CHARACTERIZATION OF POWDERY MILDEW RESISTANCE GENE AT THE *er1* LOCUS IN RESISTANT/ TOLERANT GENOTYPES OF PEA (*Pisum sativum* L.)

By

Redhima Kapoor
(J-16-MB-27)

Thesis submitted to
Faculty of Post Graduate Studies
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

School of Biotechnology
Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
Main Campus, Chatha, Jammu 180009
2018

CERTIFICATE-I

This is to certify that the thesis entitled "**Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)**" submitted in partial fulfilment of the requirements for the degree of **Master of Science in Biotechnology** to the Faculty of Post-Graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu is a record of bonafide research carried out by **Ms. Redhima Kapoor**, Registration No. **J-16-MB-27** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that such help and assistance received during the course of investigation have been duly acknowledged.

Dr. Susheel Sharma
Major Advisor

Place : Jammu

Date : 01/ 08/ 2018

Endorsed

Coordinator

School of Biotechnology

SKUAST-J, Chatha

Date: 01/ 08/ 2018

CERTIFICATE-II

We, the members of the Advisory Committee of **Ms. Redhima Kapoor**, Registration No. **J-16-MB-27**, a candidate for the degree of **Master of Science in Biotechnology**, have gone through the manuscript of the thesis entitled "**Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)**" and recommend that it may be submitted by the student in partial fulfilment of the requirements for the degree.

Dr. Susheel Sharma
Major Advisor

Place : Jammu

Date : 07/ 08/ 2018

Advisory Committee Members

Dr. R.K. Salgotra
Professor (Biotechnology)
School of Biotechnology

Dr. Moni Gupta
Associate Professor (Biochemistry)
Division of Biochemistry

Dr. S.K. Gupta (Dean's Nominee)
Professor (PBG)
Plant Breeding and Genetics

07/08/18

CERTIFICATE-III

This is to certify that the thesis entitled **"Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)"** submitted by **Ms. Redhima Kapoor**, Registration No. **J-16-WB-27** to the Faculty of Post-Graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu in partial fulfilment of the requirements for the degree of **Master of Science in Biotechnology** was examined and approved by the Advisory Committee and External(s) on 13/8/ 2018.

External Examiner

(Dr. S.M. Zargar)
Assistant Professor (Sr. Scale)
Division of Plant Biotechnology
SKUAST- Kashmir, Shalimar, Srinagar

Dr. Susheel Sharma
Assistant Professor
Major Advisor

13/8/18

Coordinator
School of Biotechnology

30/8/18

Dean
Faculty of Agriculture,
SKUAST-Jammu

ACKNOWLEDGEMENT

"It's the little things that tend to add up in a big way"

The path towards this Thesis has been circuitous. Writing this thesis would never have been accomplished and brought to a favorable conclusion without the help of those to whom I would express my heart-felt thanks.

It is an honour and great privilege to pay ineffable gratitude to my worthy, generous, venerated, unparalleled and innovative major advisor ***Dr. Susheel Sharma****, Assistant Professor, School of Biotechnology, for his presevervance, enthusiastic guidance, illuminaing inspiration and affirmative attitude to accomplish this task successfully. I am tremendously fortunate to have advisory committee members* ***Dr. R.K. Salgotra (****Professor, School of Biotechnology),* ***Dr. Moni Gupta (****Associate Professor, Biochemistry) and Dean's Nominee* ***Dr. S.K. Gupta (****Professor & Head, Division of Plant Breeding and Genetics). I thank them for supporting this thesis and giving such thoughtful feedback, always aimed at moving me forward.*

With unending modesty, I reiterate my respectful thanks and warm regards to ***Dr. Sumita Kumari*** *(Assistant Professor, School of Biotechnology) whose unfathomable concern and prudent guidance has enabled me to attain this goal.*

It is my sole prerogative to place on records my everlasting gratitude and ecstatic thanks to ***Dr. R.K. Salgotra****, Professor and Coordinator (School of Biotechnology),* ***Dr. A.K. Singh*** *(Associate Professor, School of Biotechnology),* ***Dr. Manmohan Sharma*** *(Associate Professor, School of Biotechnology),* ***Dr. G.K. Rai*** *(Assistant Professor, School of Biotechnology) and* ***Dr. Ravinder Singh*** *(Assistant Professor, School of Biotechnology).*

I do extend my thanks to the non-teaching staff of my Division for their help during the present study.

With great defence, I would like to thank Hon'ble Vice Chancellor ***Dr. Pardeep Kumar Sharma*** *for allowing me to undertake the study. I owe my sincere thanks for his kindness and generosity.*

*I would also like to thank Indian Institute of Horticultural Research (IIHR) Bangalore, National Bureau of Plant Genetic Resources (****NBPGR****) New Delhi and*

germplasm. I would also like to acknowledge the financial support received from DST SERB (No. SB/YS/LS-182/2013) for partial funding of my research work.

I am thankful my juniors ***Chahat Chopra*** *and* ***Jaspreet Singh*** *for their help and cooperation. I also cordially acknowledge the assistance extended by the field Workers* ***Neetu Bhaiya and Tarvindaram Bhaiya*** *for timely and sincere help during the course of experimentation. My thanks and appreciation also goes to my Seniors* ***Dr. Aejaz Ahmad Dar, Dr. Ritika Mahajan, Dr. Padma Lay, Ankila Salathia, Usha Kiran, Shazia Mukhtar, Diksha Bhadwal, Dr. Jebi Sudan, Dr. Meenakshi Raina, Deepika, Madhavi Sharma and Mridhu Sharma*** *for their appreciation and guidance.*

*Verbiage is not adequate to evince my indebtedness and admiration for the selfless love, unceasing efforts and illuminating guidance which I am rewarded by my parents (****Mr. Rakesh Kapoor and Mrs. Rekha Kapoor****) and my brother* ***Rishav Kapoor****. Their blessings and prayers always provide me the strength to fulfill my ambitions.*

Finally, I offer my sincere thanks & profound regards with deep sense of gratitude to all, who have helped me to accomplish this destination.

Dated: 01 / 08/ 2018

Redhima Kapoor

ABSTRACT

Title of Thesis	:	Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)
Name of Student	:	Redhima Kapoor
Admission No.	:	J-16-MB-27
Major subject	:	Biotechnology
Name &designation of Major Advisor	:	Dr. Susheel Sharma, Assistant Professor
Degree to be awarded	:	M.Sc. Biotechnology
Year of Award of Degree	:	2018
Name of University	:	Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu

Abstract

Pea (*Pisum sativum* L.) is one of the most widely grown legume food crops and represents a versatile and inexpensive protein source for animal feeding. Diseases such as powdery mildew, caused by the obligate biotrophic fungus *Erysiphe pisi* DC belonging to the ascomycete order of Erysiphales, is a serious destructive disease of the crop causing heavy yield losses. For powdery mildew resistance three genes (two recessive '*er1*', '*er2*' and one dominant '*Er3*') have been reported till date. *PsMLO1* is *Er1* and that the loss of *PsMLO1* function conditions durable broad-spectrum powdery mildew resistance in pea. To date, seven *er1* alleles (*er1-1* to *er1-7*) have been identified conferring the *er1*-resistant phenotype. In the present study, an initiative was taken to profile 37 different *Pisum* genotypes, comprising of resistant/tolerant and susceptible notified genotypes, using molecular markers linked to powdery mildew resistance genes. A set of 37 *Pisum* genotypes were sown in pots during *rabi* 2017-18 at the Research Farm of School of Biotechnology, SKUAST-J, Chatha. For molecular profiling a total of ten markers were used (Sc-OPO-18_{1200}, Sc-OPE-16_{1600}, Sc-OPO-10_{650}, AD-60, AA-374e A-5 and OPL-6: *er1* linked; ScX-17_{1400} and AA-278: *er2* linked; ScAB1_{874} *Er3* linked). For all the markers the data was highly polymorphic among the resistant and susceptible genotypes and thus might serve as a ready reference for breeders aiming introgression of powdery mildew resistance genes in pea. For the characterization of *PsMLO* locus, out of the 12 genotypes used, only five genotypes (Improved JI-1559, Bonneville, Pb-89, IC-219002 and DPPMR-09-1) were able to amplify with *PsMLO* specific markers. The sequencing of these amplified products was carried out and good contigs of length 1764 and 1778bp were obtained for IC- 219002 and DPPMR-09-1, respectively only. Based on sequence alignment analysis the sequence of IC- 219002 and DPPMR-09-1 showed that the *PsMLO*1 c-DNA had a 10-bp deletion (TCATGTTATT) corresponding to position 111–120 of the wild-type *PsMLO*1 c-DNA thus confirming the presence of *er1* allele, designated as *er1*-7. Therefore, these accessions might be used in pea breeding studies as the functional markers flanking this 10 bp deletion have been developed and can effectively be used in the MAS.

Keywords: *Pisum*, *Erysiphe*, powdery mildew linked markers, *er1, er2, Er3, PsMLO*

Signature of Major advisor

Signature of Student

CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF ABBREVIATIONS

CTAB	-	cetyl-trimethyl ammonium bromide
c-DNA	-	complementary- deoxyribonucleic acid
DNA	-	Deoxyribonucleic acid
dNTP's	-	deoxriboynucleotide triphosphate
DEPC	-	Diethyl pyrocarbonate
EDTA	-	Ethylene diamine tetra acetic acid
Fig	-	Figure
kg/ha	-	Kilogram per hectare
L	-	Litre
mg	-	milligram
mL	-	millilitre
MOPS	-	3-(N-morpholino) propanesulfonic acid
ng	-	nanogram
^{o}C	-	Degree Celsius
OD	-	Optical Density
pmol	-	Picomole
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease enzyme
rpm	-	Rotations per minute
RAPD	-	Random Amplified Polymorphic DNA
SSR	-	Simple Sequence repeat
SCAR	-	Sequence characterized amplified regions

µg	-	Microgram
µl	-	Microlitre
%	-	Percentage

CHAPTER 1

INTRODUCTION

Pisum sativum L., the common pea (also known as the garden or field pea), is an herbaceous annual in the Fabaceae (formerly Leguminosae) family with diploid chromosome number, 2n = 14 (Bouhadida *et al*., 2013) with a genome size of 5000 Mbp (Sato *et al.*, 2010), originally from the Mediterranean basin and Near East. Peas appear to have been cultivated for nearly 7,000 years. The earliest archaeological finds of peas come from Neolithic Syria, Turkey and Jordan.

The vegetable pea production amounted to 17.43 Mt worldwide (FAO, 2013) and dry pea represented the third most important pulse crop production after common bean and chickpea with 11.16 Mt produced worldwide (FAO, 2013). Dry pea ranks third to common bean and chickpea as the most widely grown in the world with more than 11 million tonnes production in 2013 (Burstin *et al.*, 2015).

Pea (*Pisum sativum* L.) was the original model organism used in Mendel's discovery of the laws of inheritance, making it the foundation of modern plant genetics. Commercial interest in peas and other pulses as a protein source (Santalla *et al.*, 2001) has been growing in recent years. But many biotic and abiotic stresses hinder in getting the potential yield/output. In order to meet these demands, the development of new high-yielding cultivars or continuous hunting for desirable traits from diverse population with resistance to various abiotic and biotic stresses on a sustainable basis is greatly desired. Further improvements through capturing genetic diversity is obligatory in order to develop crop varieties harbouring resistance to various stresses, higher yields and improved nutritive value (Able *et al*., 2007). Knowledge about germplasm diversity and genetic relationships among breeding materials are prerequisites in framing strategies for a sound crop improvement programme.

Of the various diseases affecting peas, the powdery mildew caused by *Erysiphe pisi* D.C. is a serious threat to pea cultivation in pea growing areas of the world. The powdery mildew disease, caused by obligate biotrophic fungi belonging to the

ascomycete order of Erysiphales, is common among higher plant species and severely affects the yield and the quality of many crops (Smith *et al.* 1996). The disease can hasten crop maturity and reduce total biomass yield (Gritton and Ebert, 1975; Falloon and Viljanen-Rollinson, 2001). Powdery mildew caused by *E. pisi* D.C. can result in yield losses of 25-50% of pea production (War-kentin *et al.* 1996; Fondevilla *et al.* 2011). Yield losses up to 80% under heavy infection pressure in susceptible cultivars have been reported in peas (Smith *et al.* 1996; Ghafoor and McPhee, 2012).

Farmers rely on use of fungicides to control powdery mildew which is not an environmental friendly approach. The use of resistant varieties harbouring resistant genes is the most effective and economical method of disease control (Ghafoor and McPhee, 2012; Fondevilla and Rubiales, 2012). Two single recessive genes (*er1* and *er2*) and one dominant gene (*Er3*) have been identified for powdery mildew resistance in pea germplasms to date. Most pea powdery mildew resistant cultivars rely on the presence of the recessive gene *er1*, which was first reported by Harland (1948) following the screening of germplasm collected in the town of Huancabamba, in the northern Peruvian Andes. The mechanisms of the three resistance genes have been studied at the cellular level. The *er1* gene confers systemic and immune or high- level resistance by preventing *E. pisi* from penetrating the pea epidermal cells. In contrast, resistance conferred by *er2* and *Er3* is mainly controlled by a post-penetration hypersensitive response (Fondevilla *et al.* 2006). However, complete resistance resulting from *er2* occurred only at 25^{O}C or in mature leaves (Tiwari *et al.* 1997).

The DNA markers linked to resistance genes provide an alternative to disease screening for pyramiding of powdery mildew resistance genes. Since the DNA markers are not affected by epistatic interactions, they can be used to confirm the presence of multiple resistance genes in the backcross progenies without disease screening, thereby increasing efficiency of selection and reducing time span for the introgression of resistance genes. Molecular markers linked to the three resistance genes (*er1*, *er2* and *Er3*) have been developed by several research groups. The powdery mildew resistance gene *er1* has been located to pea linkage group VI and random amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR) and simple

sequence repeat (SSR) markers tightly linked to the gene have been identified (Timmerman *et al*. 1994; Tiwari *et al*. 1998; Janila and Sharma 2004; Ek *et al*. 2005). The powdery mildew resistance gene *er2* has been reported to be located on linkage group III and SCAR and SSR markers closely linked to *er2* gene have also been identified (Katoch *et al*., 2009). Similarly for *Er3*, two SCAR markers have been reported which when used in combination can distinguish the homozygous and heterozygous individuals with high efficiency (Fondevilla *et al*., 2008)

er1 resistance has been shown to be due to loss-of-function mutations of a powdery mildew susceptibility gene belonging to the MLO gene family, referred to as *PsMLO1* (Pavan *et al*. 2010, 2011; Humphry *et al*. 2011). To date, seven *er1* alleles (*er1*-1, *er1*-2, *er1*-3, *er1*-4, *er1*-5, *er-1*-6 and *er1-7*) have been discovered. Each of these *er1* alleles corresponds to a different *PsMLO1* mutation, according to the mutation site.

In our recent studies few lines have been shown to be highly resistant to *E. pisi* (Deepika, 2016). Thus, in the present research we seek to exploit molecular marker technology for characterization of resistant/tolerant pea germplasm by using powdery mildew linked molecular markers and determine the sequence of the *PsMLO1* gene at the *er1* locus.

Based upon above considerations, the present investigation entitled, **"Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)"** was envisaged with the following objectives:-

a. To characterize resistant / tolerant pea germplasm using powdery mildew linked molecular markers

b. To characterize the resistance alleles at the *er1* locus by determining the sequence of the *PsMLO1* gene

CHAPTER 2

REVIEW OF LITERATURE

Pea (*Pisum sativum* L.) is a self-pollinated, cool-season, annual and diploid crop having 14 chromosomes (2n=14) with a genome size of about 5000 Mbp (Sato *et al*., 2010). Pea has been extensively used in early hybridization studies and was the model organism of choice for Mendel's discovery of the laws of inheritance, making pea part of the foundation of modern genetics. Pea has also been used as model for experimental morphology and physiology. Several methods are available for population improvement in pea (Muehlbauer *et al*., 1988) and analyzing variability is an important aspect. Pea (*Pisum sativum L*.) is a major pulse crop grown having protein-rich seeds and is an important component of agroecological cropping systems in diverse regions of the world (Burstin *et al*., 2015).

Powdery mildew of pea (*Pisum sativum*) caused by *Erysiphe pisi*, causing infection in all aerial green parts of the plant is one of the major destructive diseases of this crop. Ascomycete fungus *E. pisi*, the causal agent of powdery mildew of pea, is a destructive pathogen causing infection on all the above ground parts of pea plants (Singh, 2000). Identification of resistance sources and their incorporation into current cultivars remains the best strategy for controlling the diseases. Rana *et al.* (2012) aimed at screening of 761 pea germplasm for resistance to powdery mildew under natural epiphytotic conditions. Out of which 64 accessions found resistant in field screening for 2 years. There was sufficient genetic diversity and agronomic superiority in resistant accessions e.g. EC598655, EC598878, EC598704, IC278261 and IC218988, which might serve useful for plant breeders for breeding pea varieties for powdery mildew resistance and high yield.

Various management practices are adopted to control this pathogen at farmers' fields. However, with the increase in awareness regarding the hazardous effect of synthetic fungicides, attempts are being made to go "back to nature" by managing the plant diseases with ecologically acceptable management practices. Among the sustainable and non-hazardous methods, development of resistant varieties harboring resistant genes is the most important management tactic of the disease. Two single recessive genes

(*er1* and *er2*) and one dominant gene (*Er3*) have been identified for powdery mildew resistance in pea germplasms to date. Most pea powdery mildew resistant cultivars rely on the presence of the recessive gene *er1*, which was first reported by Harland (1948) following the screening of germplasm collected in the town of Huancabamba, in the northern Peruvian Andes. The *er1* locus has been mapped on linkage group VI of the pea consensus map (Timmerman *et al*. 1994).

Genetic resistance to powdery mildew has been identified in *P. sativum* lines developed from various parts of the world (Harland, 1948; Heringa *et al*. 1969; Marx 1971; Kumar and Singh, 1981; Tiwari *et al*. 1999). While the most of scientists have detailed a single recessive gene '*er1*', resistance to powdery mildew in peas (Timmerman *et al*. 1994; Vaid and Tyagi, 1997; Janila and Sharma, 2004), others announced duplicate recessive genes controlling resistance in many cultivars (Sokhi *et al*. 1979; Kumar and Singh, 1981). Heringa *et al*. (1969) revealed two autonomous powdery resistance genes, *er1* and *er2* giving distinctive resistance phenotypes; while *er1* was accounted for to confabulate a high level of protection to all plant parts, the *er2* resistance was confined just to leaves, a phenomenon likewise observed by different workers (Marx, 1986; Tiwari *et al*. 1997). While examining the histological premise of *er1*and *er2* interceded restriction, Fondevilla *et al*. (2006) announced that *er1* presents complete resistance from powdery mildew by constraining pathogen penetration, while the *er2* interceded resistance is primarily in view of decrease in penetration success supplemented by post-penetration cell death. Consolidating of resistance process intervened by *er1* and *er2* into a single genetic background is relied upon to widen the spectrum and durability of resistance adapted by each of these genes. Notwithstanding, joining of the two genes into a genetic background by conventional backcross breeding is difficult because the high level of penetration resistance mediated by *er1* will preclude the detection of *er2* in the breeding material (Fondevilla *et al*. 2006). The recessive nature of the powdery mildew resistance genes and difficulties related to the treatment of obligate pathogens like *E. pisi*, additionally entangle determination of resistant progenies in breeding programs. Recently, a dominantly inherited gene for resistance to powdery mildew, named *Er3*, was identified in *Pisum fulvum* (Fondevilla *et al. 2007*). *Er3*, unlike the gene *er2*, is not temperature dependant.

2.1. Linkage of molecular marker with various *er* genes (*er1*, *er2* and *Er3*):

Recessive gene *er1*, first described over 60 years ago, is well known in pea breeding, as it still maintains its efficiency as a powdery mildew resistance source. Sharma *et al* (2008) carried out screening of the powdery mildew-resistant cultivar 'DMR11' and its susceptible near-isogenic lines for polymorphism and revealed linkage of two RAPD primers (OPO-02 and OPU-17) to the *er* gene and a sequence characterized polymorphic region (SCAR) primer, ScOPD-10_{650} with *er* in a population of 83 F_2 plants in the order: OPU-17 - *er* - ScOPD-10_{650} - OPO-02. The markers ScOPD-10_{650} and OPU-17 being coupled with the allele causing resistance substantially increased the efficiency of marker-assisted selection in pea breeding for powdery mildew.

Similarly, there are various reports in which powdery mildew resistance gene *er1* has been located to pea linkage group VI and various random amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR) and simple sequence repeat (SSR) markers tightly linked to the gene have been identified (Timmerman *et al*., 1994; Tiwari *et al*., 1998; Janila and Sharma, 2004; Ek *et al*., 2005). Recently, highly informative cleaved amplified polymorphic sequence (CAPS), derived cleaved amplified polymorphic sequence (dCAPS), sequence tagged site (STS) and high resolution melting (HRM) markers were developed which enable the selection of each of the five *er1* alleles (Pavan *et al*., 2013).

Katoch *et al.* (2010) reported the identification of SSR and RAPD markers linked to *er2*, and conversion of one of the tightly linked RAPD markers to a SCAR marker for use in marker-assisted selection (MAS) of *er2*.

Fondevilla *et al.* (2008) in their study, on segregating F_2 population derived from the cross between a breeding line carrying the *Er3* gene, and the susceptible cultivar 'Messire', developed molecular markers linked to *Er3*. They reported that the SCAR marker SCW4637 co-segregated with the resistant gene, allowing the detection of all the resistant individuals. The SCAR marker SCAB1_{874}, in repulsion phase with *Er3*, was reported to be located at 2.8 cM from the gene and, in combination with SCW4637, was

capable to distinguish homozygous resistant individuals from heterozygous with a high efficiency.

