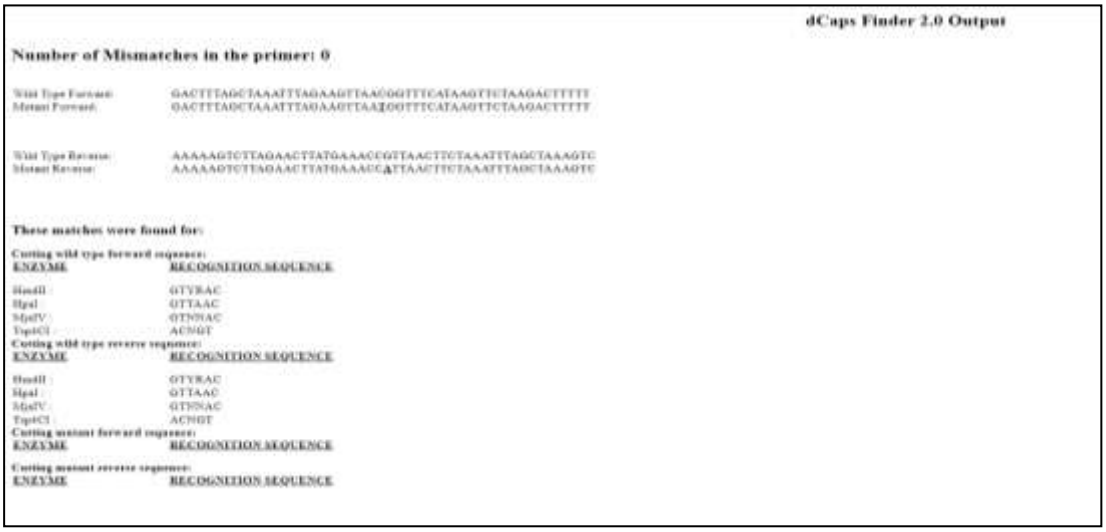


Figure 3.6: Output window of dCAPs Finder 2.0 online tool

3.4.7 dCAPs analysis and digestion protocol

Primer designing was followed by dCAPs analysis for the genotyping of population. First of all, the PCR conditions were standardized for dCAPs markers. The amplicons were first confirmed by agarose gel electrophoresis. Following the confirmation, the amplified products were then digested using a suitable enzyme along with a buffer owing to 100% activity of the enzyme and making up the volume with water (Table 3.5). The reaction set up was then incubated at optimal temperature and time in respect to the particular enzyme used.

After the digestion protocol, the digested product so obtained was analyzed by agarose gel electrophoresis methodology, using 2% agarose gel. The results so obtained were used for genotyping of the population.

Table 3.5: Reaction set up for dCAPs digestion

Components	Volume
Amplified product	5 µl
Specific Enzyme	1 µl
Nuclease-free H_2O	4 µl

3.4.8 RNA extraction

RNA of susceptible parent (Pusa 44) and resistant parent (IL274) was extracted manually. For the purpose, all microcentrifuge tubes, tips, pestle-mortars and glassware were

DEPC (Diethyl pyrocarbonate) treated prior to extraction. 1X concentration of DEPC was prepared for the purpose, by adding 1 ml of DEPC in 1 litre of autoclaved distilled water which was allowed to stand overnight at 37 °C. All the plasticwares, pestle-mortar and glassware were immersed in DEPC water overnight at 37 °C. The treatment was followed by filling the tips in tip boxes while microcentrifuge tubes were stored in jam jars. Tip boxes, jam jars and pestle-mortar were kept in hor air oven at 80°C for overnight baking which results in complete drying of moisture. After drying pestle-mortar were then wrapped in foil. Thereafter, all the material was autoclaved at 121°C for 20 minutes and complete for sterilization was achieved for RNA isolation. The intact total RNA, free from DNA and protein contaminants was isolated using TRIzol reagent (Takara) with following steps:

1. The frozen tissue samples were crushed into a fine powder using liquid nitrogen (LN_2) in pestle-mortar.
2. The fine powdered tissue was then transferred to DEPC treated 2 ml microcentrifuge tubes.
3. 1 ml of TRIzol reagent was added to each tube and it was allowed to stand for 5 minutes at room temperature.
4. To this homogenized solution, 200 µl of chloroform was added followed by vigorous mixing or light vortexing until the solution becomes milky.
5. The solution was kept still for 5 minutes at room temperature followed by its centrifugation at 12,000xg for 15 minutes at 4°C specifically.
6. The solution was then separated into three layers: top aqueous RNA containing layer, middle semisolid containing mostly DNA, and bottom organic solvent layer. This phase separation step was crucially performed by keeping tubes in ice. The aqueous layer was carefully transferred to 1.5 ml microcentrifuge tubes without disturbing the other layers.
7. An equal volume of isopropanol was added to the aqueous extract and was mixed well by inverting the tubes for 15-20 seconds. Let the mixture stand at room temperature for 10 minutes.
8. After 10 minutes, it was centrifuged at 12,000xg for 10 minutes at 4°C to get the precipitate of total RNA.
9. After centrifugation, the supernatant was discarded carefully without disturbing the RNA pellet.
10. The RNA pellet was washed twice with chilled 75% ethanol which was prepared using autoclaved DEPC water. The tubes were kept for centrifugation at 12,000xg for 10 minutes (4°C).
11. After removing and discarding the excess ethanol, the pellet was allowed to air-dry at room temperature for a short period.

12. This pellet was resuspended in autoclaved DEPC water following gentle tapping. All the extracted RNA was stored at -80°C till further usage.

3.4.9 Estimation of quantity and quality of RNA

Following the extraction protocol, the RNA was quantified to check its purity as well as intactness.

RNA quantity and quality were estimated by two methods:

a) Nanodrop method

b) Gel Electrophoresis

a) Nanodrop method

RNA quality was analysed by Thermo scientific NanoDrop™ 1000 spectrophotometer. The absorbance ratio at 260/280 nm was used to determine the RNA purity. A ratio in the range of 1.7- 2.1 depicts adequately good quality RNA. The deviation from this ratio signifies the presence of other contaminants that absorb strongly at or near 280 nm. The RNA quantity varied from 600-1000 ng/µl in different samples. Further, serial dilutions of RNA were prepared made for RT-PCR analysis.

b) Gel electrophoresis method

It is essential to confirm the quality, concentration, and integrity of isolated RNA by agarose gel electrophoresis. Before gel preparation, all the components including the flask, gel tank, casting tray, stoppers, and combs were rinsed with 0.5% SDS (Sodium Dodecyl Sulfate) followed by washing with DEPC treated water. 1.2% denaturing gel was prepared, by dissolving 1.2 gm of agarose powder in 100 ml of 1X MOPS [3-(N-Morpholino) propane sulfonic Acid] buffer by mixing 10 ml MOPS with 90 ml autoclaved DEPC water in a flask (Table 3.6). The mixture was allowed to heat in a microwave until clear gel was obtained. Further, it was slightly cooled by gentle stirring and 1.78 ml formaldehyde and 5 µl EtBr was added to obtain a denaturing RNA gel. This denaturing agarose gel was poured into gel casting tray prepared with combs and stoppers. The agarose gel was then allowed to solidify for 40-50 minutes at room temperature.

For quantification, 5µl of each RNA sample was mixed with 5 µl of 50% glycerol (prepared in DEPC water). These RNA samples were loaded into the gel kept in electrophoresis tank containing 1 X MOPS running buffer. Also, rapid mixing and loading were practised to avoid degradation of samples. It was allowed to run for about 10-15 minutes at 110 volts. The gel was visualized under UV transilluminator using photo gel documentation system and photograph of RNA samples was taken. The total RNA appropriately denatured is visualised as ribosomal RNA (28S and 18S) in 2:1 ratio. The quality of RNA was judged on the basis of three

distinct bands on gel. Based upon the two methods of quantification, normalization of the RNA sample was done to the concentration of 50ng/µl by adding adequate quantity of nuclease-free water/DEPC treated water. The stock RNA was stored at -80°C for further use.

Table 3.6: Composition of 10X MOPS Buffer

Components	Composition
MOPS (200 mM)	41.9 g
Sodium Acetate (50 mM)	8.2 g
EDTA (10 mM)	3.72 g
Double distilled water	To make 1 litre volume

3.4.10 cDNA synthesis

From the normalised RNA samples, cDNA was synthesised using PrimeScript 1st strand cDNA synthesis kit (Takara). Following the protocol, first strand cDNA synthesis was carried out using various components of kit (Table 3.7).

Primarily, template-primer mix was prepared by adding 2µl template RNA, 1µl Oligo dT primer, 1 µl dNTP mix, and 6 µl RNase free water followed by its incubation at 65°C for 5minutes and immediate cooling on ice (Table 3.8).

Table 3.7: Components of Prime Script 1st strand cDNA synthesis kit (Takara)

Kit components	Total volume
PrimeScript RT (200 U/µl)	50 µl
5X PrimeScript Buffer	200 µl
RNase Inhibitor (40 U/µl)	25 µl
dNTP Mixture (10 mM each)	50 µl
Oligo dT Primer (50 µM)	50 µl
Random 6 mers (50 µM)	100 µl
RNase free H_2O	1 ml

Table 3.8: Preparation of Template RNA-Primer mix

Components	Volume
Oligo dT Primer (50 µM)	1 µl
dNTP Mixture (10 mM each)	1 µl
Template RNA	2 µl
RNase-free dH_2O	6 µl

After 5 minutes of incubation, 4µl 5X primer script buffer, 0.5µl RNase inhibitor, 1µl PrimeScript RT, and 4.5µl RNase-free water was added to 10µl template-primer mixture and 20µl reaction was prepared. It was further incubated at 42-50°C for 30-60 minutes followed by its heating at 70°C for 15 minutes (Table 3.9). The resulting product was cooled on ice and stored at -20°C for RT-PCR assay.

Table 3.9: 1st strand cDNA synthesis

Components	Volume
Template RNA/Primer Mixture	10 µl
5X PrimeScript Buffer	4 µl
RNase Inhibitor	20 Units
PrimeScript RT	100 to 200 Units
RNase-free dH_2O	to 20 µl

3.4.11 cDNA quantification and confirmation

cDNA quantification and confirmation are essential for proceeding for expression analysis to ensure sample normalisation -

a) Quantification using nanodrop

b) Confirmation using house-keeping genes

a) cDNA Quantification -

DNA synthesized for all the samples were quantified using nanodrop method using standard ratios as in case of DNA purity assessment. Using these nanodrop values, cDNA was further normalised to 50ng/µl for each sample.

b) cDNA Confirmation -

From the normalised cDNA samples, confirmation of intact cDNA was practiced using house-keeping gene specific primer actin. The PCR reaction was set up for 10µl reaction volume each containing the reagents as given in Table 3.10. The following temperature profile (Table 3.11) was used for amplification in thermocycler. The amplified products were stored for short period at 4ºC and -20ºC for longer time. For analysis, 2% agarose gel was prepared by mixing 2 gm agarose in 0.5X TBE (Tris Borate EDTA) buffer. In this EtBr-stained agarose gel, the PCR products were loaded and then the gel electrophoresis was performed at constant voltage of 5V/cm. After 1hour, gel was visualized under UV trans-illuminator, the cDNA amplicon of 67 bp confirmed the presence and intactness of cDNA for each sample.

Table 3.10: PCR reaction for cDNA confirmation

Components of PCR reaction	Volume
cDNA	2 µl
Forward primer	1 µl
Reverse primer	1 µl
$MgCl_2$	0.4 µl
PCR Buffer	2 µl
dNTPs	2 µl
Taq polymerase	0.15 µl
Nuclease-free H_2O	1.4 µl

Table 3.11: PCR profile for amplification using Actin primer

Steps	Temperature	Time	Cycles
Initial denaturation	94ºC	5 minutes	1 cycle
Denaturation	94ºC	1 minute	35 cycles
Annealing	61ºC	1 minute	
Elongation	72ºC	1 minute	
Final extension	72ºC	7 minutes	1 cycle
Hold/Pause	4ºC	-	

3.4.12 Relative expression analysis

For the qRT-PCR, gene specific primers for the candidate genes were designed using Perl primer tool. The input sequence includes the genomic sequence entry on one side and mRNA sequence entry on the other. The primers are then selected among the displayed output according to the primer dimer conditions and specifications (Table 3.12). The primers designed for qRT-PCR comprises of an amplicon size within the range of 80-220 bp. Before cDNA confirmation, serial dilutions of cDNA were prepared uptil 3ng/µl concentration. Relative expression of all the candidate gene at 0, 8, 24, 48, and 72 hrs post inoculation with *Xoo* pathotype-7 was analysed using *Actin* as an internal control. This analysis was performed using 96-well Step One Applied Biosystem RT-PCR. The reaction consists of cDNA at 3ng/µl concentration as template along with applied biosystem SYBR green and respective gene-specific RT primers. Total reaction composition was set to 10µl, with 2µl of (3ng/µl) cDNA, 1µl (0.25 µM) primers, 4µl 1X SYBR and 2µl of nuclease-free water. The PCR conditions were: 94ºC for 3 minutes, followed by 45 cycles for 30 seconds at 94ºC, 42 seconds at 61ºC, 30 seconds at 72ºC.

Table 3.12: Real time PCR gene specific primers

Candidate gene	Forward Primer	Reverse Primer
RT_Os08g42370	GTATTTGCTTTCTGTTGGGTG	CTCATAAGTTGTCTGGTTGGT
RT_Os08g42380	GATTCATCGAGTTCACAGCTG	GATCACGCTCATGTCAAGGA
RT_Os08g42390	GCAAGTTGTTTCTCAGTATGC	GAAGAGTACAGGATATAAGCTCTG
RT_Os08g42400	GGACAAGTACTTCTTCGCCG	CAAGCAACCCTTCTTGTGCA
RT_Os08g42410	TGCATGTCTGTTGTAGAGGA	CAATGTCCTCAACCTGTGGA
RT_Os08g42420	AAAGTCGCTTACGGATCCCT	GCATGAAAGTCTTGGTCCCTG
RT_Os08g42430	GTGGAGCTCGAATCGCAGGA	TTCACCGCCACGAAGCCAAT
RT_Os08g42440	TACACTTCACTGCTCATGATGG	AAATCCCATATCTGGGTTGGT

3.4.13 Statistical analysis

Following the reaction, results were analysed by $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). The relative expression of target genes was normalized to reference gene expression for each sample. This methodology follows:

- ΔCt = Ct mean (target gene) - Ct mean (reference gene)
- $\Delta\Delta Ct = \Delta Ct$ (target sample) – ΔCt (control sample)
- $2^{-(\Delta\Delta Ct)}$ represents fold change in gene expression in stress conditions relative to the control conditions (Relative gene expression)

CHAPTER IV

RESULTS AND DISCUSSION

The present research work was conducted to fine map the BB resistance locus *xa-45(t)* identified from *O. glaberrima* (IRGC102600B) using stable RIL population obtained from a cross between Pusa 44 (susceptible parent) and IL274 (resistant introgressed line from *O. glaberrima*). The novel recessive resistance locus *xa-45(t)* was transferred to *O. sativa* and mapped on the long arm of chromosome 8 using ddRAD sequencing approach (Neelam *et al* 2019). The identified QTL spans 80 kb region on Nipponbare reference genome IRGSP-1.0 harbouring 9 candidate genes. Experiment 1 was aimed for the fine mapping of identified QTL using various molecular approaches. Experiment 2 was aimed to analyze the expression patterns of putative candidate genes responsible for providing resistance against BB. The results obtained during the present investigation have been discussed under the following headings:

4.1. Fine mapping of *xa-45(t)*

4.1.1 Phenotypic evaluation

In this study, F_6 and F_7 were raised and utilized for phenotyping. Total of 290 plants, were screened against the *Xoo* pathotypes PbXo-7 and PbXo-8 in the field of Agricultural Biotechnology for the constitutive years 2019 and 2020. The leaf blades of parents and population were inoculated at maximum tillering stage by the leaf clipping method (Kauffman *et al* 1973). The disease reaction of parental lines along with the population was evaluated after 14 days of inoculation, following the guidelines of Standard Evaluation System for Rice (IRRI 1988) and disease reaction was recorded (Figure 4.1, Table 4.1).

