"MOLECULAR MAPPING OF ASCOCHYTA BLIGHT RESISTANCE IN CHICKPEA (*Cicer arietinum* L.)"

Ph. D. THESIS

by

PRATIBHA RAMAKURI

DEPARTMENT OF BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
INDIRA GANDHI AGRICULTURAL UNIVERSIT

RAIPUR (C.G.)

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Thesis

Submitted to

Indira Gandhi Agricultural University, Raipur

by

PRATIBHA RAMAKURI

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MOLECULAR BIOLOGY AND BIOTECHNOLOGY

Roll No.: 2631 I. D. No.: AG-PG-96-12

DECEMBER' 2005

Certificate - I

This is to certify that the thesis entitled **"Molecular mapping of Ascochyta blight resistance in chickpea (*Cicer arietinum* L.)"** submitted in partial fulfillment of the requirements for the degree of "Doctor of Philosophy in Molecular Biology and Biotechnology" of the Indira Gandhi Agricultural University, Raipur (C.G.), is a record of the bonafide research work carried out by **Ms Pratibha Ramakuri** under my guidance and supervision. The students advisory committee and the Director of Instructions have approved the subject of the thesis.

No part of the thesis has been submitted for any other degree or diploma (certificate awarded etc.) or has been published / published part has been fully acknowledged. All the assistance and help received during the course of the investigations have been duly acknowledged by her.

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Certificate - II

This is to certify that the thesis entitled "**Molecular mapping of Ascochyta blight resistance in chickpea (*Cicer arietinum* L.)**" submitted by **Ms. Pratibha Ramakuri** to Indira Gandhi Agricultural University, Raipur (C.G.), in partial fulfillment of the requirements for the degree of "Doctor of Philosophy in "Molecular Biology and Biotechnology" in the Department of **Biotechnology** has been approved by the Student's Advisory Committee after oral examination in collaboration with the external examiner.

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ACKNOWLEDGEMENTS

I take this opportunity to express my deep sense of gratitude and sincere thanks to my major advisor, Dr. S. K. Katiyar, Head, Department of Biotechnology, Indira Gandhi Agricultural University, Raipur, C.G., India for his able guidance and constant encouragement during the course of this investigation. I am greatly indebted to his ceaseless help and critical suggestions rendered through out the course of study.

I extend my heartfelt thanks and sincere gratitude to my co-chairman, Dr. P. M. Gaur, Senior Scientist, (Chickpea Breeding), ICRISAT for accepting me in the organization, and providing all the facilities for my research work. I am very thankful for his invaluable suggestions, constant support and able guidance during my research work.

I express my sincere thanks to Drs. Manorama, V. K. Dubey, P. Katiyar and R. R. Saxena, members of my advisory committee for their consistent support, invaluable suggestions and encourage through out my study.

I extend my deep sense of gratitude to Dr. J. H. Crouch for providing the laboratory facilities at Applied Genomics Laboratory, ICRISAT and also for the financial support extended. I am very thankful for his invaluable suggestions and constant encouragement. I express my sincere thanks to Dr. H. K. Buhariwalla for her critical suggestions in the genotyping work.

My sincere thanks to Drs. S. Pande and G. K. Kishore for their encouragement and providing the facilities for carrying out the pathological screening. I extend my sincere thanks to Mr. Bashir and Mr. Reddy for helping me in the Pathology Laboratory at ICRISAT.

I take this opportunity extend my heartfelt thanks to Ms. K. Seetha my lab supervisor for her constant support and encouragement. I express my sincere thanks to Mr. K. Eshwar, Mr. G. S. Somaraju, Mr. A. Gafoor, Mr. M. Bryan and Mr. Narsi Reddy for their constant support in the laboratory. I am also thankful to Mr. Ramdas, Mr. Mallareddy and Ms. Bakkamma for their help rendered in the laboratory (ICRISAT).

My sincere thanks to Mr. B. V. Rao, Scientific officer, Chickpea Breeding Unit, ICRISAT, Mr. Aziz, Mr. SangaReddy, Mr. Bose, Mr. MallaReddy, Mr. Das, Mr. C. A. Selwin and Mrs. Pakiramma for their help rendered.

I express my sincere thanks to Dr. S. Chandra, Senior Scientist (Statistics and Bioinformatics) and Dr. Jayashree Balaji (Scientist, bioinformatics) for their invaluable suggestions and encouragement. I am thankful to Ms. K. Rupa and Mr. Prashant for their help rendered.

I extend my sincere gratitude and special thanks to my teachers Dr. A. S. Kotasthane, Dr. S. B. Verulkar and Dr. Girish Chandel for their constant encouragement. I am thankful to all the staff and members of Department of Biotechnology, IGAU, Raipur.

My heartfelt thanks to my class-mates Ms. P. T. Lekha and Ms. Roohi Khan for their constant support through out my study. I am thankful to Mr. M. Dhudhare and Ms. Aditi Sharma for their encouragement and help rendered in many ways. I also thank my juniors in the Department of Biotechnology and inmates of the girls hostel for their kind help and affection.

I owe my sincere thanks to my husband Dr. K. Kameswara Rao and to my daughter Srividya for their love, patience and moral support through out the course of this study and sharing all external responsibilities, which enabled me to complete this study. No words can express the deep sense of gratitude to my mother and father and father-in-law and brother for the support extended during my study. Their best wishes and blessings have always been with me through out this work. I am very thankful to Mr. K.V. V. Mohan and Mr. Gopi for helping me in typing the thesis at the fag end.

Finally, I extend my appreciation and thanks to every one who has steered my humble efforts in carrying out the research work.

Date: 20·12 2005

Pratibha Ramakuri

TABLE OF CONTENT

Chapter	Title	Page No.
I	INTRODUCTION	1-5
II	REVIEW OF LITERATURE	6-34
III	MATERIALS AND METHODS	35-54
IV	RESULTS	55-79
V	DISCUSSION	80-98
VI	SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK	99-103
	ABSTRACT	104-105
	REFERENCES	106-122
	APPENDICES	

List of Tables

Table No. No.	Title	Page
2.1	Overview of SSR, EST, SNP, and RGA markers reported in chickpea	13
2.2	Overview of genetic linkage maps generated from inter and intraspecific crosses in chickpea	15-16
2.3	Inheritance of Ascochyta blight disease in chickpea	25
2.4	Molecular markers identified for Ascochyta blight resistance QTLs in chickpea	30
3.1	List of SSR primers used for parental prescreening	43-44
3.2	List of EST and RGA primers used for parental prescreening	45
3.3	Touch down PCR profile	46
4.1	PCR amplification conditions and polymorphism between ICC4991 and ICCV04516	58-61
4.2	Proportion of polymorphic primers identified for use in mapping	62
4.3	Overview of the intraspecific linkage map of ICC4991 x ICCV04516	63
4.4	Mean Ascochyta blight disease reaction in F_2 and F_3 population ICC4991 x ICCV04516	69-70
4.5	Descriptive statistics of mean ABt disease reaction in the F_2 and $F_{2:3}$ segregating populations of F_2 (ICC4991 x ICCV04516)	72

4.6 Analysis of variance for Ascochyta blight disease reactions in $F_{2:3}$ plants 72

4.7 Association of genetic loci with Ascochyta blight disease reaction scores based on simple linear regression analysis of F_2 means 73

4.8 Map location and estimated effects of quantitive trait loci providing adult plant resistance to AB in F_2 population based on composite interval mapping 73

4.9 Association of marker loci with Ascochyta blight disease reaction scores based on simple linear regression analysis of F_3 means. 74

4.10 Map locations and estimated effects of quantitive trait loci providing seedling resistance to AB in population $F_{2:3}$ lines based on composite interval mapping 75

4.11 Mean Ascochyta blight disease reactions in F_2 validation populations (ICCV10 x ICCV04516-VP1) and ICCL87322 x ICCV04516-VP2) 76

4.12 Descriptive statistics of mean (F_2) AB disease score of AB in validation population of F_2 (ICCV10 X ICCV 04516) and F_2 (ICCL87322 x ICCV04516 78

4.13 Association of marker loci with Ascochyta blight disease reaction scores based on simple linear regression analysis of F_2 data 79

List of Figures

Figure	Title	In between Pages
3.1	Pedigree of resistant parent ICCV04516 and schematic representation of mapping populations utilized for AB QTL detection	35-36
3.2	Ascochyta blight disease screening in F_2 generation – Cut twig method	50-51
3.3	Ascochyta blight disease screening in $F_{2:3}$ generation – Seedling method	50-51
3.4	Progressive symptomatology of Ascochyta blight	50-51
4.1	Parental polymorphism of ICC4991 and ICCV04516 and genuine F_1 hybrids	55-56
4.2	Parental prescreening of ICC4991 and ICCV04516 with SSR markers	55-56
4.3a	Parental prescreening of ICC4991 and ICCV04516 with EST markers	55-56
4.3b	Parental prescreening of ICC4991 and ICCV04516 with RGA markers	55-56
4.4	Segregation of SSR markers in F_2 mapping population of ICC4991 x ICC04516 (separated on 6% PAGE)	61-62

4.5	Segregation of SSR markers in F_2 mapping population of ICC4991 x ICC04516 (separated on 8% denaturing PAGE)	61-62
4.6	Phenogram depicting segregation of SSR markers across F_2 mapping population separated by Capillary Electrophoresis ABI PRISM 3700 (Applied Biosystems)	61-62
4.7	Intraspecific genetic linkage map of Pb-7(ICC4991) x ICCV04516	62-63
4.8	Frequency distribution of disease scores for Ascochyta blight resistance in F_2 and $F_{2:3}$ populations	66-67
4.9	Graphical representation of adult plant AB resistance QTLs identified on linkage groups of ICC4991 x ICCV04516 intraspecific map	71-72
4.10	Graphical representation of seeding AB resistance QTLs identified on linkage groups of ICC4991 x ICCV04516 intraspecific map with $F_{2:3}$ means	74-75
4.11	Frequency distribution of disease scores for Ascochyta blight in F_2 populations	76-77

LIST OF ABBREVIATIONS

AB	- Ascochyta Blight
AFLP	- Amplified Fragment Length Polymorphism
ANOVA	- Analysis of Variance
BAC	- Bacterial Artificial Chromosome
BIBAC	- Binary BAC
BLUPs	- Best Liner Unbiased Predictors
bp	-base pairs
C	- Celcius
cM	- Centi Morgan
cm	- Centi meter
CAPs	- Cleaved Amplified Polymorphic sites
CIM	- Composite Interval Mapping
DAF	- DNA Amplification Fingerprinting
DAI	- Days After Inoculation
DDRT	- Differential Display Reverse Transcription
DNA	- Deoxyribose Nucleic Acid
dNTP	- deoxy nucleotide triphosphate
EDTA	- Ethylene Diamine Tetra-acetic acid
EST	- Expressed Sequence Tags
FISH	- Fluorescent In Situ Hybridization
g	- gram
h	- hour
ISSR	- Inter Simple Sequence Repeats
IM	- Interval mapping

KCl	- Potassium chloride
kg	- kilogram
λ	- Lambda
LG	- Linkage Group
LOD	- Log of Odds
LR	- Likelihood ratio
LRR	- Leucine Rich Repeats
M	- Molar
ml	- Milli liter
$MgCl_2$	- Magnesium chloride
MAB	- Marker Assisted Breeding
MAS	- Marker Assisted Selection
Mbp	- Mega base pair
mg	- Milligram
mM	- Millimolar
mm	- millimeter
NaCl	- Sodium chloride
NBS	- Nucleotide Binding Site
ng	- nanogram
PAGE	- Polyacrylamide Gel Electrophoresis
PAL	- Phenyl Alanine Ammonia Lyase
pM	- Pico Molar
PCI	- Phenol Chloroform - Isoamyl alcohol
PCR	- Polymerase Chain Reaction
PVP	- Polyvinyl pyrrolidone
QTL	- Quantitative Trait Loci
RAPD	- Random Amplified Polymorphic DNA

RE	- Restriction Enzyme
RGA	- Resistance Gene Analogues
RFLP	- Restriction Fragment Length Polymorphism
RIL	- Recombinant Inbred lines
REML	- Restricted Maximum Likelihood
SCN	- Soybean Cyst Nematode
SEM	- Standard Error of Mean
SMA	- Single marker Analysis
SSR	- Simple Sequence Repeats
STMS	- Sequence Tagged Microsatellites Repeats
SSLP	- Simple Sequence Length Polymorphism
SCAR	- Sequence characterized Amplified Regions Repeats
TAE	- Tris Acetic acid EDTA buffer
TBE	- Tris Bortae EDTA buffer
Taq	- *Thermus aquaticus*
TE	- Tris EDTA buffer
Tris-HCl	- Tris hydroxy chloride
UV	- Ultra Violet
µg	- Microgram
µl	- Microliter
µM	- Micromolar
%	- Percentage
°	- Degree

Chapter I

Introduction

CHAPTER-I

INTRODUCTION

Chickpea (*Cicer arietinum* L.) a self-pollinated diploid (2n=2x=16) is the one of the most important edible food legume in the world after common bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.) (FAO, 1994). Chickpea is grown in about 11.15 million hectares (FAOSTAT, 2005) across more than 40 countries, in tropical, subtropical and temperate regions of the world with an average productivity of 769.4 kg ha^{-1}. India is major producer contributing 67.2% of total chickpea production of the world, cultivated on 7.29 million hectares (FAOSTAT, 2005).

The genus *Cicer* belongs to the tribe Cieracea Alef. (Kupicha, 1981), comprises of 34 wild perennial, eight wild annual and one cultivated annual (*Cicer arietinum*) species (Van der Maesen, 1987). Chickpea belongs to the temperate or galegoid legume group (including *Melilotus, Trifolium, Medicago, Pisum, Vicia, Lotus, Cicer, Lens* and *Lathyrus*) of subfamily Papilionidae in the family Leguminosae. Chickpea originated in the Fertile Crescent region of Southern Turkey and adjoining Syria from its wild progenitor *Cicer reticulatum* (Van der Maesen, 1987). It is one of the first grain crops cultivated by man has been uncovered in Middle Eastern archeological sites dated to 8^{th} century B.C. (Zohary and Hopf, 2000). Chickpea includes two distinct types, "*Kabuli*" (white flower, large and cream colored seeds) and "*Desi*" (purple flower, small angular and dark seeds). *Kabuli* types have been grown traditionally in the Mediterranean basin and central Asia, while *Desi* types have been mainly produced in the Indian subcontinent, East Africa, Central Asia, and to a limited extent in the Mediterranean basin.

Chickpea is mainly used for human consumption and to a lesser extent as animal feed in developing countries. It is an important source of protein, particularly in vegetarian diets. The seeds of chickpea contain 20-30% protein, approx. 40% carbohydrates and 3-6% oil (Gil *et al.*, 1996). The mineral component is high in phosphorous (340 mg/100g), calcium (190 mg/100g), magnesium (140 mg/100g), iron (7 mg/100g) and zinc (3 mg/100g). Chickpea also contains higher amount of beneficial carotenoids such as β-carotene than in genetically engineered "Golden rice". Anti-nutritive components are nearly absent in chickpea (Williams and Singh 1987). Therefore, chickpea is considered a functional food or nutraceutical (Agharkar, 1991; McIntosh and Topping, 2000 and Charles *et al.*, 2002).

Despite its obvious nutritious value, global chickpea production has only increased by 25% over the last 25 years. Though the potential yield of chickpea is 5000 kg ha^{-1}, the average yield is very low due to abiotic and biotic constraints that limit the productivity. Pests, diseases and parasitic weeds account for the loss of nearly one fifth of global crop production. Chickpea is reported to be susceptible to over 50 pathogens in different parts of the world (Nene *et al.*, 1989). The important fungal diseases include fusarium wilt (*Fusarium oxysporum)*, Ascochyta blight (*Ascochyta rabiei),* leaf spot (*Alternaria* sp.), *rust (Uromyces ciceris-arientini),* gray mould (*Botrytis cinera*), powdery mildew *(Leviellula taurica)*, dry root rot *(Rhizoctonia bataticola),* foot rot (*Sclerotium rolfsii)* and wilt *(Verticillium albo-atrum*). Fusarium wilt and Ascochyta blight are serious diseases, which are of great economic importance causing significant yield losses. Among the insect pests pod borer *(Helicoverpa armigera)* (Smithson

et al., 1985) is serious pest besides cutworms *(Agrotis* sp.), armyworms *(Spodoptera exigua)*, groundnut aphid (*Aphis craccivora*), pea aphid (*Acyrthsosiphon pisum*), cowpea bean seed beetle (*Callosobruchus maculatus*), and adzuki bean seed beetle (*Callosobruchu. chinensis*) are also important. Among the abiotic factors drought is one of the important problems in areas where the crop is grown on residual moisture and eventually exposed to terminal drought (Johansen *et al.*, 1994). Cold stress in West Asia and North Africa (Singh, 1987) and heat and salinity stresses are also known to affect the crop (Singh *et al.*, 1994).

Progress in initial linkage studies in cultivated chickpea (*C. arietinum* L.) has been slow due to low genetic polymorphism as assessed by seed storage and protein electrophoresis (Ahmed and Slinkard, 1992), RFLP markers (Udupa *et al.*, 1993), isozymes (Labdi *et al.*, 1996). Many researchers developed and used interspecific crosses between *C. reticulatum* and *C .arietinum* for linkage analyses. DNA based markers like RAPDs and RFLPs were used for constructing the first genetic linkage map (Simon and Muehlbauer, 1997), which had low marker density. SSR markers have been used widely for developing linkage maps, because of polymorphic nature, PCR – based assay and ready portability within species. In chickpea, SSR markers have been utilized to construct both inter and intra specific linkage maps (Flandez- Galvez *et al.*, 2003a; Tekeoglu *et al.*, 2002 and Winter *et al.*, 1999) and to map genes for disease resistance and other genes of agronomic interest (Cho *et al.*, 2002, 2004; Rajesh *et al.*, 2002, 2004 ; Udupa and Baum 2003 and Winter *et al.*, 2000).

Chickpea has a moderate size genome of 750 Mbp, which is slightly larger than that of a model legume *Medicago truncatula* (530 Mbp) (Arumuganathan and Earle, 1991). An intraspecific map saturated with second generation co-dominant markers (SSR) and gene based markers (ESTs and RGAs), is essentially required, which would be quite useful to map genes conferring complex traits like disease resistance or drought tolerance.

Conventional breeding strategies in chickpea have concentrated mainly on enhancing host plant resistance to biotic and abiotic stresses. The progress has been slow in many cases as often the selection based on phenotypic expression of the trait is either difficult or unreliable. The identification of molecular markers closely linked to resistance genes is of great benefit for resistance breeding, as it allows selection based on marker genotype rather than resistance phenotype. Markers- assisted selection (MAS) for resistance genes (R) can improve the efficiency and accelerate the progress of resistance breeding. Marker-Assisted Breeding combines both classical plant breeding and advanced molecular biology techniques. Moreover, is devoid of much debated environmental risks and thereby does not require time consuming regulatory checks as with transgenics. Using MAB plant biotechnologist can pyramid or incorporate more than one resistance gene and thereby impart durable resistance to pests and diseases in crop plants.