Also the molecular linkage maps of pea, particularly those incorporating range of PCR based markers are available which can further be used in molecular characterization and MAS studies (Gilpin *et al*. 1997; Laucou *et al*. 1998; Weeden *et al*. 1998; Irzykowska, 2002; Loridon *et al,* 2005). Loridon *et al*. (2005), aimed at providing reliable and cost effective genotyping conditions, level of polymorphism in a range of genotypes and map position of newly developed microsatellite markers in order to promote broad application of these markers as a common set for genetic studies in pea. Optimal PCR conditions were determined for 340 microsatellite markers based on amplification in eight genotypes. Levels of polymorphism were determined for 309 of these markers. Compared to data obtained for other species, levels of polymorphism detected in a panel of eight genotypes were high with a mean number of 3.8 alleles per polymorphic locus and an average PIC value of 0.62, indicating that pea represents a rather polymorphic autogamous species. Data obtained from three different crosses were used to build a composite genetic map of 1,430 cM (Haldane) comprising 239 microsatellite markers. These include 216 anonymous SSRs developed from enriched genomic libraries and 13 SSRs located in genes. The markers are quite evenly distributed throughout the seven linkage groups of the map, with 85% of intervals between the adjacent SSR markers being smaller than 10 cM. There was a good conservation of marker order and linkage group assignment across the three populations.

Ek *et al.* (2005) used microsatellites (SSR) to find markers linked to powdery mildew resistance. The powdery mildew resistant pea cultivar '955180' and the powdery mildew susceptible pea cultivar 'Majoret' were crossed and F2 plants were screened with SSR markers, using bulk segregant analysis. A total of 315 SSR markers were screened out of which five showed linkage to the powdery mildew resistance gene. Instead, two of the markers can be used in combination, which would result in only 1.6% incorrectly identified plants. Thus SSR markers can be successfully used in marker-assisted selection for powdery mildew resistance breeding in pea.

2.2. Characterization of *er1* locus:

Loss-of-function alleles of plant-specific *MLO* (**M***ildew* ***R****esistance* **L***ocus* **O**) genes confer broad-spectrum powdery mildew resistance in monocot (barley) and dicot (*Arabidopsis thaliana*, tomato) plants. Humphry *et al.* (2011) reported that recessively inherited powdery mildew resistance in pea (*Pisum sativum*) *er1* plants is, in many aspects, reminiscent of *mlo*-conditioned powdery mildew immunity, yet the underlying gene had remained elusive to date. They used a polymerase chain reaction (PCR)-based approach to amplify a candidate *MLO* cDNA from wild-type (*Er1*) pea. Sequence analysis of the *PsMLO1* candidate gene in two natural *er1* accessions from Asia and two *er1*-containing pea cultivars with a New World origin revealed, in each case, detrimental nucleotide polymorphisms in *PsMLO1*, suggesting that *PsMLO1* is *Er1*. They corroborated this hypothesis by restoration of susceptibility on transient expression of *PsMLO1* in the leaves of two resistant *er1* accessions. Orthologous legume *MLO* genes from *Medicago truncatula* and *Lotus japonicus* likewise complemented the *er1* phenotype. All tested *er1* genotypes showed unaltered colonization with the arbuscular mycorrhizal fungus, *Glomus intraradices*, and with nitrogen-fixing rhizobial bacteria. Their data demonstrated that *PsMLO1* is *Er1* and that the loss of *PsMLO1* function conditions durable broad-spectrum powdery mildew resistance in pea.

Pavan *et al*. in the same year i.e. 2011, reported that genetic and phytopathological features of *er1* resistance are similar to those of barley, Arabidopsis, and tomato *MLO* powdery mildew resistance, which is caused by the loss of function of specific members of the *MLO* gene family. They described the obtainment of a novel *er1* resistant line by experimental mutagenesis with the alkylating agent diethyl sulfate. This line was found to carry a single nucleotide polymorphism in the *PsMLO1* gene sequence, predicted to result in premature termination of translation and a non-functional protein. A cleaved amplified polymorphic sequence (CAPS) marker was developed on the mutation site and shown to be fully co segregating with resistance in F_2 individuals. Sequencing of *PsMLO1* from three powdery mildew resistant cultivars also revealed the presence of loss-of-function mutations. Taken together, results reported in this study strongly indicate the identity between *er1* and *mlo* resistances and are expected to be of great.

To date, seven *er1* alleles (*er1-1* to *er1-7*) have been identified conferring the er1-resistant phenotype in pea germplasms (Humphry *et al*. 2011; Pavan *et al*. 2011, 2013; Sun *et al*. 2015a, b). Each of the seven er1 alleles corresponds to a different PsMLO1 mutation, which dependson the mutation site and pattern. All alleles except for *er1-2* and *er1-7* are the result of point mutations, including single base substitutions and deletions, in the *PsMLO1* coding sequence.

Pavan *et al*. (2013) aimed to identify functional markers which target *PsMLO1* polymorphisms directly responsible for the resistant phenotype. Highly informative cleaved amplified polymorphic sequence (CAPS), derived cleaved amplified polymorphic sequence (dCAPS), sequence tagged site (STS) and high resolution melting (HRM) markers were developed which enable the selection of each of the five *er1* alleles. Taken together, the results described provide a powerful tool for breeders, overcoming limitations of previously reported *er1*-linked markers due to the occurrence of recombination with the resistance locus and/or the lack of polymorphism between parental genotypes. The HRM marker *er1*-5/ HRM54, targeting a mutagenesis induced *er1* allele recently described by them, does not require manual processing after PCR amplification, and is therefore suitable for large-scale breeding programs based on high-throughput automated screening.

Recently, Sun *et al*. (2016) screened Chinese pea (*Pisum sativum* L.) landraces resistant *to E. pisi*, and characterized the resistance gene(s) at the *er1* locus in the resistant landraces, and developed functional marker(s) specific to the novel *er1* allele *er1-6*. The 322 landraces showed different resistance levels. Among them, 12 (3.73%), 4 (1.24%) and 17 (5.28%) landraces showed immunity, high resistance and resistance to *E. pisi*, respectively. The other landraces appeared susceptible or highly susceptible to *E. pisi*. Most of the immune and highly resistant landraces were collected from Yunnan province. To characterize the resistance gene at the *er1* locus, cDNA sequences of *PsMLO1* gene were determined in 12 immune and four highly resistant accessions. The cDNAs of *PsMLO1* from the immune landrace G0005576 produced three distinct transcripts, characterized by a 129-bp deletion, and 155-bp and 220-bp insertions, which were consistent with those of *er1*-2 allele. The *PsMLO1* cDNAs in the other 15 resistant landraces produced identical transcripts, which had a new point mutation (T/C) at

position 1121 of *PsMLO1*, indicating a novel *er1* allele, designated as *er1*-6. This mutation caused a leucine to proline change in the amino acid sequence. Subsequently, the resistance allele *er1*-6 in landrace G0001778 was confirmed by resistance inheritance analysis and genetic mapping on the region of the *er1* locus using populations derived from G0001778 × Bawan 6. Finally, a functional marker specific to *er1*-6, SNP1121, was developed using the high-resolution melting technique, which could be used in pea breeding via marker-assisted selection.

Sun *et al.* (2016) again identified a novel *er1* allele, *er1-7*, conferring pea powdery mildew resistance was characterized by a 10bp deletion in *PsMLO1* c-DNA, and its functional marker was developed and validation in pea germplam. To identify the gene conferring resistance in DDR-11, the susceptible Bawan 6 and resistant DDR-11 cultivars were crossed to produce F_1, F_2 and $F_{2:3}$ populations. Analysis of *er1*-linked markers in the F_2 population suggested that the recessive resistance gene in DDR-11 was an *er1* allele, which was mapped between markers ScOPE-16 and c5DNAmet. For characterization of *er1* allele, the c-DNA sequence of *PsMLO1* from the parent was obtained and a novel *er1* allele in DDR-11 was identified and designated as *er1-7* which has 10bp detection in position111-120. The *er1-7* allele caused a frame shift mutation, resulting in a premature termination of translation of *PsMLO1* protein. A co-dominant functional marker specific for *er1-7* was developed, InDel 111-120, seven were detected containing resistance allele *er1-7*, which was verified by sequencing their *PsMLO1* c-DNA.

CHAPTER 3

MATERIALS AND METHODS

The present study entitled **"Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)"** was carried out at Molecular Biology Laboratory and Research Farm of School of Biotechnology, Sher-e-Kashmir university of Agricultural Sciences and Technology-Jammu, Chatha during 2017-2018. Materials and methods used for conducting experiments are elucidated under the following headings:-

3.1 MATERIALS

A. The materials in the present study comprised of 37 genotypes of *Pisum* and 11 SSR, SCAR, RAPD and gene specific primers.

B. Reagents and solutions

3.2 MOLECULAR CHARACTERIZATION

3.2.1 Genomic DNA Isolation

3.2.2 DNA Quantification

3.2.3 SSR assay

3.2.3.1.1 Primers used for DNA amplification

3.2.3.1.2 Components used for PCR reaction

3.2.3.1.3 PCR amplification profile

3.2.3.1.4 SSR-PCR banding profile

3.2.4 SCAR assay

3.2.4.1.1 Primers used for DNA amplification

3.2.4.1.2 Components used for PCR reaction

3.2.4.1.3 PCR amplification profile

3.2.4.1.4 SCAR- PCR banding profile

3.2.5 RAPD assay

3.2.5.1.1 Primers used for DNA amplification

3.2.5.1.2 Components used for PCR reaction

3.1 MATERIALS

(A) Experimental material

Thirty seven genotypes of *Pisum* were used in the study for characterization of resistant/tolerant germplasm using already reported linked molecular markers. The seed material was obtained from different sources (Table. 3.1).

Table 3.1: List of genotypes along with their codes and source of procurement

S.NO.	CODE	GENOTYPE	Source
1.	Ps- 8	IC- 218991	NBPGR, New Delhi
2.	Ps- 11	IC- 219002	NBPGR, New Delhi
3.	Ps- 12 -1	IC- 219008	NBPGR, New Delhi
4.	Ps- 12 -2	IC- 219008	NBPGR, New Delhi
5.	Ps- 35	IC- 381453	NBPGR, New Delhi
6.	Ps- 49	HFP-4	NBPGR, New Delhi
7.	Ps- 51	DPPIV-2	HPKV, Palampur
8.	Ps-52	DPEPP-1	HPKV, Palampur
9.	Ps- 57	DPPMR-09-1	HPKV, Palampur
10.	Ps-68	IC-208366	NBPGR, New Delhi
11.	Ps- 73	EC- 381866	NBPGR, New Delhi
12.	Ps- 78	EC- 598729	NBPGR, New Delhi

13.	Ps- 86	Pb-89	Pau, Ludhiana
14.	Ps- 85	Improved JI-2480	HPKV, Palampur
15.	Ps- 88	Improved JI-1559	HPKV, Palampur
16.	Ps- 89	Improved JI- 1559	HPKV, Palampur
17.	Ps- 90	Improved JI- 2302	HPKV, Palampur
18.	Ps- 92	Improved JI-2480	HPKV, Palampur
19.	Ps- 87	IHR-544	IIHR, Bangalore
20.	Li	Lincoln	HPKV, Palampur
21.	Bo	Bonneville	HPKV, Palampur
22.	Ark	Arkel	HPKV, Palampur
23.	Ps- 93	Azad Pea- 1	CSAUAT, Kanpur
24.	Ps- 94	Azad Pea- 2	CSAUAT, Kanpur
25.	Ps- 96	Azad Pea- 4	CSAUAT, Kanpur
26.	Ps- 97	Azad Pea- 5	CSAUAT, Kanpur
27.	Ps- 99	Prakash	SKUAST-Jammu
28.	Ps- 98	Rachna	SKUAST-Jammu
29.	Ps- 100	Arka Sampoorna	IIHR, Bangalore
30.	Ps- 101	Arka Ajeet	IIHR, Bangalore
31.	Ps- 103	Arka Priya	IIHR, Bangalore
32.	Ps- 104	Arka Pramodh	IIHR, Bangalore
33.	Ps- 105	Arka Kartik	IIHR, Bangalore
34.	Ps- 64	Palam Priya	HPKV, Palampur
35.	Ps- 65	Palam Sumool	HPKV, Palampur
36.	Ps- 63	Palam Triloki	HPKV, Palampur
37.	Ps- 106	Pusa Shree	IARI, Pusa

Linked Molecular Markers

In the present study, 10 molecular markers linked to powdery mildew (*er1*, *er2* and *Er3*) were used which included SSRs, SCARs and RAPDs. Primers were customized from IDT (Integrated DNA technologies, USA). List of the primers along with their sequences is presented in the Table 3.2.

Table 3.2: List of ten primers along with their sequences

S. No.	Locus Name	Marker Type	Primer Sequence (5'-3')F/R	Linked gene	Reference
1.	Sc-OPO-18_{1200}	SCAR	F-CCCTCTCGCTATCCAATCC R-CCTCTCGCTATCCGGTGTG	*er1*	Tiwari et al. (1998)
2.	Sc-OPE-16_{1600}	SCAR	F-GGTGACTGTGGAATGACAAA R-GGTGACTGTGACAATTCCAG	*er1*	Tiwari et al. (1998)
3.	Sc-OPO-10_{650}	SCAR	F-GGTCTACACCTAAACAGTGTCCGT R-GGTCTACACCTCATATCTTGATGA	*er1*	Timmerman et al. (1994)
4.	AD-60	SSR	F-CTGAAGCACTTTTGACAACTAC R-ATCATATAGCGACGAATACACC	*er1*	Ek et al. (2005)
5.	AA-374e	SSR	F-GTCAATATCTCCAATGGTAACG R-GCATTTGTGTAGTTGTAATTTCAT	*er1*	Ek et al. (2005)
6.	A-5	SSR	F-GTAAAGCATAAGGGGATTCTCAT R-CAGCTTTTAACTCATCTGACACA	*er1*	Ek et al. (2005)
7.	Sc-$X17_{1400}$	SCAR	F-GGACCAAGCTCGGATCTTTC R-GACACGGACCCAATGACATC	*er2*	Katoch et al. (2010)
8.	AA-278	SSR	F-CCAAGAAAGGCTTATCAACAGG R-TGCTTGTGTCAAGTGATCAGTG	*er2*	Katoch et al. (2010)
9.	Sc-$AB1_{874}$	SCAR	F-CCGTCGGTAGTAAAAAAAACT R-CCGTCGGTAGCCACACCA	*Er3*	Fondevilla et al. (2008)
10.	OPL-6	RAPD	F/R-GAGGGAAGAG	*er1*	Tiwari et al. (1998)

(B) Reagents and Solutions

(1) Stock solutions

(I) Preparation of Stock solutions (500mL) for DNA extraction

a) 0.5 M EDTA 93.05g

b) 1M Tris-Cl 60.55g

c) 5 M NaCl 146.1g

The pH of 0.5 M EDTA and 1 M Tris-Cl was adjusted to 8.0. The solutions were autoclaved after making the final volume up to 500mL.

d) CTAB Buffer

0.5 M EDTA 20mL

1 M Tris-Cl 50mL

5 M NaCl	140mL
β- mercaptoethanol	0.2%
ddH2O	

The final volume of CTAB buffer was made up to 500mL using ddH_2O. It was then autoclaved after adjusting the pH to 8.0. β- mercaptoethanol (0.2%) was added fresh before use.

e) TE Buffer(10X)

1 M Tris-Cl	100mL
0.5 M EDTA	20mL

880mL ddH_2O was added to make 1L TE Buffer. pH was adjusted to 8.0 and autoclaved.

f) RNase (10mg/mL)- 10mL

5 M NaCl	30µL
1 M Tris-Cl	100µL
ddH_2O	9870µL
RNase	10mg

(ii) Preparation of Stock solutions for electrophoresis

a) Preparation of Buffers

1. TBE Buffer (10 X) (Stock Solution)

Tris base	108g
Boric acid	55g
0.5 M EDTA	40mL

Made volume to 1L with distilled water and then autoclaved.

2. TBE Buffer (1X TBE) (Working Solution)

100mL 10X TBE was dissolved in 900mL double distilled water.

3. DNA Loading Dye (6X)

Glycerol	30%(v/v)

Bromophenol Blue	0.25%(w/v)
Xylene cyanol	0.25%(w/v)

(b) Preparation of Agarose Gel

For DNA	0.8%
For SSR	3.0%
For SCAR	2.5%
For RAPD	1.5%

(iii) Reagents for PCR

(a) dNTPs (dATPs/dCTPs/dGTPs/dTTPs)

(b) PCR Buffer 10 X

(c) $MgCl_2$

(d) DNA polymerase

3.2 METHODOLOGY ADOPTED FOR MOLECULAR CHARACTERIZATION

The seed material was grown in pots as shown in Plate 3.1

3.2.1 Genomic DNA Isolation

The genomic DNA isolation was carried out by Doyle and Doyle (1990) method. Young leaves were taken for genomic DNA extraction. Fresh leaf material was taken and grinded to fine powder in liquid nitrogen using pestle and mortar. The powdered material was then transferred to 2mL tubes and 800µl of pre-warmed extraction buffer was added to each tube. These tubes were then incubated at 65ºC in a water bath for one hour with occasional stirring. After incubation, an equal volume of Chloroform: Isoamylalcohol (24:1) was added to each tube and slowly mixed by inverting the tubes for 5 minutes. The samples were then centrifuged at 13,000rpm for 10 minutes. After centrifugation the supernatant was transferred to another fresh autoclaved tube. After this, 0.6 volume of chilled isopropanol was added to the supernatant and stored at 4ºC for overnight. Tubes were then centrifuged at 10,000rpm for 10 minutes. The supernatant was discarded and

Plate 3.1: Growing of *Pisum* plants in pots for sampling and further seed multiplication.

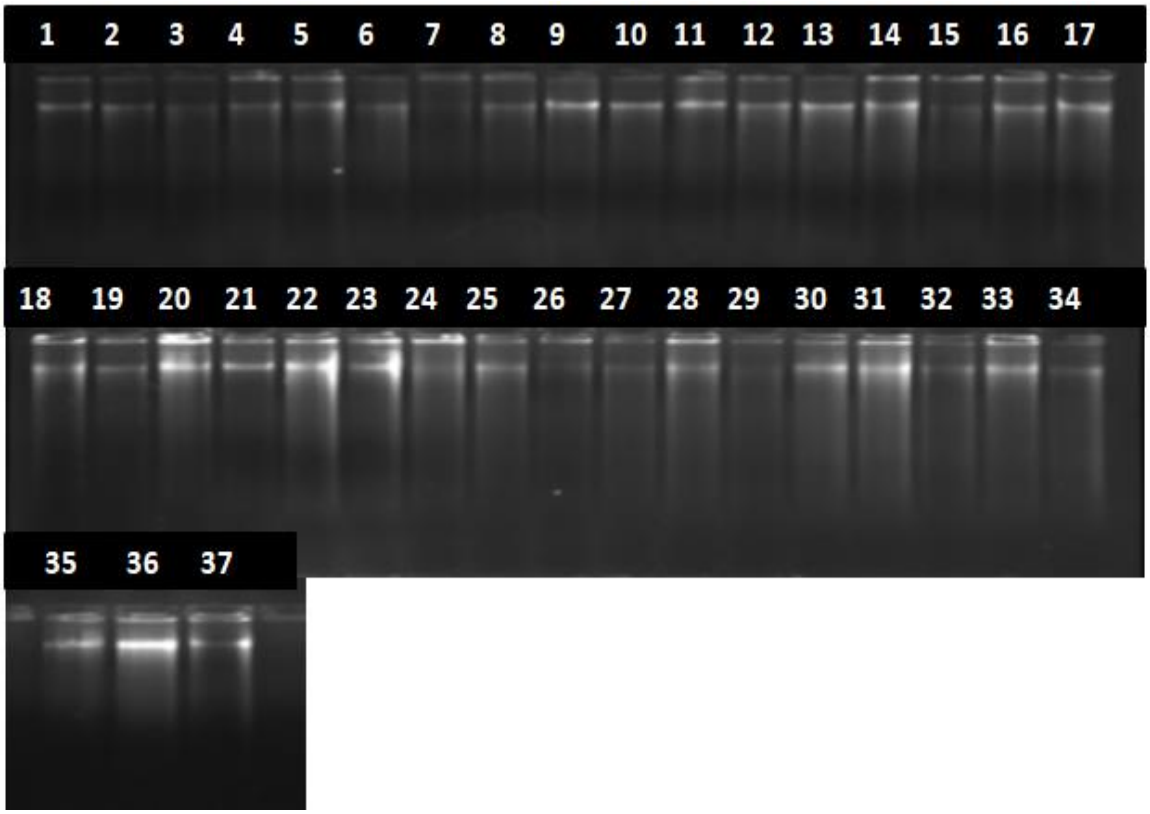


Plate 3.2: Quality DNA of different lines as observed during agarose gel electrophoresis.

the DNA pellet was washed with 70% ethanol (200µl-300µl) to remove contamination and then air dried. DNA pellets were then dissolved in 200µl-300µl of TE Buffer and kept at room temperature (37ºC) for overnight and then stored at 4ºC. For purification of DNA, 100µl of RNase was added to the each sample tube and then incubated at 37ºC for 1 hour in water bath. An equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1) was added, tilted for 10 minutes and centrifuged at 10,00rpm for 10 minutes. The supernatant was collected in another tube, 0.6 volume of chilled iso-propanol was added and centrifugation was done to get DNA pellet. DNA pellet was then washed with 70% ethanol, air dried, dissolved in TE Buffer and stored at 4ºC for further use.