Figure 4.1: Scoring of bacterial blight disease reaction after 14 days of inoculation with PbXo-7 P1 - Pusa 44; P2 - IL 274; R - Resistant plants; S - Susceptible plant

Table 4.1: Mean phenotypic data of F_6 and F_7 RIL Population

ID	Pedigree	PbXo-7	PbXo-8	ID	Pedigree	PbXo-7	PbXo-8
1	**Pusa 44**	9	9	**38**	3581-41-3-3-3-1	7	7
2	**IL274**	0	3	**39**	3581-42-3-3-3-1	7	7
3	3581-1-3-3-3-3	1	1	**40**	3581-43-3-3-3-1	5	7
4	3581-2-3-3-3-3	5	1	**41**	3581-44-3-3-3-1	5	3
5	3581-3-3-3-3-3	0	7	**42**	3581-45-3-3-3-1	5	9
6	3581-7-3-3-3-3	5	5	**43**	3581-47-3-3-3-1	1	5
7	3581-8-3-3-3-3	7	7	**44**	3581-48-3-3-3-1	1	9
8	3581-9-3-3-3-3	1	9	**45**	3581-49-3-3-3-1	1	9
9	3581-10-3-3-3-3	7	9	**46**	3581-50-3-3-3-1	5	3
10	3581-11-3-3-3-1	1	9	**47**	3581-51-3-3-3-2	5	7
11	3581-13-3-3-3-1	3	1	**48**	3581-52-3-3-3-1	5	7
12	3581-14-3-3-3-1	3	3	**49**	3581-53-3-3-3-1	1	9
13	3581-15-3-3-3-1	1	3	**50**	3581-54-3-3-3-1	1	7
14	3581-16-3-3-3-2	1	9	**51**	3581-55-3-3-3-1	9	9
15	3581-17-3-3-3-1	1	1	**52**	3581-56-3-3-3-1	1	5
16	3581-18-3-3-3-1	1	7	**53**	3581-57-3-3-3-3	7	9
17	3581-19-3-3-3-1	1	7	**54**	3581-58-3-3-3-1	1	5
18	3581-20-3-3-3-1	1	1	**55**	3581-59-3-3-3-1	1	9
19	3581-21-3-3-3-1	1	9	**56**	3581-60-3-3-3-8	1	7
20	3581-22-3-3-3-1	7	9	**57**	3581-61-3-3-3-1	9	9
21	3581-23-3-3-3-1	1	5	**58**	3581-62-3-3-3-1	1	9
22	3581-24-3-3-3-1	7	7	**59**	3581-63-3-3-3-1	7	9
23	3581-25-3-3-3-1	5	7	**60**	3581-64-3-3-3-1	1	3
24	3581-27-3-3-3-1	9	9	**61**	3581-65-3-3-3-1	7	7
25	3581-28-3-3-3-1	5	7	**62**	3581-66-3-3-3-1	5	3
26	3581-29-3-3-3-2	3	5	**63**	3581-67-3-3-3-1	0	3
27	3581-30-3-3-3-1	9	9	**64**	3581-68-3-3-3-1	1	5
28	3581-31-3-3-3-1	0	1	**65**	3581-69-3-3-3-1	1	9
29	3581-32-3-3-3-1	5	7	**66**	3581-70-3-3-3-1	5	9
30	3581-33-3-3-3-1	1	1	**67**	3581-71-3-3-3-1	7	7
31	3581-34-3-3-3-1	0	1	**68**	3581-72-3-3-3-1	5	3
32	3581-35-3-3-3-1	1	5	**69**	3581-74-3-3-3-1	1	9
33	3581-36-3-3-3-1	5	9	**70**	3581-75-3-3-3-1	5	9
34	3581-37-3-3-3-1	5	9	**71**	3581-76-3-3-3-1	5	1
35	3581-38-3-3-3-1	5	1	**72**	3581-77-3-3-3-1	3	7
36	3581-39-3-3-3-1	7	7	**73**	3581-78-3-3-3-1	9	9
37	3581-40-3-3-3-1	1	9	**74**	3581-79-3-3-3-3	3	3

Table 4.1: Contd..

ID	Pedigree	PbXo-7	PbXo-8	ID	Pedigree	PbXo-7	PbXo-8
75	3581-80-3-3-3-1	7	9	**112**	3581-117-3-3-3-1	1	9
76	3581-81-3-3-3-1	5	7	**113**	3581-118-3-3-3-3	5	9
77	3581-82-3-3-3-1	5	1	**114**	3581-119-3-3-3-1	1	9
78	3581-83-3-3-3-1	5	1	**115**	3581-120-3-3-3-1	5	5
79	3581-84-3-3-3-1	1	3	**116**	3581-121-3-3-3-1	7	9
80	3581-85-3-3-3-1	5	5	**117**	3581-122-3-3-3-1	5	5
81	3581-86-3-3-3-1	5	5	**118**	3581-123-3-3-3-1	1	7
82	3581-87-3-3-3-1	0	7	**119**	3581-124-3-3-3-1	1	7
83	3581-88-3-3-3-1	0	1	**120**	3581-125-3-3-3-1	0	1
84	3581-89-3-3-3-1	0	1	**121**	3581-126-3-3-3-1	1	9
85	3581-90-3-3-3-1	7	7	**122**	3581-127-3-3-3-1	1	1
86	3581-91-3-3-3-1	5	7	**123**	3581-128-3-3-3-1	5	9
87	3581-92-3-3-3-1	1	7	**124**	3581-129-3-3-3-1	1	1
88	3581-93-3-3-3-1	0	1	**125**	3581-130-3-3-3-1	1	1
89	3581-94-3-3-3-6	7	7	**126**	3581-131-3-3-3-1	5	9
90	3581-95-3-3-3-3	0	7	**127**	3581-132-3-3-3-1	5	1
91	3581-96-3-3-3-1	5	7	**128**	3581-133-3-3-3-1	1	1
92	3581-97-3-3-3-1	0	3	**129**	3581-134-3-3-3-1	1	1
93	3581-98-3-3-3-1	5	1	**130**	3581-135-3-3-3-1	7	7
94	3581-99-3-3-3-1	5	7	**131**	3581-136-3-3-3-1	0	1
95	3581-100-3-3-3-1	5	7	**132**	3581-137-3-3-3-1	7	7
96	3581-101-3-3-3-1	5	7	**133**	3581-138-3-3-3-1	9	9
97	3581-102-3-3-3-1	7	7	**134**	3581-139-3-3-3-1	5	7
98	3581-103-3-3-3-1	9	9	**135**	3581-140-3-3-3-1	5	7
99	3581-104-3-3-3-1	7	7	**136**	3581-141-3-3-3-1	1	9
100	3581-105-3-3-3-1	5	5	**137**	3581-142-3-3-3-1	7	7
101	Pusa 44	7	9	**138**	3581-143-3-3-3-1	1	3
102	3581-107-3-3-3-1	7	7	**139**	3581-144-3-3-3-1	1	3
103	3581-108-3-3-3-1	3	3	**140**	3581-145-3-3-3-1	5	3
104	3581-109-3-3-3-1	1	7	**141**	3581-146-3-3-3-1	7	7
105	3581-110-3-3-3-1	1	9	**142**	3581-147-3-3-3-1	7	7
106	3581-111-3-3-3-1	0	1	**143**	3581-148-3-3-3-1	5	5
107	3581-112-3-3-3-1	1	1	**144**	3581-149-3-3-3-1	7	7
108	3581-113-3-3-3-1	7	9	**145**	3581-150-3-3-3-1	7	9
109	3581-114-3-3-3-1	5	3	**146**	3581-151-3-3-3-1	7	5
110	3581-115-3-3-3-1	1	3	**147**	3581-152-3-3-3-1	1	1
111	3581-116-3-3-3-1	5	9	**148**	3581-154-3-3-3-1	5	1

Table 4.1: Contd..

ID	Pedigree	PbXo-7	PbXo-8	ID	Pedigree	PbXo-7	PbXo-8
149	3581-155-3-3-3-1	1	3	**186**	3581-194-3-3-3-1	5	7
150	3581-156-3-3-3-1	1	3	**187**	3581-195-3-3-3-4	7	7
151	3581-157-3-3-3-2	1	5	**188**	3581-196-3-3-3-1	5	5
152	3581-158-3-3-3-1	5	9	**189**	3581-197-3-3-3-1	0	1
153	3581-159-3-3-3-2	9	9	**190**	3581-198-3-3-3-1	1	1
154	3581-160-3-3-3-1	5	3	**191**	3581-199-3-3-3-1	1	7
155	3581-162-3-3-3-1	5	3	**192**	3581-200-3-3-3-1	1	5
156	3581-163-3-3-3-1	5	3	**193**	*O. glaberrima*	0	3
157	3581-164-3-3-3-1	1	7	**194**	3581-201-3-3-3-1	1	7
158	3581-165-3-3-3-1	1	1	**195**	3581-202-3-3-3-9	5	7
159	3581-166-3-3-3-1	5	5	**196**	3581-203-3-3-3-1	1	7
160	3581-167-3-3-3-1	5	5	**197**	3581-204-3-3-3-1	5	5
161	3581-169-3-3-3-1	5	5	**198**	3581-205-3-3-3-8	3	1
162	3581-170-3-3-3-1	1	1	**199**	3581-206-3-3-3-1	7	7
163	3581-171-3-3-3-1	7	7	**200**	3581-207-3-3-3-1	7	1
164	3581-172-3-3-3-1	5	5	**201**	Pusa 44	7	7
165	3581-173-3-3-3-1	1	1	**202**	3581-208-3-3-3-2	0	5
166	3581-174-3-3-3-1	1	1	**203**	3581-209-3-3-3-1	3	3
167	3581-175-3-3-3-1	5	5	**204**	3581-210-3-3-3-1	0	1
168	3581-176-3-3-3-1	7	1	**205**	3581-212-3-3-3-1	1	1
169	3581-177-3-3-3-1	7	5	**206**	3581-213-3-3-3-1	M	5
170	3581-178-3-3-3-1	9	7	**207**	3581-214-3-3-3-1	M	1
171	3581-179-3-3-3-1	5	1	**208**	3581-215-3-3-3-1	3	7
172	3581-180-3-3-3-1	1	1	**209**	3581-216-3-3-3-1	5	5
173	3581-181-3-3-3-1	1	7	**210**	3581-217-3-3-3-1	1	5
174	3581-182-3-3-3-1	1	1	**211**	3581-218-3-3-3-1	5	5
175	3581-183-3-3-3-1	5	1	**212**	3581-219-3-3-3-1	0	1
176	3581-184-3-3-3-1	1	7	**213**	3581-220-3-3-3-1	0	5
177	3581-185-3-3-3-1	1	5	**214**	3581-221-3-3-3-1	7	7
178	3581-186-3-3-3-1	1	1	**215**	3581-222-3-3-3-1	5	7
179	Pusa 44	7	7	**216**	3581-223-3-3-3-1	3	7
180	3581-188-3-3-3-1	1	1	**217**	3581-224-3-3-3-1	1	1
181	3581-189-3-3-3-1	0	1	**218**	3581-225-3-3-3-1	7	3
182	3581-190-3-3-3-1	5	3	**219**	3581-226-3-3-3-1	5	5
183	3581-191-3-3-3-1	1	7	**220**	3581-227-3-3-3-1	1	1
184	3581-192-3-3-3-1	1	7	**221**	3581-228-3-3-3-1	5	7
185	3581-193-3-3-3-1	1	1	**222**	3581-229-3-3-3-1	1	1

Table 4.1: Contd..

ID	Pedigree	PbXo-7	PbXo-8	ID	Pedigree	PbXo-7	PbXo-8
223	3581-230-3-3-3-1	1	1	**262**	3581-271-3-3-3-1	5	5
224	3581-231-3-3-3-1	3	7	**263**	3581-272-3-3-3-1	1	3
225	3581-232-3-3-3-1	1	1	**264**	3581-273-3-3-3-1	1	1
226	3581-233-3-3-3-1	1	1	**265**	3581-274-3-3-3-5	1	1
227	3581-234-3-3-3-1	7	7	**266**	3581-275-3-3-3-2	1	1
228	3581-235-3-3-3-1	1	1	**267**	3581-276-3-3-3-1	1	5
229	3581-236-3-3-3-1	1	3	**268**	3581-277-3-3-3-1	1	1
230	3581-237-3-3-3-1	1	5	**269**	3581-278-3-3-3-1	7	1
231	3581-238-3-3-3-1	1	1	**270**	3581-279-3-3-3-1	7	1
232	3581-239-3-3-3-1	1	1	**271**	3581-280-3-3-3-1	5	5
233	3581-240-3-3-3-1	1	1	**272**	3581-281-3-3-3-1	7	1
234	3581-242-3-3-3-1	5	1	**273**	3581-282-3-3-3-1	7	3
235	3581-243-3-3-3-1	7	9	**274**	3581-283-3-3-3-1	0	1
236	3581-244-3-3-3-1	1	5	**275**	3581-284-3-3-3-1	1	1
237	3581-245-3-3-3-1	5	1	**276**	3581-285-3-3-3-1	5	1
238	3581-246-3-3-3-1	1	1	**277**	3581-286-3-3-3-1	7	1
239	3581-247-3-3-3-1	7	1	**278**	3581-287-3-3-3-1	3	1
240	3581-248-3-3-3-1	1	1	**279**	3581-288-3-3-3-1	3	3
241	3581-249-3-3-3-1	1	1	**280**	3581-289-3-3-3-1	1	1
242	3581-250-3-3-3-1	1	3	**281**	3581-290-3-3-3-1	1	1
243	3581-251-3-3-3-1	1	7	**282**	3581-291-3-3-3-1	5	1
244	3581-252-3-3-3-1	7	7	**283**	3581-292-3-3-3-1	1	1
245	3581-253-3-3-3-1	1	1	**284**	3581-293-3-3-3-1	7	7
246	3581-254-3-3-3-1	5	1	**285**	3581-294-3-3-3-1	1	5
247	3581-255-3-3-3-1	1	1	**286**	3581-295-3-3-3-1	3	5
248	3581-256-3-3-3-1	0	0	**287**	3581-296-3-3-3-1	3	1
249	3581-257-3-3-3-1	0	0	**288**	3581-297-3-3-3-2	5	1
250	3581-258-3-3-3-1	1	1	**289**	Pusa 44	9	7
251	3581-259-3-3-3-1	1	7	**290**	3581-299-3-3-3-1	5	3
252	3581-261-3-3-3-1	7	7	**291**	3581-300-3-3-3-1	3	3
253	3581-262-3-3-3-1	1	1	**292**	3581-301-3-3-3-1	1	1
254	3581-263-3-3-3-1	5	5	**293**	3581-302-3-3-3-1	1	1
255	3581-264-3-3-3-1	3	7	**294**	3581-303-3-3-3-1	3	3
256	3581-265-3-3-3-1	1	3	**295**	3581-304-3-3-3-2	1	1
257	3581-266-3-3-3-1	1	7	**296**	3581-305-3-3-3-1	0	7
258	3581-267-3-3-3-1	1	1	**297**	3581-306-3-3-3-1	1	1
259	3581-268-3-3-3-1	1	3	**298**	3581-307-3-3-3-2	5	5
260	3581-269-3-3-3-2	5	5	**299**	3581-308-3-3-3-1	1	1
261	3581-270-3-3-3-1	5	5	**300**	3581-309-3-3-3-1	7	5

***ID - Population ID; *Xoo* pathotypes PbXo-7, 8 - Mean Disease score (0-9)**

4.1.2 Inheritance Study

The phenotypic evaluation of the mean data of F_6 and F_7 disease reaction represents 1:1 segregation ratio of the bacterial blight recessive gene *xa-45(t)*. The score of 0, 1 and 3 demarcates for resistant reaction while 5, 7 and 9 corresponds to the susceptible reaction. Among the 290 individuals, 152 were found resistant while 141 were susceptible which fits to 1:1 segregation ratio (Table 4.2). The calculated Chi-square value was found non-significant at 5% level of significance ($0.412 \leq 3.8\ \chi^2_{0.05,\ 1}$), thus a single gene is responsible for conferring resistance by *xa-45(t)*. The segregation of population in resistant and susceptible reactions is elaborated in the following table:

Table 4.2: Chi-square analysis of F_6 and F_7 plants showing segregation of single gene

BB Disease Score	**0-3**	**5-9**	**χ^2 (1:1)**	**$\chi^2_{(0.05,\ 1)}$**
No. of F_6 and F_7 plants (290)	152	141	0.412	3.84
Disease Reaction	Resistant	Susceptible		

4.2.1 *O. glaberrima*: Improving the rice cultivars

We identified *xa45 (t)* from *O. glaberrima* as source for providing durable resistance towards bacterial blight disease of rice. Various studies suggested that the African rice *Oryza glaberrima* (AA genome) pertains tremendous potential to act as a source for improving the cultivated rice produce. This wild accession has key advantage of its ability to grow in low input conditions (Sarla and Swamy 2005). This wild species has been used exclusively for the transfer of resistance genes to cultivated rice. Pidon *et al* (2017) reported a resistance RYMV3 from *O. glaberrima* against *Rice Yellow mottle virus*. Also accession TOG5681 (*O. glaberrima*) displays a superior role in providing resistance to several biotic and abiotic factors (Petitot *et al* 2017).