Ascochyta blight (AB) caused by *Ascochyta rabiei* (Pass.) Labr., is one of the important biotic constraints for chickpea production and causes significant loss of grain yield and quality (Gaur and Singh, 1996). Cool and wet weather conditions favour the disease development and often result in 100 % yield loss

(Reddy *et al.*, 1990; Singh *et al.*, 1992 and Singh and Reddy, 1993). The disease spreads by airborne spores and also by infected seeds, fungicide treatments to control the disease is often impractical and uneconomical (Reddy *et al.*, 1990). Consequently, breeding efforts have been focused on the development of resistant germplasm, using the host plant resistance. Developing chickpea varieties with high level of resistance to AB has been challenging because of i) non-availability of high level of resistance in the germplasm, ii) conditioning of resistance by several quantitative trait loci (QTLs), iii) considerable variability in pathogen and iv) due to emergence of new pathotypes with greater virulence-possibly due to natural recombination through the sexual cycle. Molecular markers linked to major QTLs of AB resistance can greatly facilitate marker- assisted selection (MAS) of resistance QTLs and significantly reduce the time required in development of a resistant variety. However, successful use of MAS requires tightly linked markers to QTLs of interest and their validation across populations and environments. Considering above facts the present study was taken up with the following objectives:

1. Development of an intraspecific mapping population for resistance to Ascochyta blight.
2. Generation of genetic linkage map of chickpea, using SSR, ESTs and RGA markers.
3. Molecular mapping of QTLs for Ascochyta blight resistance and identification of linked flanking markers suitable for MAS.

Chapter II

Review of Literature

CHAPTER-II

REVIEW OF LITERATURE

About 67 fungi, 3 bacteria, 22 viruses and 80 nematodes have been reported to cause diseases on chickpea (Nene *et al.*, 1996). Among these Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labrousse., is the most important foliar disease globally. It was reported for the first time from Punjab province of British India, now a part of Pakistan, where the disease was first observed in 1911 (Butler, 1918). Since then it has been reported from at least 35 countries (Nene *et al.*, 1996) and is a serious disease in many chickpea growing regions of the world. The disease is both externally and internally seed borne and also spreads by infected crop debris and airborne spores. It can occur at any growth stage and infects all the aerial parts of the plant. Disease development is favored by cool and humid weather. The typical symptom of Ascochyta blight is brown lesions at the stem base of emerged seedlings. These lesions enlarge in size and girdle the stem, which may eventually cause death of the plant. Sometimes 100 percent yield losses have been reported due to severe blighting (Nene, 1984). Severe crop losses and epidemics of the disease have been reported by several workers (Benlloch, 1941; Biggs, 1944; Kaiser, 1972; Kausar, 1965; Nene 1984; Radulescu *et al.*, 1971 and Zalpoor, 1963 and recently, Pande *et al.* (2005) gave a detailed review of this disease.

The study on **"Molecular mapping of Ascochyta blight resistance in chickpea (*Cicer arietinum* L.)"** involved developing an intraspecific linkage

map, identification of the genomic regions influencing the AB disease resistance and validation of the reported QTL markers.

2.1 Progress in development of linkage maps

2.1.1 Molecular markers for tagging and mapping of disease resistance genes in chickpea

In the past, genetic maps were based mainly on morphological and isozyme markers. But these markers are limited in number and are influenced by environment and developmental stage. Molecular markers on the other hand are large in numbers, not influenced by environment and facilitate in rapid selection of characters. Variations in the DNA sequences have been extensively studied as genetic markers for gene tagging and genome mapping in the last two decades. Several types of molecular markers have been developed and used in plants for tagging and mapping of pest and disease resistance genes.

2.1.1.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.*, 1980) are differences in the lengths of DNA fragments following digestion with sequence specific restriction endonucleases. As restriction enzymes cut DNA at specific sequences, a point mutation within the site can result in the loss or gain of recognition site, giving rise to restriction fragments of different lengths. Mutations caused by insertion, deletion, or inversion of DNA stretches can lead to length variation of DNA fragments. Restriction fragments of different lengths between genotypes can be detected on southern blots after hybridizing with a suitable labeled probe (single copy genomic or cDNA clone). RFLP markers have been used for genetic diversity studies (Udupa *et al.*, 1993) and genetic

mapping (Simon and Muehlbauer, 1997) in chickpea. But the major limitations with these markers are, need for large quantities of DNA for assay, use of radioactive labeling, laborious, relatively expensive and hazardous techniques for detection. Microsatellite-based RFLPs were used for genetic diversity studies in chickpea (Sharma *et al.*, 1995 ; Serret *et al.*, 1997 and Weising *et al.*, 1992).

2.1.1.2 Random Amplified Polymorphic DNAs (RAPDs)

This technique originally developed by Williams *et al.*, (1990) uses arbitrary decamer sequences as primers for DNA amplification. These markers are dominant because the polymorphism is due to presence or absence of a particular amplified fragment. One major advantage of these markers is that this does not need any prior sequence information. These markers have been used for phylogenic analysis of genus *Cicer* (Iruela *et al.*, 2002) as well as for genetic mapping (Cho *et al.*, 2002 ; Collard *et al.*, 2003; Santra *et al.*, 2000 and Simon and Muehlbauer 1997).

2.1.1.3 Inter-Simple Sequence Repeat (ISSR)

Inter-Simple Sequence Repeat (ISSRs) markers are detected by using anchored primers that amplify regions between simple sequence repeats. The primers are 16 –17 mer because of which, they show greater repeatability and stability of map positions in the genome. Polymorphisms are mostly of the dominant type because of changes in the anchoring nucleotides, but co-dominant types occur if length of the intervening space between the microsatellites has changed. These markers were used in many linkage maps developed in chickpea (Cho *et al.* 2002; Collard *et al.*, 2003; Fandez-Galvez *et al.*, 2003a; Ratnaparke *et al.*, 1998a and Santra *et al.*, 2000).

2.1.1.4 DNA Amplification Fingerprinting (DAF)

DNA Amplification Fingerprinting (DAF) markers employ a nucleic acid amplification technique that uses at least one primer of at least 5 nucleotides (nt) in length to produce characteristic and highly informative DNA patterns (Caetano-Anollés *et al.*, 1991). DAF can be distinguished from other genome scanning techniques by the high primer-to-template ratios, simplicity, excellent reproducibility and high multiplex ratios. Winter *et al.* (2000) and Rakshit *et al.* (2003) used these markers in their linkage analysis in chickpea.

2.1.1.5 Simple Sequence Repeats (SSR)/ Sequence Tagged Microsatellite Site (STMS) Markers

SSR or micro satellites are short tandem repeats dispersed throughout the genome. These are generally di-to-tetra- nucleotide repeats and are hyper variable. Micro satellites are flanked with unique sequences, which are highly conserved. These flanking unique sequences are analyzed and their complementary primers are synthesized. These can thus be assayed with PCR and act as co-dominant markers. Referred to as Simple Sequence Length Polymorphism (SSLP), allelic differences are usually as a result of variable number of repeat units. Though they are highly polymorphic, major limitation is the cost involved in its development. However, these are excellent markers system for developing linkage maps and have been extensively used by several researchers in chickpea (Cho *et al.*, 2002; Cho *et al.*, 2004; Collard *et al.*, 2003; Flandez-Galvez *et al.*, 2003a; Sant *et al.*, 1999 and Winter *et al.*, 1999, 2000.)

2.1.1.6 Sequence Characterized Amplified Regions (SCARs)

These markers overcome the limitations of RAPDs. In this the RAPD fragments that are linked to gene of interest are cloned and end sequenced. Based

on the terminal sequences longer primers (20 mers) are designed. These SCAR primers lead to a more specific amplification of a particular locus and are similar to STMS markers in construction and application. However, they can be converted to co-dominant markers in certain cases by digesting the amplified fragments with tetra cutting restriction enzymes. SCAR markers have been developed for a fusarium wilt resistance locus (Benko-Iseppon *et al.*, 2003) and an Ascochyta blight resistance locus (Iruela *et al.*, 2004 and Strange *et al.*, 2004).

2.1.1.7 Amplified Fragment Length Polymorphism (AFLP)

The technique was developed by Vos *et al.* (1995). In this technique, restriction fragments generated by a frequent (4 base) and a rare (6 base) cutter are anchored with oligo-nucleotide adapters of a few bases. This method generates a large number of restriction fragments facilitating the detection of polymorphism. The number of DNA fragments, which are amplified, can be controlled by choosing different base numbers and composition of nucleotides in adapters. This technique is more reliable since stringent reaction conditions are used for primer annealing and show an ingenious combination of RFLP and PCR techniques. In chickpea, Winter *et al.* (2000) used these markers in linkage map construction.

2.1.1.8 Resistance Gene Analogs (RGAs)

Resistance Gene Analogs (RGAs) or candidate resistance genes isolated by PCR amplification with degenerate oligonuleotide primers derived from conserved amino acid motifs in the Nucleotide Binding Sites (Kanazin *et al.*, 1996 and Shen *et al.*, 1998). This approach was used by Hüttel *et al.* (2002) in an effort to directly clone R-genes against *F. oxysporum* and *A. rabiei*. A series of

RGAs have been identified from both *C. arietinum* and *C. reticulatum* using two degenerate primer pairs targeting sequences in the NBS domain. Thirty of these RGAs were mapped on the reference genetic map of chickpea (Winter *et al.*, 2000). Rajesh *et al.* (2002) mapped for the first time a RGA (ptokin-2/7) to linkage group 5 (LG5) of Santra *et al.* (2000) using $F_{7\,8}$ RILs of the same cross segregating for Ascochyta blight resistance. Flandez-Galvez *et al.* (2003b) mapped 12 RGA markers, which clustered on three LGs.

2.1.1.9 Expressed Sequence Tags (EST)

These markers are developed by end sequencing of random cDNA clones. Most of these markers could be functional genes. A total of 668 ESTs are available in the Gene bank (http://www.ncbi.nlm.gov/ dbEST/ dbEST_ summary. html.) for chickpea as by May 2005. 2,860 chickpea EST sequences from substracted root library were developed at ICRISAT during 2002 (http://www.icrisat.org/gct/cpest/ home.asp).

2.1.1.10 Cleaved Amplified Polymorphisms (CAPs)

When most of the DNA markers results in monomorphic banding patterns between closely related individuals, the amplified PCR products are cleaved with restriction enzymes (often with 4-nt recognition sequence) to generate polymorphism. The markers so generated are referred to as Cleaved Amplified Polymorphisms (CAPs) markers. The RGAs can be converted to single copy PCR markers like CAPs (Konieczny and Ausubel, 1993). The CAPs were generated for RGA markers and used for genetic mapping of sugarcane mosaic virus resistance in maize (Quint *et al.*, 2002). Recently Rajesh, and Muehlbauer,

(2005) reported generation of six CAPs and dCAPs marker and fine mapping of QTL for Ascochyta blight resistance.

2.1.1.11 Single Nucleotide Polymorphism (SNP) Markers

SNPs are new generation markers that are amenable to automation and high throughput approaches. They are the most abundant of all marker systems known so far in both animals and plants. Large numbers of SNPs were developed in higher plants and are being used for SNP genotyping. SNP arises due to difference in a single nucleotide and practically they are biallelic in nature. However, the extraordinary abundance of SNP largely offsets the disadvantage of their being biallelic. According to a recent estimate, one SNP occurs every 100-300 bp in any genome. In chickpea SNPs marker development has been initiated.

The list of SSR, RGA, EST and SNP, markers reported in chickpea are given in Table 2.1.

2.1.2 Linkage studies in chickpea

Chickpea is a self-pollinated diploid (2n=2x=16) annual with a moderately sized genome of around 750 Mbp (Arumuganathan and Earle, 1991) that evolved from wild progenitor *C. reticulatum* (Ladizinsky and Alder, 1976). The cultivated chickpea (*C. arietinum* L.) was reported to have low genetic polymorphism based on seed storage protein electrophoresis (Ahmed and Slinkard, 1992), RFLP markers (Udupa *et al.*, 1993) and isozymes (Labdi *et al.*, 1996). This prompted many researchers to develop interspecific crosses between *C. reticulatum* and *C. arietinum* for linkage analysis studies. Availability of a large number of polymorphic markers is a prerequisite for taking up of genetic diversity studies or linkage analysis. DNA based markers like RAPDs (Iruela *et*

al., 2002), ISSRs (Ratnaparke *et al.*, 1998 a, b and Iruela *et al.*, 2002), microsatellite-based RFLPs (Sharma *et al.*, 1995; Serret *et al.*, 1997 and Weising *et al.*, 1992) and STMSs (Sant *et al.*, 1999; Udupa *et al.*, 1999 and Winter *et al.*, 1999, 2000) that have revealed polymorphism were used for linkage analysis. A review of the interspecific and intraspecific linkage maps constructed so far is given in Table 2.2.

Table 2.1 Overview of SSR, EST, SNP, and RGA markers reported in chickpea

Marker	Summary of the marker information	Reference
SSR	218, SSR primers designed from 389 microsatellite containing clones.	Winter *et al.*, 1999
	43 of the 53 clones from chickpea genomic libraries selected for sequencing showed the presence of microsatellites.	Huttel *et al.*, 1999
	10 SSR from genomic library of *C. arietinum* cultivar Pusa 362.	Sethy *et al.*, 2003
	233 SSR markers from BAC & BIBAC library of *C. arietinum* L. cv Hadas.	Lichtenzveig *et al.*, 2005
RGA	A series of RGAs from both *C. arietinum* and C. *reticulatum* using two degenerate primer pairs targeting sequences in the NBS domain. A total of 48 different RGAs which were members of the Toll-Interleukin Receptor (TIR)-NBS-LRR and Coiled-Coil (CC)-NBS-LRR groups designed.	Hüttel *et al.*, 2002
EST	2,858 EST sequences from substracted root library available at ICRISAT EST Database.	ICRISAT 2002 (http://www.icrisat.org/gct/cpest/home.asp)
	668 ESTs are available in the Genebank.	(http://www.ncbi.nlm.gov/dbEST/dbEST_summary.html.)
SNP	4 SNPs detected in four different loci viz, beta amylase, expansin, histone H2A and transketolase.	Buchwaldt *et al.*, 2004

2.1.2.1 Interspecific linkage maps

Interspecific populations have been used for linkage analysis of various morphological, isozyme and DNA based markers. For the first time using F_2 interspecific populations of *C. arietinum* x *C. reticulatum* and *C. arietinum* x *C. echinospermum*, 3 morphological and 26 isozyme markers were mapped on skeleton linkage map of 7 linkage groups (200 cM) (Gaur and Slinkard, 1990a.) Similarly, Kazan *et al.* (1993) developed an interspecific linkage map with 5 morphological and 23 isozyme loci distributed on 8 linkage groups covering a total map length of 257 cM.

With availability of DNA based markers Simon and Muehlbauer (1997) developed a linkage map from 9 morphological, 27 isozyme, 10 RFLP and 45 RAPD markers covering a total map length of 550 cM with 10 linkage groups.

The development of STMS markers is an important landmark in progress of chickpea linkage mapping. Winter *et al.* (1999) generated 174 STMS markers out of which 120 markers were genetically mapped on 90 recombinant inbred lines from an interspecific cross of *C. reticulatum* x *C. arietinum* distributed on 11 linkage groups covering 613 cM. An integrated molecular map of chickpea was developed using 130 F_6 derived RILs of the previously used interspecific cross of *C. arietinum* x *C. reticulatum* (Winter *et al.*, 2000). A total of 303 markers including 118 STMS, 96 DAFs, 70 AFLPs, 37 ISSRs, 17 RAPDs, 8 isozymes, 3 cDNAs, two SCARs and three loci that confer resistance to fusarium wilt, were mapped. At LOD score of 4.0, 303 markers covered 2077.9 cM map distance and distributed over eight large and eight small linkage groups.

Table 2.2 Overview of genetic linkage maps generated from inter and intraspecific crosses in chickpea

S. No.	Population	Summary of the genetic linkage map	Reference
1	F_2 intraspecific (*C. reticulatum)* F_2 interspecific (*C. arietinum* x *C .reticulatum* and C. *arietinum* x C. *echinospermum*)	The map consists of 29 markers (3 morphological and 26 isozymes) and covers 200 cM in 7 linkage groups	Gaur and Slinkard, 1990a, 1990b
2	F_2 intraspecific F_2 interspecific (*C. arietinum x C. reticulatum* and C. *arietinum x C. echinospermum*)	The map consists of 28 markers (5 morphological and 23 isozymes) and covers 257 cM in 8 linkage groups	Kazan *et al.*, 1993
3	3 F_2 populations	The map consists of 91 markers (9 morphological + 27 isozyme + 10 RFLP + 45 RAPD) in 10 linkage groups with a total distance of 550 cM, and average marker density of 6.04 cM.	Simon and Muehlbauer, 1997
4	90 RILs from (ICC 4958 *C. arietinum* x PI 489777 *C. reticulatum*)	The map consists of 120 markers grouped into 11 linkage groups with a total map length of 613 cM and an average distance of 5.47 cM.	Winter *et al.*, 1999
5	130 RILs from *C. arietinum* (ICC4958) x *C. reticulatum*) (PI489777)	The map consists of 303 markers covering 7.9 cM in 8 large and 8 small linkage groups with an average distance of 6.8 cM. A clustering of markers observed in central regions of linkage groups. The map includes 3 loci contributing to Fusarium resistance. 354 markers (118 STMS, 96 DAF, 70 AFLP, 37 ISSR, 17 RAPD, 8 isozyme, 3 cDNA, 2 SCAR).	Winter *et al.*, 2000
6	142 RILs from *C. arietinum* (FLIP84-92C) x *C. reticulatum* (PI 599072)	The map consists of 116 markers grouped into 9 linkage groups with a total map length of 981.6 cM and average marker density of 8.4 cM. 144 markers (1 morphological + 11 isozyme + 111 RAPD + 21 ISSR)	Santra *et al.*, 2000
7	RILs, from (C. *arietinum x C. reticulatum*)	The map consists of 23 linkage groups with RAPD, ISSR and morphological markers.	Hajj-Moussa, 2001

Table 2.2 (*cont....*)

S. No.	Population	Summary of the genetic linkage map	Reference
8	142 RILs from *C. arietinum* (FLIP 84-92C) x *C .reticulatum* (PI 599072)	The map consists of 167 markers and covers 1174.5 cM with 9 linkage groups, with an average marker distance of 7.0 cM. 51 markers (one RGA and 50 STMS)	Tekeoglu *et al.*, 2002
9	142 RILs from *C. arietinum* (FLIP 84-92C) x *C.reticulatum* (PI 599072)	Addition of RGA Potkin 1-2 n171 to linkage group 5 of Santra *et al.*, 2000.	Rajesh *et al.*, 2002
10	F_2 intraspecific	The map consists of 66 markers and covers 535 cM in 8 linkage groups	Flandez-Galvez *et al.*, 2003a.
11	RILs intraspecific (ICCV2) x (JG-62	The map consists of 111 markers and covers 297 cM in 14 linkage groups 68 STMS, 34 RAPD, 4 ISSR and 5 morphological markers.	Cho *et al.*, 2002
12	159 RILs from *C. arietinum* (ICC 4958) x *C .reticulatum(* PI 489777)	The map consists of 296 markers and covers 2483.3 cM in 8 large and 4 small linkage groups. The gene-specific markers derived from sequences of protein known to be involved in plant defense responses are distributed on linkage groups 3-5. 47 gene specific markers are integrated into an existing map based on SSR, AFLP, DAF, and other anonymous markers (Winter et al., 1999, 2000)	Pfaff and Kahl, 2003
13	F_2 interspecific *(C. arietinum x C. reticulatum)*	The map consists of 83 markers and covers 570 cM in 8 linkage groups 14 STMS, 54 RAPD, 9 ISSR, 6 RGA	Collard *et al.*, 2003
14	F6:7 RIL population of ILC 12272 x ILC 3279	The map consists of 52 marker loci and covers 419 cM in 8 linkage groups with an interval of 7.4 cM	Udupa and Baum, 2003
15	113 F2 RILs of a intraspecific cross PI359075 x FLIP 84-92C	The map consists of 53 marker loci and covers 318.2 cM in 8 linkage groups composed of 11 sub groups	Cho *et al.*, 2004

Rajesh *et al.* (2002) mapped for the first time a RGA (ptokin-2/7) to linkage group 5 of Santra *et al.* (2000) by using same $F_{7\,8}$ RILs mapping population as used Santra *et al.* (2000). Tekeoglu *et al.* (2002) integrated 50 STMS and 1 RGA markers extending the chickpea genome map of Santra *et al.* (2000) to 1175 cM with an average distance of 7.0 cM distributed on nine linkage groups. Further using common STMS markers as anchors 3 maps developed from 3 different mapping populations were joined and genes for Ascochyta blight resistance, fusarium wilt resistance and agronomically important traits were located on combined linkage map.