3.2.2 DNA Quantification

Quality and quantity of genomic DNA was estimated by using agarose gel electrophoresis and nano-spectrophotometric method.

Agarose gel electrophoresis

DNA of all genotypes was quantified by loading 5µl of DNA of each genotype mixed with 3µl of loading buffer on 0.8% agarose gel. Agarose gel was prepared by melting 0.8g of agarose in 100mL of 1X TBE (Tris Borate EDTA) buffer in a conical flask in the microwave for 2 minutes. It was allowed to cool for couple of minutes and then stained with ethidium bromide and stirred for sometime. The gel material was then poured in gel casting tray with combs in it and allowed to solidify for 20-25 minutes at room temperature. The electrophoresis was carried out at 80V for 1hour. It was then viewed under gel documentation system. The concentration of DNA was determined by comparing intensity of genomic DNA bands with that of known standards and the quality was assured with the intactness of the bands (Plate No. 3.1).

MySpeq/Nano-spectrophotometric method

Optical density of DNA samples was measured at 260nm and 280nm using Peq-Lab MySpeq.

a) Estimation of DNA concentration in different samples was done as follows:-

Concentration of DNA (ng/µl) = OD_{260} x 50 x Dilution factor

b) Quality analysis was done by calculating the ratio of absorption maxima at 260nm and 280nm. Ratio between 1.8 and 2.0 shows the presence of fairly pure DNA. The value less than 1.8 indicates the presence of protein contaminants and greater than 2.0 indicates the presence of RNA. DNA samples were diluted using sterilized Milli Q water.

3.2.3 SSR Assay

3.2.3.1 Primers used for DNA amplification

SSRs were selected on the basis of earlier studies (Ek et al. 2005; Katoch et al. 2010). A set of 4 SSR primers were selected for use in amplification of genomic DNA.

Primer dilution: Double distilled, autoclaved and deionized water was used to dilute primers.

3.2.3.2 Components used for PCR Reaction

DNA amplification was carried out in PCR tubes containing 25µL reaction mixture. Reaction mixture contained following components. (Table 3.3)

Table 3.3: Reagents with their concentration and quantity used for single PCR reaction in SSR Assay

S.No	Reagents	Concentration	Quantity
1	PCR Buffer	5X	2µl
2	dNTPs	10mM/each	0.2µl
3	$MgCl_2$	25Mm	0.8
4	Forward Primer	10pmole/ µl	0.3µl
5	Reverse Primer	10pmole/ µl	0.3µl
6	DNA	25ng/µl	1µl
7	*Taq* DNA polymerase	1.5U	0.2µl
8	Sterile water		4.2µl
	Total		**10µl**

3.2.3.3 PCR amplification Program

PCR tubes containing master mix and DNA template were thoroughly mixed and subjected to the thermal profile given in Table 3.4. The amplification reaction was carried out in gradient mastercycler. An initial denaturation step of 5 minutes at 94ºC was carried out in PeqLab gradient mastercycler, followed by a loop of 35 cycles each consisting of denaturation {94ºC for 1 minute}, annealing {50-60ºC for 1 minute} and extension {72ºC for 30 seconds}. The final extension was performed at 72ºC for 5 minutes. The PCR products were then stored at 4ºC.

Table 3.4: Thermal profiles used for DNA amplification of SSR markers

Steps	**Cycles**	**Temperature**	**Duration**
Initial Denaturation	1	94ºC	5min
Denaturation	35	94ºC	1min
Annealing		50-60ºC	1min
Extension		72ºC	30sec
Final Extension	1	72ºC	5min

The same reaction mixture without genomic DNA was run for each reaction to serve as a negative control.

3.2.3.4 SSR-PCR banding Profile

The amplification products were then subjected to electrophoretic separation using horizontal agarose gel electrophoresis. 3.0% agarose gel was prepared in 1X TBE buffer stained with ethidium bromide. In each PCR tube, 5µl of loading dye was added and then loaded in separate wells. 100bp DNA ladder was used as a molecular weight marker for determining the molecular weights of SSR based PCR bands. Electrophoresis was carried out at 80V for 1 hour and 30 minutes. The gel was visually examined under UV and documented using gel documentation system.

3.2.4 SCAR Assay

3.2.4.1 Primers used for DNA amplification

SCAR's were selected on the basis of earlier studies of Ek et al. (2005); Timmerman et al. (1994); Katoch et al. (2010) and Fondevilla et al. (2008) Tiwari et al. (1998). A set of 5 SCAR primers were selected for use in amplification of genomic DNA.

Primer dilution: Double distilled, autoclaved and deionized water was used to dilute primers.

3.2.4.2 Components used for PCR Reaction

DNA amplification was carried out in PCR tubes containing 10µL reaction mixture. Reaction mixture contained following components. (Table 3.5)

Table 3.5: Reagents with their concentration and quantity used for single PCR reaction in SCAR Assay

S.No	Reagents	Concentration	Quantity
1	PCR Buffer	5X	2µl
2	dNTPs	10mM/each	0.2µl
3	$MgCl_2$	25Mm	0.8
4	Forward Primer	10pmole/ µl	0.3µl
5	Reverse Primer	10pmole/ µl	0.3µl
6	DNA	25ng/µl	1µl
7	*Taq* DNA polymerase	1.5U	0.2µl
8	Sterile water		4.2µl
	Total		**10µl**

3.2.4.3 PCR amplification Program

PCR tubes containing master mix and DNA template were thoroughly mixed and subjected to the thermal profile given in Table 3.6. The amplification reaction was carried out in gradient master cycler. An initial denaturation step of 5 minutes at 94ºC was carried out in PeqLab gradient mastercycler, followed by a loop of 35 cycles each consisting of denaturation {94ºC for 1 minute}, annealing {50-60ºC for 1 minute} and

extension {72ºC for 2 minutes}. The final extension was performed at 72ºC for 10 minutes. The PCR products were then stored at 4ºC.

Table 3.6: Thermal profiles used for DNA amplification in SCAR Assay

Steps	Cycles	Temperature	Duration
Initial Denaturation	1	94ºC	5min
Denaturation	35	94ºC	1min
Annealing		50-60ºC	1min
Extension		72ºC	2 min
Final Extension	1	72ºC	10min

The same reaction mixture without genomic DNA was run for each reaction to serve as a negative control.

3.2.4.4 SCAR-PCR banding Profile

The amplification products were then subjected to electrophoretic separation using horizontal agarose gel electrophoresis. 2.5% agarose gel was prepared in 1X TBE buffer stained with ethidium bromide. In each PCR tube, 5µl of loading dye was added and then loaded in separate wells. 100bp DNA ladder was used as a molecular weight marker for determining the molecular weights of SCAR based PCR bands. Electrophoresis was carried out at 80V for 1 hour and 30 minutes. The gel was visually examined under UV and documented using gel documentation system.

3.2.5 RAPD Assay

3.2.5.1 Primers used for DNA amplification

RAPD was selected on the basis of earlier studies Tiwari *et al*. (1998). A RAPD primer was selected for use in amplification of genomic DNA.

Primer dilution: Double distilled, autoclaved and deionized water was used to dilute primers.

3.2.5.2 Components used for PCR Reaction

DNA amplification was carried out in PCR tubes containing 10µL reaction mixture. Reaction mixture contained following components. (Table 3.7)

Table 3.7: Reagents with their concentration and quantity used for single PCR reaction for RAPD Assay

S.No	Reagents	Concentration	Quantity
1	PCR Buffer	5X	2µl
2	dNTPs	10mM/each	0.2µl
3	$MgCl_2$	25Mm	0.8
4	Primer	10pmole/ µl	0.6µl
5	DNA	25ng/µl	1µl
6	*Taq* DNA polymerase	1.5U	0.2µl
7	Sterile water		4.2µl
	Total		**10µl**

3.2.5.3 PCR amplification Program

PCR tubes containing master mix and DNA template were thoroughly mixed and subjected to the thermal profile given in Table 3.8. The amplification reaction was carried out in gradient master cycler. An initial denaturation step of 5 minutes at 94ºC was carried out in PeqLab gradient mastercycler, followed by a loop of 35 cycles each consisting of denaturation {94ºC for 1 minute}, annealing {29-38ºC for 1 minute} and extension {72ºC for 2 minutes}. The final extension was performed at 72ºC for 10 minutes. The PCR products were then stored at -20ºC.

Table 3.8: Thermal profiles used for DNA amplification in RAPD Assay

Steps	Cycles	Temperature	Duration
Initial Denaturation	1	94ºC	5min
Denaturation	35	94ºC	1min
Annealing		29-38ºC	1min
Extension		72ºC	2 min
Final Extension	1	72ºC	10min

The same reaction mixture without genomic DNA was run for each reaction to serve as a negative control.

3.2.5.4 RAPD-PCR banding Profile

The amplification products were then subjected to electrophoretic separation using horizontal agarose gel electrophoresis. 1.5% agarose gel was prepared in 1X TBE buffer stained with ethidium bromide. In each PCR tube, 5µl of loading dye was added and then loaded in separate wells. 100bp DNA ladder was used as a molecular weight marker for determining the molecular weights of RAPD based PCR bands. Electrophoresis was carried out at 110V for 1 hour and 30 minutes. The gel was visually examined under UV and documented using gel documentation system.

3.3 METHODOLOGY ADOPTED FOR *er1* ALLELE CHARACTERIZATION

The tissue for the samples was taken from the already grown plant material for our first objective.

3.3.1. RNA Isolation:-

Chemicals Required:

a) Liquid Nitrogen
b) TRIzol® (Invitrogen, Life Technologies, USA)
c) Chloroform
d) Isopropanol
e) Ethanol 70%
f) Diethyl pyrocarbonate treated water (DEPC)

Procedure for RNA isolation:

The leaf sample was crushed in liquid nitrogen and nearly 50 mg-100 mg of this sample was taken in a micro centrifuge tube. 1ml of TRIzol reagent was added to the sample and it was homogenized using a homogenizer till a suspension was formed and mixed properly. The sample was incubated at room temperature for 5 min and then centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge at 4^0C. The supernatant obtained was then transferred to another tube and 200µl of chloroform was added to it. It was then shaken vigorously to mix the contents properly in the tube and then was incubated in ice for 5 minutes. The sample was then centrifuged at 10,000 rpm for 15 minutes at 4^0C. The top most layer of the supernatant was transferred to another tube and 500µl of isopropanol was added. It was then incubated at room temperature for about 15-20 minutes. After incubation it was then centrifuged for 20 minutes at 10,000 rpm at 4^0C. The supernatant obtained was discarded and the pellet was given 75% ethanol wash (750µl of 75% absolute ethanol mixed with 250µL DEPC water). The pellet was air dried and dissolved 20µL DEPC water.

3.3.2 RNA quantity and quality check

RNA gel was checked on 1% agarose through horizontal electrophoresis technique.

1% of agarose was prepared for 60mL volume. 0.6gm of agarose was added to 50ml of DEPC water. This was further cooled and 6 ml of 10X MOPS was added with continuous swirling. After that 4 ml of Formaldehyde was added. Thereafter the gel was casted on tray. Then 2µL of DEPC water, 1µL of RNA loading & 2µL of RNA sample was added to PCR tubes. These PCR tubes were then incubated at 70^oCfor 10 min and loaded on the gel. And run at 1X MOPS buffer (prepared with100ml MOPS and 900mL DEPC).

Total RNA concentration was estimated using MySpec spectrophotometer. The absorbance was checked at 260 and 280nm. The ratio for A260/A280 was close to 2.0.

DNase Treatment:-

In order to remove genomic DNA contamination from RNA sample, total RNA was treated with DNase (DNA-freeTM kit; Ambion-TURBO DNA-freeTM, Life

Technologies, USA) as per the given protocol. Following reagents were added in the order as shown in table 3.9:

Table 3.9: Reagents and their quantity used for DNase treatment

S.No.	Components	Volume
1.	DNase buffer	2µL
2.	DNase enzyme	1µL
3.	RNA	10µg
4.	Total	20µL

This reaction mix was incubated for 45 minutes at 37^{o}C, after that 2.2µL of DNase inactivator was added to the mix and was kept at room temperature for 2 minutes. After that it was centrifuged at 5,000 rpm for 2 minutes. The supernatant was taken out which is DNAse treated RNA.

3.3.3 c-DNA Synthesis:

c-DNA was synthesized by using ImProm-II ™ Reverse Transcription System (Promega, Madison, USA) with an anchored oligo dT15 primer and 1µg of DNAse treated RNA as template. The following reagents were added in the order as in table 3.10.

Table 3.10: Reagents and their quantity used for c-DNA synthesis

Mix I

Components	Volume (µl)
5X Buffer	4
$MgCl_2$	3.8
dNTP's	1
rRNAsin	0.5
DEPC Water	4.7
Total	14

Mix II

Components	**volume (µl)**
DNase treated RNA	2.4
Oligo dT primer	1
DEPC water	1.6
Total	5

Mix II was incubated in PCR machine at 70^{O}C for 5 minutes. After incubation, it was given a quick chill in liquid nitrogen. Again the mixture was incubated at 25ºC for 2 minutes. Then mix I was added to mix II. This mixture was again incubated at 25ºC for 5 minutes. After this RT enzyme was added (1µL) to the mixture. After this the mix is incubated at 42ºC for 75 minutes and then at 70ºC for 15 minutes.

3.3.3 *PsMLO* assay

3.3.3.1 Primers used for c-DNA amplification

A house-keeping gene β-tubulin (Jose *et al*. 2009) was used to check the quality of c-DNA. The amplified products using specific primers of β-tubulin were checked on 2% agarose through horizontal electrophoresis technique. 50bp DNA ladder was used as a molecular weight marker for determining the molecular weights of β-tubulin based PCR bands. For *PsMLO* amplification primers were designed using Primer3 software http://primer3.ut.ee/. Two overlapping *PsMLO* gene specific markers *PsMLOA* and *PsMLOB* were used for amplification of c-DNA. To amplify the full target gene forward primer of *PsMLOA* and reverse primer of *PsMLOB* (labeled as *PsMLOAB*) were used. The sequence of all these primers alongwith their allele size is given in table 3.11.

Primer dilution: Double distilled, autoclaved and deionized water was used to dilute primers.

Table 3.11: list of primers used for c-DNA amplification

S.No.	Locus Name	Marker Type	Primer Sequence (5'-3')F/R	Allele Size (bp)
1.	β-tubulin	Gene specific	F: GCTCCCAGCAGTACAGGACTCT R: TGGCATCCCACATTTGTTGA	60
2.	*PsMLOA*	Gene specific	F: AAAATGGCTGAAGAGGGAGTT R: TGCACCCTTGATTACTTCTCC	1080
3.	*PsMLOB*	Gene specific	F: ATCTGGCTCTTCACAGTGCTT R: CTTGCATTTTCATACCCTATGATT	1065
4.	*PsMLOAB*	Gene specific	F: AAAATGGCTGAAGAGGGAGTT R: CTTGCATTTTCATACCCTATGATT	1875

3.3.3.2 Components used for PCR reaction

c-DNA amplification was carried out in PCR tubes containing 10µL reaction mixture. Reaction mixture contained following components. (Table 3.12)

Table 3.12: Reagents with their concentration and quantity used for single PCR reaction for c-DNA amplification

S.No.	Reagents	Concentration	Quantity
1	PCR Buffer	5X	2µl
2	dNTPs	10mM/each	0.2µl
3	$MgCl_2$	25Mm	0.8
4	Forward Primer	10pmole/ µl	0.3µl
5	Reverse Primer	10pmole/ µl	0.3µl
6	c-DNA	25ng/µl	1µl
7	*Taq* DNA polymerase	1.5U	0.2µl
8	Sterile water		4.2µl
	Total		**10µl**

3.3.3.3 PCR amplification profile

PCR tubes containing master mix and c-DNA template were thoroughly mixed and subjected to the thermal profile given in Table 3.13. The amplification reaction was carried out in gradient master cycler. An initial denaturation step of 5 minutes at 95ºC was carried out in PeqLab gradient mastercycler, followed by a loop of 35 cycles each consisting of denaturation {95ºC for 1 minute}, annealing {55-60ºC for 30 seconds} and extension {72ºC for 45 seconds}. The final extension was performed at 72ºC for 10 minutes. The PCR products were then stored at -20ºC.

Table 3.13: Thermal profiles used for c-DNA amplification with *PsMLO* primers and β-tubulin

Steps	Cycles	Temperature	Duration
Initial Denaturation	1	95ºC	5min
Denaturation	35	95ºC	30sec
Annealing		55-60ºC	45sec
Extension		72ºC	2 min
Final Extension	1	72ºC	10 min

The same reaction mixture without c-DNA was run for each reaction to serve as a negative control.

3.3.3.4 *PsMLO* banding profile

The amplification products were then subjected to electrophoretic separation using horizontal agarose gel electrophoresis. 2% agarose gel was prepared in 1X TBE buffer stained with ethidium bromide. In each PCR tube, 5µl of loading dye was added and then loaded in separate wells. 100bp ladder was used as a molecular weight marker for determining the molecular weights of gene specific based PCR bands. Electrophoresis was carried out at 80V for 1 hour and 30 minutes. The gel was visually examined under UV and documented using gel documentation system.

Gel Elution

The gel slice containing desired DNA fragment was excised by using a clean razor blade. Slice was placed into pre-weighed 1.5mL tube and weighed. Weight of gel slice was recorded. Then 1:1 volume of Binding buffer was added to the gel slice (Volume: weight). The sample was incubated for 10min at 60^{O}C until the gel was

completely dissolved in buffer. The tubes were mixed by inversion after few minutes to facilitate melting process. Then 800µLof solublised gel solution was transferred to GeneJET purification column and centrifuged for 1min. The flow-through was discarded and column was placed back into the same collection tube.

100µL of Binding Buffer was added to GeneJET purification column and centrifuged for 1min. The flow-through was discarded and column was placed back into the same collection tube. Now 700µl of Wash Buffer was added to the GeneJET purification column and centrifuged for 1min. The flow-through was discarded and column was placed back into the same collection tube. The empty GeneJET purification column was then centrifuged for another 1min so that residual wash buffer is completely removed. Then GeneJET purification column was transferred to a clean 1.5mL microcentrifuge tube. 50µL of Elution Buffer was added to the center of purification column membrane and centrifuged for 1min. GeneJET purification column was then discarded and sent for Sequencing.

3.3.3.5 Sequencing

The PCR product sequencing was outsourced from AgriGenome Labs Pvt Ltd using Sanger's sequencing method. The sequencing of the PCR as well as purified products was done in both direction (forward and reverse).

3.3.3.6 Sequence analysis

The trimmed forward and reverse sequences were then used for pairwise alignment and then contigs were prepared using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/page2.html). Pairwise alignment as well as multiple sequence alignment for the DNA alongwith DNA of *PsMLO* was performed online through clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and then the alignment was viewed using jalview (http://www.jalview.org/). The DNA was translated to protein using the translation tool of expasy (https://web.expasy.org/translate/) and then again the protein sequences were pairwise as well as multiple sequences aligned. The resulting sequences were compared and analysed with *PsMLO* cDNA sequences of wild-type pea cultivar Sprinter (susceptible to *E. pisi*; NCBI accession number: FJ463618.1).

CHAPTER 4

RESULTS

The present study entitled, "Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)" was conducted to profile the resistant/ tolerant and susceptible germplasm of pea using powdery mildew linked molecular markers and to characterize the *er1* locus. A set of thirty seven *Pisum* genotypes was sown during *rabi* 2017-18 at School of Biotechnology, SKUAST-J, Chatha. The data on various aspects of the present study have been given under various headings and sub-heading:-

4.1 Molecular profiling of resistant/tolerant and susceptible germplasm

- 4.1.1 Genomic DNA isolation and quantification
- 4.1.2 PCR amplification with molecular markers linked to *er1*locus
- 4.1.3 PCR amplification with molecular markers linked to *er2* locus
- 4.1.4 PCR amplification with molecular markers linked to *Er3* locus

4.2 Characterization of *er1* locus

- 4.2.1 RNA isolation and quantification
- 4.2.2 RNA quantity and quality check
- 4.2.3 c-DNA synthesis and quality check
- 4.2.4 PCR/Gene amplification of *PsMLO*
- 4.2.5 Sequence analysis

4.1 Molecular profiling of resistant/tolerant and susceptible germplasm

In the present study, thirty seven genotypes of pea (*Pisum* spp.) were used to evaluate their assumed linkage with powdery mildew using ten different molecular markers (SSR, SCAR and RAPD) reported to be closely linked with *er1*, *er2* and *Er3*. The associated molecular markers used in the present study have been given in table 3.2. For molecular characterization of thirty seven genotypes, genomic DNA was extracted and quantified using rUV-Vis spectrophotometer and agarose gel electrophoresis. Then

PCR amplification was carried out using linked markers (SSR, SCAR and RAPD). The experimental results obtained from the molecular analysis of these genotypes are elaborated as under:

4.1.1 Genomic DNA isolation and quantification

4.1.2 PCR amplification with molecular markers linked to *er1*locus

4.1.3 PCR amplification with molecular markers linked to *er2* locus

4.1.4 PCR amplification with molecular markers linked to *Er3* locus

4.1.1 Genomic DNA isolation and quantification

The genomic DNA was extracted from all the 37 genotypes using CTAB method of DNA extraction. The details of DNA extraction method followed are given in the materials and methods chapter. For extraction of DNA, young leaves from 4 weeks old pea plants were collected and processed.