4.2.2 Bacterial blight recessive genes in plant breeding

BB is one of the major constraints to rice production. In nature, the environment conditions favor the spread and proliferation of *Xoo*. Effective strategies to combat this stress involves exploitation of already available resistance mechanism of rice wild species. These genetic resources have potential in improving the disease management protocols demanded in present era. The present genetic study proves that the resistance conferred by *xa45 (t)* is recessive in nature. Till date, 46 BB resistance genes have been identified among which *xa5* (Blair *et al* 2003), *xa8* (Sidhu *et al* 1978), *xa9* (Yoshimura *et al* 1983; Sun *et al* 2004; Xiang *et al* 2006), *xa13* (Yoshimura *et al* 1995, Zhang *et al* 1996; Chu *et al* 2006), *xa15* (Noda and Ohuchi 1989), *xa19* (Taura *et al* 1991), *xa20* (Taura *et al* 1992), *xa24* (Mir & Khush 1990; Khush & Angeles 1999), *xa25* (Gao *et al* 2001,2005), *xa26* (Lee *et al* 2003; Sun *et al* 2004), *xa28* (Lee *et al* 2003), *xa31* (Wang *et al* 2009), *xa33* (Korinsak *et al* 2009), *xa34* (Chen *et al* 2011), *xa41* (Hutin *et al* 2015), *xa42* (Busungu *et al* 2016), *xa44* (Kim 2018) and *xa45* (Neelam *et al* 2019) are the recessive genes.

4.3 Genotyping

The *xa-45(t)* QTL was further analyzed for narrowing the 80 kb region harboring 9 candidate genes. These genes own diverse functions in plant metabolism. The list of genes with their possible functionality is described in table below (Table 4.3).

Table 4.3: Candidate genes

Candidate genes	Function associated
LOC_Os08g42360	Hypothetical protein
LOC_Os08g42370	Zinc finger DHHC domain-containing protein, putative, expressed
LOC_Os08g42380	Mitochondrial import inner membrane translocase subunit Tim, putative, expressed
LOC_Os08g42390	Glycerophosphoryl diester phosphodiesterase family protein, putative, expressed
LOC_Os08g42400	No apical meristem protein, putative, expressed
LOC_Os08g42410	Transketolase, putative, expressed
LOC_Os08g42420	Expressed protein
LOC_Os08g42430	Membrane-associated DUF588 domain-containing protein, putative, expressed
LOC_Os08g42440	CCT/B-box zinc finger protein, putative, expressed, involved in flowering

4.3.1 Fine mapping

The experiment for fine mapping, initiated by the designing of locus specific primers for 9 candidate genes. PCR amplification was performed for these overlapping locus specific markers to amplify in Pusa 44 and IL274 in triplicates. The amplicons were outsourced for sequencing at Agri genome. The results were obtained in the format of AB1 files and chromatograms. Chromatograms were manually checked for estimating the quality and accuracy of sequences (Figure 4.2).

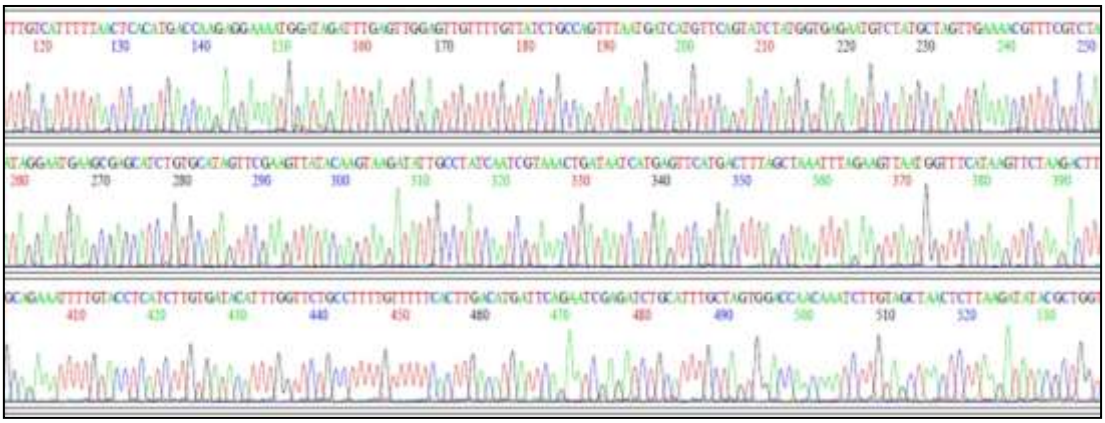


Figure 4.2: Representation of chromatographs obtained from sequencing of candidate genes

For the analysis of sequencing data, first step was to align forward and reverse sequence of a single amplicon and remove any ambiguity found at any end of the amplicon (Figure 4.3). The continuous sequence so obtained, was used for the development of contig by adding the neighboring amplicon sequence stretch. All these steps were performed using DNA Baser Assembler offline tool.

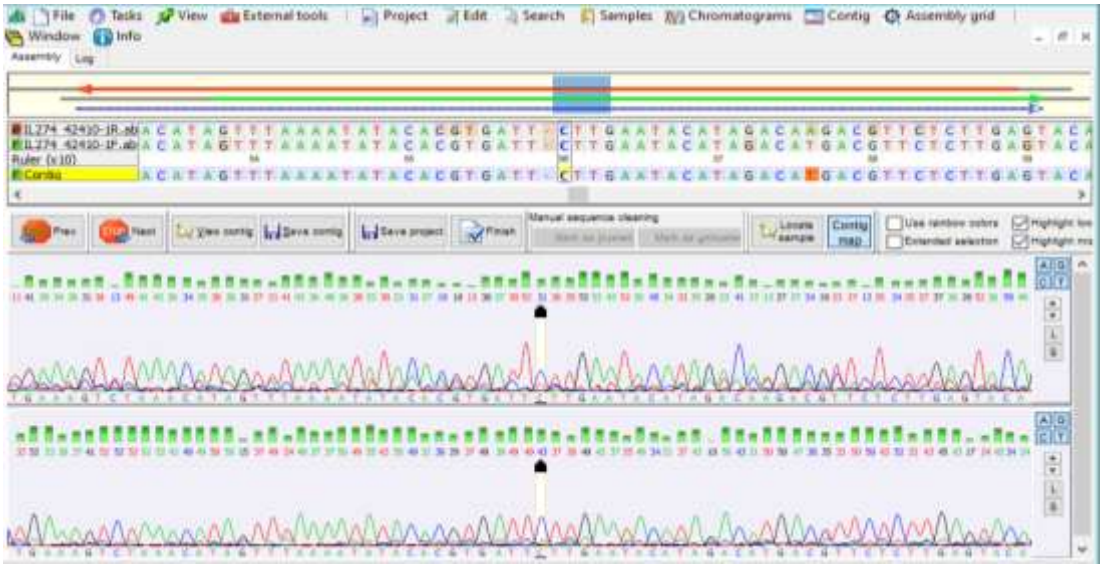
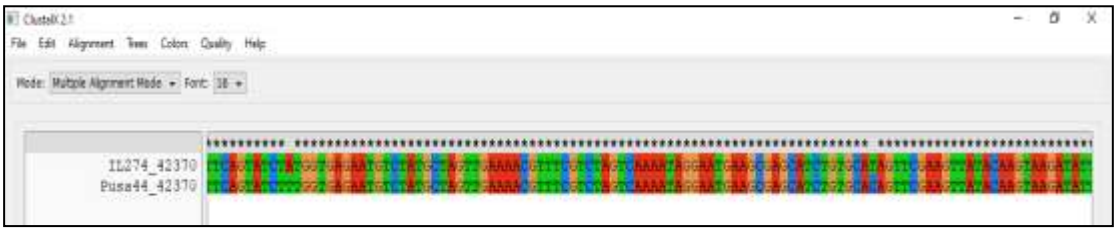
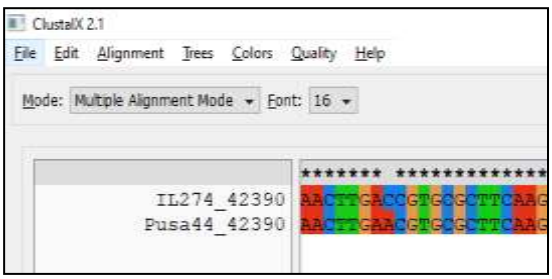


Figure 4.3: Representative screenshot of contig generation from forward and reverse sequence of single primer

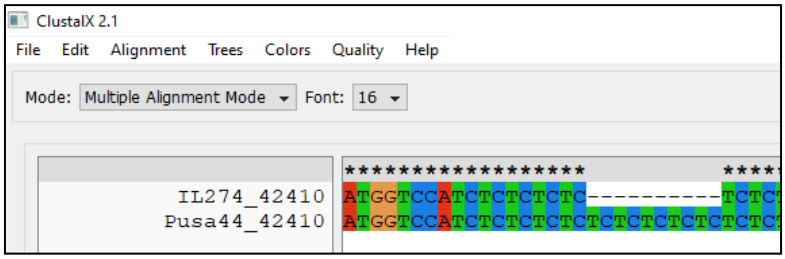
The contigs obtained of all the candidate genes for Pusa 44 and IL274 were then further used for identification of variations among resistant and susceptible parents with respect to reference sequence of Nipponbare. For finding SNPs or Indels, the sequences were aligned using Clustal X 2.1 offline software. The results revealed the presence of 4 SNPs at LOC_Os08g42370, a SNP and an Indel corresponding to LOC_Os08g42390, LOC_Os08g42400 having 2 SNPs and Indels, 3 SNPs and 10 bp deletion in LOC_Os08g42410, 1 SNP pertaining to LOC_Os08g42420 and LOC_Os08g42440 comprised of 2 SNPs and an Indels (Table 4.4) whereas no SNPs were obtained for the LOC_Os08g42360, LOC_Os08g42380 and LOC_Os08g42430. The SNPs so found, concluded to the fact that among 9 candidate genes, 7 can be considered putative candidate genes for further analysis. Among the 18 SNPs so found, 7 putative SNPs as well as Indels were selected in correspondence to IL274. The putative SNPs were selected in a way that the nucleotide base difference is present in forward and reverse sequence of the amplicon. Also, for the SNP selection was proceeded as per the quality of the base sequenced, by manually observing the chromatographs (Table 4.5). Figure 4.4 depicts the ClustalX result depiction of putative SNPs and Indels of candidate genes.



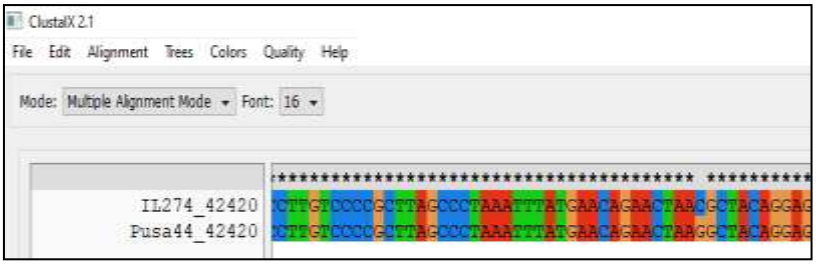
LOC_Os08g42370



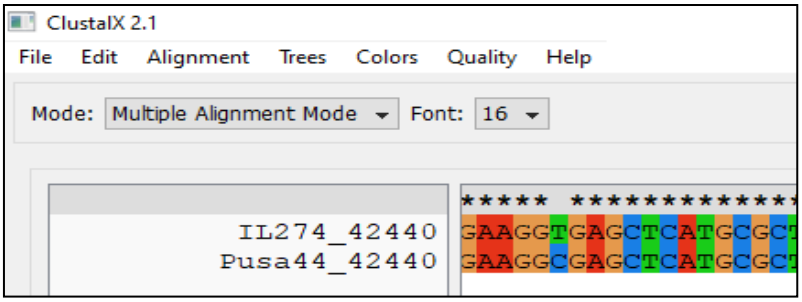
LOC_Os08g42390 **LOC_Os08g42400**



LOC_Os08g42410



LOC_Os08g42420



LOC_Os08g42440

Figure 4.4: Selected putative SNPs and Indels

Table 4.4: SNPs and Indels identified in different candidate genes

Locus_id	SNPs identified in IL274/Pusa44
LOC_Os08g42360	No SNP found
LOC_Os08g42370	T/G, A/T, T/C, T/C
LOC_Os08g42380	No SNP found
LOC_Os08g42390	C/A, A/-
LOC_Os08g42400	T/G, A/G, A/-, AT/--
LOC_Os08g42410	A/C, A/T, T/G, ----------/CTCTCTCTCT
LOC_Os08g42420	C/G
LOC_Os08g42430	No SNP found
LOC_Os08g42440	A/-, T/C, T/C

Table 4.5: Putative SNPs and Indels selected in candidate genes

Locus_id	Putative SNPs and Indels
LOC_Os08g42370	A/T, T/C
LOC_Os08g42390	C/A
LOC_Os08g42400	T/G
LOC_Os08g42410	----------/CTCTCTCTCT
LOC_Os08g42420	C/G
LOC_Os08g42440	T/C

4.3.2 SNP-based markers

The filtered 7 SNPs, were further used for designing dCAPs (derived-Cleaved Amplified Polymorphic Sequences) markers. The primers carried a specific restriction site in correspondence to the SNP of IL274. These restriction sites were used for the fragmentation of the amplicon with the specified enzyme with respect to the candidate gene (Table 4.6). For initiating the dCAPs analysis, firstly the amplification conditions for dCAPs markers were standardized. The PCR amplification of parental lines was first analyzed using agarose gel electrophoresis before the enzymatic digestion. The size differentiation among the parental lines was observed. Thus, the analysis was carried forward for the population. The digestion patterns so obtained were used for the genotyping of the population (Figure 4.5, 4.6 and 4.7).