An interspecific linkage map was constructed using an F_2 population from *C. arietinum* (Lasseter) x *C .echinospermum* (PI 527930) comprising of 8 linkage groups and covering a map distance of 570 cM. This map incorporated 83 molecular markers (14 STMS, 54 RAPD, 9 ISSR and 6 RGA) (Flandez- Galvez *et al.*, 2003a).

2.1.2.2 Intraspecific linkage maps

Genetic map constructed from an interspecific cross may not represent the true recombination distance (cM) and map order of the cultivated genome. Due to uneven recombination of homeologous chromosomes during meiosis, DNA markers for linkage analysis would have a high degree of segregation distortion resulting in biased estimation of the marker distance (Flandez-Galvez *et al.*, 2003a). Interspecific maps suffer from another disadvantage that the polymorphic loci identified may be monomorphic between the closely related genotypes and thus have little direct application in intraspecific breeding programs. Because of these limitations construction of intraspecific linkage maps has gained momentum recently.

An intraspecific linkage map spanning 543.5 cM with an average interval of 8.1 cM was constructed using an F_2 population of cross ICC12004 x Lasseter. Fifty-one STMS, 3 ISSR and 12 RGA loci were mapped into eight linkage groups (Flandez-Galvez *et al.*, 2003a).

Cho *et al.*, (2002) constructed an intraspecific linkage map using 76 F_{10} derived RILs from ICCV2 x JG-62. The linkage map covered a distance of 297.5 cM comprising 14 linkage groups that consisted of 55 STMS, 20 RAPDs, 3 ISSRs and two morphological markers.

Udupa and Baum (2003) constructed an intraspecific genetic linkage map from a mapping population from $F_{6:7}$ RILs of cross between ILC1272 x ILC3279. 52 STMS marker loci were distributed into 8 linkage groups covering a total map length of 419 cM with an interval of 7.4 cM between two loci.

Cho *et al.* (2004) constructed a genetic linkage map using RILs from an intraspecific cross of PI359075 (I) x FLIP 84-92c. Fifty-three STMS marker loci were mapped to eight linkage groups composed of 11 subgroups covering 318.2 cM of chickpea genome.

2.2 Ascochyta blight in chickpea and mapping AB resistance QTLs

2.2.1 Pathogen *Ascochyta rabiei* (Pass.) Labr of chickpea

Ascochyta rabiei (Pass.) Labr., the causal agent of blight was first named *Zythea rabiei* by Passerini on the basis of its unicellular pycnidiospores. Labrousse (1931) suggested the name *Ascochyta rabiei* because of its ability to produce 2-4% single septate spores, which is now accepted by majority of Pathologists and Commonwealth Mycological Institute. Kovachevski (1936) observed the sexual stage of fungus *Mycosphaerella rabiei* (Syn. *Didymella*

rabiei (Kovachevski) Ax) on an over wintered straw in Bulgaria. In a detailed study, Trapero–Casas and Kaiser (1992) clearly proved the relationship between the perfect state and imperfect state of *A. rabiei* under both field and laboratory conditions and confirmed the identity of the perfect state as *Didymella rabiei.* The perfect state has been found in a number of other countries (Nene, 1982 and Nene and Reddy, 1987).

The anamorph (asexual) *Ascochyta rabiei*, is characterized by the formation of spherical or pear shaped black fruiting bodies called pycnidia. The pycnidium contains numerous hyaline unicellular and occasionally bicellular spores, pycnidiospores or conidia developed on short conidiophores (stalks) embedded in a mucilaginous mass. Pycnidiospores are oval to oblong, straight or slightly bent at one or both ends and measures 6-12 x 4-6 mm (Nene, 1982).

The telomorph, *Didymella rabiei* (Kovacheski) v.avr is a bipolar heterothallic ascomycete and requires the pairing of two compatible mating types (MAT-1 and MAT-2) for successful sexual reproduction. The two mating types are widely distributed in several major chickpea growing areas of the world (Armstrong *et al.,* 2001). The telomorph is characterized by perithecia occurring on the crop residue of chickpea that had over wintered in the field. The perithecia appear dark brown to black, globose or applanate, with perceptible beak and ostioles and vary in size from 76-152 mm x 120-250 mm. Cylindrical to clavate, curved and pedicellate asci measuring from 48-85 mm x 8-22 mm develop inside the perithecia (Armstrong *et al.,* 2001). Eight ascospores measuring 12-22 mm x 5–6 mm form in each asci. Ascospores are usually monotrichous and rarely distichous, ovoid, constricted at the septum and divided into two very unequal cells (Haware, 1987).

2.2.2 Molecular analysis of *Ascochyta rabiei* (Pass.) Labr.

Genetic diversity analysis in plant pathogen populations is necessary to understand co-evolution in plant pathosystems (McDonald *et al.*, 1989). However, evaluating genetic diversity in the field requires a set of highly discriminating, selectively neutral and reliable criteria for genotype analysis. Pathogen variability of *Ascochyta rabiei* has been demonstrated by many authors and occur in several regions as in North Africa, Middle East, India and USA (Gowen *et al.*, 1989; Kaiser, 1973 and Mmbaga, 1997). *A. rabiei* is known for variation in its morphology (Grewal, 1984), pathogenicity (Gowen *et al.*, 1989 and Porta-Puglia, 1996) and phytotoxin production (Alam *et al.*, 1989 and Hohl *et al.*, 1990). According to differential set used, Vir and Grewal (1974a) found 10 pathotypes among field isolates from India. Six races (pathotypes) were identified among 50 isolates from Syria (Reddy and Kabbabeh, 1985). Therefore, any identification based on these characters is difficult and suffers from several disadvantages. Biological pathotyping is time-consuming and labor-intensive and its reproducibility is often poor.

In recent years, DNA polymorphisms have increasingly been used to complement traditional markers in the analysis of genetic identity, variability and relatedness in fungi. A high level of genetic variation in *A. rabiei* population has been noted when utilizing both DNA markers and morphological characters. For example, extensive genetic diversity within *A. rabiei* from Tunisia based on molecular techniques such as RFLP and RAPD have been shown to be reliable tools for characterization of *Ascochyta rabiei* populations (Morjane *et al.*, 1994 and Weizing *et al.*, 1991). Using a microsatellite sequence $(GATA)_4$ as a probe,

diagnosed *A. rabiei* pathotypes found in Syria (Hamza *et al.*, 2000). Similar results were found in Dutch (Klein-Bolting, 1992) and Italian isolates (Fischer *et al.*, 1995) using RAPD markers. Significant genetic variation within *A. rabiei* isolates of Indian origin based on morphological and cultural variation has also been observed (Singh, 1990 and Ambardar and Singh, 1996). Comparative studies of virulence cluster analysis and RFLP analysis revealed that DNA polymorphism is independent of virulence. Similarly Chongo *et al.* (2004), indicated weak association between RAPD and pathotype groups.

A DNA marker ($ubc756_{1.6\ kb}$) specific to Indian isolates was identified by Santra *et al.* (2001). In another study, Taylor *et al.* (2002) reported a very small amount of molecular variation using (STMS) markers, among *A. rabiei* isolates collected from throughout Australia over several years. Study of genetic diversity of *Ascochyta rabiei* in Canada was based on virulence tests and RAPD markers (Chongo *et al.*, 2004). Canadian isolates were grouped into 14 pathotypes using eight chickpea differentials. RAPD analysis of 39 Canadian isolates and 20 from different countries revealed considerable genetic diversity. The levels of DNA variability and virulence among isolates showed that the population of *A. rabiei* in Canada was highly diverse.

In many chickpea growing regions several patho- and genotypes of the fungus may coexist in the same field or even in the same lesion (Jamil *et al.*, 2000; Morjane *et al.*, 1994 and Peever *et al.*, 2004). Since random mating may occur between different pathotypes of the fungus carrying different mating type alleles (Barve *et al.*, 2003), genetic recombination may contribute to genotypic diversity and provide the fungus with an additional means to adapt to newly introduced resistant germplasm (Peever *et al.*, 2004).

2.2.3 Disease screening

Different methods were applied for assessment of disease severity. Testing under controlled glasshouse or growth chamber conditions (Millán *et al.*, 2003; Singh *et al.*, 1992 and Udupa and Baum, 2003) combined with field screening (Cho *et al.*, 2004) would very much help to improve the reproducibility of the results, since severity and spread of the disease are highly dependent on environmental conditions, especially on humidity (which may change from year to year). Indeed, Cho *et al.* (2004) observed dramatic increases in severity of blight symptoms, if 100 % relative humidity was maintained for more than two days after inoculation, as compared to normal greenhouse conditions. Further, different loci may contribute to resistance at different points of the life cycle of the plant (Collard *et al.*, 2003).

2.2.4 Host plant resistance

Host plant resistance is most effective, economical and environmentally sound means of controlling the disease. Progress in breeding blight resistant cultivars has been slow because of absence of durable source of resistance. In view of both importance of Ascochyta blight resistant cultivars in stabilizing chickpea production and frequent breakdown of resistant sources identified, a large-scale evaluation of world germplasm collection maintained in gene banks at ICRISAT and ICARDA was undertaken. A total of 19342 germplasm accessions of chickpea (12749 desi and 6594 kabuli types) were evaluated for resistance to six races of *Ascochyta rabiei* during 1979 to 1991. Only three desi (ICC4475, ICC6328 and ICC12004) and two kabuli (ILC200 and ILC6482) accessions were resistant in both field and greenhouse evaluations. Another 6 desi accessions and

3 kabuli accessions were resistant in repeated field tests but tolerant in green house evaluations (Singh and Reddy, 1993).

In another initiative to identify sources of resistance to the 6 races of AB reported from Lebanon and Syria, 1069 germplasm accessions and breeding lines were screened against the 6 races during 1985-86. Of total lines, 47, 27 29, 8, 13 and 4 (ILC2056, ILC2956, ILC3856 and ILC5928) were resistant to races 1, 2, 3, 4, 5 and 6, respectively (Singh, 1990).

The evaluation of the world collection of chickpea germplasm resulted in identification of lines with high and broad based resistance (Singh and Reddy, 1996). Further the resistant sources have been utilized in comprehensive breeding programs to develop around hundred resistant varieties, for commercial production. But Ascochyta blight continues to be a major biotic constraint limiting the productivity of chickpea globally. An insight into the genetics and inheritance of resistance and pathogen diversity is required to improve the breeding efforts to produce cultivars with durable resistance.

2.2.5 Inheritance of resistance to ascochyta blight in chickpea

Resistance to Ascochyta blight is partial or incomplete and the existence of immunity has not been confirmed (Lichtenzveig *et al.*, 2002). Inheritance of Ascochyta blight resistance seems to be complex and does not fit a simple gene-for-gene interaction. Depending upon the resistance source, fungal isolate and screening method used, monogenic, oligogenic and quantitative inheritance of Ascochyta blight resistance has been reported (Table 2.3).

The initial studies on inheritance of Ascochyta blight resistance identified a single dominant gene (Acikgoz and Demir, 1984; Eser, 1976; Hafiz and Ashraf,

1953; Singh and Reddy, 1983; Tewari and Pandey, 1986 and Vir *et al.*, 1975) or a single recessive gene (Acikgoz and Demir, 1984; Singh and Reddy, 1983 and Tewari and Pandey, 1986.) for resistance. Several studies later identified oligogenic inheritance, e.g., two dominant complementary (Singh *et al.*, 1992) and two recessive complementary genes (Nene and Sheila, 1992), two recessive genes with additive gene action (Kusmenglo, 1990), two dominant complementary genes with interallelic interaction (Dey and Singh, 1993) and three recessive and complementary major genes with several modifiers (Tekeoglu *et al.*, 2000).

Subsequent studies reported that ascochyta blight is quantitatively inherited. Most of these studies used recombinant inbred lines (RILs) for study of inheritance and molecular mapping of Ascochyta blight resistance (Cho *et al.*, 2004; Flandez-Galvez *et al.*, 2003b; Santra *et al.*, 2000; Tekeoglu *et al.*, 2004 and Udupa and Baum, 2003).

The segregating RIL populations have been extensively used for mapping Ascochyta blight resistance genes/QTLs. Inheritance of Ascochyta blight resistance was studied in three RIL mapping populations (two intraspecific and one interspecific) for two years in the same location at Pullman (USA). It was reported that three recessive and complementary major genes with several modifiers conferred AB resistance. Absence of one of the two major genes resulted susceptibility, whereas the presence of modifiers determined the degree of resistance (Tekeoglu *et al.*, 2000).

Table 2.3 Inheritance of Ascochyta blight disease in Chickpea

Gene action	Reference	Year	Cultivars/Population
Monogenic			
Single dominant gene	Hafiz & Asraf	1953	F8, F10.
	Vir *et al*	1975	1-13
	Eser *et al*	1976	Code no 72-92
	Singh & Reddy	1983	ILC72, ILC183, ILC200, ICC4935
	Acikgoz	1984	ILC200, ILC201
	Tewari & Pandey	1986	P 1252-1, EC26446, PG 82-1
Single recessive gene	Singh & Reddy	1983	ILC 191
	Acikgoz	1984	72012, ILC195, NEC 138-1
	Tewari & Pandey	1986	BRG-8
Oligogenic			
Two recessive genes with additive gene action	Kusmenglo	1990	F_2 and F_3 families
Complementary dominant genes	Nene and Sheila	1992	-
	Dey and Singh	1994	GLG84.38, GLG84094
Complementary recessive genes	Singh *et al.*,	1992	-
One dominant and one recessive	Dey and Singh	1994	ICC19468
Three recessive and complementary major genes with several modifiers	Tekeoglu *et al.*,	2000	RIL of intraspecific PI359075 (11) XFLIP 84-82C, BlancoLechoso X Dwelley and Interspecific FLIP 84-92 (3) *(C. arietinum)* X PI 599072 (*C .reticulatum*)
Quantitative Inheritance			
Two major QTLs	Santra *et al.*,	2000	$F_{5:6}$ FLIP 84-92 (3) (*C. arietinum*) X PI 599072 *C. reticulatum*)
Seven QTLs (3 major QTLs and four minor QTLs)	Flandez-Galvez *et al.*,	2003a	F_2 intraspecific population of cross ICC12004 X Lasserter
Four QTLs (2 QTLs for seedling resistance and 2 for adult plant) *ar* 1(major locus against pathotypes I) and *ar2a* and *ara2b* (two independent recessive major loci with complementary)	Udupa and Baum	2003	F_2 Lasserter (*C .arietinum*) X PI 527930 (*C. echinospermum*) $F_{6:7}$ RILs population from an intraspecific of ILC1272 X ILC3279
Two major QTLs (same QTLs Santra *et al.*, 2000 were identified in different locations)	Tekeoglu *et al.*,	2004	$F_{5:6}$ FLIP 84-92 (3) (*C. arietinum*) X PI 599072 (*C. reticulatum*)
Five QTLs (two QTLs to pathotypes II and one QTLs for pathotypes I and putative single gene *Ar19* (or *Ar 21d*) against pathotypes I	Cho *et al.*,	2004	RILs from an intraspecific crossPI359075 x FLIP 84-92C

Santra *et al.* (2000) further studied the same interspecific RIL population used by Tekeoglu *et al.* (2002) and detected three Ascochyta blight resistance QTLs *viz.* QTL1, QTL2 and QTL3 with a LOD score of 17.23, 7.31 and 3.04, on linkage groups 6, 1 and 4 respectively. QTL-1 accounted for an estimated 42.5 % and 41.4% of variation in blight reaction in two consecutive years. The markers UBC733b and UBC181a flanking QTL1 were 10.9 cM apart on linkage group 6, whereas Dia4 and UBC836 flanking QTL2 were spaced on 5.9 cM apart on LG1. UBC681a and UBC 858b markers flanking QTL 3 were 11.7 cM apart on linkage group 4. QTL-I and QTL-2 together accounted for 50.3% and 45% of variation in two years of evaluation. Further these two loci were considered likely to coincide the two recessive genes reported by Kusmenoglu (1990) and Tekeoglu *et al.* (2000).

Flandez-Galvez *et al.* (2003b) reported 7 QTLs conditioning AB resistance. In their study, the first three QTLs (1, 2 and 3) identified from glass house and field trials corresponded with AB resistance QTLs mapped in *Cicer arietinum* x *Cicer reticulatum* populations by Santra *et al.* (2000). These are the major QTLs and have been detected across different population types, resistance sources and infection conditions. Four additional minor QTLs (4, 5, 6 and 7) were identified as having significant effect in the field under natural AB infection. All the RGA and ISSR markers were mapped in the QTL regions 1, 3, 4 and 6. CLRRinv and TA146 flanked the strongest QTL (QTL3) at an interval of 0.1 cM. The QTL 5 flanked by TS12 and TR56 (1.9 cM) and QTL 7 flanked by M44sp and TA28 (7.6 cM) were also reported in the same study. The flanking

STMS marker TA146 to QTL3 is a ready to use marker for gene pyramiding together with other tightly linked STMS markers for QTL 7, i.e. TS 12, TR56, M44sp and TA28 to breed chickpea cultivars with durable resistance to Ascochyta blight.

Rakshit *et al.* (2003) identified three DNA Amplification Fingerprinting Markers, OPSO6-1, OPS03-1 and OPKO6-5, linked to QTLs for Ascochyta blight resistance using the same mapping population and Ascochyta data set of Tekeoglu *et al.* (2000) and Santra *et al.* (2000). Using the RIL mapping population of Winter *et al.* (2000), these markers were localized on LG-4, and linked OPSO6-1 and OPS03-1 at a distance of 4.1 cM and 25.1 cM away from UBC733B on either side. While third marker OPKO6-5 was placed at 30cM away from UBC733b at the distal end of LG-4A. OPSO3-1 marker was also found tightly linked to STMS markers, STMS11 GA24 and GA47, which enabled to localize major Ascochyta blight resistance locus QTL1 (Santra *et al.*, 2000 and Tekeoglu *et al.*, 2000) on LG-4.