Quality and quantity of DNA was estimated using agarose gel electrophoresis and spectrophotometric method (mySpec). Intensity of intact bands was used to estimate the quality of DNA. The quantity of DNA extracted was determined by comparing intact DNA bands with known standards using 0.8% agarose gel. The same set of DNA was then also subjected to calculate absorption of DNA samples at 260 and 280 nm. The ratio of absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) was used to estimate the quality and quantity of the extracted DNA. The concentration of genomic DNA varied from 275 to 2132 ng/µL and absorbance ratio varied from 1.798 to 2.053 (Plate 3.2) which indicated a good quality of DNA. The DNA was then diluted to desired working concentration of 50ng/ µL and then again checked on agarose gel and was found to be of good quality (Plate 3.2).

Table 4.1: Quantity and quality analysis of 37 pea genotypes using mySpec

S .No.	Codes	Genotype	Concentration of DNA(ng/ µl)	Absorbance ratio (260/280nm)
1.	Ps- 8	IC- 218991	567 ng/µl	1.942
2.	Ps- 11	IC- 219002	878 ng/µl	1.864
3.	Ps- 12 (1)	IC- 219008	498 ng/µl	1.995
4.	Ps- 12 (2)	IC- 219008	339 ng/µl	2.012
5.	Ps- 35	IC- 381453	443 ng/µl	1.925
6.	Ps- 49	HFP-4	434 ng/µl	1.857
7.	Ps- 51	DPPIV-2	786 ng/µl	1.945
8.	Ps-52	DPEPP-1	432 ng/µl	1.972
9.	Ps- 57	DPPMR-09-1	487 ng/µl	2.009
10.	Ps- 68	IC- 208366	532 ng/µl	1.987
11.	Ps- 73	EC- 381866	1109 ng/µl	2.034
12.	Ps- 78	EC- 598729	587 ng/µl	1.926
13.	Ps- 86	Pb-89	435 ng/µl	1.853
14.	Ps- 85	Improved JI-2480	498 ng/µl	1.798
15.	Ps- 88	Improved JI-1559	567 ng/µl	1.956
16.	Ps- 89	Improved JI-1559	454 ng/µl	1.999
17.	Ps- 90	Improved JI-2302	875 ng/µl	2.053
18.	Ps- 92	Improved JI-2480	434 ng/µl	1.987
19.	Ps- 87	IIHR544	445 ng/µl	1.801
20.	Li	Lincoln	987 ng/µl	1.986
21.	Bo	Bonneville	557 ng/µl	1.847
22.	Ark	Arkel	686 ng/µl	1.899
23.	Ps- 93	Azad Pea- 1	978 ng/µl	1.985
24.	Ps- 94	Azad Pea- 2	1095 ng/µl	1.798
25.	Ps- 96	Azad Pea- 4	275 ng/µl	1.895
26.	Ps- 97	Azad Pea- 5	576 ng/µl	1.899
27.	Ps- 99	Prakash	523 ng/µl	2.009
28.	Ps- 98	Rachna	421 ng/µl	1.987
29.	Ps- 100	Arka Sampoorna	1076 ng/µl	2.003
30.	Ps- 101	Arka Ajeet	1132 ng/µl	1.895
31.	Ps- 103	Arka Priya	1198 ng/µl	1.823
32.	Ps- 104	Arka Pramodh	987 ng/µl	1.965
33.	Ps- 105	Arka Kartik	565 ng/µl	2.005
34.	Ps- 64	Palam Priya	2000 ng/µl	1.960
35.	Ps- 65	Palam Sumool	2132 ng/µl	2.001
36.	Ps- 63	Palam Triloki	2123 ng/µl	1.853
37	Ps- 106	Pusa Shree	1564 ng/µl	2.009

4.1.2 PCR amplification with primers linked to *er1*locus

In the present study ten molecular markers were used, out of which seven were linked to *er1* locus (Table 3.2). The markers linked to *er1* locus are: Sc-OPO-18, Sc-OPE-16, Sc-OPO-10, AD-60, AA-374e A-5 and OPL-6.

Sc-OPO-18$_{1200}$:

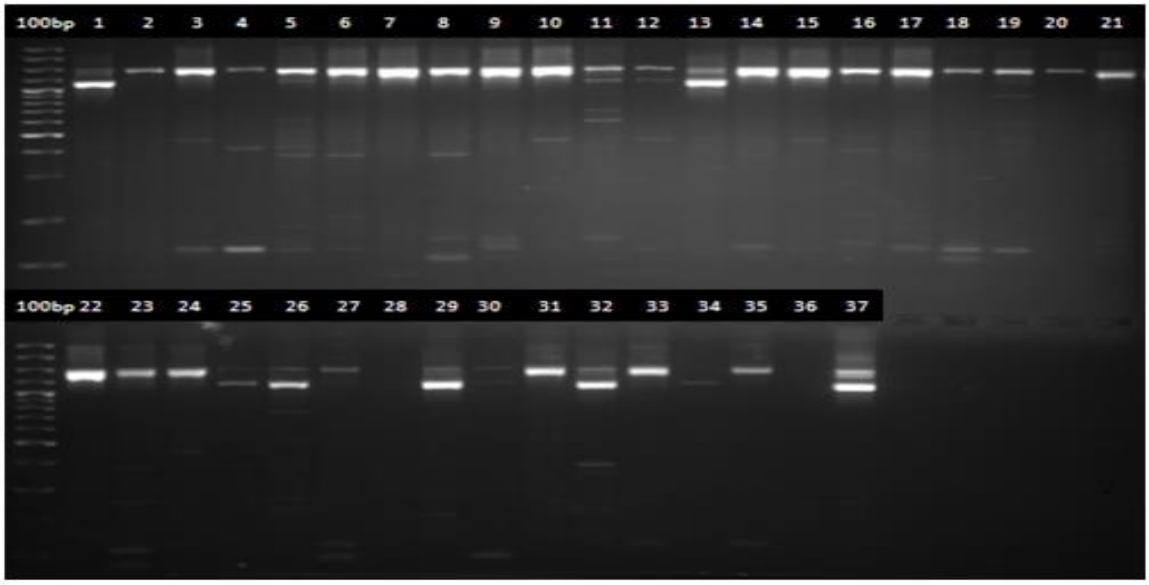
Sc-OPO-18$_{1200}$ is a SCAR marker and has been reported to be located at 0.0 cM distance from *er1* locus (Tiwari *et al.* 1998). Sc-OPO-18 is linked in coupling (*trans* to *er-1*). This primer was optimized at annealing temperature of 53ºC. Sc-OPO-18 marker was used to amplify genomic DNA of 37 genotypes by setting the concentration of the components of PCR mixture and thermal profile as mentioned in materials and methods. The expected and observed allele size was 1200bp (Plate 4.1). Only eleven genotypes amplified the preferred band size; Ps-8, Ps-73, Ps-78, Ps-86, Ps-96, Ps-97, Ps-100, Ps-101, Ps-104, Ps-64, Ps-106.

Sc-OPE-16$_{1600}$

Sc-OPE-16$_{1600}$ is a SCAR marker and is located at 4.0 cM distance from *er1* locus (Tiwari *et al*. 1998). Sc-OPE-16 is linked in repulsion (*cis* to *er-1*) phase. This primer was optimized at annealing temperature of 50ºC. The expected and observed allele size was 1600bp (Plate 4.2). All the genotypes subjected to ScOPE-16 assay amplified the expected band except for seven genotypes *viz.*, Ps-11, Ps-12, Ps-35, Ps-51, Li, Ps-98 and Ps-65.

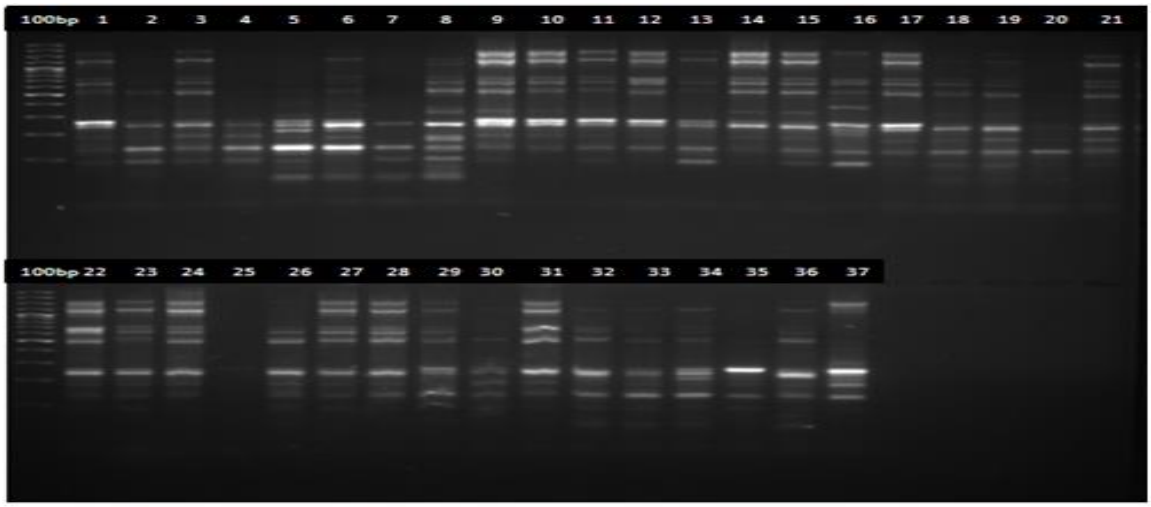
Sc-OPO-10$_{650}$

Sc-OPO-10$_{650}$ is also a SCAR marker. The marker is located at 2.1cM distance from *er1* locus in *cis* with *er1* (Timmerman *et al*. 1994). This primer was optimized at annealing temperature of 55ºC. The expected allele size was 650bp (Plate 4.3). The PCR amplification resulted for amplicons of size 650 bp in all the genotypes except for Ps-93 and Ps-96.



1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.1: Banding profile of Sc-OPO-18_{1200}



1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.2: Banding profile of Sc-OPE-16_{1600}

AD-60

AD-60 is a SSR marker located at 10.4 cM distance from *er1* locus Ek *et al.* (2005). The expected allele size was 220bp and was optimized at annealing temperature of 55.8ºC. AD-60 was also used to amplify genomic DNA of 37 genotypes by setting the concentration of the components of PCR mixture and thermal profile as mentioned in materials and methods. The results were highly polymorphic. The expected band was observed in five genotypes and was polymorphic to rest of the thirty two accessions (Plate 4.4).

AA-374e

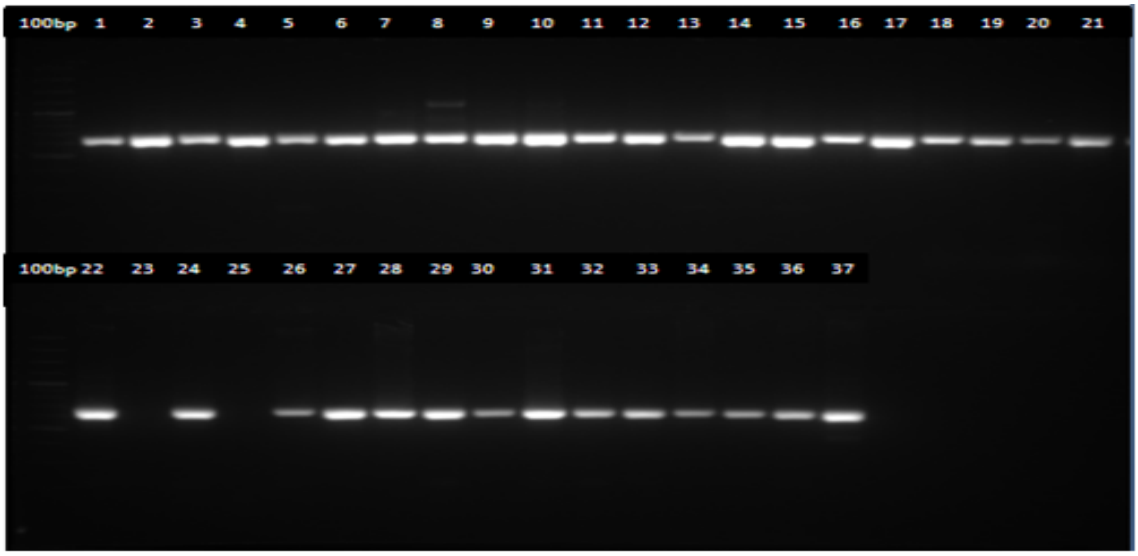
AD-374e is a SSR marker located at 11.6cM distance from *er1* locus with an expected amplicons of 300 bp (Ek *et al*. 2005). This primer was optimized at annealing temperature of 56ºC. The observed allele size was also 300bp (Plate 4.5). The results were highly polymorphic. Most of the genotypes amplified the expected band size and were polymorphic to Ps-35, Ps-51, Ps-52, Ps-89, Ps-94, Ps-101, Ps-103, Ps-104, Ps-64, Ps-63 and Ps-106.

A-5

A-5 is also a SSR marker located at 14.9cM distance from *er1* locus (Ek et al. 2005). A-5 marker was used to amplify genomic DNA of 37 genotypes by setting the concentration of the components of PCR mixture and thermal profile as mentioned in materials and methods. This primer was optimized at annealing temperature of 56ºC. The expected allele size was 900bp and the results were highly polymorphic (Plate 4.6). All genotypes amplified the expected amplicons except for accessions *viz.,* Ps-12(2), Ps-85, Ps-92, Ps-87 and Li.

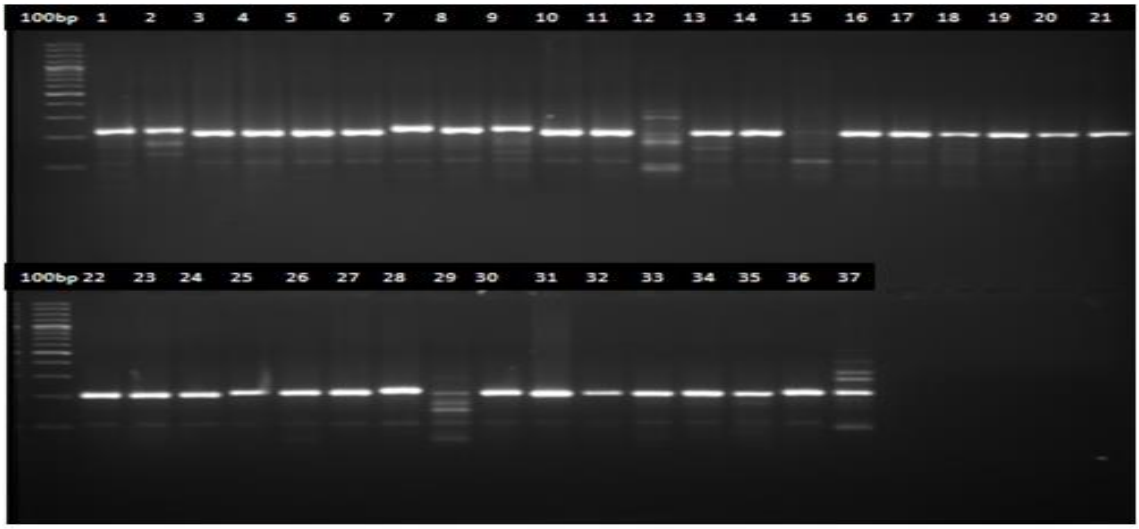
OPL-6

OPL-6 is a RAPD marker and is located at 2cM distance from *er1* locus (Tiwari *et al*. 1998). The RAPD marker could not be optimized for amplification and thus was not used in the present study.



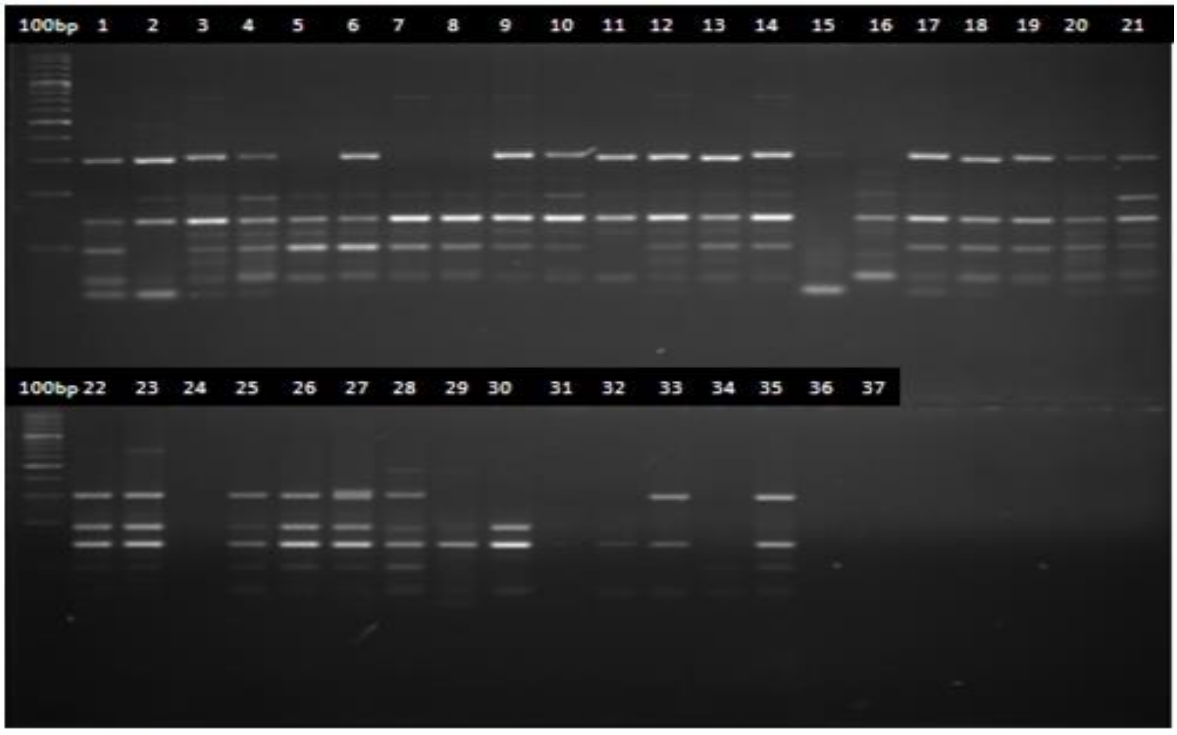
1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.3: Banding profile of Sc-OPO-10_{650}



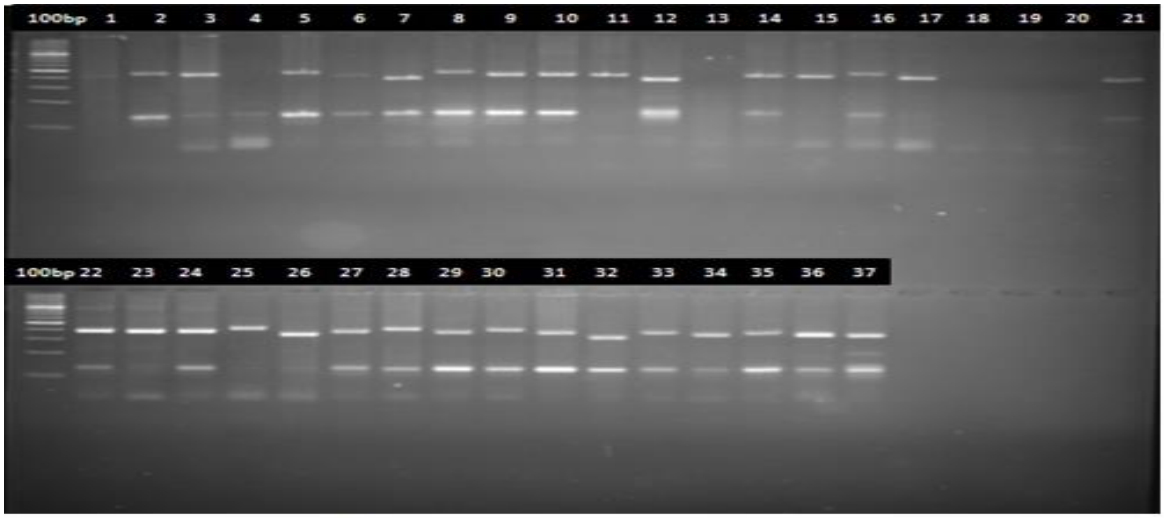
1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.4: Banding profile of AD-60



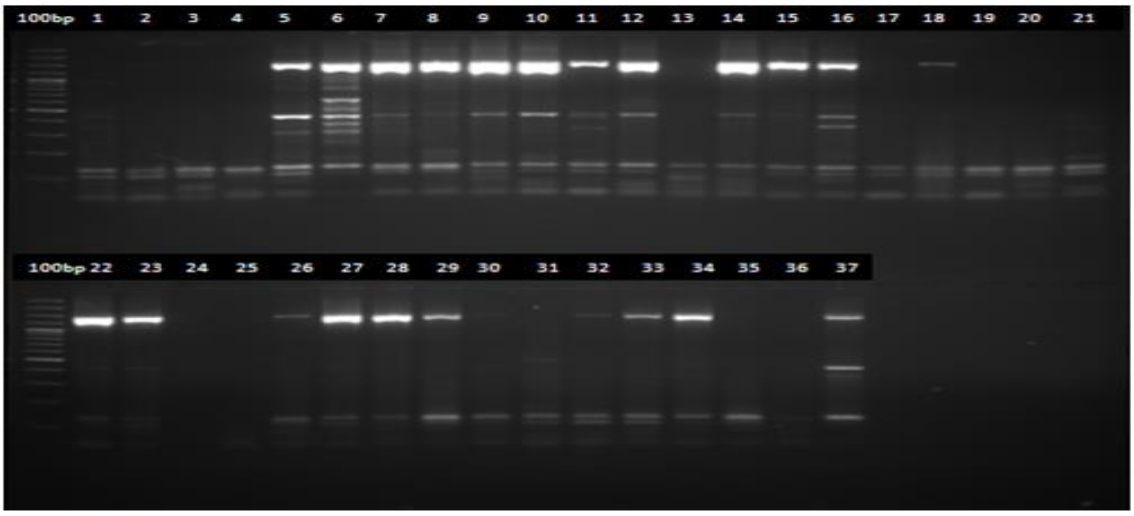
1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.5: Banding profile of AA-374e



1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.6: Banding profile of A-5



1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.7: Banding profile ScX-17

4.1.3 PCR Amplification with primers linked to *er2* locus

In the present study 10 markers were used, out of which two were kinked to *er2* locus. The markers linked to *er2* locus are: ScX-17 and AA-278.