Table 4.6: Enzymes used with respect to the restriction site present in the dCAPs primers designed from the putative SNPs

Locus_id	Enzyme	Incubation Temperature
LOC_Os08g42370	*NlaIII*	37 °C
LOC_Os08g42390	*AcuI*	-
LOC_Os08g42400	*SwaI*	25 °C
LOC_Os08g42420	*HgaI*	37 °C
LOC_Os08g42440	*HpyCHIV*	37 °C

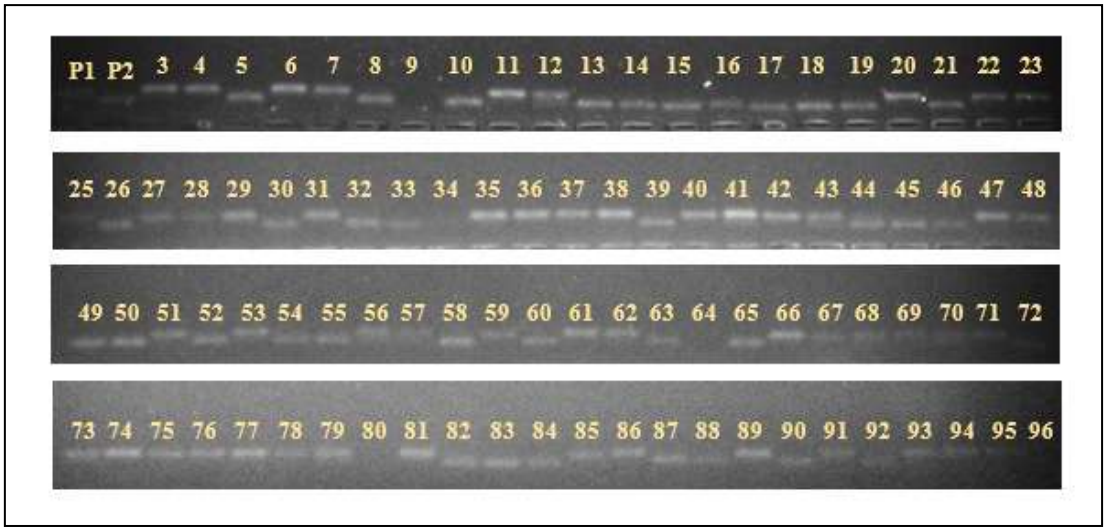


Figure 4.5: LOC_Os08g42400 digestion using *SwaI*; P1 - Pusa 44, P2 - IL274

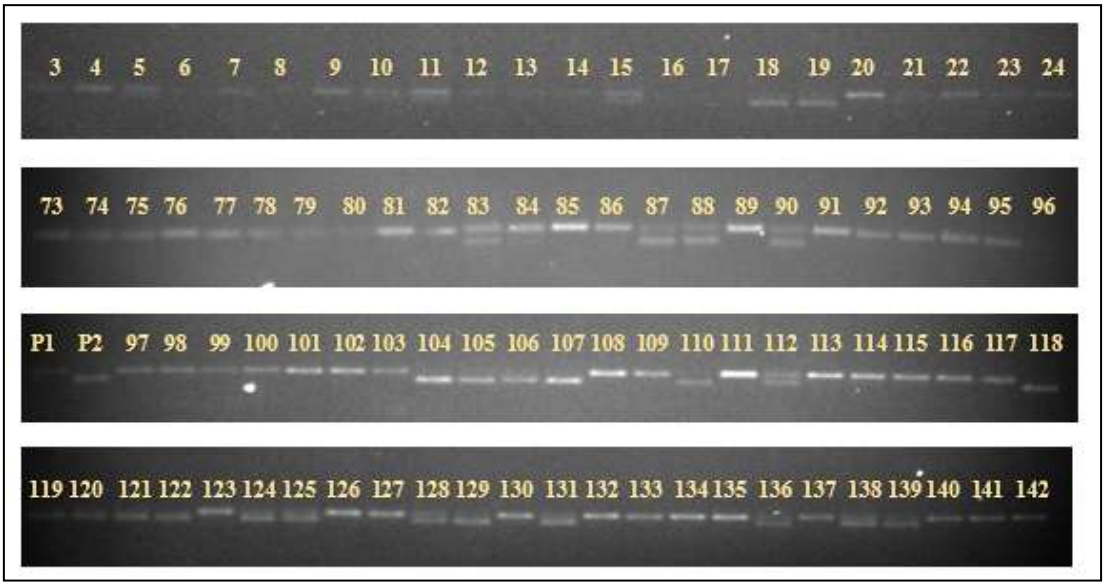
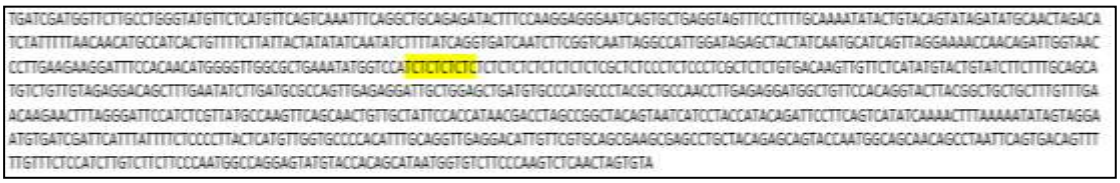


Figure 4.6: LOC_Os08g42420 digestion using *HgaI*; P1 - Pusa 44, P2 - IL274


TGATCGATGGTTCTTGCCTGGGTATGTTCTCATGTTCAGTCAAATTTCAGGCTGCAGAGATACTTTCCAAGGAGGGAATCAGTGCTGAGGTAGTTTCCTTTTGCAAAATATACTGTACAGTATAGATATGCAACTAGACA
TCTATTTTTAACAACATGCCATCACTGTTTTCTTATTACTATATATCAATATCTTTTATCAGGTGATCAATCTTCGGTCAATTAGGCCATTGGATAGAGCTACTATCAATGCATCAGTTAGGAAAACCAACAGATTGGTAAC
CCTTGAAGAAGGATTTCCACAACATGGGGTTGGCGCTGAAATATGGTCCA
TCTCTCTCTCTCTCTCGCTCTCCCTCTCCCTCGCTCTCTGTGACAAGTTGTTCTCATATGTACTGTATCTTCTTTGCAGCATGTCTGTTGTAGAGGACAGCTTTGAATATCTTGATGCGCCAGTTGAGAGGATTGCTGGA
GCTGATGTGCCCATGCCCTACGCTGCCAACCTTGAGAGGATGGCTGTTCCACAGGTACTTACGGCTGCTGCTTTGTTTGAACAAGAACTTTAGGGATTCCATCTCGTTATGCCAAGTTCAGCAACTGTTGCTATTCCACC
ATAACGACCTAGCCGGCTACAGTAATCATCCTACCATACAGATTCCTTCAGTCATATCAAAACTTTAAAAATATAGTAGGAATGTGATCGATTCATTTATTTTCTCCCCTTACTCATGTTGGTGCCCCACATTTGCAGGTTG
AGGACATTGTTCGTGCAGCGAAGCGAGCCTGCTACAGAGCAGTACCAATGGCAGCAACAGCCTAATTCAGTGACAGTTTTTGTTTCTCCATCTTGTCTTCTTCCCAATGGCCAGGAGTATGTACCACAGCATAATGGTG
TCTTCCCAAGTCTCAACTAGTGTA

(a)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TTGTTTCTCCATCTTGTCTTCTTCCCAATGGCCAGGAGTATGTACCACAGCATAATGGTGTCTTCCCAAGTCTCAACTAGTGTA

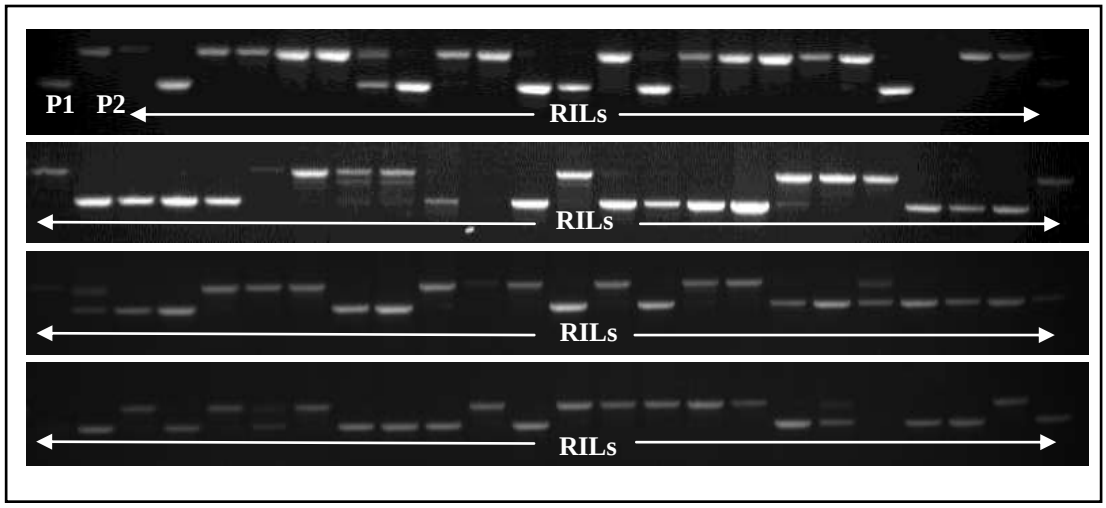
(b)

population which revealed correspondence to the STS marker of LOC_Os08g42410 and also to the phenotype of the individuals in F_7 population (Figure 4.9).

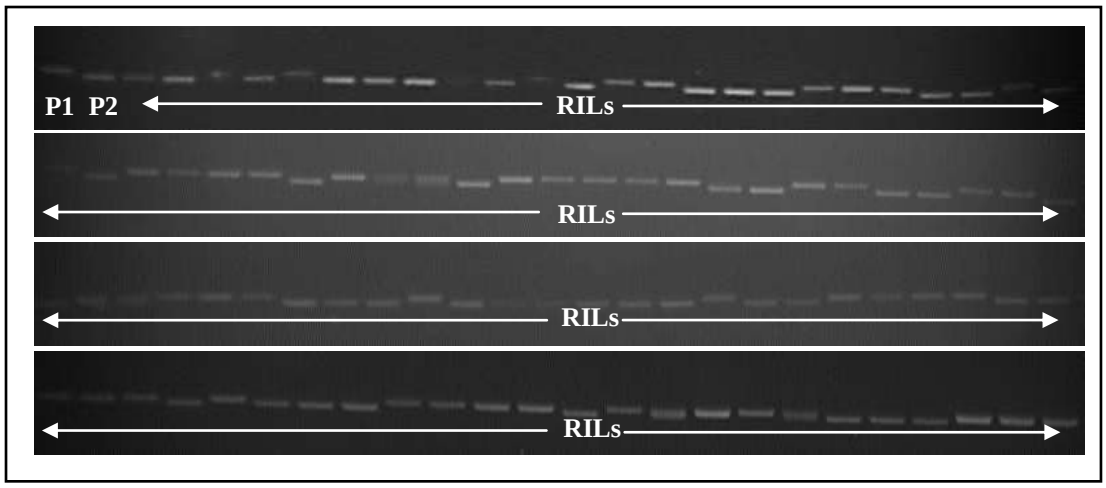
Table 4.7: Deletion LOC_Os08g42410 marker sequence

Marker	Sequence
Del_42410_F	**GTTGGCGCTGAAATATGGTC**
Del_42410_R	**ACAAAGCAGCAGCCGTAAGT**

The deletion LOC_Os08g42410 marker along with the LOC_Os08g42410 STS marker was applied on whole F_7 population. The results reveal the same genotypic behaviour of two marker in the population devised. The genotypic behaviour of the two markers shows segregation in 1:1 ratio for resistant and susceptible individuals. The genotypic data for the candidate genes on the RIL population is illustrated in following table (Table 4.8).



(a)



(b)

Figure 4.9: LOC_Os08g42410 specific markers (a) STS LOC_Os08g42410, (b) Deletion LOC_Os08g42410 marker; applied on RIL population

Table 4.8: Genotyping

ID	42350	42370	42400	Phenotype	42410	42420	42440
1	S	S	S	S	S	S	S
2	R	R	R	R	R	R	R
3	S	S	S	S	S	S	S
4	S	S	S	S	S	S	S
5	R	R	R	R	R	R	R
6	S	S	S	S	S	S	S
7	S	S	S	S	S	S	S
8	R	R	R	R	R	R	R
9	S	S	S	S	S	S	S
10	R	R	R	R	R	S	S
11	S	S	S	R	R	R	R
12	S	S	S	S	S	S	S
13	R	R	R	R	R	S	S
14	R	R	R	R	R	S	S
15	R	R	R	R	R	R	R
16	R	R	R	R	R	R	R
17	R	R	R	R	R	R	R
18	R	R	R	R	R	R	R
19	R	R	R	R	R	R	R
20	S	S	S	S	S	S	S
21	R	R	R	R	R	S	S
22	S	S	S	S	S	S	S
23	S	S	S	S	S	S	S
24	S	S	S	S	S	S	S
25	S	S	S	S	S	S	S
26	S	S	S	R	S	S	S
27	S	S	S	S	S	S	S
28	R	R	R	R	R	R	R
29	S	S	S	S	S	S	S
30	R	R	R	R	R	R	R
31	R	R	R	R	R	R	R
32	R	R	R	R	R	R	R
33	S	S	S	S	S	S	S
34	S	S	S	S	S	S	S
35	S	S	S	S	S	S	S
36	S	S	S	S	S	S	S
37	R	R	R	R	R	R	R

Table 4.8: Contd..

ID	42350	42370	42400	Phenotype	42410	42420	42440
38	S	S	S	S	S	S	S
39	S	S	S	S	S	S	S
40	S	S	S	S	S	S	S
41	S	S	S	S	S	S	S
42	S	S	S	S	S	S	S
43	R	R	R	R	R	S	S
44	R	R	R	R	R	S	S
45	R	R	R	R	R	S	S
46	S	S	S	S	S	S	S
47	S	S	S	S	S	S	S
48	S	S	S	S	S	S	S
49	R	R	R	R	R	S	S
50	R	R	R	R	R	S	S
51	S	S	S	S	S	S	S
52	R	R	R	R	R	R	R
53	S	S	S	S	S	S	S
54	R	R	R	R	R	R	R
55	R	R	R	R	R	R	R
56	R	R	R	R	R	R	R
57	M	M	H	S	R	M	M
58	R	R	R	R	R	R	R
59	S	S	S	S	S	S	S
60	R	R	R	R	R	H	S
61	S	S	S	S	S	S	S
62	S	S	S	S	S	S	S
63	R	R	R	R	R	S	S
64	R	R	R	R	R	S	S
65	R	R	R	R	R	M	S
66	S	S	S	S	S	S	S
67	S	S	S	S	S	S	R
68	S	S	S	S	S	S	R
69	R	R	R	R	R	S	M
70	S	S	S	S	S	S	R
71	S	S	S	S	S	R	R
72	R	R	R	R	R	R	R
73	S	S	S	S	S	S	S
74	S	S	S	S	S	S	S

Table 4.8: Contd..

ID	42350	42370	42400	Phenotype	42410	42420	42440
75	S	S	S	S	S	S	S
76	S	S	S	S	S	S	S
77	S	S	S	S	S	S	R
78	S	S	S	S	S	S	S
79	S	S	S	R	S	S	S
80	S	S	S	S	S	S	S
81	S	S	S	S	S	S	S
82	R	R	R	R	R	R	R
83	R	R	R	R	R	R	R
84	R	R	R	R	R	R	R
85	S	S	S	S	S	S	S
86	S	S	S	S	S	S	S
87	R	R	R	R	R	R	R
88	R	R	R	R	R	R	R
89	S	S	S	S	S	S	S
90	R	R	R	R	R	R	R
91	S	S	S	S	S	S	S
92	R	R	R	R	R	R	R
93	S	S	S	S	S	S	R
94	S	S	S	S	S	S	R
95	S	S	S	S	S	S	R
96	S	S	S	S	S	S	S
97	S	S	S	S	S	S	S
98	S	S	S	S	S	S	S
99	S	S	S	S	S	S	S
100	S	S	S	S	S	S	S
101	S	S	S	S	S	S	S
102	S	S	S	S	S	S	S
103	S	S	S	R	S	S	S
104	R	R	R	R	R	R	R
105	R	R	R	R	R	R	R
106	R	R	R	R	R	R	R
107	R	R	R	R	R	R	R
108	S	S	S	S	S	S	S
109	S	S	S	S	S	S	S
110	R	R	R	R	R	R	R
111	S	S	S	S	S	S	S

Table 4.8: Contd..

ID	42350	42370	42400	Phenotype	42410	42420	42440
112	R	R	R	R	R	R	R
113	S	S	S	S	S	S	S
114	R	R	R	R	R	R	R
115	S	S	S	S	S	S	S
116	S	S	S	S	S	S	S
117	S	S	S	S	S	S	S
118	R	R	R	R	R	R	R
119	R	R	R	R	R	R	R
120	R	R	R	R	R	R	R
121	R	R	R	R	R	R	R
122	R	R	R	R	R	R	R
123	S	S	S	S	S	S	S
124	S	S	S	R	R	R	R
125	S	S	S	R	R	R	R
126	S	S	S	S	S	S	S
127	S	S	S	S	S	S	S
128	R	R	R	R	R	R	R
129	R	R	R	R	R	R	R
130	S	S	S	S	S	S	S
131	R	R	R	R	R	R	R
132	S	S	S	S	S	S	S
133	S	S	S	S	S	S	S
134	S	S	S	S	S	S	S
135	S	S	S	S	S	S	S
136	R	R	R	R	R	R	R
137	S	S	S	S	S	S	S
138	R	R	R	R	R	R	R
139	R	R	R	R	R	R	R
140	S	S	S	S	S	S	S
141	S	S	S	S	S	S	R
142	S	S	S	S	S	S	S
143	S	S	S	S	S	S	S
144	S	S	S	S	S	S	S
145	S	S	S	S	S	S	S
146	S	S	S	S	S	S	S
147	R	R	R	R	R	R	R
148	S	S	S	S	S	S	S

Table 4.8: Contd..