Mapping of RGAs facilitates localization of disease and pest resistance genes in plants. RGA marker RGAptokin1-2 $_{171}$ was mapped on LG5 of interspecific cross *C. arietinum* (FLIP 84-9c) x *C. reticulatum* (PI 599072) and by comparative mapping it was further positioned on LG-3 of integrated map of *Cicer* (Winter *et al.*, 2000). However, it could not be associated to blight resistance major QTLs (Rajesh et *al.*, 2002).

Tekeoglu *et al.* (2002) reported QTLs conferring resistance to Ascochyta blight on LG8 (QTL1) and LG4 (QTL2). Only one STMS marker was linked to QTL1 for blight resistance on LGVII (GAA47) and five STMS markers were mapped within QTL2 (TA72, TA2, TS45, TA146 and GA2).

A QTL was detected in a genomic region saturated with RAPD markers using ILC3279 as source of resistance in an intraspecific cross (Millán *et al.*, 2003). A SCAR marker tightly linked to this QTL have been developed (Iruela *et al.*, 2004 and Strange *et al.*, 2004), and STMS analysis revealed that this QTL could be the same as QTL-2 of Santra *et al.* (2000), since it was linked to the same markers TA72 and TA146.

Collard *et al.* (2003) used an interspecific F_2 population derived from a cross between a susceptible chickpea cultivar *C. arietinum* (Lasseter) and a resistant *C. echinospermum* (PI527930) accession to generate a preliminary linkage map of low density. The F_2 population was evaluated for seedling and stem resistance in glasshouse trials. Interval mapping and single-point analysis identified two QTLs for seedling resistance and two QTLs for adult plant resistance. Markers X LRR_{520}, STMS11, GA 2, UBC836, UBC 77c, Cs34a, Cs5c and TR 20 were in the vicinity of two QTLs for seedling resistance as well as one QTL for adult plant resistance co-localized on LG2. Markers CS44, CS39b and Cs54b flanked the other adult plant resistance QTL on LG 1.

The chickpea landrace ILC3279 has resistance to pathotypes I and II of Ascochyta blight pathogen. Using a set of intraspecific RILs derived from a cross between susceptible accession ILC1272 and resistant accession ILC 3279, microsatellite markers were identified for a major locus (ar1 mapped on LG 2), which confer resistance to pathotypes I, and two independent recessive loci (*ar2a* mapped on LG 2 and *ar2b* mapped on LG 4) with complementary gene action conferring resistance to pathotypes II. The markers are GA16 linked to ar1 and *ar2a* on LG 2 and TA130, TA72, TR20, TS72 and TS104 are linked to *ar2b* on

LG4 (Udupa and Baum, 2003). This was the first study to employ defined *A. rabiei* pathotypes (I and II) in a controlled greenhouse environment for scoring of disease symptoms.

Tekeoglu *et al.* (2004) using the RIL population of interspecific cross *C.arietinum* (FLIP 84-9c) x *C. reticulatum* (PI 599072) studied by Tekeoglu *et al.*, 2000 confirmed and validated the two QTLs previously identified at Pullman (USA) in another environment at Eskisehir (Turkey). This study proved that the makers associated with these QTLs could be used for marker-assisted selection as they were confirmed across environments.

Cho *et al.* (2004) screened intraspecific RIL population of cross PI35905 x FLIP 84-92 with single isolates (Ar19 and ar21d) of and also with a mixture of ten isolates of pathotypes I in field and glasshouse conditions. A total of five QTLs were detected on the genetic linkage map constructed with 53 STMS markers. Two QTLs for resistance to pathotype I (Ar19 and Ar21d) were co-located between linkage GA20 and GA16 on LG2A+6B, with LOD scores of 3.08 and 2.66, respectively. These two QTLs were postulated to be a single gene designated as *Ar19* (or *Ar21d*). Another QTL for resistance to pathotype I was identified on LG2B between TA37 and TA200 with a LOD score of 3.69. One QTL for blight resistance in the field was mapped on LG4 A between GA24 and GAA47 with LOD score of 4.17 co-located in the same region along with another QTL identified from a mixture of pathotypes II isolates in the growth chamber with a LOD score of 2.83.

A summary of QTLs identified for Ascochyta blight and the linked markers is given in Table 2.4.

Table 2.4 Molecular markers identified for Ascochyta blight resistance on QTLs in chickpea

Pathotype	Genetic locus/ QTL	Linkage group	Flanking markers identified	Reference
-	QTL 1 QTL 2 QTL 3	6 1 4	UBC 733b & UBC 181a UBC 836 b & Dia UBC 681a & UBC 858b	Santra *et al.* (2000)
-	QTL 1 QTL 2	8 4	GAA 47 TA 72s TA 2 TS 54 TA 146 and GA 2	Tekeoglu *et al.* (2002)
-	QTL 1 QTL 2 QTL 3 QTL 4 QTL 5 QTL 6 QTL 7	2 2 3 1 2 4 6	XL RRb & XLRRinv TS 12 &TR56 UBC 858 TA3a & TS 45 TA 146 & CLRRinv TA140 b & PTOFENa M44sp & TA 28	Flandez-Galvez *et al.* (2003b)
-	Seedling resistance QTL 1 QTL 2 Adult plant resistance QTL 1 QTL 2	 2 2 2 1	 $XLRR_{520}$ STMS11 GA 2 UBC836 UBC 77c Cs34a Cs5c Tr20 STMS11 GA 2 UBC836 UBC 77c CS44 CS39b Cs54b	Collard *et al.* (2003))
-		5	1-RGAPtokin1-2 $_{171}$	Rajesh *et al.* (2002)
-	Linked to QTLs reported by Santra *et al.*, 2000.		OPS 06-1 OPS 03 –1 OPK 06 5	Rakshit *et al.* (2003)
-	QTL 1	4	SC/OPK13$_{602}$ SC/OPM02 $_{935}$	Millan *et al.* (2003)
I II II	*ar1* *ar2a* *ar2b*	2 2 4	GA16 GA 16 TA130 TA72 TR20 TS72	Udupa and Baum (2003)
I I II	*Ar19 /Ar21d* QTL3 QTL 4 and 5	2B-6B 2 B 4A	GA20 GA 16 TA37 TA200 GA24 GAA47	Cho *et al.* (2004)

2.2.6 Biochemical basis of Ascochyta blight resistance

Initial studies of biochemical comparisons between resistant and susceptible cultivars showed a higher peroxidase and catalase activity and more L-cysteins and phenolic contents after inoculation of the resistant ones (Vir and Grewal, 1974a, b). Upon infection of various biotic agents (e.g. fungi and bacteria) several higher plants rapidly synthesize antibiotic compounds termed as phytoallexins (Ingham, 1972), which are believed to play a significant role in defense of higher plants against phyto pathogenic fungi). Koster *et al.* (1983) showed that in chickpea and other legumes isoflavons occur mainly as an isoflavone 7- O, glucoside, and 6 malanoate. Accumulation of such antifungal compounds appears to be an important trait of a resistant plant (Kue and Rush, 1985 and Tani and Mayama, 1982).

Weigand *et al.* (1986) reported a high level of phytoalexins, medicarpins and maackain in resistant plants. In chickpea strong accumulation of the pterocarpan phytoallexins, maackain and medicarpin were observed upon inoculation with spores of *Ascochyta rabiei* or when treated with different elicitors (Barz and Mackenbrock, 1994). Alam and Strange (1995) purified maackain, medicarpin and formononetin from germinating seeds of chickpea and Farhat *et al.*, (1996) identified these compounds in the stem of different chickpea cultivars against *Ascochyta rabiei*. Kunzuru *et al.* (1996) first recorded phytoallexin formation by chickpea in 1966, they showed that an antifungal compound cicerin was produced when spore suspension of *A. rabiei* were incubated in the seed cavity of detached pods.

2.2.7 Molecular basis of Ascochyta blight resistance

The functional genomic studies of chickpea for elucidating the genes involved in resistance to the Ascochyta blight disease have been initiated.

Chalcone synthase (CHS) a key enzyme in the biosynthesis of flavonoids. The flavanoids are able to protect the plants from detrimental effects of UV light and also their importance as antibiotic phytoallexins during plant –pathogen interaction is well established. Isolation and sequencing of pCAHS-1 a cDNA encoding a chalcone synthase from chickpea infected with *Ascochyta rabiei* (Hanselle *et al.*, 1999) and phenyl alanine ammonia-lyase (PAL) (Hein *et al.*, 2000) another enzyme important in defense response was reported.

The resistant parents FLIP 84-92 of *C. arietinum* and PI 489777 of *C. reticulatum* which have been extensively used in developing populations for Ascochyta blight resistance QTLs, were used for functional genome analysis. The Differential Display Reverse Transcription analysis and subsequent cloning of differentially expressed DDRT products showed 87% and 86% similarity with serine hydroxymethyl transferase and aldolase of pea indicating their probable role in defense response against Ascochyta blight pathogen (Rajesh *et al.*, 2004).

2.3 Validation of QTL markers

Pathogens and insects are known to overcome resistance provided by single genes. Durability of resistance has been increased in several crops by incorporating genetic diversity of major resistance genes. Marker assisted selection (MAS) is most useful for traits where phenotypic evaluation is expensive or difficult, particularly for those polygenic traits with low heritablity that are highly effected by the environment (Nienhuis *et al.*, 1987). MAS offer

many advantages like reducing the number of generations, selection based on genotypes rather than phenotypes and overall lowering of cost. MAS can be successfully employed for pyramiding or incorporating more than one resistance gene and thereby impart durable resistance to pests and diseases in crop plants.

MAS is now routinely used in breeding of major cereals like rice bacterial blight resistance (Ahmadi *et al.* 1992; Huang *et al.*, 1997; Sanchez *et al.*, 2000 and Yoshimura *et al.*, 1995) submergence tolerance (Xu *et al.*, 2004), maize (Ribaut *et al.*, 1997), barley (Laurie *et al.*, 1995 and Thomas, 2003). In the legume crops successful examples of MAS also have been reported, soybean, soybean cyst nematode (SCN) resistance (Cregan *et al.*, 1999), seed weight (Hoeck *et al.*, 2003), common bean, bacterial blight resistance (Yu *et al.*, 2000), lentil ascochyta blight resistance in (Taran *et al.*, 2003). The efficiency of MAS or MAB depends on the size of population, the number of markers used, the distance between loci, the genomic region containing the desired quantitative trait loci (QTL) and the experimental design used. However successful use of MAS requires tightly linked marker to QTLs of interest and their validation across population and environments. Validation of QTL markers is critical precursor to routine use in applied breeding programs. At least four levels of validation can be envisaged using a different population from the same cross, a half-sib population, a population from one or more closely related parental genotypes and a population from distantly related parental genotypes. Phenotyping in a number of different environments to simultaneously detect environmental (E) effects and QTL x E interactions for the putative QTL. Validation of QTLs is a prerequisite to Marker assisted selection (MAS), however only a fraction of QTLs identified

for important plant traits have been independently tested for validation. Paulo *et al.*, 2004 validated QTLs for fusarium head blight and kernel discoloration in barely in validation populations developed.

Chapter III

Materials & Methods

CHAPTER-III

MATERIALS AND METHODS

The present investigation on "Molecular mapping of Ascochyta blight resistance in chickpea (*Cicer arietinum* L.)" was carried out at International Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, Hyderabad, India. Patancheru is located at an altitude of 545 m above mean sea level, latitude 17°32' N and longitude 78°16' E. The research materials used and the methods followed are furnished in this chapter.

3.1 Materials

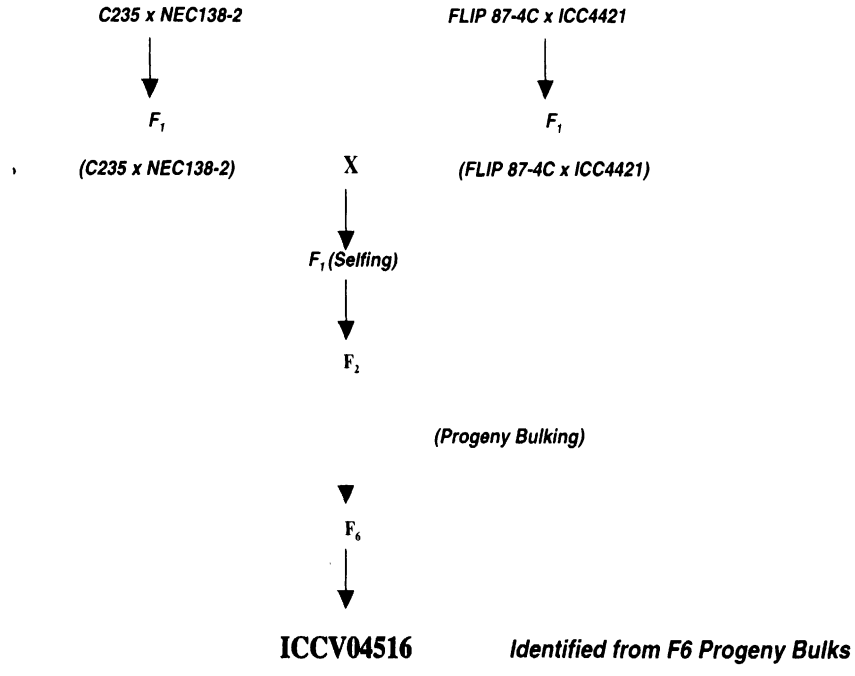
The materials consisted of F_2 and $F_{2:3}$ mapping population (n=179) derived from a cross between ICC4991 x ICCV04516. Genotyping using SSR, EST and RGA markers was carried out in F_2 lines for constructing a genetic linkage map. Identification of AB resistance QTL was attempted in both the generations. F_2 mapping population was used to identify adult plant resistance (80 days old) while, $F_{2:3}$ lines were used for detecting seedling resistance (14 days old) under controlled conditions. Two F_2 validation populations (n=94) of ICCV10 x ICCV04516 and ICCL87322 x ICCV04516 were used for validating the reported (published) QTL markers. Chickpea breeding Unit at ICRISAT provided the seed materials for the present investigation.

3.1.1 Development of intraspecific mapping population

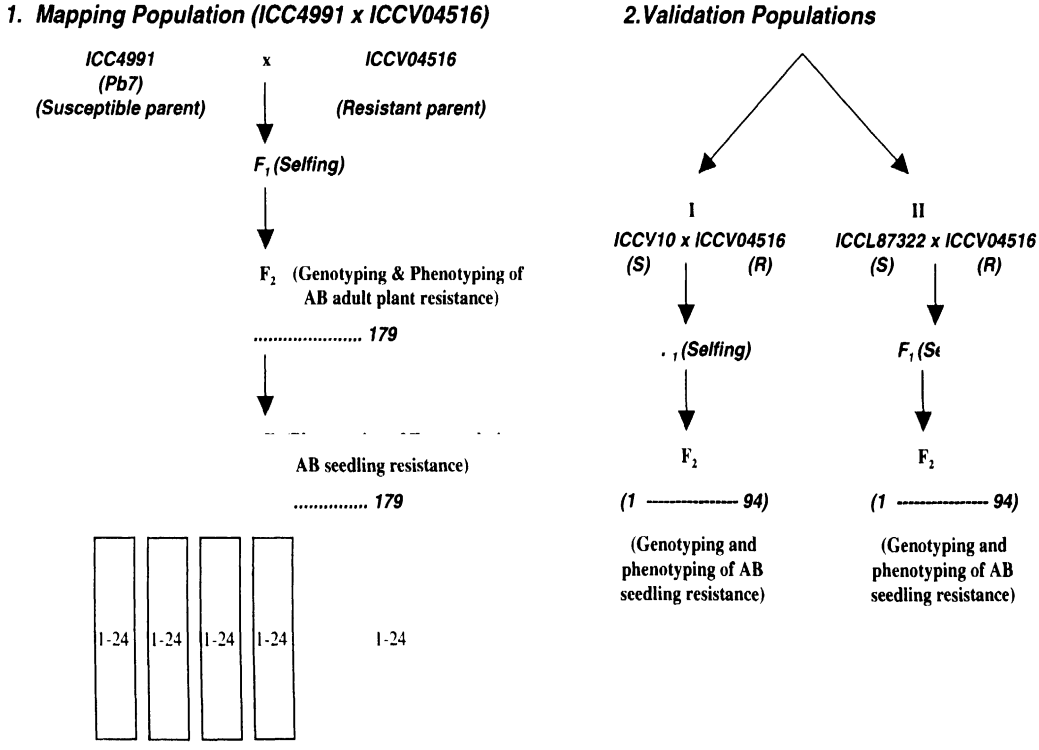
In the present study, one of the objectives was to develop an intraspecific F_2 mapping population. Schematic representation of selection of the resistant donor parent and mapping populations developed for this study is given in Figure 3.1.

Figure 3.1 Pedigree of resistant parent ICCV04516 and Schematic representation of mapping populations utilized for AB QTL detection.

A. Pedigree of resistant parent ICCV04516



B. Intraspecific Mapping Population used for QTL analysis



3.1.1.1 Selection of parents and crossing

Pb-7 (ICC4991) a highly, susceptible and a standard susceptible check commonly used for Ascochyta blight screening with a disease score 9.0 was crossed with a resistant parent ICCV04516 with a consistent disease score between 3.0 to 4.0 from glass house screening at seedling stage against AB. The resistant parent was selected from F_6 progeny bulks of a double cross (C235 x NEC 138-2) x (FLIP87-4C x ILC 4421) developed at ICRISAT. The crossing was attempted during winter 2003.

3.1.1.2 F_2 and $F_{2:3}$ mapping population

F_1s were selfed during summer 2004 in the glass house (temperature 25 $\pm$ 2 °C, relative humidity 70-80% and normal daylight conditions) to obtain an F_2 population. A population of 179 F_2 plants was raised in cups containing vermiculite base in the glass house. DNA was extracted from 14 days old seedlings before transferring them to field. The F_2 plants were grown in the field with spacing of 30 cm between plants and 60 cm between rows and standard package of practices were followed through out the crop period. The F_2 plants were selfed to obtain the $F_{2:3}$ mapping population for phenotyping against AB. The weather conditions that prevailed during the crop growth period are given in Appendix I.

3.1.1.3 Validation populations

The susceptible parent Pb-7 (ICC4991) though a susceptible check for AB screening is an obsolete cultivar. Therefore, crossing of the resistant parent with present day cultivars (ICCV10 and ICCL 87322) was attempted, in order to develop validation populations. Two susceptible parents ICCV 10 with average

disease score of (8.0 to 9.0) and ICCL87322 with average disease score of 9.0 were crossed with resistant parent ICCV04516. The F_1s were selfed to obtain F_2 populations for validating the earlier reported AB resistance QTL markers.

3.2 Methods

3.2.1 F_2 Genotyping

3.2.1.1 Isolation of total genomic DNA

Total genomic DNA was isolated from 14 days old individual F_2 seedlings using CTAB-based high throughput DNA extraction protocol (Mace *et al.*, 2004).

3.2.1.1.1 Sample preparation

20-30 mg of leaf tissue (3 young pinnules) from each plant was collected and placed in 12 x 8 well stripe tubes with stripe caps (Marsh Biomarket, USA) in a 96 deep well plate together with two 4 mm stainless steel grinding balls (Spex Centri Prep, USA).

3.2.1.1.2 CTAB extraction

- 450 µl of preheated (65^{0}C) extraction buffer (100 mM Tris-HCl [pH 8.0], 1.4 mM NaCl, 20 mM EDTA, CTAB [2% w/v], β-mercaptoethanol [0.03% v/v] was added to each sample and secured with 8 stripe caps.
- The samples were thoroughly ground in a Geno Grinder 2000 (Spex Centri Prep, USA) at 500 strokes /minute for 10 minutes.
- The samples were incubated for 10 minutes in a 65°C water bath with occasional mixing.