ScX-17$_{1400}$

ScX-17 is a SCAR marker located at 2.6cM distance from *er2* locus (Katoch et al. 2010). ScX-17 is linked in repulsion (*cis* to *er2*) phase. This primer was optimized at annealing temperature of 60^{o}C. ScX-17 marker was also used to amplify genomic DNA of 37 genotypes by setting the concentration of the components of PCR mixture and thermal profile as mentioned in materials and methods. The expected and observed allele size was 1400bp and the results were highly polymorphic (Plate 4.7). All genotypes amplified the expected amplicon except for Ps-8, Ps-11, Ps-12(1), Ps-12(2), Ps-86, Ps-90, Ps-87, Li, Bo, Ps-94, Ps-96 and Ps-63.

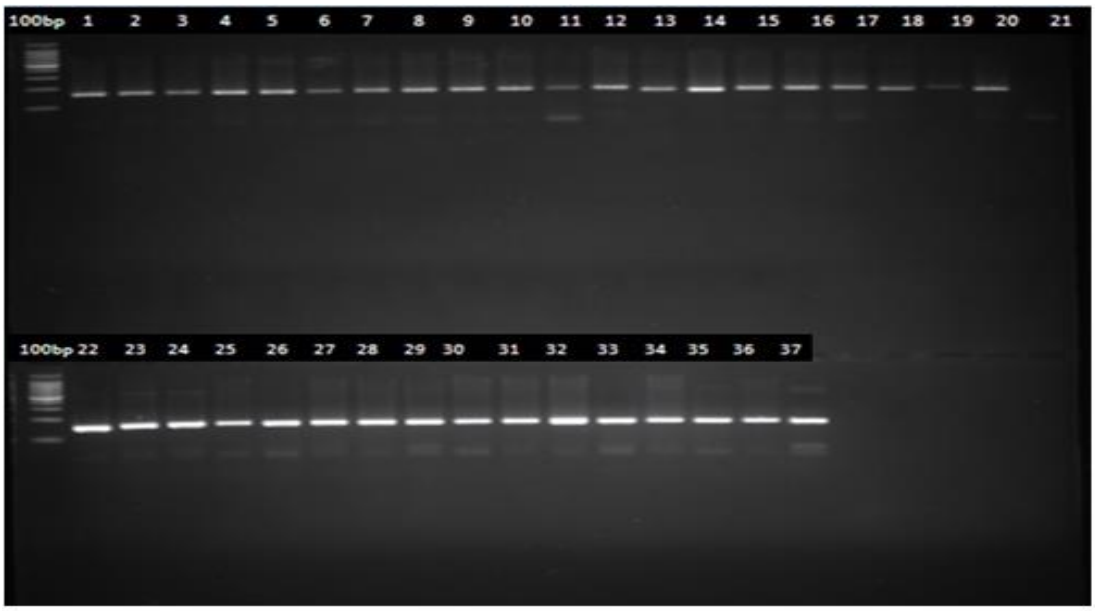
AA-278

AA-278 is SSR marker located at 14.9cM distance from *er2* locus (Katoch et al. 2010). The expected and observed allele size was 155bp (Plate 4.8). The results were highly polymorphic. All genotypes were amplified. The expected band was observed in nineteen genotypes and was polymorphic with eighteen accessions.

4.1.4 PCR Amplification with primers linked to *Er3* locus

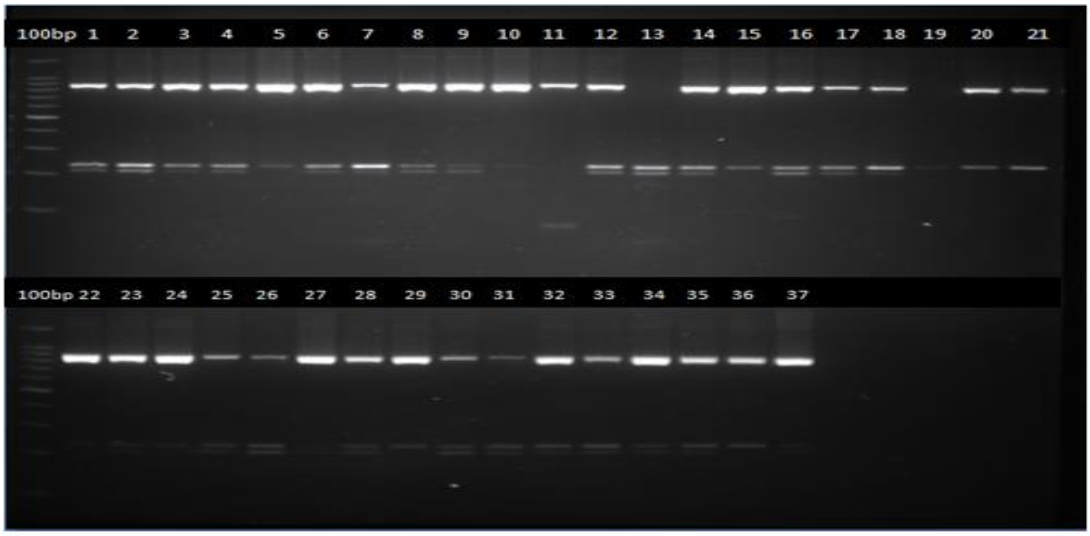
ScAB1$_{874}$

ScAB1$_{874}$ is a SCAR marker and is located at 2.8cM distance from *Er3* locus (Fondevilla *et al.* 2008). ScAB is linked in repulsion (*cis)* phase to *Er3*. This primer was optimized at annealing temperature of 55^{o}C. ScAB1 marker was used to amplify genomic DNA of 37 genotypes by setting the concentration of the components of PCR mixture and thermal profile as mentioned in materials and methods. The expected and observed allele size was also 874bp (Plate 4.9). All the genotypes amplified the expected amplicons except for Ps-86 and Ps-87.



1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.8: Banding profile AA-278



1	IC-218991	11	EC-381866	20	Lincoln	29	Arka Sampoorna
2	IC-219002	12	EC-598729	21	Bonneville	30	Arka Ajeet
3	IC-219008(1)	13	Pb-89	22	Arkel	31	Arka Priya
4	IC-219008(2)	14	Improved JI-1559	23	Azad Pea-1	32	Arka Pramodh
5	IC-381453	15	Improved JI-1559	24	Azad Pea-2	33	Arka Kartik
6	HFP-4	16	Improved J11559	25	Azad Pea-4	34	Palam Priya
7	DPPIV-2	17	Improved JI-2302	26	Azad Pea-5	35	Palam Sumool
8	DPEPP-1	18	Improved JI-2480	27	Prakash	36	Palam Triloki
9	DPPMR-09-1	19	IIHR-544	28	Rachna	37	Pusa Shree
10	IC-208366						

Plate 4.9: Banding profile ScAB1

4.2 Characterization of *er1* locus

In the present study, twelve genotypes of pea (*Pisum* spp.) were used to characterize the resistance alleles at the *er1* locus. Out of these twelve genotypes nine were resistant/ tolerant and three were susceptible known notified varieties. For characterization of *er1* locus of these twelve genotypes, RNA was extracted and quantified using my-Spec series of microvolume UV-Vis spectrophotometer and agarose gel electrophoresis. The RNA was converted to c-DNA and was confirmed with a house keeping gene. Then PCR amplification was carried out using *PsMLO* gene derived markers, the products were sequenced and analysis was done. The experimental results obtained from the molecular analysis of these genotypes are elaborated as under:

4.2.1 RNA isolation and quantification

4.2.2 c-DNA synthesis

4.2.3 PCR amplification (*PsMLO*)

4.2.4 Sequencing

4.2.5 Sequence analysis

4.2.1 RNA Isolation and Quantification

The RNA was extracted from all the 12 genotypes using Trizol method of RNA extraction. The details of RNA extraction method followed are given in the materials and methods chapter. Quality and quantity of RNA was estimated using agarose gel electrophoresis and spectrophotometric method (MySpec). Intensity of intact bands was

used to estimate the quality of RNA. The quantity of RNA extracted was determined by comparing intact RNA bands with known standards using 1% agarose gel. The same set of RNA was then subjected to calculate absorption of RNA samples at 260 and 280 nm. The ratio of absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) was used to estimate the quality and quantity of the extracted RNA (Plate 4.10). Overall good quality of RNA was isolated. The RNA was then diluted to desired working concentration of 50ng/µL.

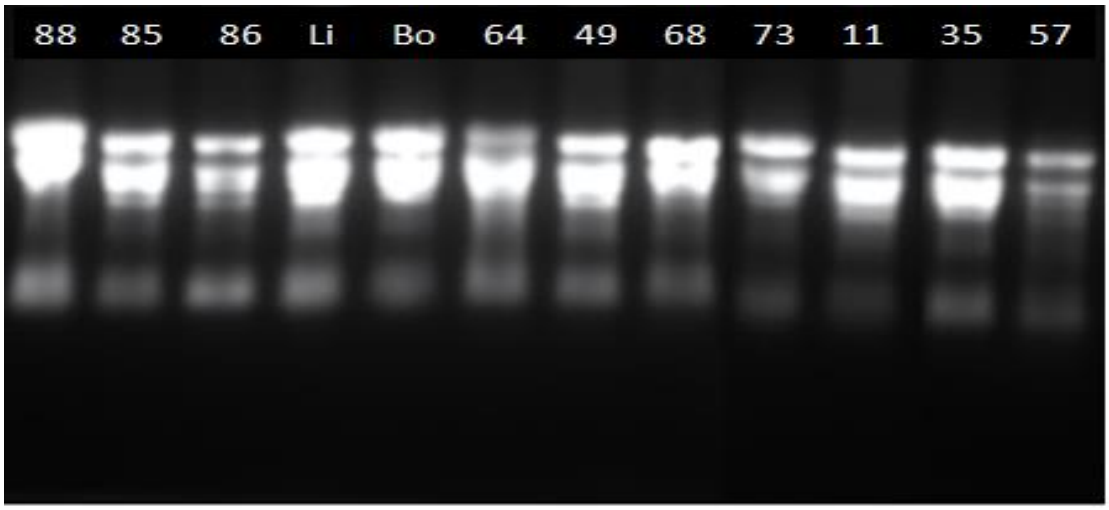


Plate 4.10: Quality RNA of different lines as observed during agarose gel electrophoresis

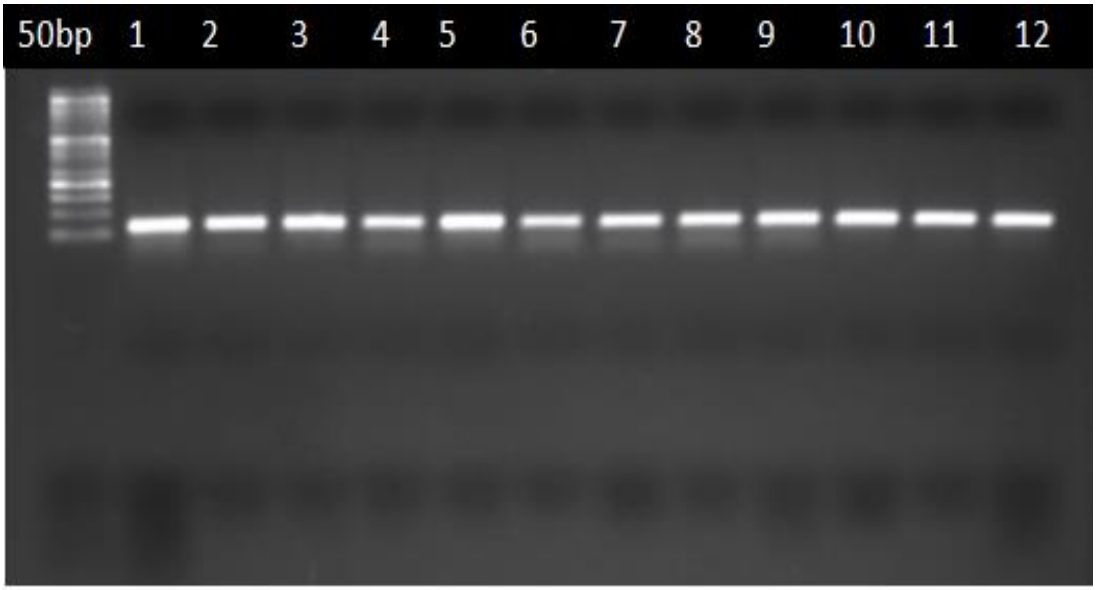


Plate 4.11: Quality c-DNA of different lines as observed during agarose gel electrophoresis with β- tubulin.

Table 4.2: Quantity analysis of RNA sample of 12 pea genotypes using mySpec

S .No.	Code	Genotype	Concentration of RNA(ng/ µl)	Absorbance ratio (260/280nm)
1.	Ps- 88	Improved JI-1559	6032.855	1.846
2.	Ps- 85	Improved JI-2480	8168.426	2.686
3.	Ps- 86	Pb-89	3395.940	2.575
4.	Li	Lincoln	2230.929	2.531
5.	Bo	Bonneville	3444.211	2.996
6.	Ps- 64	Palam Priya	1839.824	3.846
7.	Ps- 49	HFP- 4	1901.224	2.128
8.	Ps- 68	IC- 208366	3330.583	2.053
9.	Ps- 73	EC- 381866	8535.622	2.726
10.	Ps- 11	IC- 219002	848.740	1.958
11.	Ps- 35	IC- 381453	873.746	1.925
12.	Ps- 57	DPPMR-09-1	1696.095	2.559

4.2.2 c-DNA synthesis

c-DNA was synthesized using ImProm-II^{TM} reverse transcription system (Promega, Madison, USA) with an anchored oligo dT15 primer and 1µg of DNAse treated RNA as template. The quantity of c-DNA was estimated using spectrophotometric method (MySpec). The set of c-DNA samples was then subjected to calculate absorption at 260nm and 280 nm to estimate the quality and quantity of the extracted c-DNA. Overall good quality of c-DNA was synthesized (Plate 4.11). The absorbance ratio of c-DNA varied from 1.2 to 1.9.

The confirmation of c-DNA was done using housekeeping gene β-tubulin (Jose *et al*. 2009). The allele size of β-tubulin was 60bp. All the genotypes amplified the expected band size of 60 bp for the house keeping gene β-tubulin (Plate 4.11) thus suggesting an overall good quality of c-DNA synthesis.

Table 4.3: Quantity analysis of c-DNA sample of 12 pea genotypes using mySpec

S .No.	Code	Genotype	Concentration of c-DNA(ng/ µl)	Absorbance ratio (260/280nm)
1.	Ps- 88	Improved JI-1559	5111.768	1.513
2.	Ps- 85	Improved JI-2480	1633.418	1.126
3.	Ps- 86	Pb-89	2406.966	1.460
4.	Li	Lincoln	1889.655	1.078
5.	Bo	Bonneville	1746.574	1.965
6.	Ps- 64	Palam Priya	3760.925	1.630
7.	Ps- 49	HFP- 4	6983.294	1.935
8.	Ps- 68	IC- 208366	3903.821	1.773
9.	Ps- 73	EC- 381866	4987.908	1.701
10.	Ps- 11	IC- 219002	4919.266	1.641
11.	Ps- 35	IC- 381453	4987.908	1.701
12.	Ps- 57	DPPMR-09-1	2356.588	1.308

4.2.3 PCR amplification (*PsMLO*)

For *PsMLO1* gene amplification, primers reported by Pavan et al., (2013), were used and we couldn't amplify the full gene with those primers. Therefore, two overlapping markers designated as *PsMLO*A and *PsMLO*B were designed (Plate 4.12) using the Primer 3 software and customized for the full gene amplification. However, to amplify the full target gene of *PsMLO* forward primer of *PsMLO*A and reverse primer of *PsMLO*B (labeled as *PsMLO*AB) were also used in the present study.

Out of the 12 genotypes used in the study, only five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) were able to amplify for both *PsMLO*A and *PsMLO*B. The expected allele size of *PsMLO*A was 1080bp and the annealing was optimized at 53^{o}C. For *PsMLO*A a single amplicon of expected size i.e. 1080bp was observed in all the five genotypes (Plate 4.13). For *PsMLO*B the expected amplicon size was 1065bp and the annealing temperature was optimized at 51^{o}C. For *PsMLO*B a single band of expected size was observed for Bonneville, and IC- 219002. Three amplicons were observed for JI-1559 and two each in Pb-89 and DPPMR-09-

Pisum sativum MLO1 (MLO1) mRNA, complete cds

GenBank: FJ463618.1

GenBank Graphics

>FJ463618.1 Pisum sativum MLO1 (MLO1) mRNA, complete cds

AAAACAACAAGTCAAAAAAGAAAGAAAAAATGGCTGAAGAGGGAGTTAAGGAACGAACTTTGGAA
GAAACACCAACTTGGGCTGTTGCAGTTGTGTGTCTTGTGTTGCTAGCTGTTTCAATCTTAATTGA
ACATATTATTCATGTTATTGGAAAGTGGTTGAAGAAGAGAAACAAAAATGCTCTTTATGAAGCTT
TGGAAAAGATCAAAGGAGAGCTTATGCTACTAGGATTCATATCCTTGCTTCTAACTGTCTTCCAA
GATAATATTTCTAAAATATGCGTATCACAAAAAATTGGATCAACTTGGCATCCTTGTTCCACTTC
AAACACAAAGGCCAAGGCTAAATCTGATGAATCATTAGACTATAAAACCAACAATGATAGAAAAC
TCTTGGAGTATTTTGATCCTATTCCTCGGAGAATTCTTGCTACAAAAGGATATGATAAATGTTTT
GATAAGGGTCAAGTTGCATTAGTTTCTGCATATGGAATTCACCAACTCCATATATTCATTTTTGT
GCTGGCACTATTTCATATCCTTCAATGTATAATAACATTAACTTTGGGAAGAATCAAGATGAGGA
AGTGGAAGACTTGGGAAGATGAGACAAGAACAGTTGAATATCAATTTTATAATGATCCTGAGAGG
TTTAGGTTTGCAAGGGACACAACATTTGGAAGAAGGCACTTGAGCATGTGGGCTCAGTCACCTAT
TTTGTTATGGATTGTTAGCTTCTTCAGACAATTCTTTGGATCTATCAGTAGAGTTGATTATATGG
CTCTTAGGCATGGATTTATCATGGCTCATCTTCCTCCAGGACATGATGCACAATTTGATTTCCAA
AAGTATATAAGTAGATCAATTGAAGAGGATTTTAAAGTTGTTGTAGGAATAAGTCCAACTATCTG
GCTCTTCACAGTGCTTTTCCTTCTTACAAATACTCATGGGTGGTATTCTTATTATTGGCTTCCA
TTTCTTCCACTAATTGTAATCTTATTAGTTGGTGCTAAGTTACAAATGATCATAACAAAAATGGG
ATTAAGGATTCAAGACAGAGGAGAAGTAATCAAGGGTGCACCTGTGGTTGAGCCTGGAGATCACC
TTTTCTGGTTCAATCGTCCTCACCTTCTTCTCTTCACGATTCATCTTGTTCTCTTTCAGAATGCC
TTTCAACTTGCATTTTTTGCTTGGAGTACATATGAGTTTTCCATAACCTCTTGCTTCCACAAAAC
AACTGCAGATAGTGTCATTAGAATCACTGTAGGGGTTGTAATACAAACTCTATGTAGCTATGTGA
CTTTGCCTCTTTATGCTCTAGTCACACAGATGGGATCAACCATGAAACCAACCATTTTCAACGAA
AGAGTGGCAACAGCGCTTAAGAACTGGCACCACACAGCCAAAAAGCAGGTAAAACAGAGCAACCA
CTCAAACAACACGACACCGTATTCAAGCAGGCCATCAACCCCAACACATGCCATGTCTCCTGTTC
ACCTGCTCCATAGACACACTGCTGGAAACAGCGACAGTCTACAAACTTCTCCGGAAAAGTCTGAT
TATAAAAATGAACAGTGGGATATTGAAGGAGAAGGACCAACTTCCCTAAGAAACGATCAAACAGG
GCAACATGAGATTCAAATAGCGGGTGTCGAGTCATTTTCGTCAACCGAATTGCCGGTTAGAATTA
GACATGAAAGCACCTCTGGTTCAAAAGATTTTTCTTTCGAGAAGCGCCACTTAGGGAGCAATTAG
AATTGTAGGTATTGATAACCAGTTCAATGTATACCAATTAGGTACATTCTTGCAGATAAAGATAG
AGGAACTCCTTCTAAGAATGGAGTGTAAATTTGTTGAGGTAGCAGCTTGATTTGTGGATATAATC
ATAGGGTATGAAAATGCAAGACTATATTTTGTAAAAAAAAAAAAA

Plate 4.12: Primer designing of *PsMLO*A and *PsMLO*B

Forward primer

Reverse primer

1(Plate. 4.14). The desired band of expected size i.e. 1065bp from JI-1559, Pb-89 and DPPMR-09-1 of 1065bp size was eluted using Thermo Scientific, GeneJET Gel Extraction Kit. The expected allele size of *PsMLO*AB was 1875bp and the annealing temperature was optimized at 50^{o}C (Plate 4.15). Only four genotypes (Improved JI-1559, Bonneville, IC- 219002 and DPPMR-09-1) were able to amplify for *PsMLO*AB but the amplicons were quite faint. For *PsMLO*AB a single amplicon of desired size was observed for three genotypes: Bonneville, IC- 219002 and DPPMR-09-1, whereas three amplicons were observed for JI-1559 genotype. The desired band for improved JI-1559 with allele size 1875bp was then eluted by using Thermo scientific, GeneJET Gel Extraction Kit.