ID	42350	42370	42400	Phenotype	42410	42420	42440
149	S	S	S	R	S	S	S
150	S	S	S	R	S	S	S
151	R	R	R	R	R	R	R
152	S	S	S	S	S	S	S
153	S	S	S	S	S	S	S
154	S	S	S	S	S	S	S
155	S	S	S	S	S	S	S
156	S	S	S	S	S	S	S
157	R	R	R	R	R	R	R
158	R	R	M	R	R	R	R
159	S	S	S	S	S	S	S
160	S	S	S	S	S	S	S
161	R	R	R	S	R	R	R
162	R	R	R	R	R	R	R
163	S	S	S	S	S	S	S
164	S	S	S	S	S	S	S
165	R	R	R	R	R	R	R
166	R	R	R	R	R	R	R
167	S	S	S	S	S	S	R
168	S	S	S	S	S	S	S
169	S	S	S	S	S	S	S
170	S	S	S	S	S	S	S
171	S	S	S	S	S	S	S
172	R	R	R	R	R	R	R
173	R	R	R	R	R	R	R
174	R	R	R	R	R	R	R
175	S	M	S	S	S	S	S
176	R	R	R	R	R	R	R
177	R	R	R	R	R	R	R
178	R	R	R	R	R	R	R
179	S	S	S	S	S	S	S
180	R	R	R	R	R	R	R
181	R	R	R	R	R	R	R
182	S	S	M	S	S	S	S
183	R	R	R	R	R	H	R
184	R	R	R	R	R	S	S
185	R	R	R	R	S	S	S

Table 4.8: Contd..

ID	42350	42370	42400	Phenotype	42410	42420	42440
186	S	S	S	S	S	S	S
187	S	S	S	S	S	S	S
188	S	S	S	S	S	S	S
189	R	R	R	R	R	R	R
190	R	R	R	R	R	R	R

***ID: Population ID, R: Resistant, S: Susceptible, 42410: STS marker and Del LOC_Os08g42410**

Table 4.9: Recombinant lines for LOC_Os08g42420 and LOC_Os08g42440

ID	42350	42370	42400	Phenotype	42410	42420	42440
10	R	R	R	R	R	S	S
13	R	R	R	R	R	S	S
14	R	R	R	R	R	S	S
21	R	R	R	R	R	S	S
43	R	R	R	R	R	S	S
44	R	R	R	R	R	S	S
45	R	R	R	R	R	S	S
49	R	R	R	R	R	S	S
50	R	R	R	R	R	S	S
60	R	R	R	R	R	S	S
63	R	R	R	R	R	S	S
64	R	R	R	R	R	S	S
65	R	R	R	R	R	S	S
67	S	S	S	S	S	S	R
68	S	S	S	S	S	S	R
70	S	S	S	S	S	S	R
71	S	S	S	S	S	R	R
77	S	S	S	S	S	S	R
93	S	S	S	S	S	S	R
94	S	S	S	S	S	S	R
95	S	S	S	S	S	S	R
141	S	S	S	S	S	S	R
167	S	S	S	S	S	S	R
184	R	R	R	R	R	S	S

***Pink color indicates recombinants for** LOC_Os08g42420 and LOC_Os08g42440 whereas blue colour indicates recombinants for the LOC_Os08g42440.

Table 4.10: Recombinant lines for LOC_Os08g42350, LOC_Os08g42370 and LOC_Os08g42400

ID	42350	42370	42400	Phenotype	42410	42420	42440
11	S	S	S	R	R	R	R
124	S	S	S	R	R	R	R
125	S	S	S	R	R	R	R

The genotypic data when compiled showed different behaviour with respect to each gene. From the 190 individuals, at most three lines were clarifying the break point with respect to LOC_Os08g42350, LOC_Os08g42370 and LOC_Os08g42400 (Table 4.10). These break points show distant behaviour as compared to *xa-45(t)* resistance pattern and thus cannot be considered candidate for the same. In addition, 15 individuals showed non correspondence of LOC_Os08g423420 and LOC_Os08g42440 collectively to the phenotypic behaviour (Table 4.9). Also, 9 progenies demarcate a single locus LOC_Os08g42440 for not considering further for candidacy (Table 4.9). Following this data analysis, the genotype of LOC_Os08g42410 was recorded for 300 individuals, from which the genotype of 277 individuals validates the phenotypic behaviour governed by *xa-45(t)* specific pathotype, PbXo-7. The identification of SNPs and Indels has helped in evaluation of genotypic behaviour of all candidate genes with development of SNP and Indel-based marker. The conclusion drawn from the genotypic analysis of the population clearly reveals the correspondence of LOC_Os08g42410 with the phenotypic data. This proves LOC_Os08g42410 to be putative gene among the 9 candidate genes in the destined region of *xa-45(t)*.

Post the recombinant analysis, it was observed that the phenotype for some individuals was governed by other QTL of chromosome 11. This was found in the vicinity of *Xa21*, thus the genotyping of these lines with *Xa21* depicts the phenotype governance with QTL of chromosome 11 (Table 4.11).

Table 4.11: Selected lines phenotypic evaluation governed with another QTL of chromosome 11

ID	Phenotype	42410	*Xa21*	ID	Phenotype	42410	*Xa21*
26	R	S	R	**258**	R	S	R
79	R	S	R	**264**	R	S	R
103	R	S	R	**266**	R	S	R
149	R	S	R	**275**	R	S	R
150	R	S	R	**293**	R	S	R

Table 4.11: Contd..

ID	Phenotype	42410	*Xa21*	ID	Phenotype	42410	*Xa21*
161	S	R	S	**295**	R	S	R
198	R	S	R	**297**	R	S	R
240	R	S	R	**299**	R	S	R
249	R	S	R				

The genotypic evaluation clearly reveals the correspondence of LOC_Os08g42410 with the phenotypic data. This proves LOC_Os08g42410 to be putative gene among the 9 candidate genes in the destined region of *xa-45(t)*.

4.4 Novelty of *xa-45(t)*

The recessive BB gene *xa-45(t)* shares the same chromosome (on long arm of rice chromosome 8) with another recessive gene *xa13*. For the purpose of demarcation of *xa13* from *xa45(t)* phenotypic and genotypic data was analysed on 1000 RILs. The IRBB13, a NIL carrying (*xa13* gene) is found susceptible against the *Xoo* pathotype PbXo-8 whereas the *xa45(t)* confers resistance to PbXo8, indicating novelty of the gene. We inoculated the whole population with the *Xoo* pathotype PbXo-8. The RIL population showed segregation for the disease reaction (Table 4.12) which again indicated that the *xa45(t)*is a novel gene conferring durable resistance to the *Xoo* pathotypes prevalent in Punjab. We also got three recombination break points with *xa13* gene in the population while recording the data against the *Xoo* pathotype PbXo-7 (ID-11,124,288). In addition, the difference in the promoter region of *xa13* gene was observed between IRBB13 and IL 274 which further proves that these genes are different (Figure 4.10).

Table 4.12: Differential reaction of PbXo- 7 and 8 in the RIL population

ID	VII	VIII	ID	VII	VIII	ID	VII	VIII	ID	VII	VIII
5	0	7	**64**	1	5	**184**	1	7	**12**	5	3
8	1	9	**65**	1	9	**191**	1	7	**35**	7	1
10	1	9	**69**	1	9	**192**	1	5	**41**	7	3
14	1	9	**72**	3	7	**194**	1	7	**46**	7	3
16	1	7	**82**	0	7	**196**	1	7	**62**	7	3
17	1	7	**87**	1	7	**202**	0	5	**71**	5	1
19	1	9	**90**	0	7	**208**	3	7	**74**	5	3
21	1	5	**104**	1	7	**210**	1	5	**77**	5	1
26	3	5	**105**	1	9	**213**	0	5	**78**	5	1
32	1	5	**112**	1	9	**216**	3	7	**93**	5	1
37	1	9	**114**	1	9	**224**	3	7	**109**	5	3
43	1	5	**118**	1	7	**236**	1	5	**127**	5	1
44	1	9	**119**	1	7	**243**	1	7	**148**	5	1

Table 4.12: Contd..

ID	VII	VIII	ID	VII	VIII	ID	VII	VIII	ID	VII	VIII
45	1	9	**121**	1	9	**251**	1	7	**154**	7	3
49	1	9	**136**	1	9	**255**	3	7	**155**	5	3
50	1	7	**151**	1	5	**257**	1	7	**156**	7	3
52	1	5	**157**	1	7	**267**	1	5	**168**	7	1
54	1	5	**173**	1	7	**285**	1	5	**171**	5	1
55	1	9	**176**	1	7	**286**	3	5	**175**	5	1
56	1	7	**177**	1	5	**296**	0	7	**182**	5	3
58	1	9	**183**	1	7	**4**	5	1	**200**	7	1
218	7	3	**246**	5	1	**272**	7	1	**277**	7	1
234	5	1	**269**	7	1	**273**	7	3	**282**	9	1
239	7	1	**270**	7	1	**276**	5	1	**290**	9	3

***ID - Population ID; VII and VIII - Mean disease score for PbXo- 7 and 8 respectively**

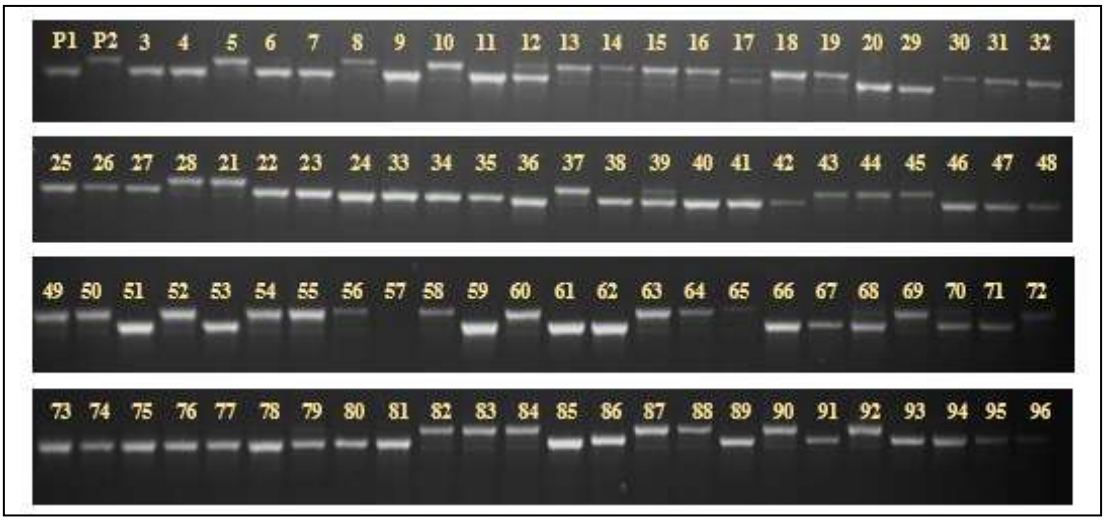


Figure 4.10: *xa13* promoter primer applied on population

Thus, analysis validates that genic interactions come into play for providing resistance against PbXo-7,8.

4.5 Relative expression analysis through qRT-PCR

The results for fine mapping were further validated using the relative gene expression analysis *viz.* qRT-PCR analysis.

4.5.1 RNA extraction and analysis

RNA sampling is the first step for this experiment which was proceeded for the two biological replicates of parental lines. It was conducted at 0, 8, 24, 48, 72 and 96 hours of post inoculations. RNA extraction was followed by quality and integrity check, validates with two predominant fragments, 28 S and 18 S RNA (Figure 4.11). The agarose gel results show good

quality of all the RNA samples without degradation. The RNA was then, quantified using nanodrop spectrophotometer for the $A_{260/280}$ ratio (Table 4.12). Prior to this, cDNA was synthesised and it was verified using endogenous control *Actin* gene specific primers harbouring 67 bp amplicon. It confirms the authenticity of cDNA (Figure 4.12).

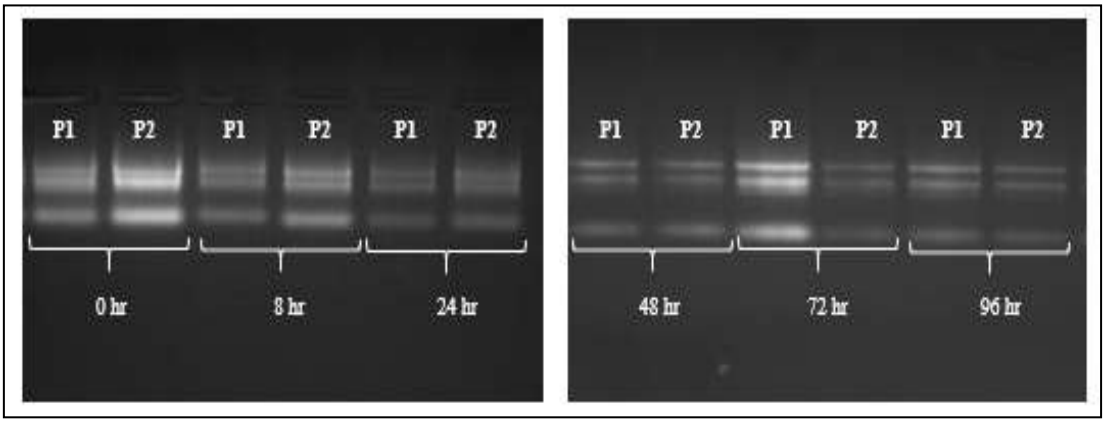


Figure 4.11: RNA quantification, P1 - Pusa 44; P2 - IL274

Table 4.13: RNA quantity using nanodrop spectrophotometer

Sample ID	Ratio	ng/µl	Sample ID	Ratio	ng/µl	Sample ID	Ratio	ng/µl
P1-0	2.02	636	**P1-24**	1.99	303	**P1-72**	1.87	900.5
P1-0	1.93	1003	**P1-24**	2.33	1024	**P1-72**	2.06	1032
P2-0	1.97	1628	**P2-24**	1.92	1534	**P2-72**	1.88	864.7
P2-0	1.90	1244	**P2-24**	2.14	1322	**P2-72**	1.99	883
P1-8	2.2	952	**P1-48**	2.06	1167	**P1-96**	2.13	934
P1-8	2.01	1456	**P1-48**	1.99	1023	**P1-96**	2.04	1185
P2-8	1.97	875	**P2-48**	2.0	846	**P2-96**	2.11	1235
P2-8	2.16	1096	**P2-48**	2.1	679	**P2-96**	1.99	1476

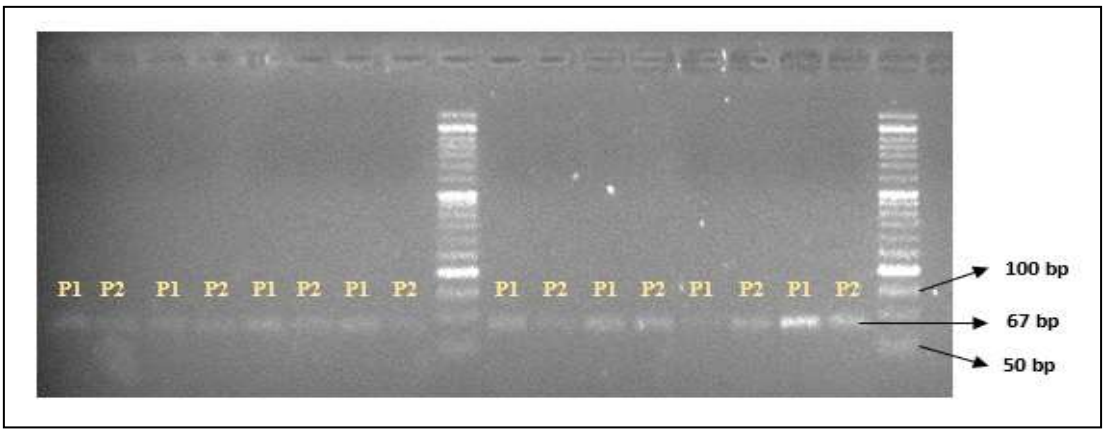


Figure 4.12: Confirmation of cDNA for parental lines (P1-Pusa 44 and P2- IL274) using biological replicates of RNA at different intervals, with *Actin* gene specific primers

4.5.2 Calibration of qRT-PCR and generation of standard curves

Standard curve was generated by preparing the first standard (ST1) by preparing 3-fold dilutions of all the cDNA templates. From ST1, sequential serial dilutions were prepared till 6 standards (ST-6) for relative gene quantitation. The curve signifies linear pattern of C_t values over the range of dilutions prepared (Figure 4.13).