3.2.1.1.3 Solvent extraction

- 450 µl of chloroform : isoamylalcohol (24:1) was added to each sample and inverted twice to mix.

- The 96 well plates were centrifuged (Sigma 4K15C) at 5000 rpm for 15 minutes.
- Using filter tips 300 µl of aqueous layer was transferred to fresh tubes.

3.2.1.1.4 Initial DNA precipitation and RNase treatment

- 0.7 volumes of isopropanol (stored at -20° C) was added to each sample and centrifuged at 6200 rpm for 15 minutes.
- Supernatant was decanted from each sample and pellet was air dried for 30 minutes.
- Low salt TE (10 mM Tris, 0.1mM EDTA [pH 8.0] was added to each sample and each sample was treated with 3 µl of RNase (10 mg/ml) and incubated at 37 ^{0}C for 30 minutes to obtain pure DNA samples free from RNA.

3.2.1.1.5 Solvent Extraction

- 200 µl phenol-chloroform-isoamylalcohol (PCI 25:24:1) was added to each sample and inverted twice to mix and the plate was centrifuged at 5000 rpm for 5 minutes.
- Aqueous layer was transferred to fresh tubes.
- 200 µl of chloroform-isoamylalcohol (24:1) was added to each sample and inverted twice to mix and the plates were centrifuged at 5000 rpm for 5 minutes.
- Aqueous layer was transferred to fresh tubes.

3.2.1.1.6 Purification

- 315 µl ethanol acetate solution (absolute ethanol and 3M sodium acetate pH 5.2) was added to each sample and placed in –20°C for 10 minutes.
- The samples were centrifuged at 6000 rpm for 10 minutes for pelleting DNA.

- Supernatant was decanted from sample and pellet was washed with 70% ethanol.
- The plate was centrifuged at 6200 rpm for 5 minutes.
- Supernatant was decanted from sample and air dried for approximately one hour.
- The pellet was resuspended in 100 µl of low salt TE and stored at 4^0 C.

3.2.1.2 DNA Quantification

DNA quality and quantity was assessed on a 0.8% agarose gel. One microlitre of DNA sample, was loaded on 0.8% agarose gel and electrophoresis was carried out for half an hour at 100 volts. The DNA was observed under UV gel documentation (UVI Gel Documentation). The amount of fluorescence is proportional to the total mass of DNA. The quantity of DNA in the sample was estimated by comparing the fluorescent yield of the sample with that of a series of standards (lambda DNA).

3.2.1.3 Stocks and solutions

a. Extraction buffer (2% CTAB)

100 mM Tris (MW 121.14)	12.1 g
1.4 mM NaCl (MW 58.44)	81.8 g
20 mM EDTA (MW 372.24)	7.45 g
CTAB	20.0 g

First CTAB was dissolved, followed by NaCl and EDTA in distilled water. The pH was adjusted to 8.0 and volume was made up to 1000 ml.

b. 1M Tris (pH 8.0)

Trizma base	121.1 g

121.1 g of Tris was dissolved in distilled water, pH was adjusted to 7.0 using concentrated HCl and the volume was made up to 1000 ml and autoclaved.

c. TE buffer

Trizma base	1.21 gm
EDTA (disodium salt)	0.372 gm

pH was set to 8.0 and final volume was adjusted to one liter and autoclaved.

d. 10X loading buffer

Bromophenol blue	40 mg (final vol. 0.4%)
Xylene cyanole	40 mg (final vol. 0.4%)
Glycerol	5 ml

The final volume was adjusted to 10 ml with distilled water; 1.5 ml was aliquoted to micro centrifuge tubes and heated in boiling water. Cooled for 10 minutes and stored at 4^0C.

e. 3M Sodium acetate

204.12 gm of sodium acetate was dissolved in 350 ml of distilled water and pH was adjusted to 5.2 and final volume was made up to 500 ml and autoclaved.

f. 50X TAE

Trizma bas	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA (pH 8.0)	100 ml

The volume was made up to 1000 ml and autoclaved.

g. 10X TBE (Tris Borate EDTA buffer)

109 g of Trizma base, 55 g of boric acid and 40 ml of 0.5M EDTA pH 8.0 were dissolved in 800 ml distilled water and the solution was made up to the volume to 1000 ml. The buffer was autoclaved and stored at 4^0 C. To prepare working solution of 1X stock solution was diluted 10 times.

h. APS (Ammonium persulphate)

100 mg of APS was dissolved in 10 ml distilled water.

i. Bind silane buffer

Bind silane	1.5 ml
Acetic acid	5.0 ml
Ethanol	993.5 ml

1.5 ml of bind silane and 5ml of acetic acid were dissolved in 993.5 ml of ethanol.

j. Acrylamide | Bisacrylamide (29:1)

29 ml acrylamide and 1 ml bisacrylamide were mixed.

k. Orange loading dye

0.5 M EDTA	10 ml
5M NaCl	1 ml
Glycerol	50 ml
Distilled water	39 ml

Orange dye powder was added until the colour was sufficiently dark and the volume was made up to 100 ml.

l. RNAse A (10 mg / ml)

100 mg RNAase A was dissolved in 10 ml of 10 mM Tris pH 7.5, 15 mM NaCl. Heated in boiling water for 15 min and was cooled slowly to room temperature. Dispensed into aliquots and stored at - 20^0 C.

m. 5M NaCl

292.2 g of NaCl was dissolved in distilled water and volume was made up to 1000 ml and autoclaved.

n. 1M $MgCl_2$

20.33 g $MgCl_2$ $6H_2O$ (MW 203.30) was dissolved in distilled water and the final volume was made up to 100 ml and autoclaved.

o. 0.5M EDTA pH 8.0

186.12 g EDTA (MW 372.24) was dissolved in 750 ml of distilled water. pH was adjusted to 8.0 using NaOH pellets. The volume was made up to 1000 ml.

3.2.1.4 Parental screening

The parents of the mapping population Pb-7 (ICC4991) and ICCV 04516 were screened with 232 Chickpea SSR (Table 3.1 and Appendix II), 108 EST and 15 chickpea RGAs markers (Table 3.2 and Appendix III) for identification of the polymorphic markers. Further a subset of 24 ESTs and 15 chickpea RGAs were cleaved at restriction sites to develop CAPs. Restriction digestions were carried at a concentration of 2.5 U of restriction enzyme per one microlitre of PCR product. Digestions were carried out according to manufacturers (New England Biosystems) instructions for each restriction enzyme.

3.2.1.5 PCR amplification

The optimized concentrations of the different PCR reagents were determined for each primer using adapted Cobb and Clarkson 5 grid optimization protocol. (Cobb and Clarkson 1994). PCR amplification was achieved in a 5µl reaction volume containing 10 to 15 ng of genomic DNA, 0.2 to 0.6 pico moles of forward and reverse primer, 0.1 to 0.25 mM of each dNTP, 1.0 to 3.5 mM $MgCl_2$, 0.1 to 0.5 U of Bioline Taq DNA Polymerase and 1x Buffer (provided by manufacturer). Amplification was achieved in 384 well plates using Perkin Elmer Gene-Amp PCR Sys 9700 (Norwalk Conn.) thermal cycler. The touch down temperature profiles used for PCR amplification are given in Table 3.3.

Table 3.1 List of SSR primers used for parental screening

S. No.	Marker	S. No.	Marker	S. No.	Marker
1	CaSTMS2	41	GA105	81	TA27
2	CaSTMS4	42	GA117	82	TA28
3	CaSTMS5	43	GA137	83	TA29
4	CaSTMS6	44	GAA39	84	TA30
5	CaSTMS7	45	GAA40	85	TA30s
6	CaSTMS8	46	GAA43	86	TA34
7	CaSTMS9	47	GAA46	87	TA36
8	CaSTMS10	48	GAA58	88	TA37
9	CaSTMS11	49	GA102	89	TA38
10	CaSTMS12	50	GA108	90	TA39
11	CaSTMS13	51	GA119	91	TA42
12	CaSTMS14	52	GA129	92	TA43
13	CaSTMS15	53	GA148	93	TA44x
14	CaSTMS16	54	GAA41	94	TA45
15	CaSTMS19	55	GAA42	95	TA46
16	CaSTMS20	56	GAA44	96	TA47
17	CaSTMS21	57	GAA45	97	TA53
18	CaSTMS22	58	GAA50	98	TA57
19	CaSTMS23	59	GAA51	99	TA59
20	CaSTMS24	60	GAA54	100	TA64
21	CaSTMS25	61	GAA60	101	TA65
22	CaSTMS28	62	TA1	102	TA70
23	GA2	63	TA2	103	TA71
24	GA4	64	TA3	104	TA72
25	GA6	65	TA4	105	TA76s
26	GA9	66	TA5	106	TA78
27	GA8	67	TA6	107	TA80
28	GA11	68	TA8	108	TA87
29	GA13	69	TA9	109	TA89
30	GA14	70	TA11	110	TA93
31	GA16	71	TA11(s)	111	TA96
32	GA17	72	TA12	112	TA103
33	GA20	73	TA13	113	TA103II
34	GA21	74	TA14	114	TA104
35	GA22	75	TA18	115	TA106
36	GA24	76	TA20	116	TA108
37	GA26	77	TA21	117	TA110
38	GA31	78	TA22	118	TA113
39	GA33	79	TA23	119	TA114
40	GA34	80	TA25	120	TA116

Cont...

S. No.	Marker	S. No.	Marker	S. No.	Marker
121	TA117	161	TAA107	201	TS10
122	TA118	162	TAA137	202	TS16
123	TA120	163	TAA169	203	TS17
124	TA122	164	TAA170	204	TS17x
125	TA125	165	TAA194	205	TS19
126	TA127	166	TAASH	206	TS23
127	TA130	167	TR1	207	TS24
128	TA132	168	TR2	208	TS29
129	TA135	169	TR3	209	TS35
130	TA136	170	TR5	210	TS36
131	TA140	171	TR7	211	TS38
132	TA141	172	TR8	212	TS39
133	TA142	173	TR11	213	TS43
134	TA144	174	TR13	214	TS45
135	TA146	175	TR14	215	TS46
136	TA153	176	TR17	216	TS47
137	TA158	177	TR18	217	TS53
138	TA159	178	TR19	218	TS54
139	TA167	179	TR19R	219	TS54II
140	TA176	180	TR20	220	TS58
141	TA179	181	TR24	221	TS58s
142	TA180	182	TR26	222	TS62
143	TA186	183	TR28	223	TS68
144	TA189	184	TR29	224	TS71
145	TA191	185	TR31	225	TS72
146	TA194	186	TR32	226	TS74
147	TA196	187	TR33	227	TS79
148	TA198	188	TR35	228	TS83
149	TA199	189	TR40	229	TS84
150	TA200	190	TR42	230	TS14
151	TA203	191	TR43	231	TS15
152	TA206	192	TR43II	232	TS129
153	TAA55	193	TR44		
154	TAA56	194	TR45		
155	TAA57	195	TR55		
156	TAA58	196	TR56		
157	TAA59	197	TR58		
158	TAA60	198	TR59		
159	TAA61	199	TR60		
160	TAA104	200	TS5		

Table3.2 List of EST and RGA primers used for parental screening

S. No.	Marker	S. No.	Marker	S. No.	Marker	S. No.	Marker
	ESTs					RGAs	
1	AGLC1	41	AGLC41	81	AGLC81	1	RGAA
2	AGLC2	42	AGLC42	82	AGLC82	2	RGAB
3	AGLC3	43	AGLC43	83	AGLC83	3	RGAC
4	AGLC4	44	AGLC44	84	AGLC84	4	RGAD
5	AGLC5	45	AGLC45	85	AGLC85	5	RGAE
6	AGLC6	46	AGLC46	86	AGLC86	6	RGAF
7	AGLC7	47	AGLC47	87	AGLC87	7	RGAG
8	AGLC8	48	AGLC48	88	AGLC88	8	RGAH
9	AGLC9	49	AGLC49	89	AGLC89	9	JB1
10	AGLC10	50	AGLC50	90	AGLC90	10	JB2
11	AGLC11	51	AGLC51	91	AGLC91	11	JB3
12	AGLC12	52	AGLC52	92	AGLC92	12	JB4
13	AGLC13	53	AGLC53	93	AGLC93	13	JB5
14	AGLC14	54	AGLC54	94	AGLC94	14	JB6
15	AGLC15	55	AGLC55	95	AGLC95	15	JB7
16	AGLC16	56	AGLC56	96	AGLC96		
17	AGLC17	57	AGLC57	97	AGLC97		
18	AGLC18	58	AGLC58	98	AGLC98		
19	AGLC19	59	AGLC59	99	AGLC99		
20	AGLC20	60	AGLC60	100	AGLC100		
21	AGLC21	61	AGLC61	101	AGLC101		
22	AGLC22	62	AGLC62	102	AGLC102		
23	AGLC23	63	AGLC63	103	AGLC103		
24	AGLC24	64	AGLC64	104	AGLC104		
25	AGLC25	65	AGLC65	105	AGLC105		
26	AGLC26	66	AGLC66	106	AGLC106		
27	AGLC27	67	AGLC67	107	AGLC107		
28	AGLC28	68	AGLC68	108	AGLC108		
29	AGLC29	69	AGLC69				
30	AGLC30	70	AGLC70				
31	AGLC31	71	AGLC71				
32	AGLC32	72	AGLC72				
33	AGLC33	73	AGLC73				
34	AGLC34	74	AGLC74				
35	AGLC35	75	AGLC75				
36	AGLC36	76	AGLC76				
37	AGLC37	77	AGLC77				
38	AGLC38	78	AGLC78				
39	AGLC39	79	AGLC79				
40	AGLC40	80	AGLC80				

Table 3.3 Touch down temperature profiles

Cp65-60			
	Temperature^{0}C		
Initial Denaturation	95	3 min	
Denaturation	94	20 sec	5 cycles
Annealing	65	20 sec	
Extension	72	30 sec	
Denaturation	94	20 sec	30 cycles
Annealing	59	20 sec	
Extension	72	30 sec	
Final Extension	72	20 min	

Cp60-55			
	Temperature^{0}C	Time	
Initial Denaturation	95	20 min	
Denaturation	94	20 sec	5 cycles
Annealing	60	20 sec	
Extension	72	30 sec	
Denaturation	94	20 sec	30 cycles
Annealing	56	20 sec	
Extension	72	30 sec	
Final Extension	72	20 min	

Cp55-45			
	Temperature^{0}C	Time	
Initial Denaturation	95	3 min	
Denaturation	94	20 sec	5 cycles
Annealing	55	20 sec	
Extension	72	30 sec	
Denaturation	94	20 sec	30 cycles
Annealing	49	20 sec	
Extension	72	30 sec	
Final Extension	72	20 min	

3.2.1.6 Electrophoresis

The amplified products were separated using

A) Non-denaturing Poly Acrylamide Gel Electrophoresis (PAGE)

B) Denaturing Poly Acrylamide Gel Electrophoresis (PAGE)

C) Capillary Electrophoresis (ABI PRISM 3700 DNA Sequencer)

3.2.1.6.1 PolyAcrylamide Gel Electrophoresis

a. Gel casting

- The glass plates were thoroughly cleaned, twice with double distilled water and twice with 70% ethanol.
- Few drops of Repel- silane were applied to back-plate and evenly spread for easy separation of the back plate from gel.
- Few drops of Bind- silane were applied to glass plate and thoroughly spread over entire surface to prevent from dislodging of gel during staining.
- The gel was casted using the following composition.

b. Composition of 6% gel matrix Biorad Sequi-Gen Unit (37.5 cm x 30 cm)

Distilled water	52.5 ml
10X TBE	7.5 ml
Acryalmide/Bisacrylamide of (29:1)	15.0 ml
Ammonium Per Sulphate	450 µl
TEMED	100 µl

c. Electrophoresis

The polymerized gel was pre run for 10 minutes at 650V in 0.5X TBE buffer. Loading dye (orange juice) was added to PCR products and 2.0 µl of

the mix was loaded on the gel and DNA was separated at 650V for 3.5 hours. 100 bp marker (50 ng/ul) was always loaded on first and last lanes to ensure proper sizing of the amplified PCR product. The gel plate was carefully removed and subjected to silver staining.

d. Silver staining

The protocol involves staining and destaining the gel in a set of solutions as follows with gentle shaking at 60-70 rpm:

- Gel was rinsed in distilled water for 2-3 minutes.
- The gel was then soaked in 1.5 liter of 0.1% CTAB for 20 minutes.
- Incubated in 1.5 litres of 0.3% ammonia solution for 15 minutes.
- Transferred to freshly prepared staining solution (0.1% silver nitrate and 6 ml of 1N NaOH, which was titrated with 6–8 ml of 25% ammonia solution until the cloudy suspension became clear) for 15 minutes.
- The gel was placed in developer solution (1.5% sodium carbonate and 400 µl of formaldehyde) and was gently shaken until bands were visualized.
- Finally the gel plate was placed in the fixer solution containing 1.5% glycerol for two minutes.

3.2.1.6.2 Denaturing PolyAcrylamide Gel Electrophoresis

The denaturing gels were prepared and run under similar conditions as of non-denaturing gels with the following changes.

- The gel matrix contained 7.5 mM urea along with the other ingredients.

- Equal volumes of amplified products and loading buffer were denatured at 94^0 C for five minutes and snap cooled on ice before loading on to the gel.
- The electrophoresis was carried out at a constant temperature of 50^{0}C and 100 watts.

The gels were scanned in Umax-Scanner (Umax Mirage II).

3.2.1.6.3 Capillary Electrophoresis

PCR amplification was achieved according to the conditions described in 3.2.1.5. using fluorescent-labeled primers (Fam, Pet, Ned and Vic). PCR amplification products (1.0 ul each) were multiplexed and denatured for 5 minutes at 94^{0}C using high Hidye-formamide along with the standard LIZ (500) and loaded onto ABI PRISM 3700 (96 well capillary). CE was carried out with support of the software GeneScan Analysis version 3.7 (Applied Bio systems). Fragment sizes were calculated by comparison with internal standard GeneScan-500 LIZ using Genotyper Version 3.7 software (Applied Biosystems). Four primers were multiplexed in a single well of 384 well plate based on pre-determined sizes of amplification products.

3.2.1.7 Data scoring

The polymorphic markers were scored across segregating population. Data was recorded as A for the susceptible allele (band) and B for the resistant allele (band) and H for the heterozygotes manually on PAGE. In case of CE the peaks were analyzed using ABI PRISM Genotyper Version 3.7 software (Applied Biosystems).

3.2.2 F_2 and $F_{2:3}$ screening against Ascochyta blight disease

3.2.2.1 Cut twig method

Three twigs with a minimum of five pinnules were collected from each 80 day old F_2 plants. The twigs were wrapped with a cotton plug and transferred to test tubes (15 x 100 mm) containing fresh tap water. The test tubes were transferred to growth room maintained at 20 ± 1°C with ~1500 lux light intensity for 12 h a day. The cut twigs were inoculated by foliar spray of the inoculum using hand-operated atomizer. Thereafter, 100% RH was provided for the initial 4 days (24 h) after inoculation (DAI) and later 100% RH was maintained for 6-8 h a day until 10 DAI. The disease was scored when the susceptible check Pb-7 (ICC4991) completely dried out i.e., on 10 DAI on a 1-9 scale Figure 3.2.