4.2.4 Sequencing

The amplified products of five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) with *PsMLO*A, *PsMLO*B and *PsMLO*AB were outsourced for sequencing from AgriGenome Labs Pvt Ltd. The lab performed Sanger's

method for sequencing of the amplicons of these five genotypes. The sequencing for all the samples was done in both the directions (forward and reverse). However, the sequencing results were not that good and the final trimmed sequences were shorter than the expected respective sizes. In some of the samples, the sequencing could not be carried out. However, we were able to build contigs from the overlapping sequences of respective genotypes using pair-wise sequence alignment.

4.2.5 Sequence analysis

All the trimmed sequences (including forward and reverse sequences of *PsMLO*A , *PsMLO*B and *PsMLO*AB) of the samples pertaining to respective five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) were imported to BioEdit, aligned using the overlapping regions and then finally the contigs were generated using the CAP Assembly programme of BioEdit (http://www.mbio.ncsu.edu/BioEdit/page2.html). The same contigs of DNA were also used for translation using the translation tool of expasy

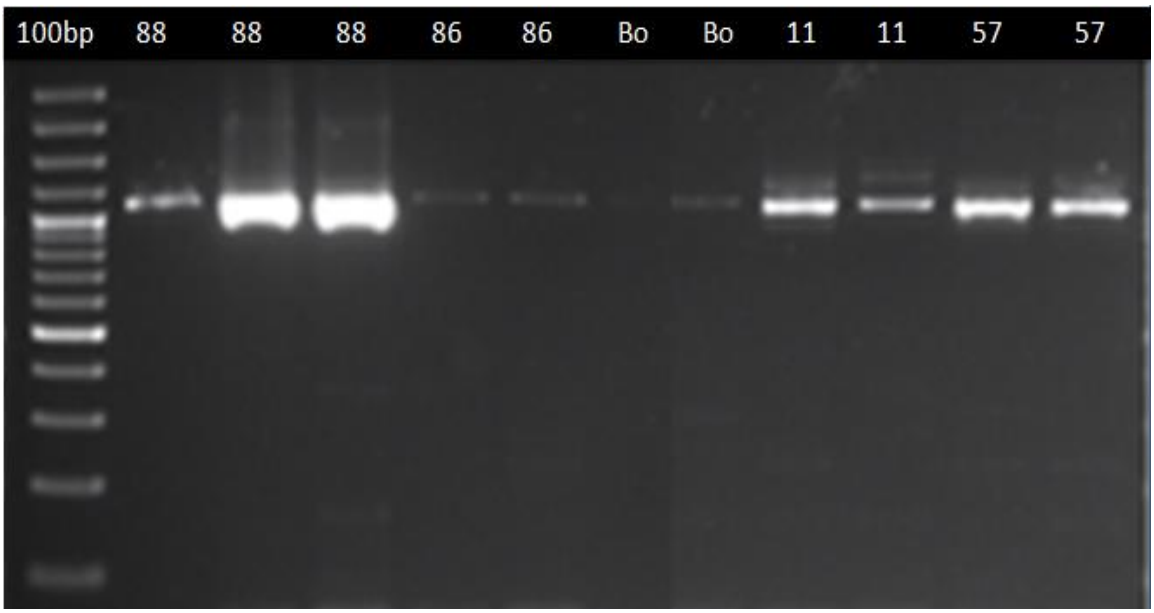


Plate 4.13: Banding profile of *PsMLO*A

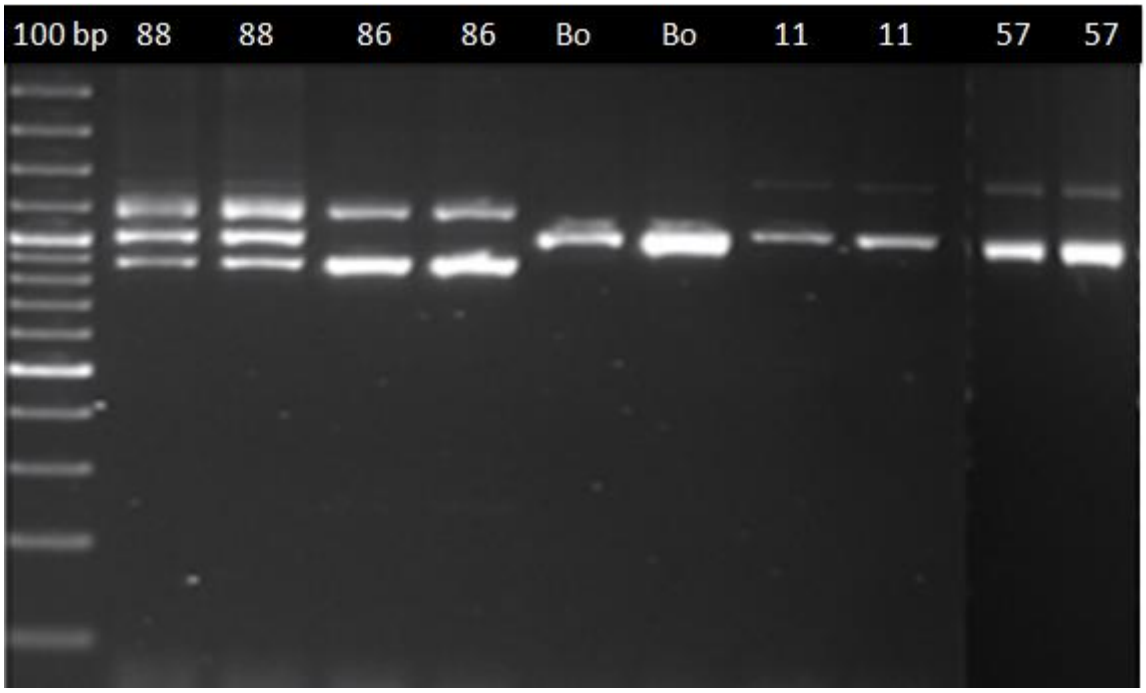


Plate 4.14: Banding profile of *PsMLO*B

(https://web.expasy.org/translate/). The DNA sequences as well as the translated sequences were then pair aligned with the *PsMLO* cDNA sequences of wild-type pea cultivar Sprinter (susceptible to *E. pisi*; retrieved from NCBI accession number: FJ463618.1). The contigs could not be formed for Pb89 and Improved JI 1559 as the sequence length was too less and fragmented. The contig length of IC- 219002 and DPPMR-09-1 was 1764 and 1778bp, respectively. A contig of good length for Bonneville could not be constructed therefore a partial sequence of two small fragments of 196bp and 1089bp length were used in the present study. Sequence alignment analysis showed that c-DNA sequence of both the genotypes *viz*., IC- 219002 and DPPMR-09-1 had a 10-bp deletion (TCATGTTATT) corresponding to position 111–120 of the wild-type PsMLO c-DNA (Plate 4.16, 4.17, 4.18, 4.19). A single base substitution G → A was also observed at positions 1734bp in both the geotypes. As expected 100 per cent similarity was found with the Bonneville partial sequence (Plate 4.20 and 4.21). Then the multiple sequence alignment was also carried out using the clustal omega programme (Plate 4.22 and 4.23).

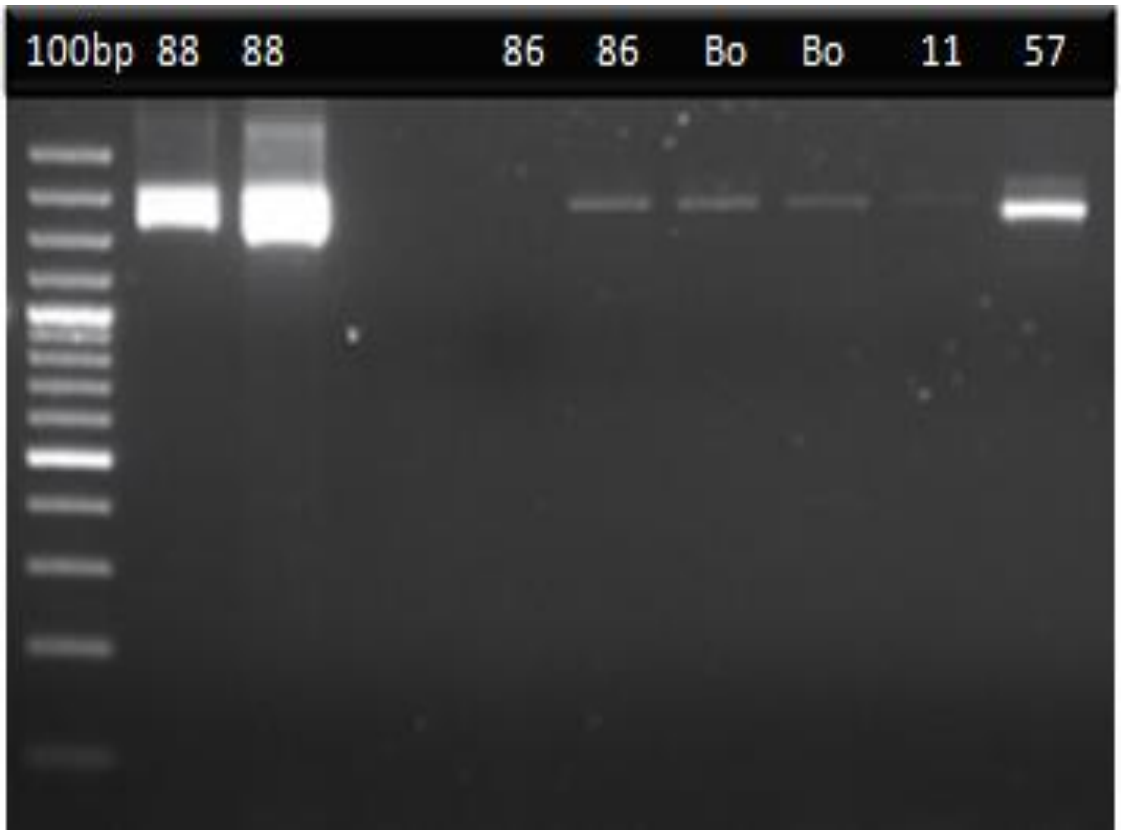


Plate 4.15: Banding profile of *PsMLO*AB

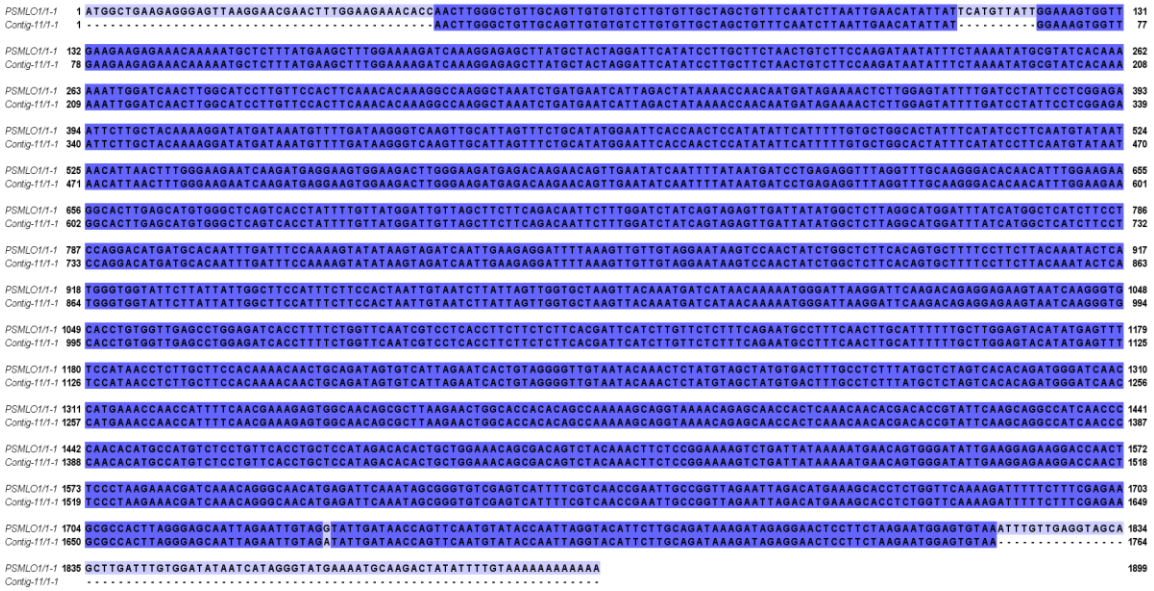


Plate 4.16: Pair wise alignment of c-DNA of IC- 219002 with *PsMLO* cDNA sequences of wild-type pea cultivar Sprinter

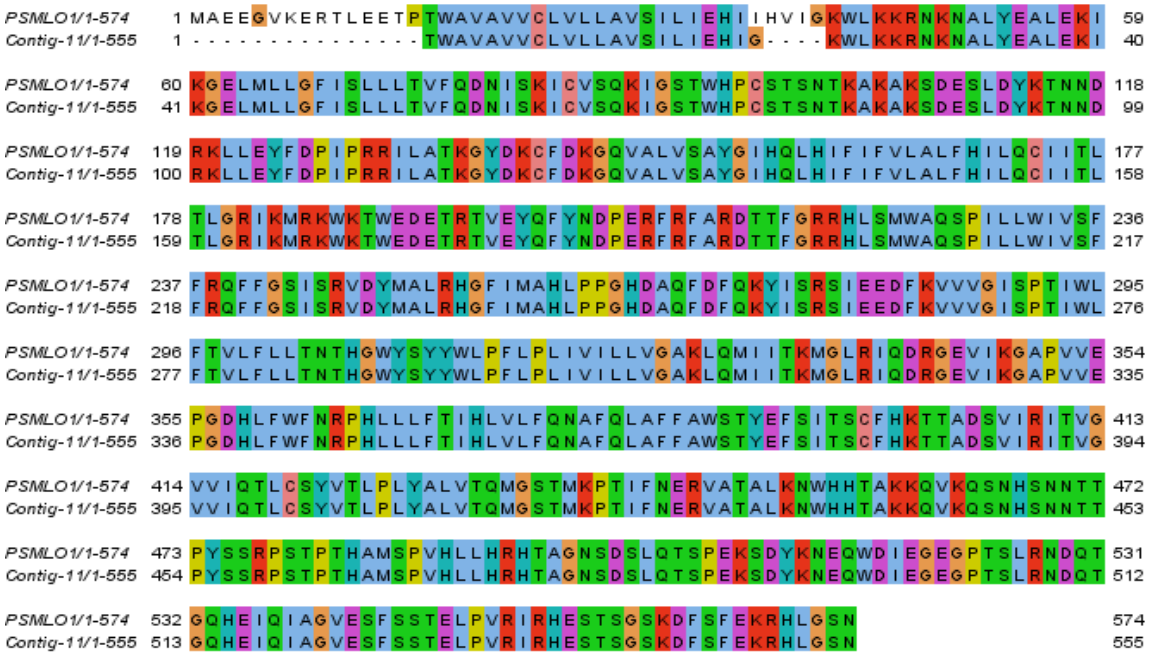


Plate 4.17: Pair wise alignment of translated c-DNA of IC- 219002 with *PsMLO* residues of wild-type pea cultivar Sprinter

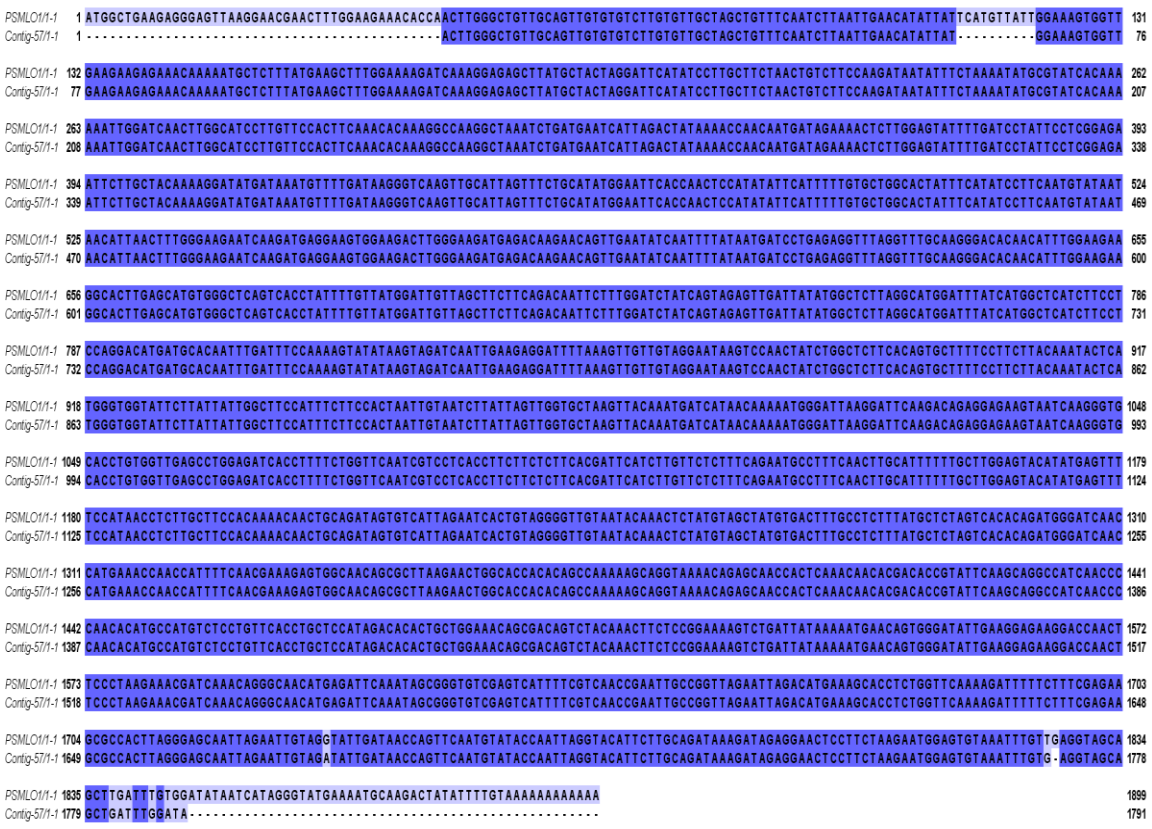


Plate 4.18: Pair wise alignment of c-DNA of DPPMR-09-1 with *PsMLO* cDNA sequences of wild-type pea cultivar Sprinter

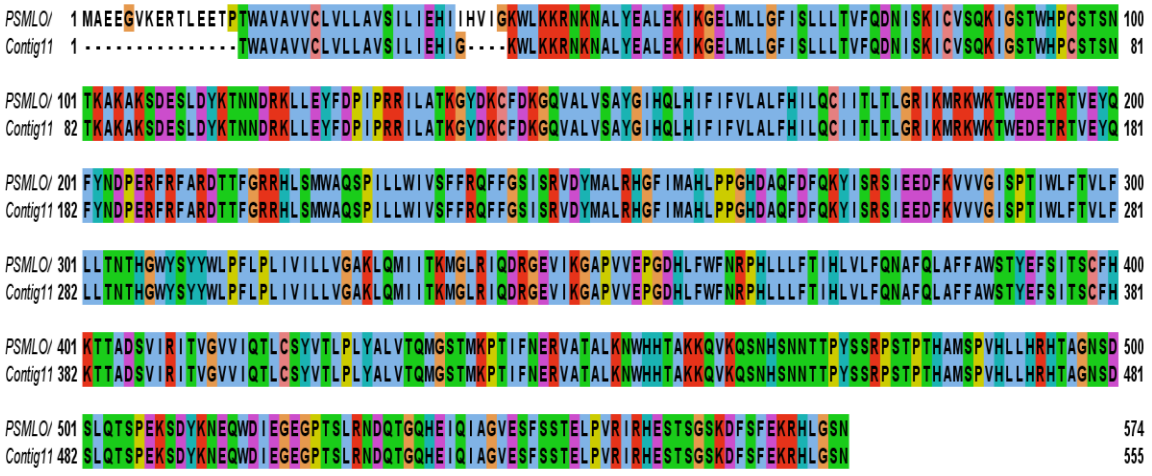


Plate 4.19: Pair wise alignment of translated c-DNA of DPPMR-09-1 with *PsMLO* residues of wild-type pea cultivar Sprinter