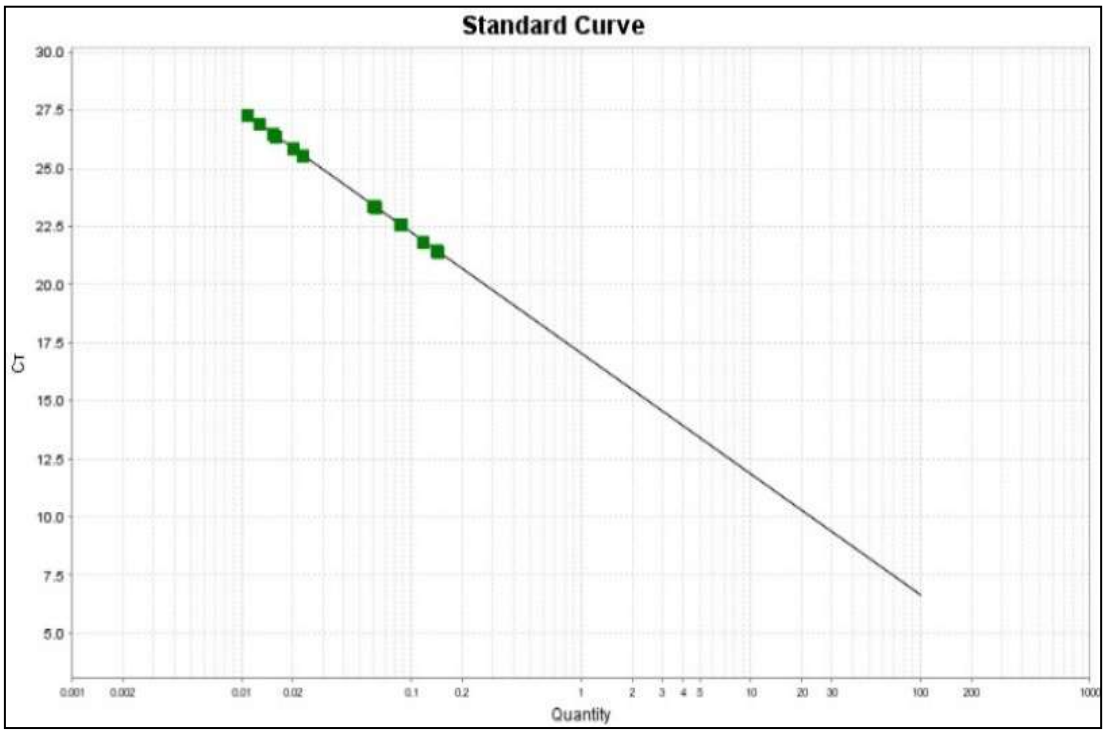


Figure 4.13: Standard curve analysis of *Actin*

4.5.3 Expression studies

Using cDNA as a template, the primers designed for qRT-PCR analysis for all the candidate genes, were applied onto the parental lines. Melt curves so obtained, signifies single peak for each primer owing to the stringent specificity of the reaction (Figure 4.14). Secondly, the amplification plots signifying the sigmoidal pattern of amplification confirms the error free run process (Figure 4.15). Figure 4.16 and Figure 4.17 represents the melt curves and amplification plots for all the candidate genes. Following these quality checks for analysis, comparative transcript level was assessed using delta delta C_t ($\Delta\Delta C_t$) method devised by Livak and Schmittgen (2001). The analysis included two biological replicates along with three technical replicates each. The fold change was evaluated following standard calculations, fold change of value 1 signifies the threshold for control samples. Fold change values are evaluated for values more than threshold value 1.

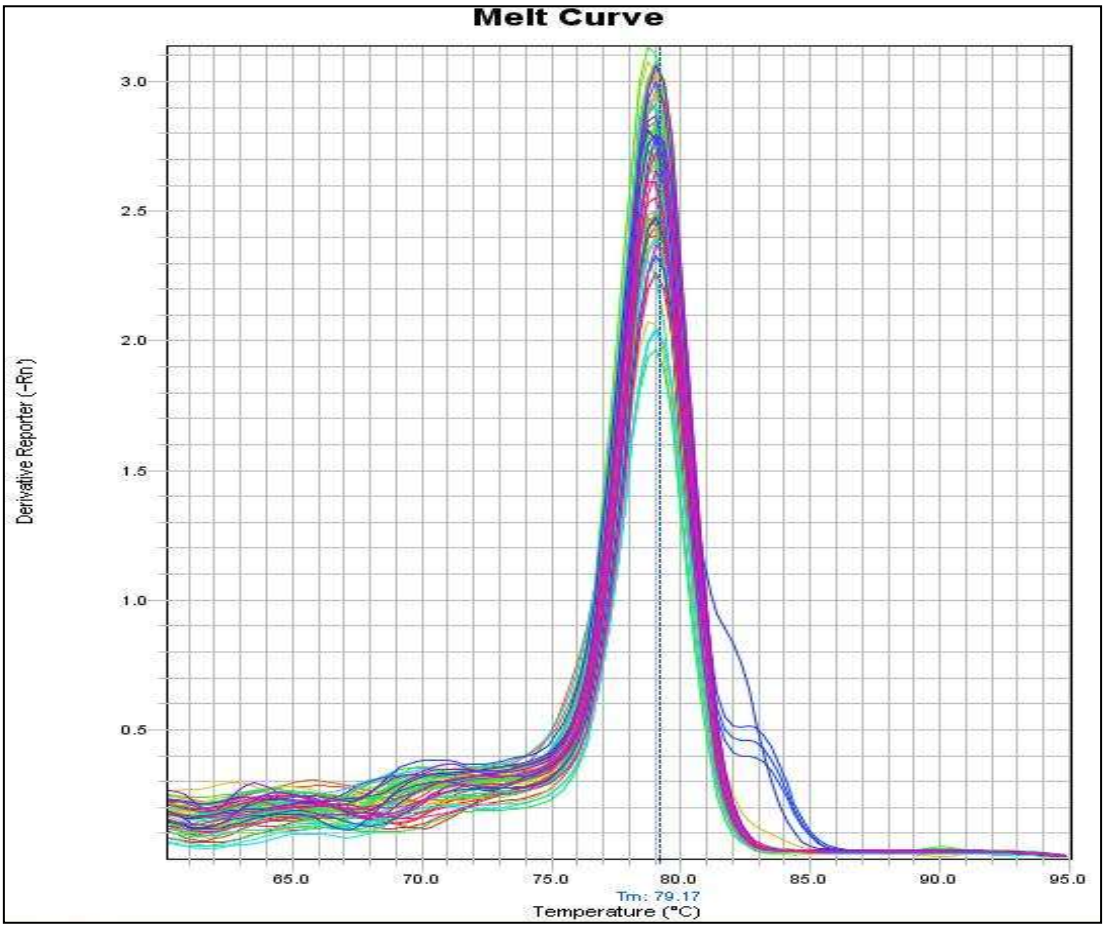


Figure 4.14: Melt Curve depiction of *Actin*

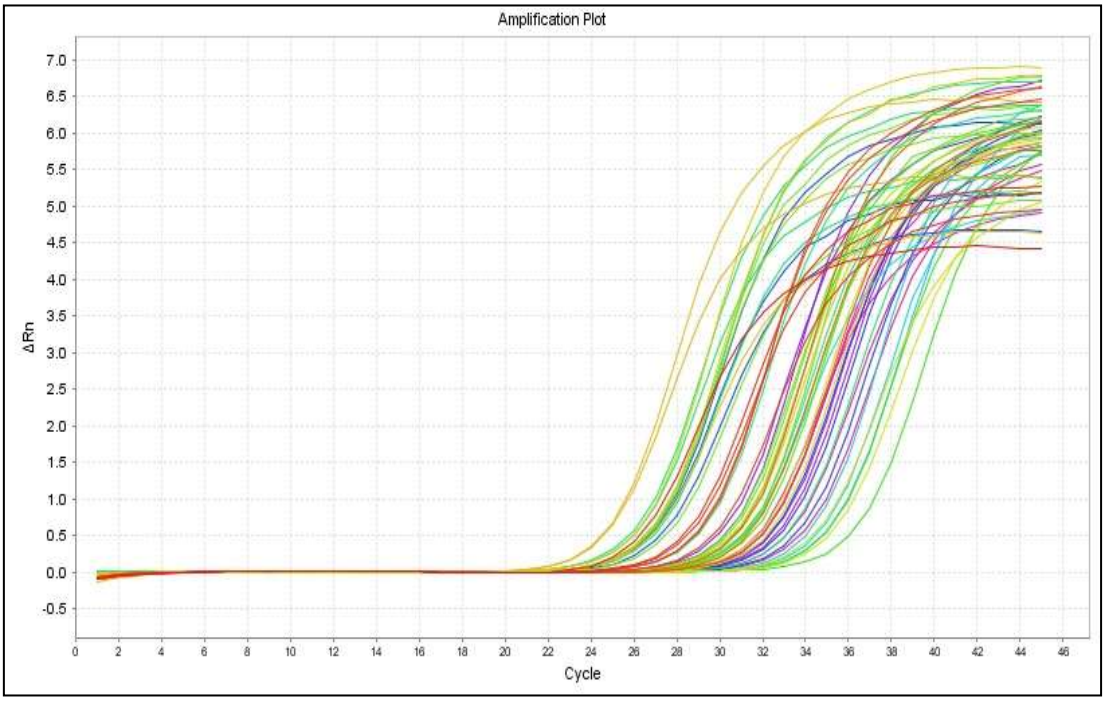


Figure 4.15: Amplification plot depiction of *Actin*

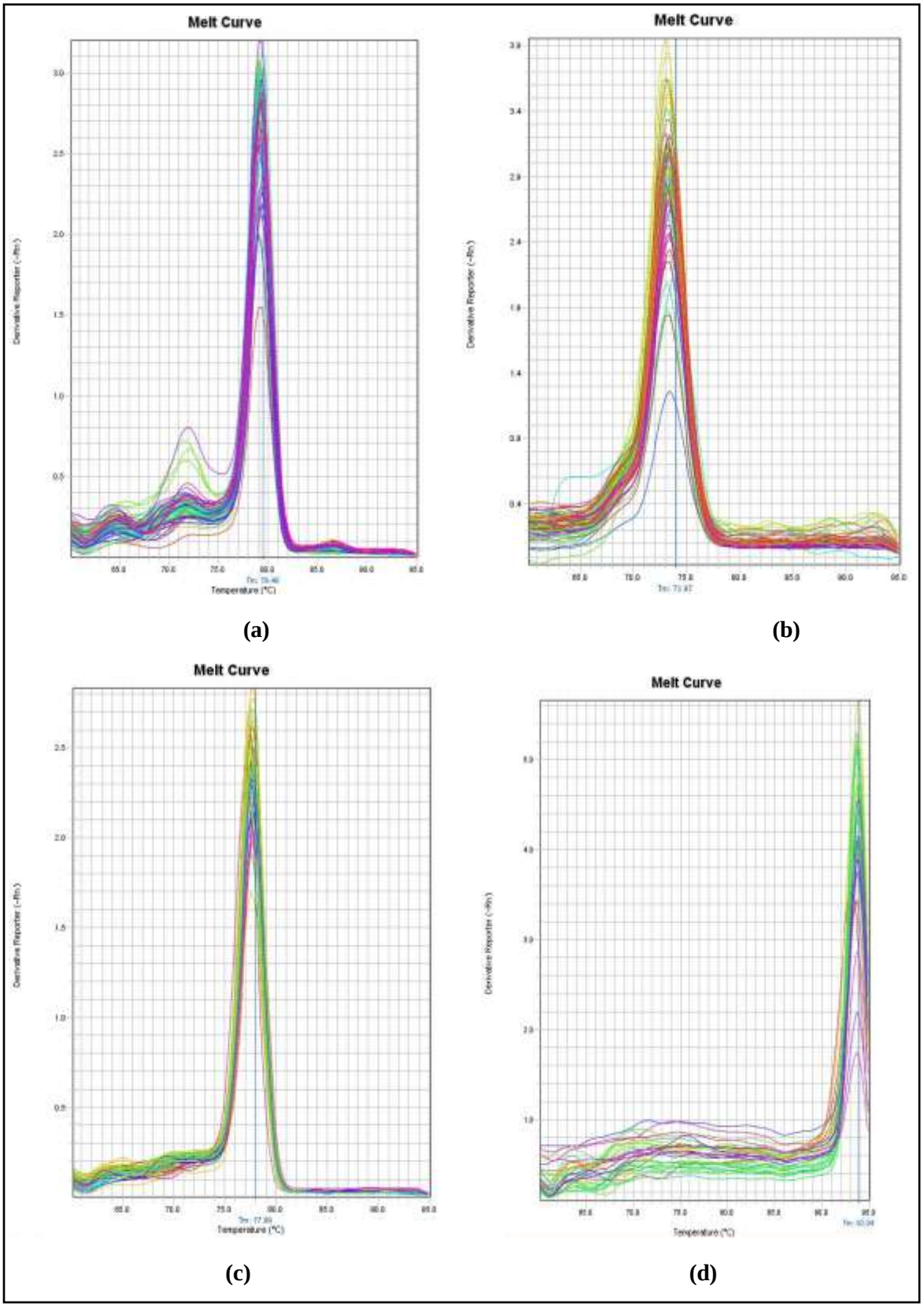


Figure 4.16: Melt Curve analysis for candidate genes (a) LOC_Os08g42370 (b) LOC_Os08g42380 (c) LOC_Os08g42390 (d) LOC_Os08g42400