3.2.2.2 Seedling method

Seedlings of the F_3 families (24 plants each) along with the susceptible check Pb-7 (ICC 4991) were raised in 40 x 30 x 5 cm plastic trays filled with sand and vermiculite mixture (10:1), in greenhouse at 25 ± 3°C and 12-13 h photoperiod. Ten-day-old seedlings were transferred to a growth room maintained at 20 ± 1°C with ~1500 lux light intensity for 12 h a day. The seedlings were inoculated by foliar spray of the inoculum using hand-operated atomizer. Inoculated plants were allowed to partially dry for 30 min to avoid dislodging of the spores. Thereafter, 100% RH was provided for the initial 4 days (24 h) after inoculation (DAI) and later 100% RH was maintained for 6-8 h a day until 10 DAI. The disease was scored when the susceptible check Pb-7 (ICC4991) completely dried out i.e., on 10 DAI on a 1-9 scale Figure 3.3.

Figure 3.2 F_2 Ascochyta blight disease screening -cut twig method

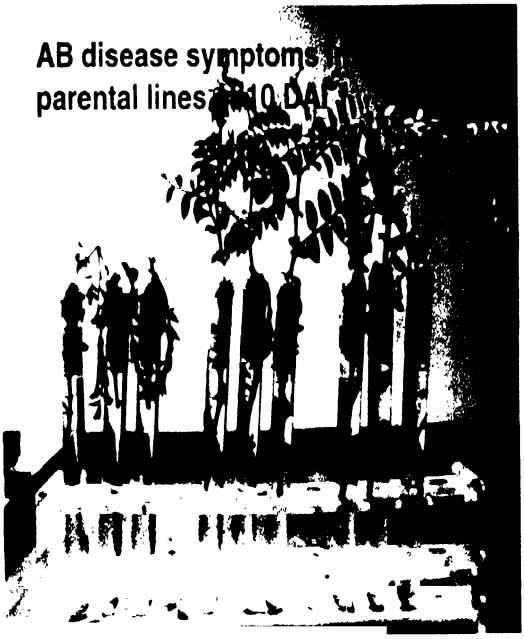
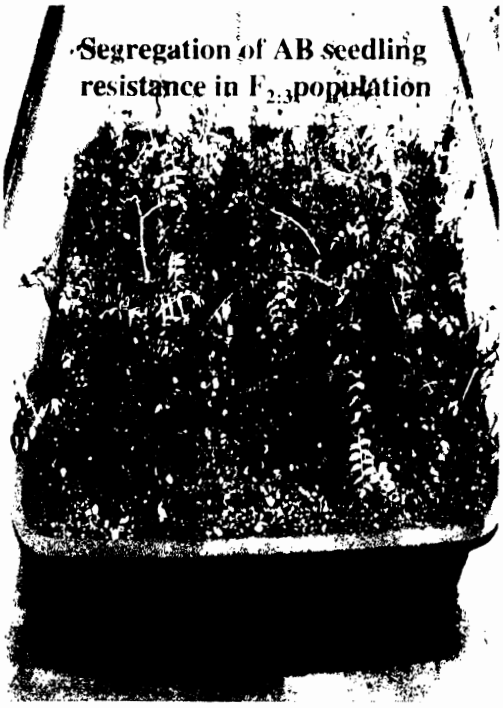


Figure 3.3 $F_{2:3}$ Ascochyta blight disease screening seedling method



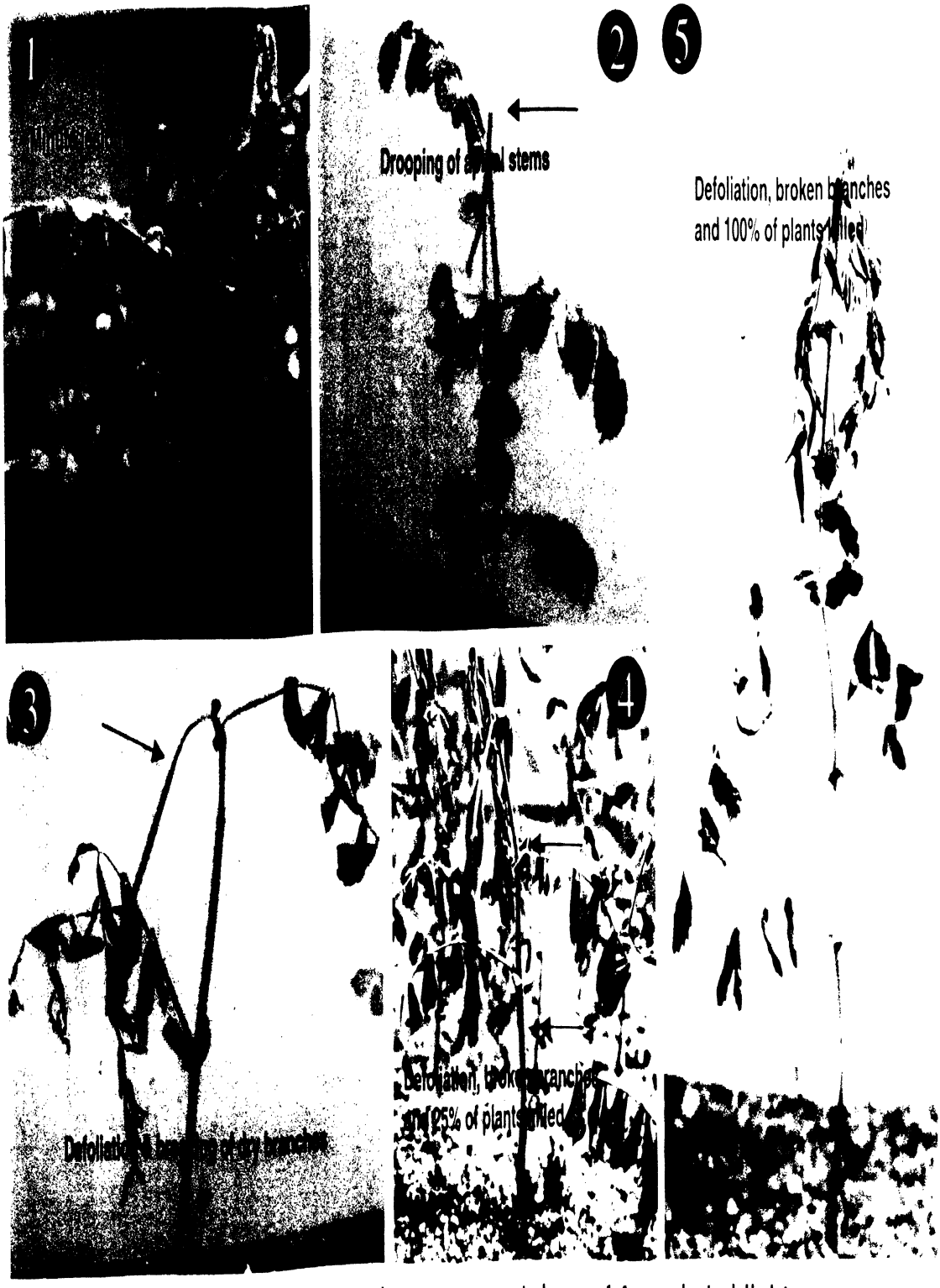


Figure 3.4 Progressive symptomatology of Ascochyta blight

3.2.2.3 Inoculum preparation

Single spore isolate of a virulent culture of *A. rabiei* collected from infected chickpea plants in Hissar was multiplied separately on sterile seeds of kabuli chickpea genotype ICCV 88901. Chickpea seeds were soaked overnight in water, autoclaved at 121°C for 25 min, and inoculated with 1 cm diameter actively growing culture of *A. rabiei* on CDA. Inoculated seeds were incubated for 8 days at 20°C and 12 h photoperiod. Profusely sporulated seeds were stirred in sterile distilled water to facilitate the release of pycnidiospores into water and filtered through a muslin cloth. The pycnidiospore concentration in the suspension was adjusted to 5×10^4 spores/ml and used as inoculum.

3.2.2.4 Disease scoring

Disease scoring: 1. No symptoms 2. Minute lesions prominent on the apical stem 3.Lesions up to 5 mm size and slight drooping of the apical stem 4. Lesions obvious on all plant parts and clear drooping of apical stem 5. Lesions obvious on all plants/parts, defoliation initiated and breaking and drying of branches slight to moderate 6. Lesions as in 5, defoliation, broken, dry branches common, some plants killed 7. Lesions as in 5, defoliation, broken, dry branches very common, up to 25% of the plants killed 8. Symptoms as in 7 but up to 50% of the plants killed 9. Symptoms as in 7 but up to 100% of the plants killed. Based on the disease score, the plants were categorized for their reaction to Ascochyta blight infection as follows: 1 = immune (I); 1.1-3 = resistant (R); 3.1-5 = moderately resistant (MR), 5.1-7 = susceptible (S), and 7.1-9 = highly susceptible (HS) Figure 3.4.

3.3 Statistical analysis

3.3.1 Parameters of variability assessment

a. Mean

Mean value ($\overline{X}$) of disease reaction was computed dividing the sum of the observed values by the corresponding number of observations.

$$\overline{X} = \sum X_{IJ} / N$$

where,

X_{ij} = observation in the i th treatment and j th replication, and

N = total number of observations.

b. Range

It is difference between lowest and highest mean disease reaction values.

c. Standard deviation

The standard deviation is the square root the arithmetic average of the squares of deviations measured from mean.

$$\sigma = \sqrt{\Sigma d^2 / N-1}$$

where

Σd^2 = sum of square of the deviations measured from arithmetic mean.

N = total number of observations.

d. Standard error

Standard error of mean was calculated for mean disease reaction from the corresponding mean square error values from the analysis of variance.

$$S.E.(m) = \sqrt{\sigma^2_e / r}$$

where

σ^2_e = estimated mean sum of squares

S.E. (m) = the standard error of the mean, and

r = the number of replications.

e. Analysis of Variance (ANOVA)

Analysis of variance was performed on the data from $F_{2:3}$ mean disease score using completely randomized design. Best Linear Unbiased Predictors (BLUPs) of the random effect were computed in restricted maximum likelihood (REML) Variance Components Analysis from Genstat 8.0 with replicates as fixed model and genotypes as random effects.

Table 3.4 Analysis of variance

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	F ratio
Treatment	(t-1)	TrSS	TrMS	TrMS/EMS
Error	t(r-1)	ESS	EMS	
Total	(tr-1)	TSS		

where, r = number of replications and t = number of treatments or genotypes

f. Coefficient of Variation:

Genotypic and phenotypic coefficients of variation for disease reaction was computed using the following formulae.

$$\text{Genotypic coefficient of variation (G.C.V)} = \frac{\sqrt{\sigma^2_g}}{\bar{x}} \times 100$$

$$\text{Phenotypic coefficient of variation (P.C.V.)} = \frac{\sqrt{\sigma^2_p}}{\bar{x}} \times 100$$

where $\bar{x}$ is the mean of the disease reaction observed in the $F_{2:3}$ lines.

g. Heritablity (in broad sense):

Heritablity in broad sense was calculated using the formula

$$h^2 b = \frac{\sigma^2_g}{\sigma^2_p}$$

3.3.2 Linkage map construction

The segregating markers were mapped in the F_2 population. The genetic linkage map was constructed using Join Map 3.0 Software (Van Ooijen and Voorrip, 2001) based on principle described by Stam (1993). LOD scores and pair wise recombination values were converted to genetic distances using the Kosambi (1944) mapping function. A LOD score > 3.0 were used to create linkage groups.

3.3.3 QTL Mapping

The Ascochyta blight disease resistance score of F_2 and the mean disease score of each $F_{2:3}$ family was analyzed to detect and map the quantitative trait loci (QTLs) influencing the disease resistance using the software QTL Cartographer Version 2.0(Wang *et al.*, 2003). Single markers analysis, interval mapping and composite interval mapping strategies were used for detecting QTLs. The phenotypic variance explained by the QTLs was calculated by simple regression analysis using Genstat 8.0.

Chapter IV

Results

CHAPTER-IV

RESULTS

The present investigation on **"Molecular mapping of Ascochyta blight resistance in chickpea (*Cicer arietinum L.*)"** was carried out with three major objectives -i) Construction of intraspecific genetic linkage map ii) Detection of the QTL regions responsible for AB resistance against an Indian virulent isolate of Hissar and iii) Validation of the reported markers linked to AB QTLs markers in the populations developed using the resistant parent ICCV04516.

4.1 Construction of an intraspecific genetic linkage map

4.1.1 Development of an intraspecific mapping population

Pb-7 (ICC4991) a cultivar highly susceptible to AB and commonly used as susceptible check for AB screening with a disease score of 9.0 was crossed with a resistant parent ICCV04516, which shared a consistent disease score between 3.0 to 4.0 in AB resistance screening under controlled environment. The resistant parent was selected from F_6 progeny bulks of a double cross (C235 x NEC138-2) x (FLIP87-4C x ILC 4421) developed at ICRISAT (Figure 3.1). SSR markers were used to identify genuine F_1 hybrids (Figure 4.1). F_2 seeds from a single F_1 plant were used to obtain an F_2 mapping population. The F_2 plants were raised under field conditions and used for genotyping and phenotyping. Harvest from individual F_2 plants were used to obtain $F_{2:3}$ progenies.

4.1.2 Parental screening

The parents Pb-7 (ICC4991) and ICCV04516 were screened with available 232 chickpea SSR (Table 3.1), 108 EST and 15 chickpea RGAs primer pairs (Table 3.2) for detection of polymorphic markers (Figure 4.2, Figure 4.3a and Figure 4.3b).

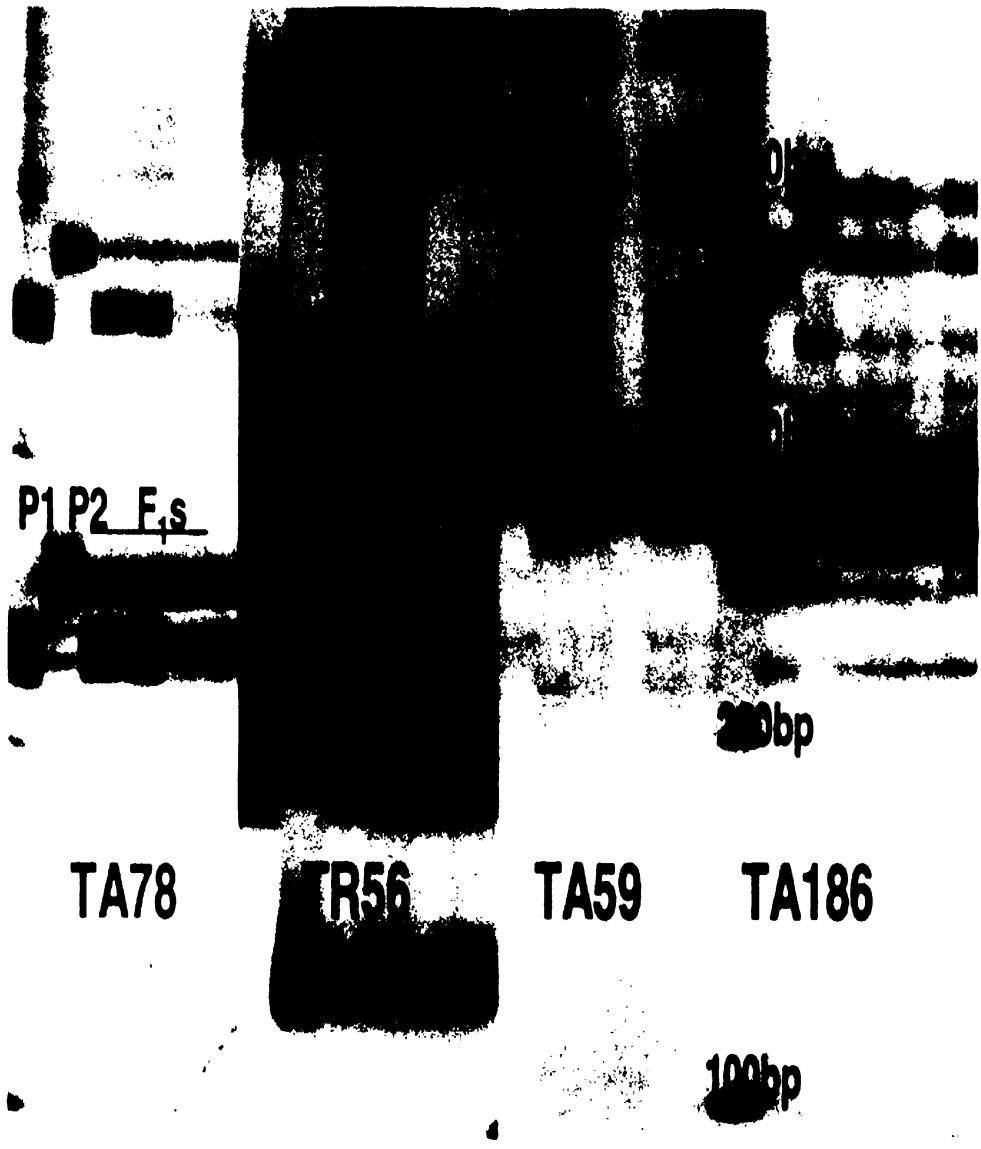


Figure 4.1 Parental polymorphism of ICC4991 and ICCV04516 and genuine F_1 hybrids