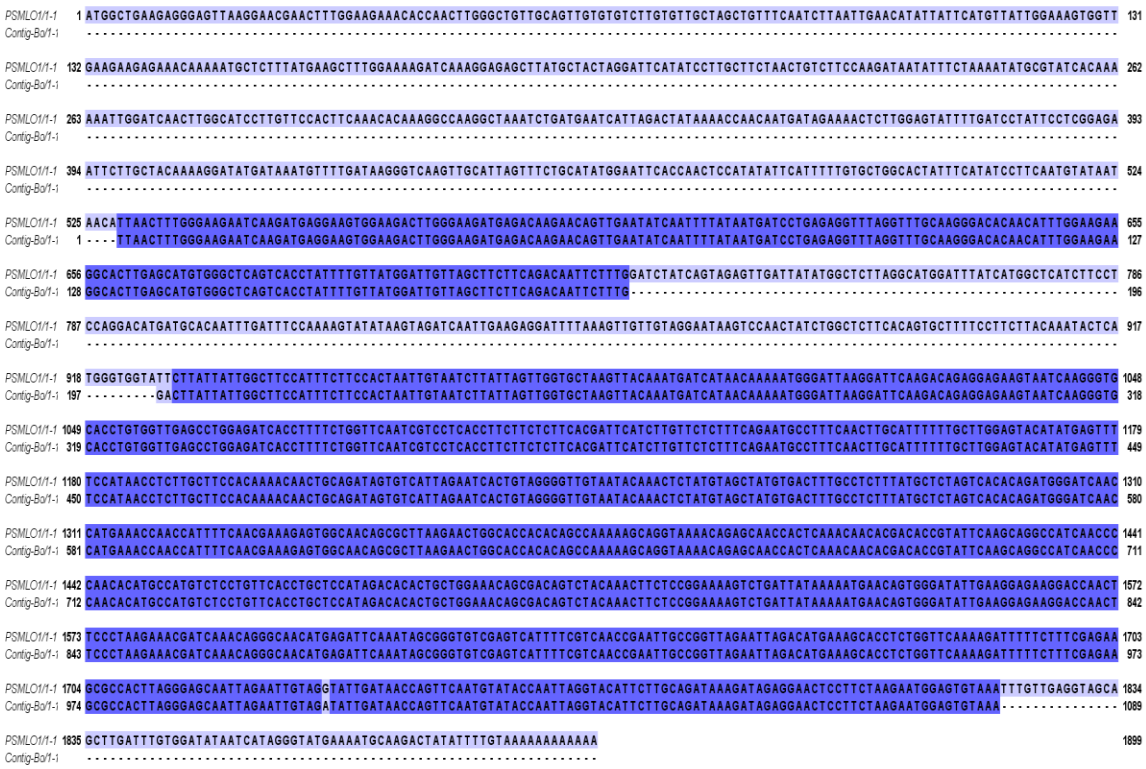


Plate 4.20: Pair wise alignment of c-DNA of Bonneville with *PsMLO* cDNA sequences of wild-type pea cultivar Sprinter

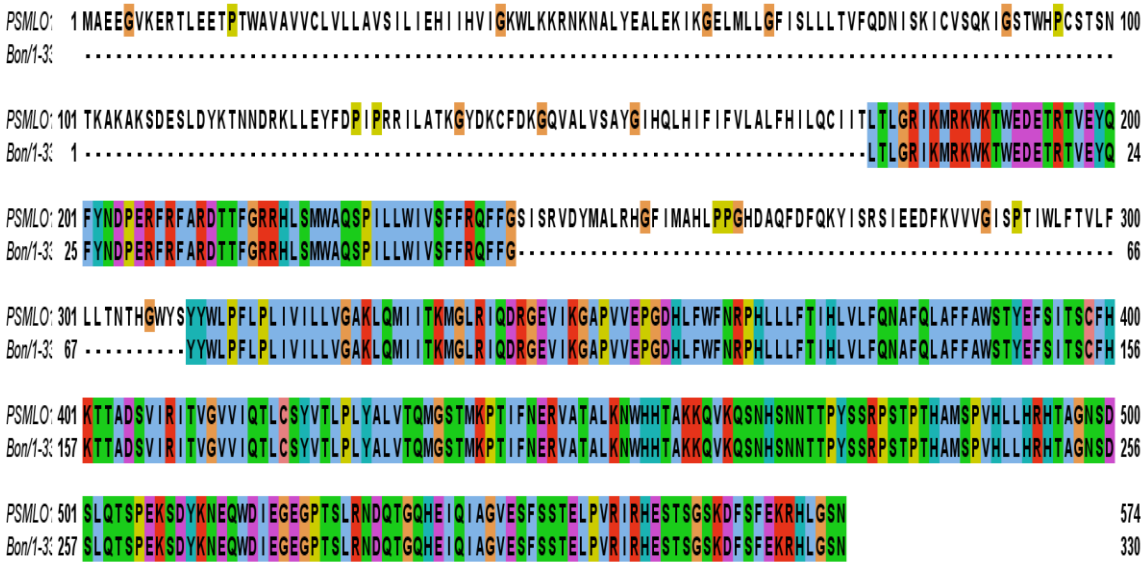
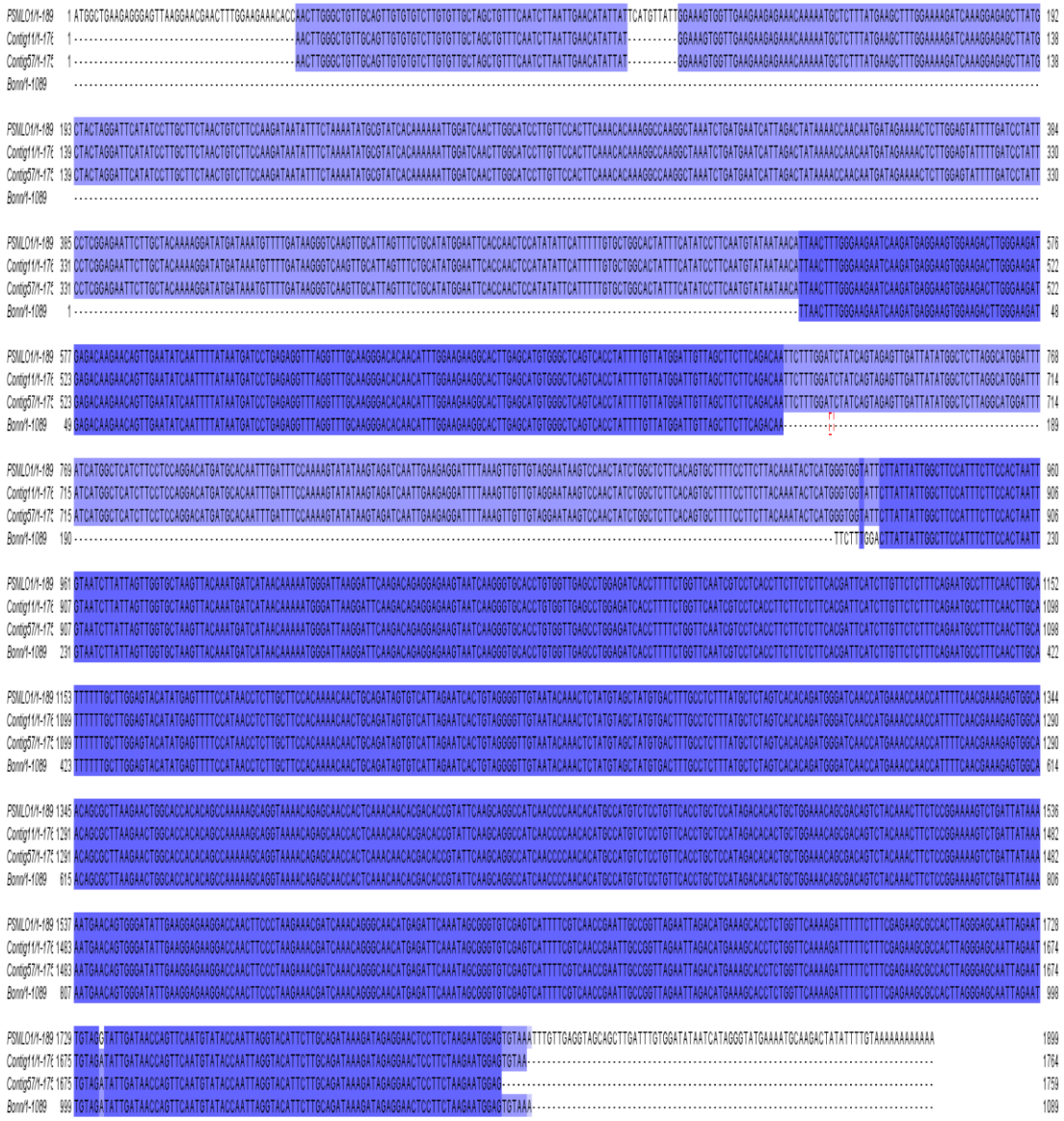
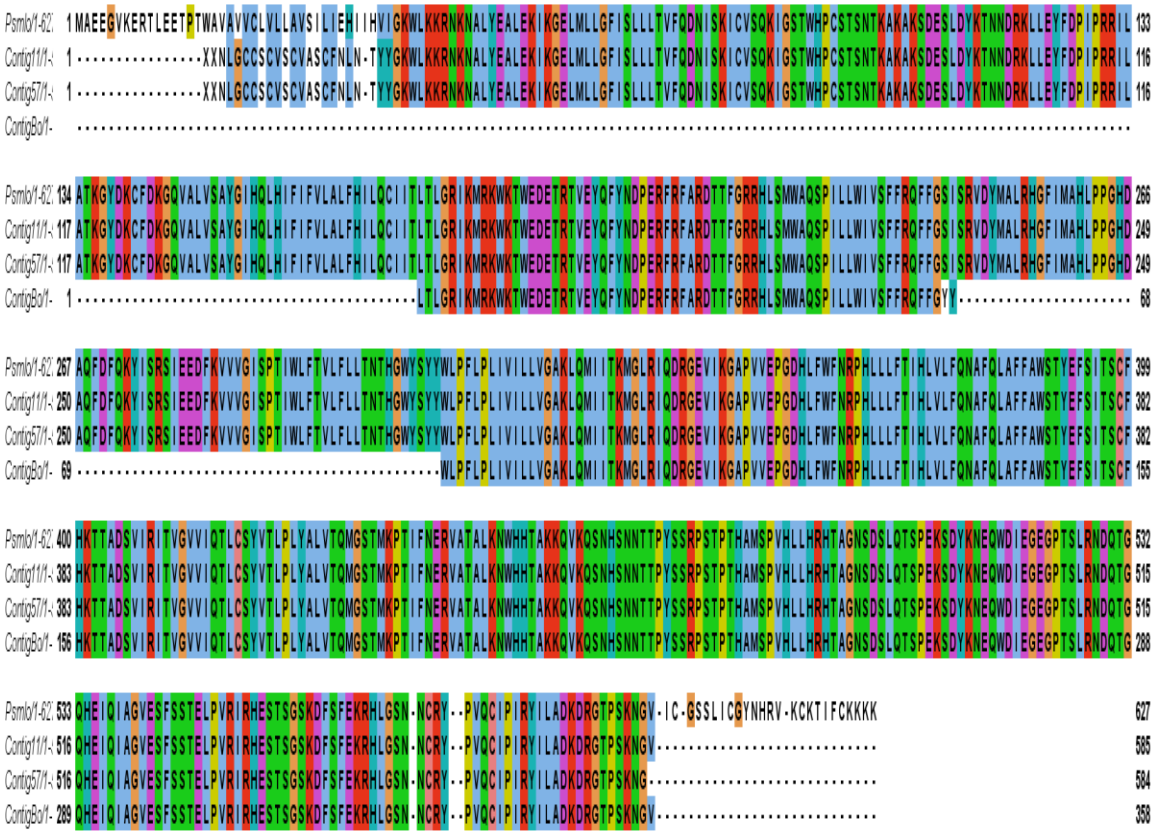


Plate 4.21: Pair wise alignment of translated c-DNA of Bonneville with *PsMLO* residues of wild-type pea cultivar Sprinter



Pate 4.22: Multiple Sequence Alignment of cDNA *PsMLO* cDNA sequences of wild-type pea cultivar Sprinter



Pate 4.23: Multiple Sequence Alignment of translated c-DNA of DPPMR-09-1 with *PsMLO* residues of wild-type pea cultivar Sprinter

CHAPTER 5

DISCUSSION

Pea (*Pisum sativum* L.) is one of the most widely grown legume food crops and represents a versatile and inexpensive protein source for animal feeding. Pea is used in rotation with cereals and oil crops and increases soil fertility by fixing atmospheric nitrogen. Commercial interest in peas and other pulses as a protein source (Santalla *et al.*, 2001) has been growing in recent years. In order to meet these demands, the development of new high-yielding cultivars with resistance to various abiotic and biotic stresses on a sustainable basis is greatly desired. Diseases such as powdery mildew, caused by the obligate biotrophic fungus Erysiphe pisi DC belonging to the ascomycete order of Erysiphales, are a strong limitation for the crop causing yield losses of 25–50% (Munjal *et al*. 1963; Warkentin *et al*. 1996). Therefore, further improvements in capturing genetic diversity in respect of various biotic and abiotic stresses is obligatory in order to develop crop varieties harbouring resistance to these stresses, higher yields and improved nutritive value (Able *et al*., 2007).

Now a day, with the development and availability of an array of molecular markers, marker assisted selection (MAS) has become possible for traits both governed by major genes as well as quantitative trait loci (QTLs). For the recessive traits like powdery mildew resistance the gene introgression through conventional process further complicates the breeding programme. For powdery mildew resistance three genes (two recessive '*er1', 'er2'* and one dominant '*Er3*') have been reported till date. *er1* confers complete resistance by limiting pathogen penetration whereas *er2* and *Er3* resistance is mainly based on post penetration hypersensitive response (Fondevilla, 2006). *er1* has been exploited extensively because of its stability and durable powdery mildew resistance. To date, seven *er1* alleles (*er1-1* to *er1-7*) have been identified conferring the *er1*-resistant phenotype in pea germplasms (Humphry *et al*. 2011; Pavan *et al*. 2011, 2013; Sun *et al*. 2015a, b and Sun *et al*.2016). Therefore, in the present study we sought to exploit molecular marker technology for characterization of resistant/tolerant pea germplasm by using powdery mildew linked molecular markers and determine the sequence of the *PsMLO1* gene at the *er1* locus of some resistant/tolerant genotypes.

5.1 Molecular characterization:

For characterization of resistant/tolerant pea germplasm using powdery mildew linked molecular markers, a total of ten markers were used, out of which seven were linked to *er1*, two were linked to *er2* and one was linked to *Er3* locus (Tiwari *et al.* 1998; Timmerman *et al*. 1994; Janila & Sharma, 2004; Ek *et al.* 2005; Katoch *et al.* 2010; Fondevilla *et al.* 2008). The markers linked to *er1* locus are: Sc-OPO-18_{1200}, Sc-OPE-16_{1600}, Sc-OPO-10_{650}, AD-60, AA-374e A-5 and OPL-6. The markers linked to *er2* locus are: ScX-17_{1400} and AA-278.The marker linked to *Er3* locus is ScAB1_{874}.

Sc-OPE-16 and Sc-OPO-10 are SCAR markers and have been linked in *cis* to *er1* at 4.0 and 3.4 cM distance from *er1* locus (Tiwari *et al*. 1998; Timmerman *et al.* 1994). The expected and observed allele size was 1600bp for Sc-OPE-16. All the genotypes subjected to ScOPE-16 assay amplified the expected band except for seven genotypes viz., Ps-11, Ps-12, Ps-35, Ps-51, Li, Ps98 and Ps-65. For Sc-OPO-10 marker the expected and observed allele size was 650bp and all the genotypes amplified except for Ps-93 and Ps-96. Since the markers are linked in *cis* to *er1*, the presence of the amplicon might be linked to *er1*. Sc-OPO-18_{1200} is linked in *trans* to *er1* at a distance of 0.0 cM from *er1* (Tiwari *et al*. 1998) and the expected and observed allele size was 1200bp. All the genotypes amplified the preferred band size except for Ps-98 and Ps-36, where no band was present. Here, the presence of amplicon shows possible linkage with *Er1*. The SCAR markers linked in coupling and repulsion phase when used in combination are capable of distinguishing homozygous individuals from heterozygous individuals with high efficiency and thus can act as co-dominant markers (Fondevilla *et al*. 2008)

AD-60, AA-374e and A-5 are SSR markers located at 10.4, 11.6 and 14.9 cM distance from *er1* locus with an expected amplicon of 220, 300 and 900bp, respectively (Ek *et al*. 2005). All the SSRs showed polymorphic results with resistant and susceptible parents and thus can be included in the breeding programmes after validation. Simultaneous use of flanking markers (AD60/AA374e and A5 in this case) is estimated to result in increased selection efficiency comparable to that achieved by single marker (Katoch *et al*. 2010); therefore these markers can be used in combination for better selection.

A second resistant gene to powdery mildew, *er2*, was reported after about five decades and also inherited recessively (Heringa *et al*. 1997). ScX-17_{1400}, a SCAR marker, and AA-278, a SSR marker, located at 2.6 and 14.9cM distance, respectively from *er2* locus (Katoch *et al*. 2010). The results for these two markers were highly polymorphic and can be used for incorporating *er2* after proper validation. These two markers flank the *er2* and therefore an effective selection can be carried out for the gene.

Recently, another resistance gene has been identified in *P. fulvum* accessions, a relative of garden pea. The new resistance gene is dominant and designated as *Er3* (Fondevilla *et al*. 2007). ScAB1 is a SCAR marker linked in repulsion (cis) phase, located at 2.8cM distance from Er3 locus (Fondevilla *et al*. 2008). All genotypes amplified expected band size except for Ps-86 and Ps-87. Combining of resistance mechanisms mediated by *er1*, *er2* and *Er3* into a single genetic background is expected to broaden the spectrum and durability of resistance conditioned by each of these genes.

Many workers have characterized the pea germplasm using RAPD, ISSR and SSR markers (Choudhury *et al.* 2007; Kapila *et al*. 2011; Simioniuc *et al*. 2002; Baranger *et al.* 2004; and Smykal *et al.* 2008) for the purpose of genetic diversity studies. But, we have characterized the germplasm using already reported powdery mildew linked markers. The major goal of this objective was to make available a ready reference of marker profile (powdery mildew linked) of resistant and susceptible notified varieties. This information can aid the breeders in selecting suitable donors and recipients aiming to improve field and garden pea in respect to powdery mildew resistance. However, the marker results in the present study are not in consonance with powdery mildew reaction as reported and that may be because of the fact that the reported researchers have carried out linkage studies with different genotypes. Moreover, the genotypes which we have characterized and have resulted in polymorphism in corroboration with earlier studies need to be validated in recombinant population before proceeding for introgression studies.

5.2 Characterization of *er1* locus

For the characterization of *PsMLO* locus, the RNA was extracted from all the 12 genotypes using Trizol method of RNA extraction. Quality and quantity of RNA was

estimated using agarose gel electrophoresis and spectrophotometric method (mySpec). The RNA extracted from these 12 genotypes was then used for c-DNA synthesis and was confirmed using housekeeping gene β-tubulin (Jose *et al*. 2009). All the genotypes amplified the expected band size of 60 bp for the house keeping gene β-tubulin suggesting an overall good quality of c-DNA synthesis. The c-DNA was then amplified with *PsMLO* gene specific primers to characterize the *er1* locus.

For full gene amplification, two overlapping markers designated as *PsMLO*A and *PsMLO*B were used. However, in present study, the forward primer of *PsMLO*A and reverse primer of *PsMLO*B (labeled as *PsMLO*AB) were also used. Out of the 12 genotypes used in the study, only five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) were able to amplify for both *PsMLOA* and *PsMLOB*.

The amplified products of five genotypes with *PsMLOA*, *PsMLOB* and *PsMLOAB* were outsourced for sequencing from AgriGenome Labs Pvt Ltd. The lab performed Sanger's method for sequencing of the amplicons in both the directions (forward and reverse). However, the sequencing results were not that good and the final trimmed sequences were way shorter than the expected respective sizes. All the trimmed sequences (including forward and reverse sequences of *PsMLO*A , *PsMLO*B and *PsMLO*AB) of the samples pertaining to respective five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) were imported to BioEdit, aligned using the overlapping regions and then finally the contigs were generated. The contigs could not be formed for Pb89 and Improved JI 1559 as the sequence length was too less and fragmented. Though three amplicons were observed in the line JI 1559, showing the presence of three transcripts but the sequencing results pertaining to this accession were not good. Sun *et al*, 2016 have also reported appearance of three transcripts in the cDNA sequences of *PsMLO1* of the accession G0005576, characterized by a 129-bp deletion, and 155-bp and 220-bp insertions in the wild-type *PsMLO1* of pea cv. Sprinter. This indicated that the resistance in G0005576 was conferred by *er1* allele, *er1-2*. Similar, studies can be carried out in our germplasm also to validate the presence of *er1* allele

The contig length of IC- 219002 and DPPMR-09-1 was 1764 and 1778bp, respectively. A contig of good length for Bonneville could not be constructed therefore a

partial sequence of two small fragments of 196 and 1089bp length were used in the present study. The DNA sequences as well as the translated sequences were then pair aligned with the *PsMLO* c-DNA sequences of wild-type pea cultivar Sprinter (susceptible to *E. pisi*; retrieved from NCBI accession number: FJ463618.1). Based on sequence alignment analysis *PsMLO* c-DNA of resistance lines, the sequence of IC- 219002 and DPPMR-09-1 showed that the *PsMLO*1 c-DNA had a 10-bp deletion (TCATGTTATT) corresponding to position 111–120 of the wild-type *PsMLO*1 c-DNA (Plate 4.16-4.19). On the contrary, whatever partial sequence we have generated in Bonneville, the *PsMLO* c-DNA from Bonneville was identical to that of the wild-type pea cultivar Sprinter, indicating that Bonneville contains the susceptible gene *Er1* (Plate 4.20-4.21).

The small-fragment deletion mutation in *PsMLO*1 has already been reported by Sun *et al* (2016), which suggested that resistance in IC- 219002 and DPPMR-09-1 is conferred by *er1* allele, designated as *er1*-7 (Plate 4.16-4.19). In this study, the 10-bp deletion in *er1-7* caused a frame shift at codon 37 leading to premature termination of translation at codon 85 and this resulted in a severely truncated protein (Plate 4.16-4.19). However, a single base substitution was also observed at positions 1734bp for G → A in all the accessions (IC- 219002, DPPMR-09-1 and Bonneville), however, it was well beyond the stop codon. The point mutations have earlier also been reported but they were inside the transcript and have lead to multi allelic events.