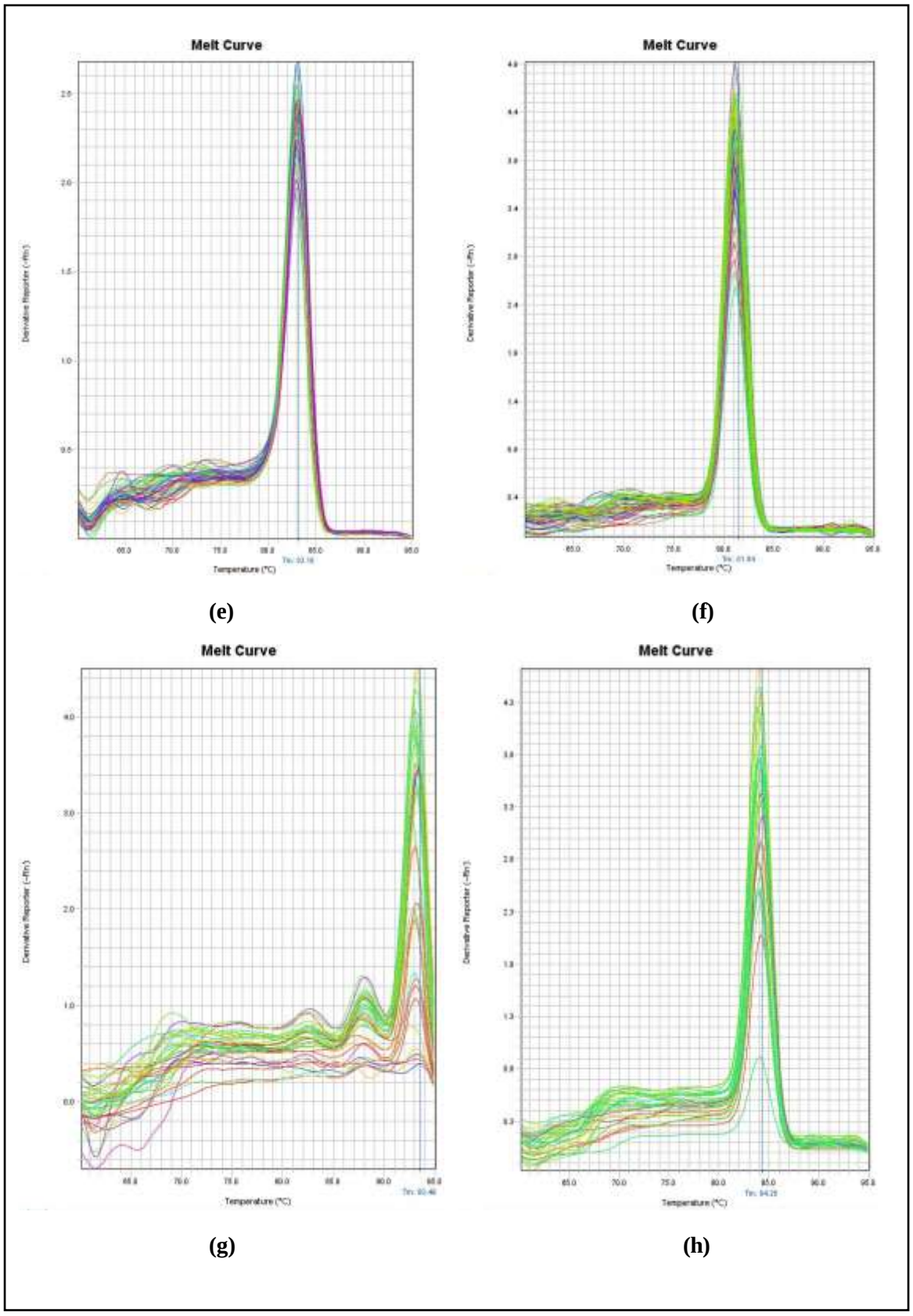
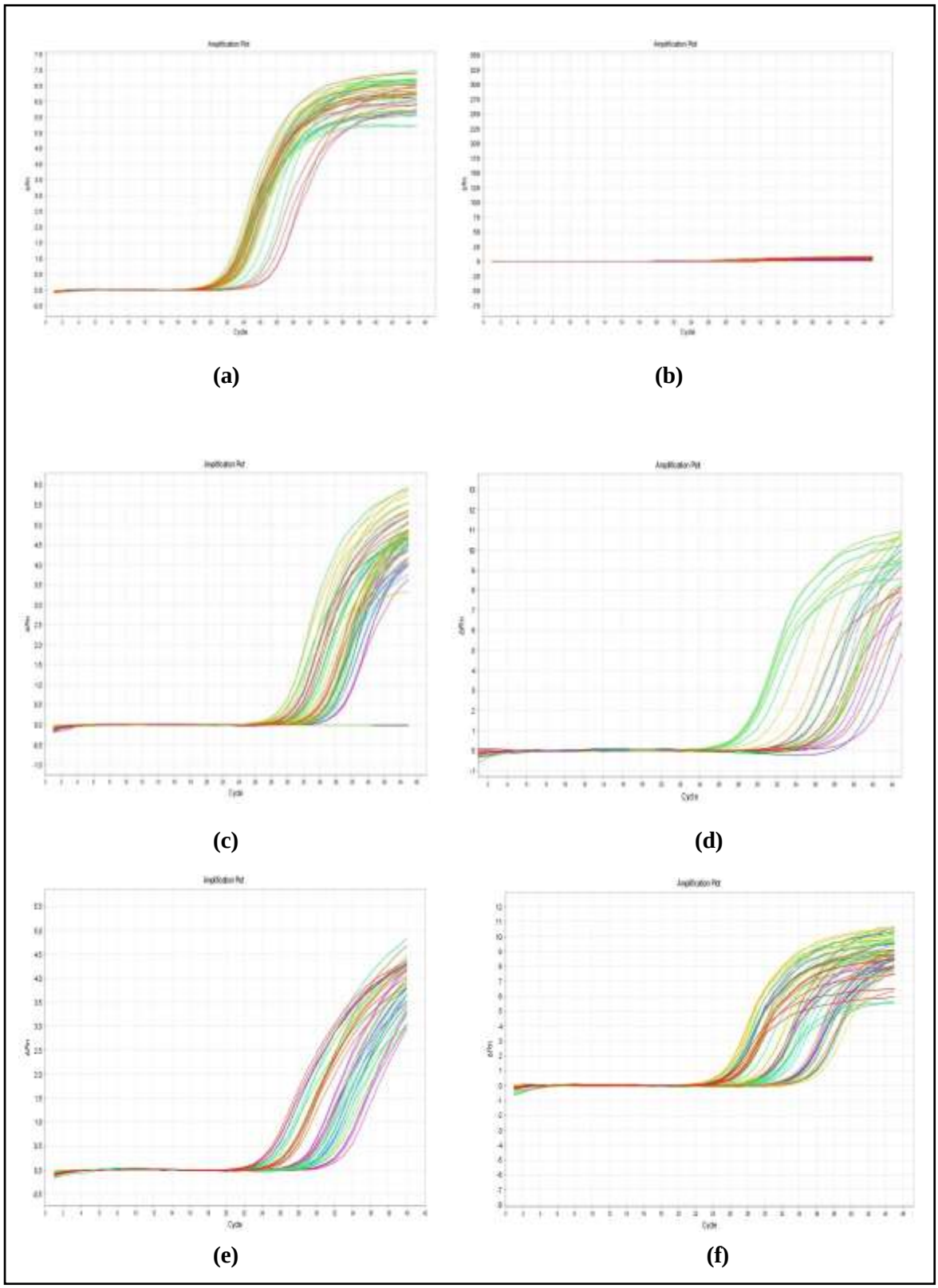


Figure 4.16 Contd..: Melt Curve analysis for candidate genes (e) LOC_Os08g42410 (f) LOC_Os08g42420 (g) LOC_Os08g42430 (h) LOC_Os08g42440

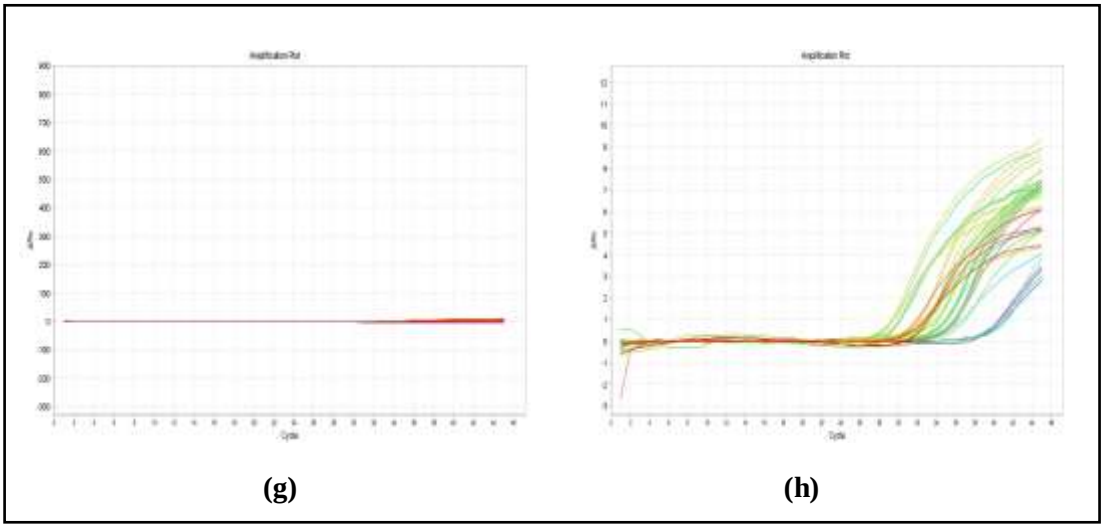


(a) **(b)**

(c) **(d)**

(e) **(f)**

Figure 4.17: Amplification plots for candidate genes (a) LOC_Os08g42370 (b) LOC_Os08g42380 (c) LOC_Os08g42390 (d) LOC_Os08g42400 (e) LOC_Os08g42410 (f) LOC_Os08g42420



(g) (h)

Figure 4.17 Contd..: Amplification plots for candidate genes (g) LOC_Os08g42430 (h) LOC_Os08g42440

4.5.4 Candidate gene specific qRT-PCR analysis

1. LOC_Os08g42370: The fold change analysis with respect to this gene reveals upregulation of transcript starting from 24 hours post inoculation. The maximum transcript abundance is observed at 48 hours post infection (Figure 4.18).

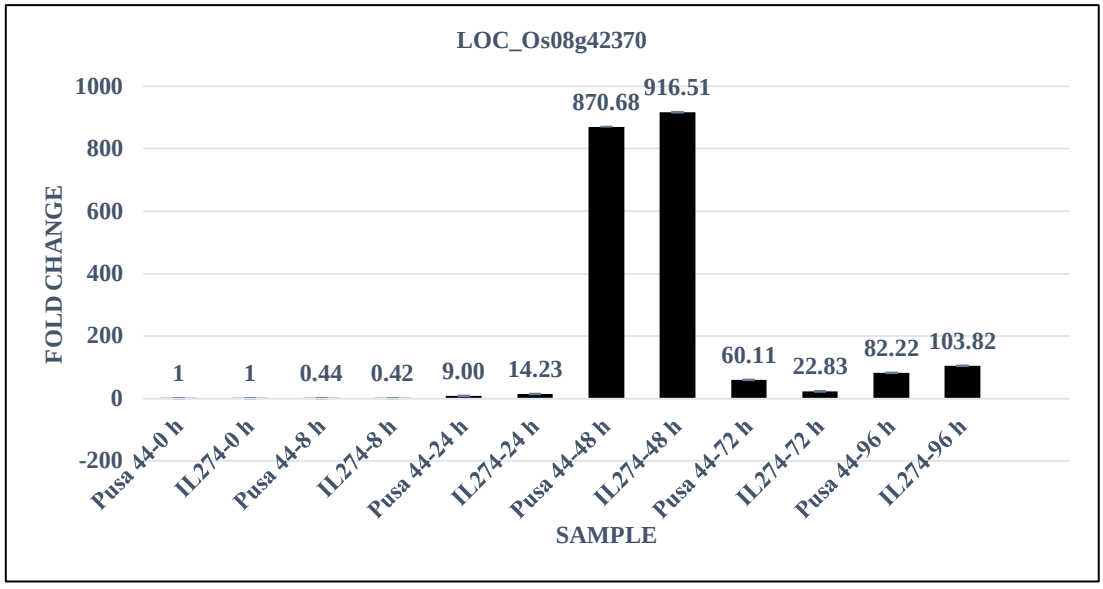


Figure 4.18: Fold Change depiction for LOC_Os08g42370

2. LOC_Os08g42380: This gene cannot be validated due to inconvenience in obtaining the amplification for the respective primers.

3. LOC_Os08g42390: The $\Delta\Delta C_t$ method used for calculating the fold change for the expression of transcripts of this gene is depicted in graphical manner in Figure. The results indicated the abundance of transcripts post inoculations starting from 8 hours to 96 hours

(Figure 4.19). The relative abundance was observed for the susceptible and resistant parents owing to the fact that these transcripts are not involved in molecular mechanism conferring resistance governed by *xa-45(t)*.

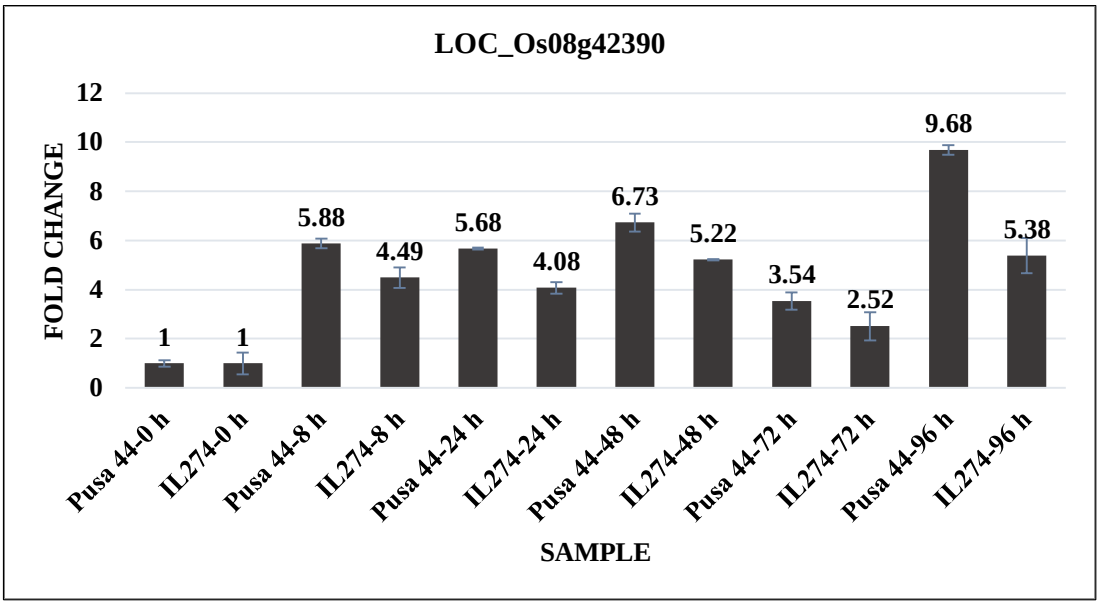


Figure 4.19: Fold Change depiction for LOC_Os08g42390

4. LOC_Os08g42400: The fold change evaluated for this gene represents down regulation of this gene after inoculations (Figure 4.20). The downregulation was found consistent at all the time intervals.

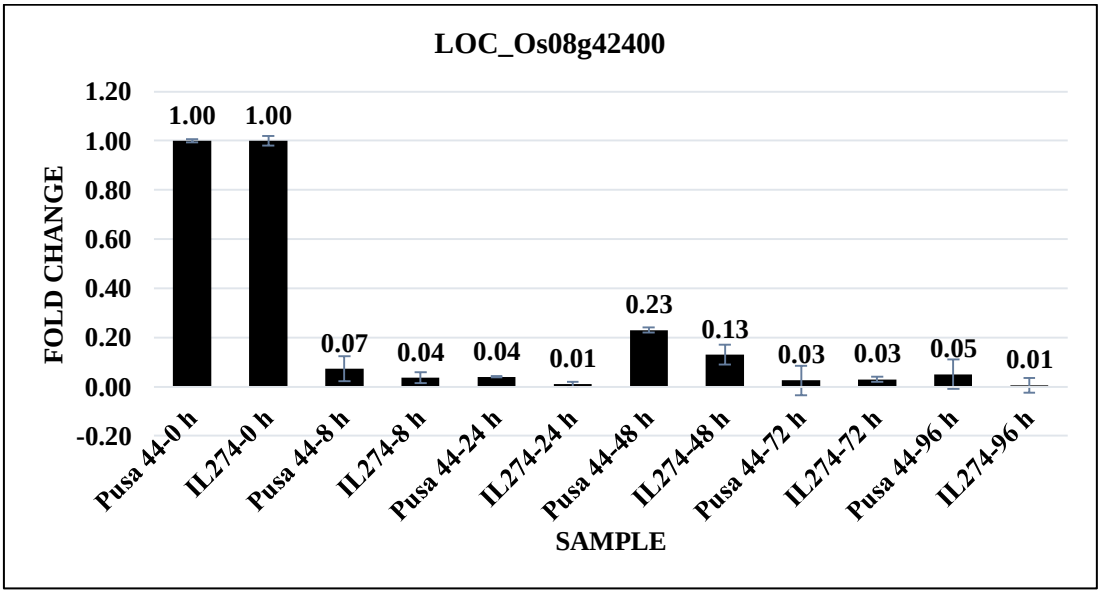


Figure 4.20: Fold Change depiction of LOC_Os08g42400

5. LOC_Os08g42410: This gene has been confirmed as the putative gene for *xa-45(t)* following the experiments targeted for fine mapping. The qRT-PCR was targeted especially for the validation of results of this gene. The relative abundance of transcripts was indicated

gradually increasing for the resistant post inoculation starting from 8 hours. The maximum expression of transcript of this gene was recorded at 72 hours of time interval after inoculation. It corresponded to 4.46-fold upregulation at this time interval. These results however validate the findings of fine mapping experiment (Figure 4.21).