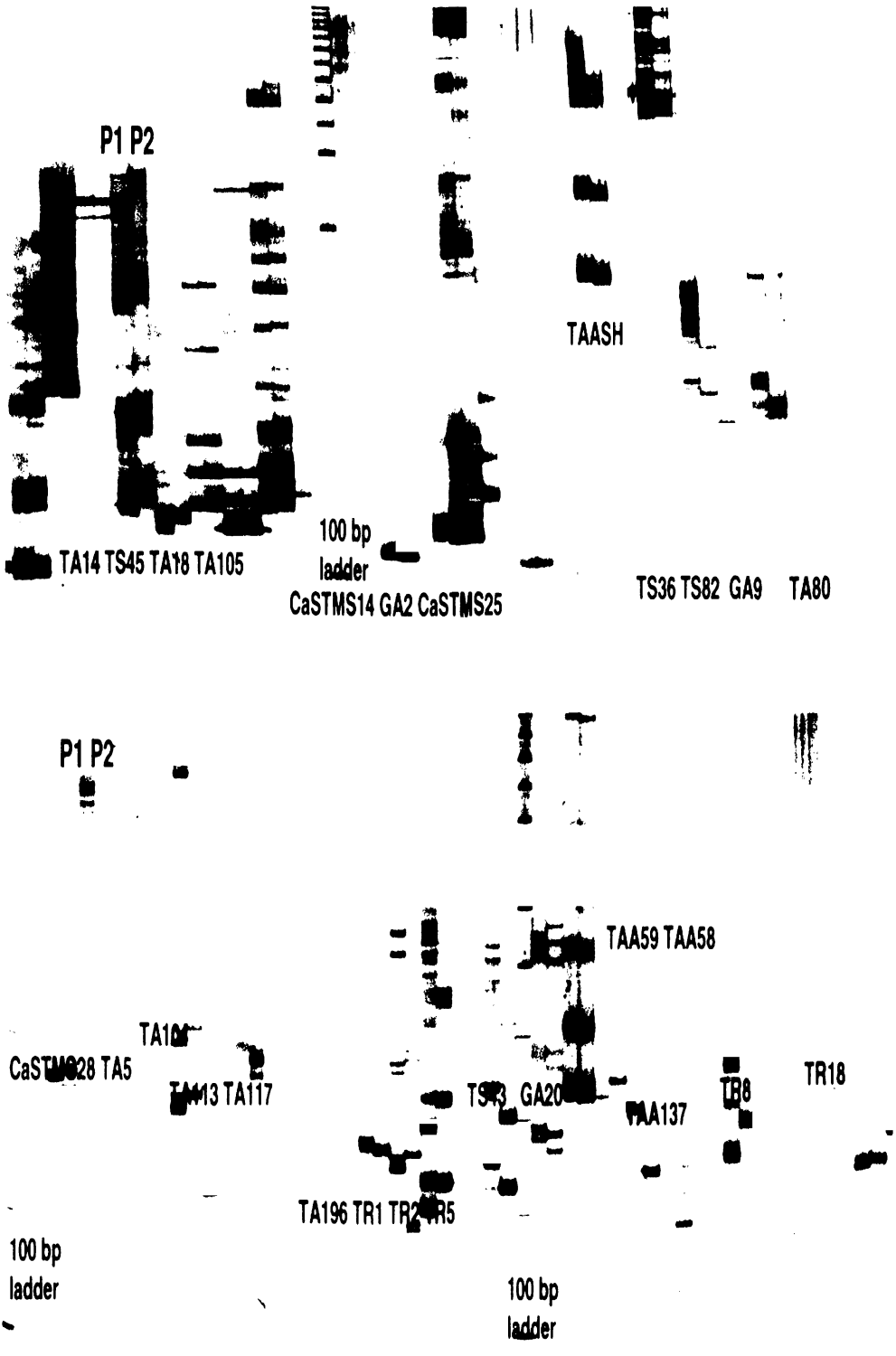


Figure 4.2 Parental prescreening of ICC4991 and ICCV04516 with SSR markers

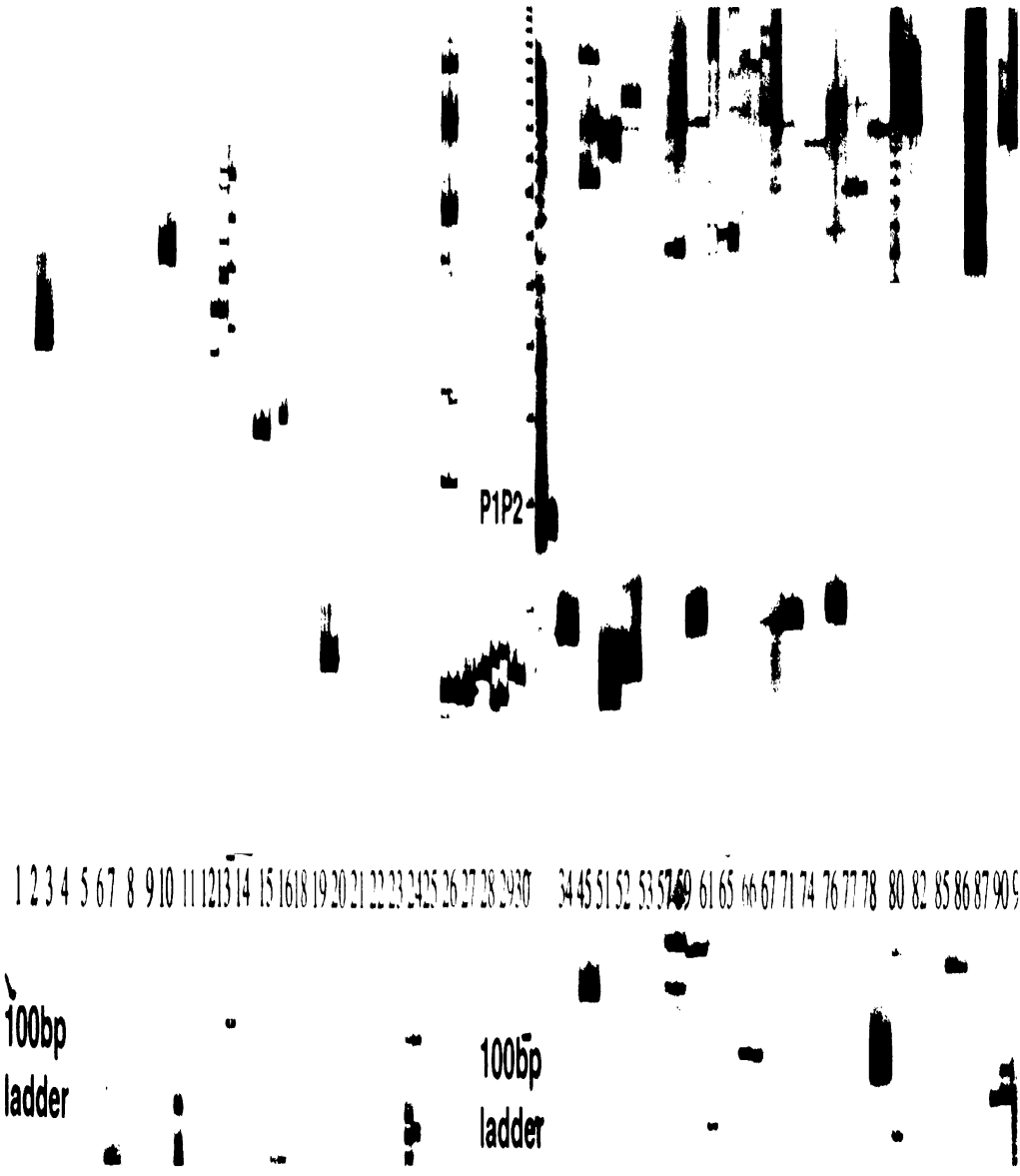


Figure 4.3a Parental prescreening of ICC4991 and ICCV04516 with EST markers

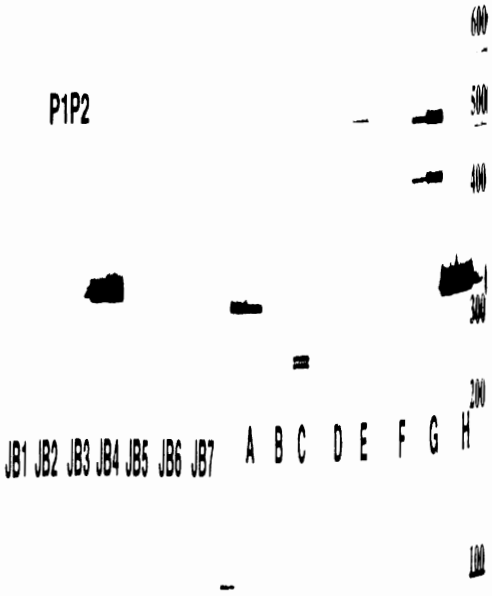


Figure 4.3b Parental prescreening ICC4991 and ICCV04516 with RGA markers

4.1.2.1 Simple Sequence Repeats

The initial screening of the 232 SSR markers gave amplification with 198 primer pairs. Of these, 106 markers were polymorphic, however, 96 of 232 (41.38%) distinct and highly reproducible SSR markers were used as genetic markers for linkage analysis. The amplified product size ranged from 100- 550 base pairs. In most of the cases (75%), the primer pairs amplification resulted in a single polymorphic band between the two parents and the rest of the primer pairs amplified more than two bands, which segregated identically across the populations. The PCR amplification conditions, size of polymorphic parental bands and the mode of electrophoretic separation for the polymorphic SSR and EST markers used for the linkage analysis are given in Table 4.1.

4.1.2.2 Expressed Sequence Tags

A set of 108 ESTs was screened for parental polymorphism. The polymorphism detected was very minimal as only three markers (2.7%) were polymorphic. These were AGLC 011, AGLC29 and AGLC66 (Figure 4.3a).

4.1.2.3 Resistance Gene Analogues

A set of 15 RGAs (JB1 to JB7 and Rga A to H) was screened between the parents, all of which were monomorphic (Figure 4.3b).

4.1.2.4 Cleaved Amplification Polymorphic sites

As very low polymorphism was detected with ESTs and no polymorphism with RGAs, PCR product was cleaved with restriction enzymes for generation of CAPs. All the 15 chickpea RGAs and a sub set of 24 ESTs were subjected to CAPs analysis. The PCR product was cleaved with restriction enzymes deduced from *in-silico* restriction maps developed using Sequencher software (http:// www.genecodes.com/). Only two RGAs, JB7 with Aci I and

RgaH with restriction enzyme Hinf I, generated polymorphism. A set of 24 ESTs namely AGLC 34, 45, 51, 52, 53, 57, 59, 61, 65, 66, 67, 71, 74, 76, 77, 78, 80, 82, 85, 86, 87, 90, 94 and 103 were subjected to restriction digestion. Out of these three ESTs, namely AGLC53 with Aci I, AGLC59 with HpyCH4 V, and AGLC87 with Hae III, were found polymorphic. However, the CAPs generated were not used for the linkage analysis.

4.1.3 F_2 Genotyping

The segregation of the polymorphic markers across the mapping population was analyzed using the PCR conditions described in Table 4.1. The polymorphic markers were separated on non-denaturing PAGE, denaturing PAGE (Figure 4.4) and 4.5) and by capillary electrophoresis ABI 3700 (Figure 4.6).

4.1.4 Inheritance of the markers and linkage analysis

A total of 99 polymorphic markers (96 SSRs and 3 ESTs) were used for linkage analysis (Table 4.1). Each segregating marker was tested for goodness of fit to the expected 1:2:1 ratio by χ 2 test ($P < 0.05$). Sixty-three markers gave a good fit to the expected segregation ratio. Thirty-six markers showed χ 2 values significant at 5% percent level showing segregation distortion (Appendix 6). However, all markers were used for the linkage analysis in spite of the distorted segregation.

A total of 84 markers, (63 markers with 1:2:1 Mendelian inheritance and 19 markers with distorted segregation) were mapped into eight major and two minor linkage groups. Fifteen markers (15.5%) out of 99 remained unmapped. The proportions of polymorphic markers identified for linkage analysis are given in Table 4.2.

Table 4.1 PCR amplification conditions and polymorphism between ICC4991 and ICCV04516

S. No.	Marker	PCR Program	Primer pm	dNTPs mM	$MgCl_2$ mM	Taq Units	DNA ng	Pb-7 (ICC4991) bp	ICCV04516 bp
1	TA108	65-60	0.3	0.15	1.25	0.3	10	300	290
2	TA180	65-60	0.2	0.15	3	0.2	10	230/330	245/345
3	TA113	60-55	0.3	0.15	2.5	0.3	10	220	215
4	TA2	65-60	0.3	0.15	2.5	0.3	10	160	200
5	TA200	65-60	0.5	0.15	1.5	0.5	10	330/430	325/425
6	TA5	60-55	0.5	0.15	3	0.2	10	210	240
7	TA110	65-60	0.3	0.15	1.5	0.2	10	290	280
8	TA78	65-60	0.2	0.15	2.5	0.2	10	235/335	250/350
9	TA59	65-60	0.2	0.15	2.5	0.2	10	285/385	280/380
10	TA64	65-60	0.5	0.15	1.5	0.2	10	285/385	305/405
11	TA117	60-55	0.2	0.15	2.5	0.2	10	285/385	275375
12	TA127	60-55	0.2	0.15	2.5	0.2	10	250/350	265/365
13	TA28	65-60	0.4	0.1	1.5	0.4	10	400	380
14	TA53	65-60	0.2	0.15	3	0.4	10	230/305	250/290
15	TR1	60-55	0.2	0.15	2.5	0.2	10	255/355	225/325
16	TR2	60-55	0.2	0.15	2.5	0.2	10	290/280	285/265
17	GA16	65-60	0.15	0.15	2	0.2	10	285	275
18	GA6	65-60	0.5	0.15	1.5	0.2	10	180/210/260	185/215/265
19	TA71	65-60	0.2	0.15	2.5	0.2	10	280	290
20	TA96	65-60	0.16	0.15	3	0.3	10	315/295	300/400
21	CaSTMS15	65-60	0.5	0.1	1.25	0.2	10	290/390	280/380
22	TA176	65-60	0.2	0.15	2.5	0.2	10	310	320
23	TAA104	65-60	0.2	0.15	3	0.2	10	210	260
24	TAA169	65-60	0.2	0.15	2	0.2	10	250/270	255/275
25	TA186	65-60	0.16	0.15	3	0.2	10	270	290

S. No.	Marker	PCR Program	Primer pm	dNTPs mM	$MgCl_2$ mM	Taq Units	DNA ng	Pb-7 (ICC4991) bp	ICCV04516 bp
26	TA106	65-60	0.2	0.15	2.5	0.3	10	270	290
27	TA14	65-60	0.4	0.15	1.5	0.4	10	310-	308
28	TA11(s)	65-60	0.5	0.15	2.5	0.8	10	215/225	210/220
29	TA18	65-60	0.4	0.1	1.5	0.3	10	160/260	175/275
30	TAASH	65-60	0.2	0.15	1.5	0.2	10	510	500
31	TA203	65-60	0.3	0.15	3	0.4	10	310/410	275/375
32	TA146	60-55	0.6	0.2	2.5	0.6	15	205	185
33	TA80	60-55	0.4	0.15	1.5	0.2	10	270	240
34	TAA170	60-55	0.4	0.2	3	0.3	10	315	300
35	TS54	60-55	0.4	0.15	2	0.4	15	295	240
36	TS82	60-55	0.5	0.15	2.5	0.5	10	185/285	190/290
37	CaSTMS10	60-55	0.5	0.15	2	0.4	10	195	190
38	CaSTMS28	60-55	0.5	0.1	1.5	0.4	10	300	320
39	TR35	65-60	0.4	0.15	1.5	0.4	10	320	300
40	TA27	65-60	0.4	0.1	1.5	0.2	10	280	275
41	TA120	65-60	0.5	0.2	1.5	1	15	225	210
42	TA103	65-60	0.5	0.2	2.5	0.5	15	145	150
43	TAA59	65-60	0.2	0.2	2	0.3	10	215/295	250/350
44	TA132	60-55	0.5	0.1	2.5	0.8	15	210/300	220/310
45	TA194	65-60	0.5	0.1	0.75	0.5	15	275	260
46	TR19	65-60	0.2	0.15	2	0.3	10	285	290
47	TR31	65-60	0.2	0.15	3	0.2	10	265	270
48	TR43	65-60	0.3	0.1	2	0.2	10	350	410
49	TA 142	55-45	0.4	0.2	2.5	0.5	10	150	160
50	TA34	55-45	0.4	0.15	2.5	0.5	10	280	275/280

S. No.	Marker	PCR Program	Primer pm	dNTPs mM	$MgCl_2$ mM	Taq Units	DNA ng	Pb-7 (ICC4991) bp	ICCV04516 bp
51	TS29	55-45	0.5	0.1	1.5	0.5	10	450	400
52	TA39	55-45	0.4	0.15	3	0.5	10	420	405
53	TAA57	65-60	0.3	0.15	1.25	0.2	10	380	295
54	GAA44	65-60	0.5	0.15	1	0.6	10	310	300
55	GA102	65-60	0.2	0.15	1	0.6	10	330/370/380	360/390/400
56	AGLC11	60-55	0.4	0.15	1.5	0.2	10	280	275
57	AGLC29	60-55	0.4	0.15	1.5	0.2	10	320/360	325/370
58	AGLC66	60-55	2	0.1	1	0.2	10	580/600	600/620
59	TA43	65-60	0.2	0.15	1	0.2	10	220	200
60	TA22	65-60	0.4	0.15	2.5	0.4	10	300/400	335/425
61	TS5	65-60	0.4	0.2	2	0.3	10	465	445
62	TS24	65-60	0.5	0.15	1	0.3	10	305	330
63	TA21	65-60	0.5	0.15	2	0.4	10	400	390
64	TS53	65-60	0.5	0.15	2	0.4	10	250	290
65	TA118	65-60	0.5	0.2	1.5	1	15	230	250
66	TS71	65-60	0.5	0.2	1.5	1	10	295/395	280/380
67	TR5	60-55	0.8	0.2	1.5	1	10	330	325
68	TAA137	60-55	0.4	0.15	1	0.6	5	240	260
69	GA9	60-55	0.3	0.15	2	0.3	15	210	200
70	TR20	65-60	0.2	0.15	2.5	0.3	10	168.2	171.3
71	TA8	60-55	0.5	0.15	3	0.3	15	229.8	214.8
72	GA34	65-60	0.2	0.15	2.5	0.2	10	160	154.2
73	CaSTMS25	65-60	0.16	0.15	3	0.2	10	172.8	177.3
74	TR 8	60-55	0.5	0.1	1.5	0.5	10	218.7	213.8
75	TR40	55-45	0.4	0.1	1.5	0.5	10	235.8	246.6

S. No.	Marker	PCR Program	Primer pm	dNTPs mM	$MgCl_2$ mM	Taq Units	DNA ng	Pb-7 (ICC4991) bp	ICCV04516 bp
76	TS43	60-55	0.4	0.15	3	0.3	15	315/415	250/350
77	TS45	65-60	0.4	0.15	3	0.3	10	245	250
78	GA26	65-60	0.2	0.15	2.5	0.2	10	305	320
79	TA25	60-55	0.3	0.15	1.25	0.2	10	319.3	224.7
80	TR60	60-55	0.4	0.2	1.5	0.4	10	240	242.9
81	TR14	60-55	0.8	0.2	1.5	1	15	192	194.8
82	TR3	65-60	0.3	0.1	1.25	0.4	10	246	250
83	TAA194	65-60	0.1	0.15	2	0.2	10	238.2	226
84	TA159	60-55	0.8	0.2	1.5	1	15	229.6	267.5
85	TS36	60-55	0.5	0.2	2.5	0.5	10	129.3	185.6
86	TR55	55-45	0.4	0.15	1.5	0.5	10	469.7	478.5
87	TR29	65-60	0.3	0.15	1.5	0.4	10	207.9	205
88	TS84	65-60	0.4	0.1	1.5	0.3	10	229	214
89	TA144	65-60	0.5	0.2	1.25	0.2	10	268.9	241.7
90	GA20	60-55	0.2	0.15	2.5	0.2	10	229	214
91	TA116	65-60	0.4	0.15	1.5	0.4	10	205	200
92	TS46	55-45	0.3	0.15	3	0.5	10	287	290
93	GAA60	65-60	0.5	0.15	1	0.6	10	330	315
94	TAA55	65-60	0.5	0.15	1.25	0.5	15	370/450	310/405
95	TA37	65-60	0.4	0.2	2	0.2	10	325	320
96	TR58	65-60	0.5	0.15	2.5	0.5	10	375	385
97	TA196	60-55	0.5	0.2	2	0.5	10	235	210
98	TA72	60-55	0.8	0.2	1.5	1	15	300	295
99	STMS21	65-60	0.5	0.2	1	0.2	10	170	175