At the transcription level, the point mutations in *er1*-1 (C → G) and *er1*-5 (G → A) have been reported to introduce a termination codon (TGA) in exon 1 and 5 of *PsMLO*1, respectively, which is predicted to result in the production of a severely truncated *PsMLO*1 protein. The mutation in *er1*-6 (T → C) has been reported to lead to a single amino acid substitution (L replaced by P) at position 374, which produces a novel *PsMLO*1 protein that is inactive. There has also been reported a frame shift mutation in *er1*-3 (ΔG) due to a missing base, which also results in a single amino acid substitution (G replaced by E) at codon 288 and a premature termination signal at codon 307. However, *er1*-2 produces three *PsMLO*1 transcripts, which are characterized by a 129-bp deletion and 155-bp and 220-bp insertions (Humphry *et al*. 2011; Wang *et al*. 2015; Sun *et al*. 2015b).

Therefore, we can conclude that the resistance in IC- 219002, and DPPMR-09-1 is conferred by an *er1* allele, *er1*-7, which has earlier been reported by Sun *et al* (2016) and has been caused by a 10-bp deletion in the wild-type *PsMLO*1 cDNA. Pavan *et al*. (2016) have reported this allele in the pea cultivar 'DDR-11' (China Genebank accession number: G0003967) which was introduced from India and has been reported to be immune to Chinese *E. pisi* isolates (Wang *et al*. 2013). Therefore, our accessions may have been evolved from DDR11 or vice versa. These accessions might be used in pea breeding studies as the functional markers flanking this 10 bp deletion have been developed (Sun *et al.* 2016) and can effectively be used in the MAS.

CHAPTER 6

SUMMARY AND CONCLUSION

Pea (*Pisum sativum* L.) is one of the most widely grown legume food crops and represents a versatile and inexpensive protein source for animal feeding. For the improvement of any crop the investigations on resistance to various abiotic and biotic stresses on a sustainable basis is greatly desired. Diseases such as powdery mildew, caused by the obligate biotrophic fungus *Erysiphe pisi* DC cause heavy yield losses in the pea crop. The use resistant varieties harbouring resistant genes are the most effective and economical methods of disease control. For powdery mildew resistance, two single recessive genes (*er1* and *er2*) and one dominant gene (*Er3*) have been identified in pea germplasms to date. The DNA markers linked to resistance genes provide an alternative to disease screening for pyramiding of powdery mildew resistance genes. Molecular markers linked to the three resistance genes (*er1*, *er2* and *Er3*) have been developed by several research groups. Most pea powdery mildew resistant cultivars rely on the presence of the recessive gene *er1* which has been reported to be stable and provide complete resistance against powdery mildew. *er1* has been reported to be loss of function and, to date, seven *er1* alleles (*er1*-1, *er1*-2, *er1*-3, *er1*-4, *er1*-5, *er-1*-6 and *er1-7*) have been discovered. Each of these *er1* alleles corresponds to a different *PsMLO1* mutation, according to the mutation site.

Thus, in the present research we seek to exploit molecular marker technology for characterization of resistant/tolerant pea germplasm by using powdery mildew linked molecular markers and determine the sequence of the *PsMLO1* gene at the *er1* locus.

For characterization of resistant/tolerant pea germplasm using powdery mildew linked molecular markers, a total of ten markers were used, out of which seven were linked to *er1*, two were linked to *er2* and one was linked to *Er3* locus. The markers linked to *er1* locus are: Sc-OPO-18_{1200}, Sc-OPE-16_{1600}, Sc-OPO-10_{650}, AD-60, AA-374e A-5 and OPL-6. The markers linked to *er2* locus are: ScX-17_{1400} and AA-278.The marker linked to *Er3* locus is ScAB1_{874}. A good quality of DNA was isolated from 37 genotypes used in the study and assay with the reported markers was carried out. The major goal of

this objective was to make available a ready reference of marker profile (powdery mildew linked) of resistant and susceptible notified varieties. For all the markers the expected amplicons were observed and the data was highly polymorphic among the resistant and susceptible genotypes. This information can aid the breeders in selecting suitable donors and recipients aiming to improve field and garden pea in respect to powdery mildew resistance. However, the marker results in the present study need to be validated in recombinant population before proceeding for introgression studies.

For the characterization of *PsMLO* locus, the RNA was extracted from all the 12 genotypes using Trizol method of RNA extraction. Quality and quantity of RNA was estimated to be good using and was then used for c-DNA synthesis. The c-DNA was confirmed using housekeeping gene β-tubulin and all the genotypes amplified the expected band size of 60 bp suggesting an overall good quality of c-DNA synthesis. The c-DNA was then amplified with *PsMLO* gene specific primers to characterize the *er1* locus. Two overlapping markers designated as *PsMLO*A and *PsMLO*B were used for the full gene amplification. The forward primer of *PsMLO*A and reverse primer of *PsMLO*B (labeled as *PsMLO*AB) were also used in the present study. Out of the 12 genotypes used in the study, only five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) were able to amplify for both *PsMLOA* and *PsMLOB*.

The amplified products of five genotypes were outsourced for sequencing from AgriGenome Labs Pvt Ltd. Though, the sequencing results were not that good and the final trimmed sequences were way shorter than the expected respective sizes. All the trimmed sequences (including forward and reverse sequences of *PsMLO*A , *PsMLO*B and *PsMLO*AB) of the samples pertaining to respective five genotypes (Improved JI-1559, Bonneville, Pb-89, IC- 219002 and DPPMR-09-1) aligned and contigs were generated. A good contig length 1764 and 1778bp was constructed for IC- 219002 and DPPMR-09-1, respectively only. Based on sequence alignment analysis, the sequence of IC- 219002 and DPPMR-09-1 showed that their *PsMLO*1 c-DNA had a 10-bp deletion (TCATGTTATT) corresponding to position 111–120 of the wild-type *PsMLO*1 c-DNA. On the contrary, the partial *PsMLO* c-DNA from Bonneville was identical to that of the wild-type pea cultivar Sprinter, indicating that Bonneville contains the susceptible gene. The small-

fragment deletion mutation in *PsMLO*1 has already been reported and has been designated as *er1*-7. In this study, the 10-bp deletion in *er1-7* caused a frame shift at codon 37 leading to premature termination of translation at codon 85 and this resulted in a severely truncated protein. However, a single base substitution was also observed at positions 1734 bp for G – A in all the accessions (IC- 219002, DPPMR-09-1 and Bonneville), but it was well beyond the stop codon. The same deletion of 10 bp has also been reported as allele *er-1-7* in Indian line 'DDR-11' therefore, our accessions might have been evolved from DDR11 or vice versa. These accessions might be used in pea breeding studies as the functional markers flanking this 10 bp deletion have already been developed. Thus from our studies it can be concluded that:

- For all the markers the data was highly polymorphic among the resistant and susceptible genotypes.
- Characterization of already known resistance linked markers with the various notified varieties of field and garden pea will serve as a ready reference for breeders aiming improvement of pea. This information can aid the breeders in selecting suitable donors and recipients aiming to improve pea in respect to powdery mildew resistance.
- The results of the study have lead to identification of *er-1-7* allele in IC- 219002 and DPPMR-09-1 conferring a complete resistance to powdery mildew.
- These accessions may be used in pea breeding studies as the functional markers flanking this 10 bp deletion have already been developed and can effectively be used in the MAS.

Thus, the results of the present study may assist for further utilization of the germplasm in pre-breeding programmes for more stable/broad resistance.

REFERENCES

Able, J.A., Langridge, P. and Milligan, A.S. 2007. Capturing diversity in the cereals: many options but little promiscuity. *Trends in Plant Sciences*, **12(2):** 71-79.

Baranger, A., Aubert, G., Arnau, G., Laine, A.L., Deniot, G., Potier, J., Weinachter, C., Tallemand, J. and Burstin, J. 2004. Genetic diversity within *Pisum sativum* using protein and PCR based markers. *Theoretical and Applied Genetics*, **108:** 1309-1321

Bouhadida, M., Srarfi, F., Saadi, I., Kharrat, M. 2013. Molecular chareacterization of pea (*Pisum sativum* L.) using microsatellite markers. *Applied Chemistry* **5(1):** 57-61. Doi: 10.9790/5736-0515761.

Burstin, J., Salloignon, P., Chabert-Martinello, M., Magnin-Robert, J.B., Siol, M., Jacquin, F. 2015. Genetic diversity and trait genomic prediction in pea diversity panel. *BMC Genomics*, **16:** 105.

Choudhury, P. R., Tanveer, H., Dixit, G. H. 2007. Identification and detection of genetic relatedness among important varieties of pea (*Pisum sativum* L.) grown in India. *Genetica,* **130:** 183-191.

Doyle, J.L. and Doyle, J.J. 1990. Isolation of plant DNA from fresh tissue. *Focus*, **12:** 13-15

Deepika. 2016. Molecular and morphological characterization of pea genotypes. M.Sc. thesis. Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu. Jammu and Kashmir, India.

Ek, M., M. Eklund, R. Von Post, C. Dayteg, T. Henriksson, P. Weibull, A. Ceplitis, P. Isaac, and S. Tuvesson. 2005. Microsatellite markers for powdery mildew resistance in pea (*Pisum sativum* L.). *Hereditas* **142:** 86—91

F.A.O. Statistical Database, Food and Agriculture Organization (FAO) of the United Nations, Rome. 2013. (http://www.fao.org.StatisticalDatabase).

Falloon RE, Viljanen-Rollinson SLH. 2001. Powdery mildew. In: Kraft JM, Plfleger FL (eds) Compendium of pea diseases and pests. *American Phytopathological Society*, St. Paul, pp 28–29

Fondevilla S, Carver TLW, Moreno MT, Rubiales D. 2006. Macroscopic and histological characterisation of genes *er1* and *er2* for powdery mildew resistance in pea. *European Journal of Plant Pathology*, **115:**309–321.

Fondevilla S, Carver TLW, Moreno MT, Rubiales D. 2007a. Identification and characterisation of sources of resistance to *Erysiphe pisi* Syd. in *Pisum* spp. *Plant Breeding Journal*, **126:**113–119.

Fondevilla S, Cubero JI, Rubiales D. 2011. Confirmation that the *Er3* gene, conferring resistance to *Erysiphe pisi* in pea, is a different gene from *er1* and *er2* genes. *Plant Breeding Journal*, **130:** 281–282

Fondevilla S, Rubiales D. 2012. Powdery mildew control in pea: a review. *Agronomy Sustainable Development*, **32:** 401–409.

Fondevilla, S., Rubiales, D., Moreno, M. T., Torres A. M. 2008. Identification and validation of RAPD and SCAR markers linked to the gene *Er3* conferring resistance to *Erysiphe pisi* DC in pea. *Molecular Breeding*, **22:**193–200.

Fondevilla S, Torres AM, Moreno MT, Rubiales D. 2007b. Identification of a new gene for resistance to powdery mildew in *Pisum fulvum*, a wild relative of pea. *Breeding Science*, **57:** 181–184.

Ghafoor A, McPhee K. 2012. Marker assisted selection (MAS) for developing powdery mildew resistant pea cultivars. *Euphytica*, **186:**593–607.

Gilpin, B.J., Mccallum, J.A., Frew, T.J., and Timmerman-Vaughan, G.M. 1997. A linkage map of pea (*Pisum sativum* L.) genome sequence tags (ESTs). *Theoretical and Applied Genetics*, **95:** 1289-1299.

Gritton ET, Ebert RD. 1975. Interaction of planting date and powdery mildew on pea plant performance. *American Society for Horticultural Science*, **100:**137–142

Harland SC (1948) Inheritance of immunity to mildew in Peruvian forms of *Pisum sativum*. *Heredity,* **2:**263–269

Heringa, R.J., Norel, A., Tabelaar, M.F. 1969. Resistance to powdery mildew (*Erisyphe polygoni* D.C.) in peas (*Pisum sativum* L.). *Euphytica* **18(2):** 163-169.

Humphry M, Reinstädler A, Ivanov S, Bisseling T, Panstruga R. 2011. Durable broad-spectrum powdery mildew resistance in pea er1 plants is conferred by natural loss-of-function mutations in *PsMLO1*. *Molecular Plant Pathology Journal,* **12:** 866–878.

Irzkowska, L., Wolko, B. and Swiecicki, W.K. 2001. The genetic linkage maps of pea (*Pisum sativum* L.) based on molecular, biochemical and morphological markers, *Pisum Genetics Association,* **33:** 13-18

Janila, P. and Sharma, B. 2004. RAPD and SCAR markers for powdery mildew resistance gene er in pea. *Plant Breeding,* **123:** 271/274.

Jose, V.D., Roman, B., Nadal, S., Gonzâlez-Verd, C.I. 2010. Evaluation of candidate reference genes for expression studies in Pisum sativum under different experimental. *Planta,* **232:** 145-153.

Kapila, K.,Naryal, S. and Dhiman, K.C. 2011. Analysis of genetic diversity among garden and field pea genotypes of Higher Indian Himalayas. *Journal of Plant Biochemistry and Biotechnology*, 21**(2):** 286-291

Katoch, V., Sharma. S., Pathania. S., Banayal, D. K., Sharma, S. K., Rathour, R. 2010. Molecular mapping of pea powdery mildew resistance gene *er2* to pea linkage group III. *Molecular Breeding*, **25(2):** 229-237/doi 10.1007/s11032-009-9322-7

Kumar, H. and Singh, R. B. 1981. Genetic analysis of adult plant resistance to powdery mildew in peas (*Pisum sativum* L.). *Euphytica,* **30:** 147/151.

Laucou, V., Hauronge, K., Ellis, N and Rameau. C. 1998. Genetic mapping in pea.1. RAPD-based genetic linkage map of Pisum sativum. *Theoretical and Applied Genetics,* **97:** 905-915.

Loridon,K., K. McPhee, J. Morin,P. Dubreuil, M. L. Pilet-Nayel, G. Aubert, C.Rameau, A.Baranger, C.Coyne, I.Lejeune-Henaut, and J. Burstin. 2005. Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). *Theoretical and Applied Genetics*. **111**: 1022—1031.

Marx, G.A. 1971. New linkage relations for chromosome III of *Pisum*. *Pisum Newsletter*, **3:** 18–19.

Marx, G.A. 1986. Location of *er* proving elusive. *Pisum Newsletter, **18:*** 39–41

Muehlbauer, F.J., Redden, R.J., Nassib, A.M., Robertson, L.D., and Smithson, J.B. 1998. Population improvement in pulse crops: an assessment of methods and techniques. In: Summerfield, R.J. (ed.). *World Crops: Cool Season Food Legumes, Kluwer Academic Publishers,* 943-966.

Munjal, R. L., Chenulu, V. V., Hora, T. S. 1963. Assessment of losses due to powdery mildew (*Erisiphe polygoni*) on pea. *Indian Phytopathology*, **19:**260–267

Pavan, S., Schiavulli, A., Appiano, M., Marcotrigiano, A. R., Cillo, F., Visser, R. G. F., Yuling Bai, Y., Lotti, C., Luigi-Ricciardi, L. 2011. Pea powdery mildew *er1* resistance is associated to loss-of-function mutations at a MLO homologous locus. *Theoretical and Applied Genetics*, **123:**1425–1431.

Pavan, S., Schiavulli, A., Appiano, M., Miacola, C., Visser, R. G. F., Bai, Y. L., Lotti, C., Luigi-Ricciardi, L. 2013. Identification of a complete set of functional markers for the selection of *er1* powdery mildew resistance in *Pisum sativum* L. *Molecular Breeding Journal,* **31:**247–253.

Rana, J.C., Banyal, D.K., Sharma, K.D., Sharma, M.K., Gupta, S.K. and Yadav, S.K. 2013. Screening of pea germplasm for resistance to powdery mildew. *Euphytica*, **189:** 271-282.

Santalla, M., Amurrio, J.M., De Ron, A.M. 2001. Food and feed potential breeding value of green, dry and vegetable pea germplasm. *Canadian Journal of Plant Sciences*, **81:** 601-610.

Sato, S., Isobe, S., Tabata, S. 2010. Structural analysis of the genomes in legumes. *Current Opinion in Plant Biology*, **13:** 146-152.

Sharma, A. Singh, G., Singh, Y., and Sood, S. 2008. Detection of genetic components of variation for biometrical traits in garden pea. *International Journal of Vegetable Sciences*, **14:** 67-81.

Simioniuc, D., Uptmoor, R., Friedt, W. and Ordon, F. 2002. Genetic diversity and relationships among pea cultivars by RAPDs and AFLPs. *Plant Breeding Journal*, **121:** 429-435.

Singh, U.P. and Singh, H.B. 1983. Development of *Erysiphe pisi* on susceptible and resistant cultivars of pea. *Transactions of the British Mycological Society*, **81:** 275-278.

Smith PH, Foster EM, Boyd LA, Brown JKM. 1996. The early development of *Erysiphe pisi* on *Pisum sativum* L. *Plant Pathology Journal*, **45:**302–309

Smykal, P., Aubert, G., Burstin, J., Coyne, C.J., Jarkovsky. J., Flavell, A.J and Griga, M. 2008. Genetic diversity and population structure of pea (*Pisum sativum*) varieties derived from combined retrotransposon, microsatellite and morphological marker analysis. *Theoretical and Applied Genetics*, **117:** 413-424.

Sun, S., Fu, H., Wang, Z., Duan, C., Zong, X., Zhu, Z. 2015a. Discovery of a novel *er1* allele conferring powdery mildew resistance in Chinese pea (*Pisum sativum* L.) landraces. PLoS one. doi:10.1371/journal.pone.0147624.

Sun, S., Fu, H., Wang, Z., Duan, C., Wang, X., Zhu, Z. 2015b. Resistance to powdery mildew in the pea cultivar Xucai 1 is conferred by the gene *er1*. *Crop Journal*, **3:** 489–499.

Sun, S., Deng, D., Wang, Z., Duan, C.,Wu, X., Wang, X., Zong, X. and Zhu, Z. 2016. A novel *er1* allele and the development and validation of its functional marker for breeding pea (*Pisum sativum* L.*)* resistance to powdery mildew. *Theoretical and Applied Genetics*, **129:** 909–919.

Taran, B., T. Warkentin, D. J. Somers, D. Miranda, A. Vandenberg, S. Blade, S. Woods, D. Bing, A.Xue, D. DeKoeyer, and G. Penner. 2003. Quantitative trait loci for lodging resistance, plant height and partial resistance to mycosphaerella blight in field pea (*Pisum sativum L.*). *Theoretical and Applied Genetics* . **107**:1482—1491.

Timmerman, G. M., Frew, T. J., Weeden, N. F. et al. 1994. Linkage analysis of er-1, a recessive *Pisum sativum* gene for resistance to powdery mildew fungus *(Erysiphe pisi* D.C.). / *Theoretical and Applied Genetics*, **88:** 1050/1055.

Tiwari KR, Penner GA, Warkentin TD. 1999. Identification of AFLP markers for powdery mildew resistance gene *er2* in pea. *Pisum Genetics Association,* **31:**27–29.

Tiwari, K. R., Penner, G. A. and Warkentin, T. D. 1997. Inheritance of powdery mildew resistance in pea. *Canadian Journal of Plant Sciences*, **77:** 307/310.

Tiwari, K. R., Penner, G. A. and Warkentin, T. D. 1998. Identification of coupling and repulsion phase RAPD markers for powdery mildew resistance gene, *er-1* in pea. / Genome **41:** 440/444.

Vaid, A., Tyagi, P. D. 1997. Genetics of powdery mildew resistance in pea. *Euphytica,* **96:**203–206.

Wang, Z., Bao, S., Duan, C., Zong, X., Zhu, Z. 2013. Screening and molecular identification of resistance to powdery mildew in pea germplasm. *Acta Agronmica Sinica*, **39:**1030–1038

Warkentin, T. D., Rashid, K. Y., Xue, A. G. 1996. Fungicidal control of powdery mildew in field pea. *Canadian Journal of Plant Sciences* 76:933–93

Weeden, N.F., Ellis, T.H.N., Timmerman-Vaughan, G.M., Swiecicki, W.K., Rozov, S.M. and Berdnikov, V.A. 1998. A consensus linkage map of Pisum sativum. *Pisum Genetics Association*, **30: 1-3.**

CURRICULUM VITA

Name of the student	**:**	Ms. Redhima Kapoor
Father's Name	**:**	Mr. Rakesh Kapoor
Mother's Name	**:**	Mrs. Rekha Kapoor
Nationality	**:**	Indian
Date of Birth	**:**	19-11-1994
Address	**:**	H. No. 54 Bakshi Nagar, Roulki, Jammu
Email id	**:**	redhimakapoor994@gmil.com

EDUCATIONAL QUALIFICATIONS

Bachelor's Degree	:	B.Sc. (Hons.) Biotechnology
University and year of Award	:	SKUAST-J (2016)
OGPA	:	7.76/10
Master's Degree	:	M.Sc. Biotechnology
OGPA	:	8.30/10.00

CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the external examiner and the Advisory Committee have been duly incorporated in the thesis entitled **"Characterization of powdery mildew resistance gene at the *er1* locus in resistant/ tolerant genotypes of pea (*Pisum sativum* L.)"** submitted by **Ms. Redhima Kapoor**, Regd. No. **J-16-MB-27**.

Dr. Susheel Sharma
Major Advisor

Place: Jammu

Date: 13/ 08/ 2018

Coordinator

School of Biotechnology