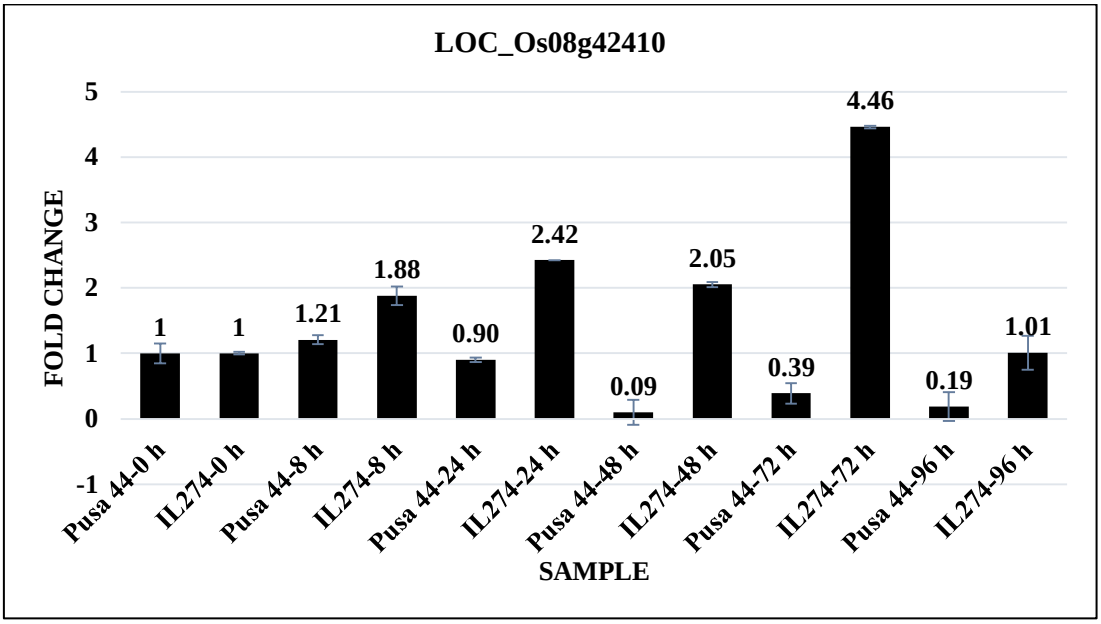


Figure 4.21: Fold Change depiction for LOC_Os08g42410

6. LOC_Os08g42420: The fold change assay for this gene reveals a gradual abundance of transcript at 8 and 24 hours post inoculations of only Pusa 44, susceptible parent. It is followed by significant decrease in transcript level post 48 hours of inoculations. This pattern of transcript expression is futile, because this does not correspond to the phenotypic behaviour for the resistance governed by *xa-45(t)* (Figure 4.22).

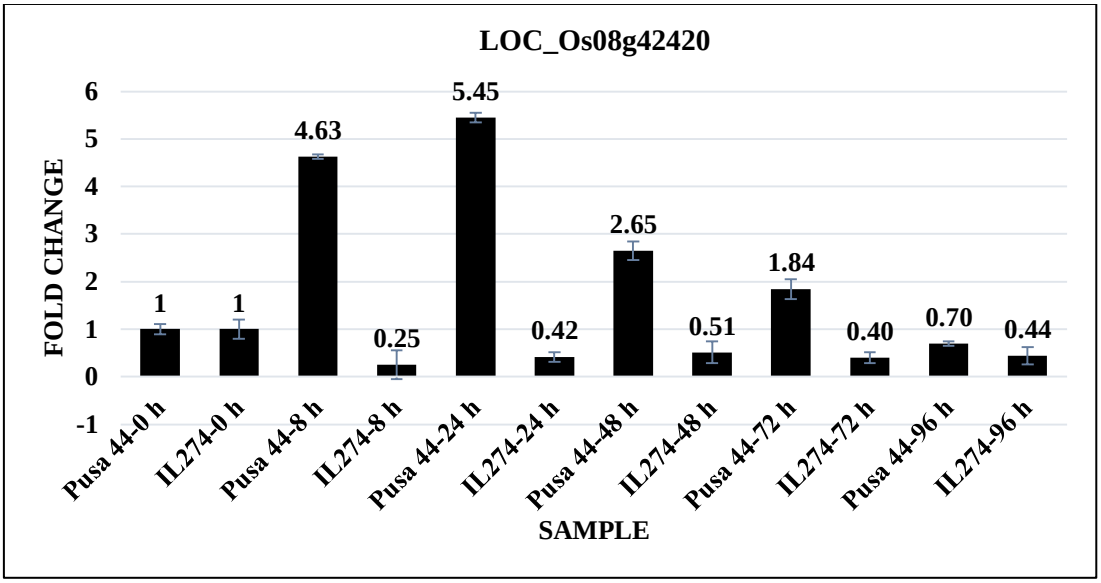


Figure 4.22: Fold Change depiction for LOC_Os08g42420

7. LOC_Os08g42430: This gene cannot be validated due to inconvenience in obtaining the amplification for the respective primers.

8. LOC_Os08g42440: The data for transcript abundance of this gene do not represents any correspondence with the resistance behaviour governed by *xa-45(t)* (Figure 4.23).

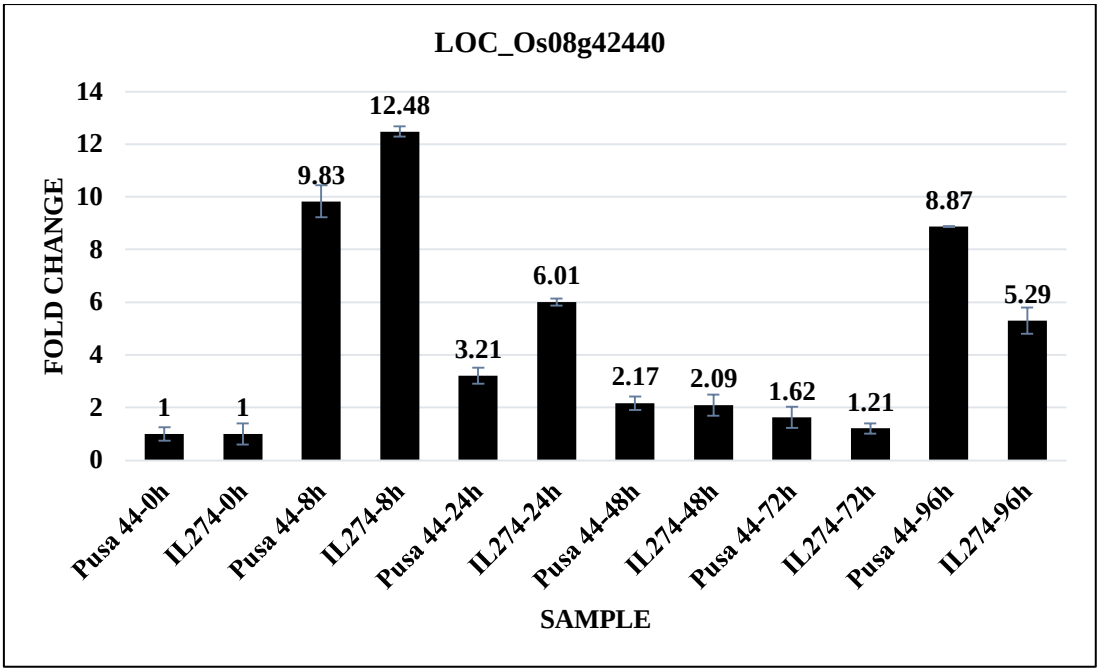


Figure 4.23: Fold Change depiction for LOC_Os08g42440

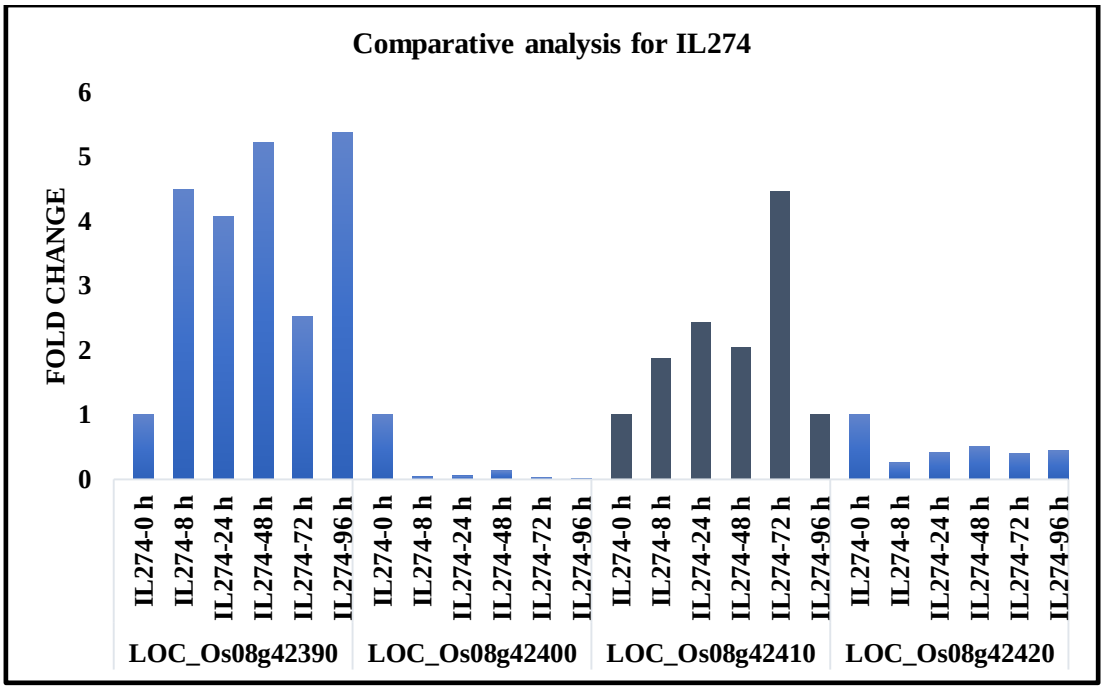


Figure 4.24: Comparative depiction for candidate gene with respect to the genotype IL274 at different time intervals post inoculations

Therefore, these results infer that the LOC_Os08g42410 is the putative gene among the 80 kb region, involved in providing resistance (Figure 4.24). The findings of fine mapping study have been well clarified with the expression analysis experiment. Thus, LOC_Os08g42410 is proven gene for the *xa-45(t)* at DNA as well as at transcript abundance level.

4.6 Functional role of the LOC_Os08g42410

The putative function of LOC_Os08g42410 belongs to the transketolase activity. Various studies have clearly demonstrated the role of transketolase in plant growth, development as well as physiological processes. Many studies reveal the role of transketolase especially in photosynthetic activities of plants. Anciently Henkes *et al* (2001) reported the downregulation of transketolase enzyme in tobacco transformants which further inhibits ribulose-1,5-bisphosphate, confirming its role in photosynthesis. Similarly, in rice plants decreased amount of transketolase was observed under salt stress that ultimately hampers photosynthetic activity of seedlings (Kim *et al* 2005). Transketolase enzymatic activity is found highly profound in signalling cascades and reactive oxygen species (ROS) production on the onset of disease reaction. Tunc-Ozdemir *et al* (2009) verdicts the role of transketolase in production of cytosolic NADPH that further promotes ROS production during stress conditions. Fernandez *et al* (2014) reported role of transketolase in providing resistance in rice against rice blast disease. Various reports suggest the protective role of transketolase for other crops like *Zea mays* (Rapala- Kozik *et al* 2008) and *Solanaceum oleracea* (Kaiser 1976; Takabe *et al* 1980).

4.7 Conclusions

Among the various recessive genes, present study was targeted for *xa-45(t)*. The experiments were focused on devising the gene responsible for the disease reaction governed by *xa-45(t)*. The fine mapping experiment involved the phenotypic and genotypic evaluation of RIL population developed from Pusa 44 and IL274 (BC_3F_8). The phenotyping assay with respect to PbXo-7 reveals the inheritance pattern of population, corresponding to the fact that a single gene is conferring this resistance. Fine mapping strategies revealed that among the different genes 13 SNPs and 5 Indels were identified form which 6 SNPs and 1 Indel were considered putative for dCAPs analysis and development of Indel markers. The genotypic pattern through dCAPs markers finally concluded that a single locus LOC_Os08g42410 is considered as the putative gene behind *xa-45(t)*. The putative function of LOC_Os08g42410 belongs to the transketolase activity. Transketolase enzymatic activity is highly profound in

signalling cascades and ROS production on the onset of disease reaction further strongly supports its candidacy towards *xa-45(t)*. The phenotyping with PbXo-7, PbXo-8 and promoter differences obtained in *xa13* gene clearly shows that *xa-45(t)* is not allelic to *xa13* rather a novel gene.

CHAPTER V

SUMMARY

Bacterial blight is one of the most serious diseases of rice causing huge yield losses. The deployment of breeding and molecular techniques for the development of resistant varieties has been devised as the best strategy for the purpose of effective disease management. It includes exploitation of already present germplasm in the form of wild cultivars. The gene pool of wild species of rice has been opening new avenues in the field of research at unprecedent pace. Studies suggest that these wild relatives are exclusive reservoir of many resistance genes, which can be beneficial when combined with the elite traits. At PAU, the *O. glaberrima* acc. 102600B has displayed resistant reactions against PbXo 1-10 pathotypes prevalent in Punjab. A novel recessive QTL, *xa-45(t)* has been already identified from population devised from *O. glaberrima* derived introgression line and Pusa 44 on chromosome 8.

For the genetic studies, the F_6 and F_7 RIL population developed from a cross between Pusa 44 and IL274 were subjected to phenotypic evaluation against PbXo-7 pathotype. Around 1100 individuals were raised and used for phenotypic evaluations under field conditions of School of Agricultural Biotechnology field, PAU, Ludhiana in the 2019-2020 Kharif season. The disease reaction was recorded in terms of the length of diseased lesion. Following it, mean disease score was allotted to each individual. Scoring was followed in the pattern of, 0 for immune, 1 for highly resistant, 3 for resistant, 5 for moderately susceptible, 7 for susceptible and 9 for highly susceptible reactions. Among the 1100 individuals, a sub-population of 300 individuals was chosen for further analysis. The statistical analysis included the Chi square analysis for goodness of fit, which depicted the segregation ratio of population in 1:1 at 5% level of significance. The ratio validates the role of single gene in the response of *xa-45(t)*.

Following the phenotypic assay, various strategies were employed for fine mapping of the 80 kb region which harbors 9 candidate genes. The segregation pattern reveals the significant relevance of a single gene conferring resistance governed by *xa-45(t)*. Subsequently, for the genotypic assay, outsource sequencing of the PCR products of parental lines from the overlapping primers of all the candidate genes was performed. The sequences so obtained were utilized for the identification of variations among the two parents for 9 candidate genes. The putative SNPs were filtered and analyzed using dCAPs analysis. From the 190 individuals, at most three lines were clarifying the break point with respect to LOC_Os08g42350, LOC_Os08g42370 and LOC_Os08g42400. In addition, 15 individuals showed non correspondence of LOC_Os08g423420 and LOC_Os08g42440 collectively to

the phenotypic behaviour. Also, 9 progenies demarcate a single locus LOC_Os08g42440 for not considering further for candidacy. Apart from SNPs, an Indel was confirmed for LOC_Os08g42410 genotypically by designing of specified marker. The genotypic data of LOC_Os08g42410 clearly reveals its correspondence with the disease reaction of the individuals. Thus, among the 9-candidate gene, LOC_Os08g42410, approximately a 5 kb long gene is to be considered as a putative gene in the region. The putative function of this gene belongs to transketolase activity. Its role has been verified under various biotic and abiotic stress conditions for many crops.

The relative expression analysis following qRT-PCR was used to validate the role and to explore the behavior of putative gene at different time intervals from the *Xoo* inoculation. All candidate genes were included in qRT-PCR analysis. Evaluation of transcript abundance of LOC_Os08g42370, LOC_Os08g42390 and LOC_Os08g42400 shows overexpression of these genes post BB inoculation for the resistant as well as susceptible parent. Following this, the expression of LOC_Os08g42400 shows downregulation of this gene post inoculation for both parental lines. The candidacy of these genes has been nullified by qRT-PCR analysis. In addition, qRT-PCR analysis verifies the role of LOC_Os08g42410 under pathogen infection. The results summarize the upregulation of LOC_Os08g42410 transcripts from 8 hours to 72 hours post inoculation. Whole study cumulates to the fact that LOC_Os08g42410 is considered to be the putative gene among the 9 candidate genes specified for *xa-45(t)*. The putative function of this gene is transketolase activity. Various studies are reported for the role of transketolase under biotic and abiotic stresses. The markers specific to LOC_Os08g42410 can be effectively used for various breeding approaches for the transfer of this BB resistance gene to elite cultivars in order to improve the agriculture produce.

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