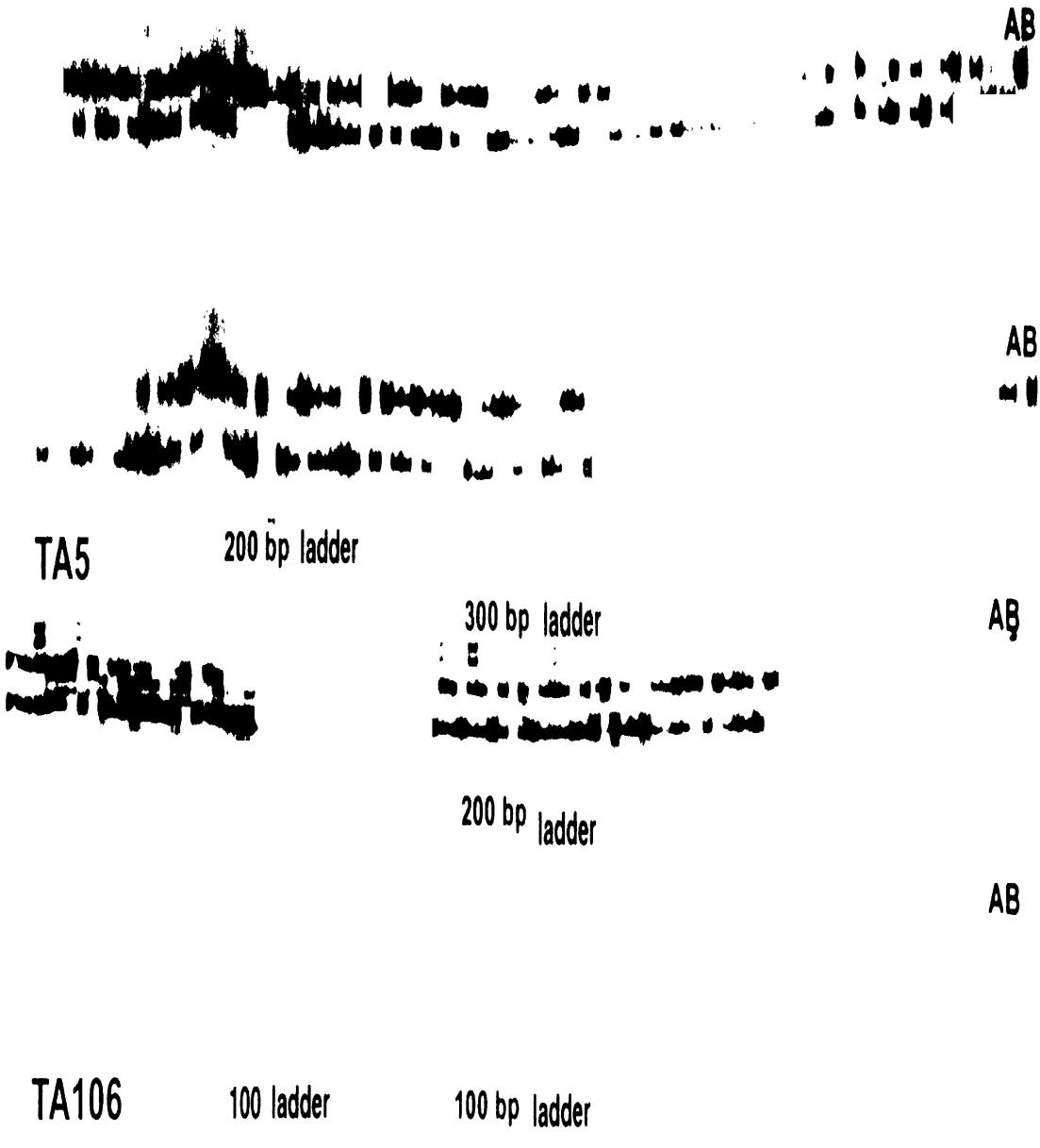


Figure4.4 Segregation of SSR markers in F_2 mapping population of ICC4991 x ICC04516 (separated on 6% PAGE)

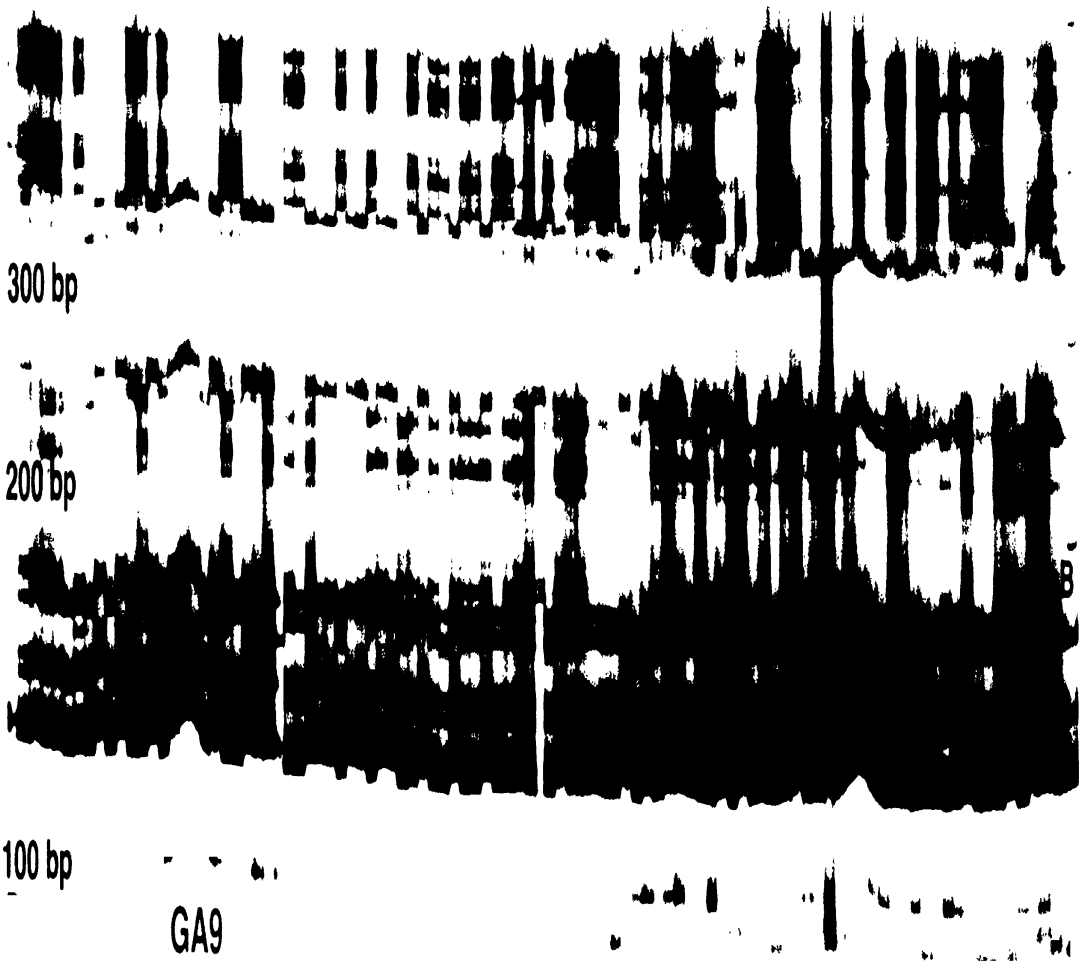


Figure4.5 Segregation of SSR markers in F_2 mapping population of ICC4991 x ICC04516 (separated on 8% denaturing PAGE)

Figure4.6 Phenograms depicting segregation of SSR markers across F_2 mapping population separated by Capillary Electrophoresis ABI PRISM 3700 (Applied Biosystems)

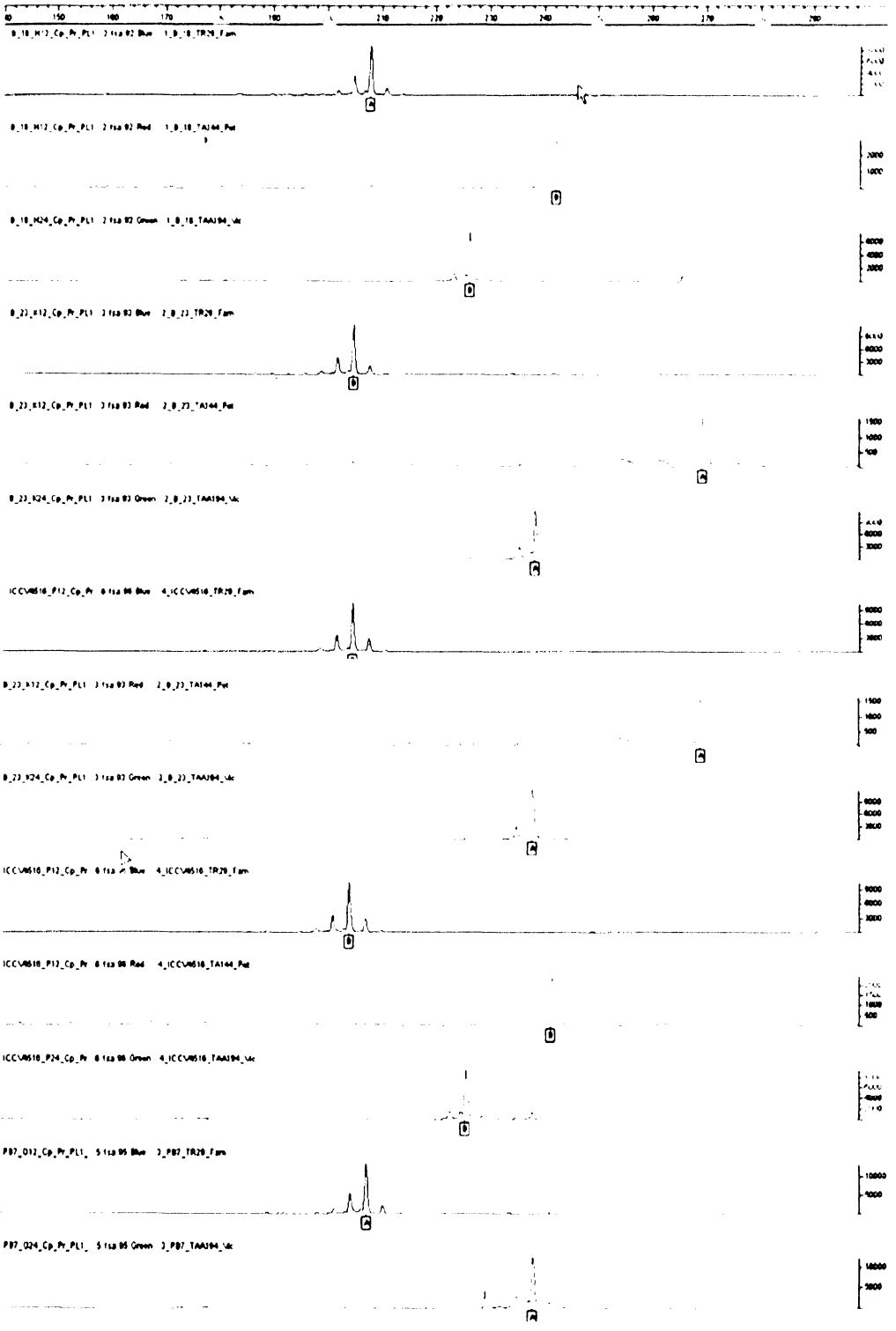


Table 4.2 Proportion of polymorphic markers identified for use in mapping

Marker type	No. of markers screened	Polymorphic markers	No. of markers used for mapping
SSR	232	96 (41.37%)	82
EST	108	3 (2.78%)	2
RGA	15	0 (0%)	-
Total	355	99	84

4.1.5 General features of linkage map

An intraspecific genetic linkage map of Pb-7 (ICC4991) x ICCV04516 was constructed using Kosambi mapping function with SSR and EST markers (Figure 4.7) and Appendix VI. The markers were included on the map only if the LOD value obtained was > 3.0. The total map length spanned a distance of 724.4 cM with an average marker density of 8.62 cM. 82 SSR markers and 2 EST markers were distributed into ten linkage groups (8 major and 2 minor groups), however, eight major linkage groups were assigned corresponding to the basic chromosome number of chickpea. LG5A and LGB5B are sub groups of LG5. Similarly LG8A and LG8B are considered to be part of LG8.

The linkage groups were numbered after comparative study of the previously published intraspecific chickpea maps of Udupa and Baum *et al.* (2003) and Millan *et al.* (2003). The general features of the map are summarized in Table 4.3 and Figure 4.7.

4.1.5.1 Linkage group 1

The markers TR8, TA113, TA8, TA203, TR53, TS71, GAA44 and AGLC11 were evenly distributed at average marker density of 9.67. AGLC11 a new EST marker was placed at the distal end of the linkage group # 1 for the first time.

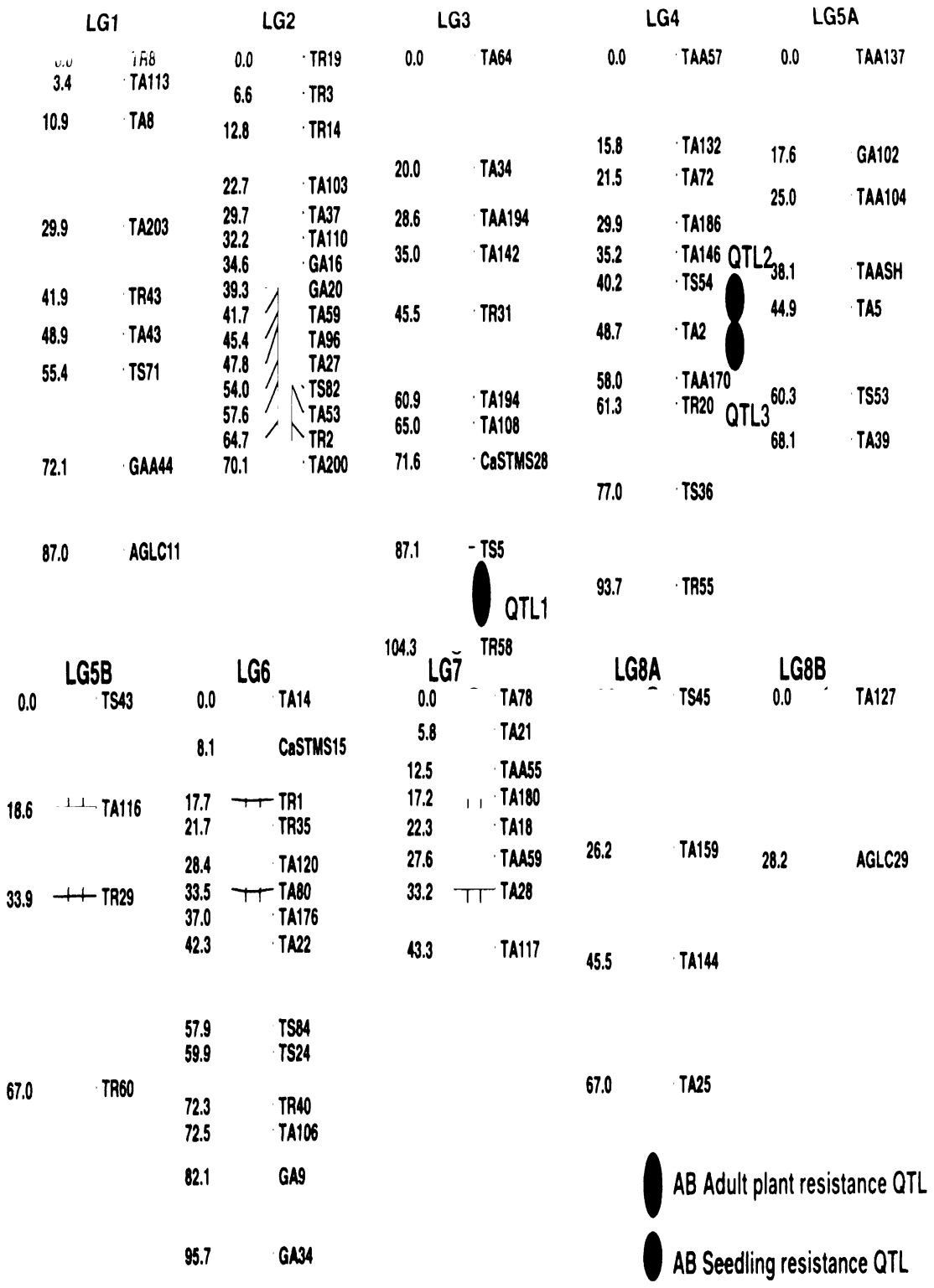


Figure 4.7 Intraspecific genetic linkage map of Pb-7(ICC4991) x ICCV04516

4.1.5.2 Linkage group 2

The markers included in this linkage group were TR19, TR3, TR14, TA103, TA37, TA110, GA16, GA20, TA59, TA96, TA27, TS82, TA53, TR2 and TA200 with an average density of 4.67 cM. LG-2 was of most dense group among all, with even distribution of markers.

Table 4.3 Overview of the intraspecific linkage map of ICC4991 x ICCV04516

Linkage group	Size cM	Segregation of mapped markers		Total number of mapped markers	Average marker density	Remarks
		Mendelian segregation	Distorted segregation			
LG1	87	8	1	9	9.67	-
LG2	70.1	11	4	15	4.67	Densest LG
LG3	104.3	6	4	10	10.43	Longest LG
LG4	93.7	7	3	11	8.51	-
LG5A	68.1	6	1	7	9.7	Sub group of LG5
LG5B	67.0	3	1	4	16.75	Sub group of LG5
LG6	95.7	11	3	14	6.83	2nd largest LG
LG7	43.3	7	1	8	5.41	2nd densest LG
LG8A	67.0	2	2	4	16.75	Sub group of LG8
LG8B	28.2	2	0	2	14.1	Sub group of LG8

4.1.5.3 Linkage group 3

The markers included in this linkage group were TA64, TA34, TAA194, TA142, TR31, TA194, TA108, CASTMS28, TS5 and TR58. The average marker density of this linkage group was 10.43 cM. This was the longest linkage group spanning a distance of 104.3 cM.

4.1.5.4 Linkage group 4

The linkage group 4 spanned a distance of 93.7 cM and consisted of TAA57, TA132, TA72, TA186, TA146, TS54, TA2, TAA170, TR20, TS36 and TR55 markers.

4.1.5.5 Linkage group 5 (Linkage group 5A and Linkage group 5B)

The linkage group 5 included two subgroups LG5A and LG5B. LG5A spanned a distance of 68.1 cM. The markers included in this subgroup were TAA57, GA102 TAA104, TAASH, TA5, TS53 and TA3. Only four markers TS43, TA116, TR29 and TR60 were distributed on the second sub group LG5B at average density of 16.75. Udupa and Baum (2003) and Millan *et al.* (2003) assigned TS43 to LG5. TS43 and TR29 were designated on LG 5 by Winter *et al.* (2000). Therefore, LG5B was designated as sub group of LG 5. Though these two sub groups were in the same grouping node in the joinmap linkage analysis, these could not be joined due to insufficient linkage.

4.1.5.6 Linkage group 6

LG6 is the second longest group spanning a distance of 95.7 cM including 14 markers. TA14, CaSTMS15, TR1, TR35, TA120, TA80, TA176, TA22, TS84, TS24, TR40, TA106, GA9 and GA34 were mapped at an average density of 6.83 cM.

4.1.5.7 Linkage group 7

The markers TA78, TA21, TAA55, TA180, TA18, TAA59, TA28 and TA117 were placed in this linkage group covering a distance of 43.3 cM at an average density of 5.41 cM. This linkage group was the second dense group after LG2.

4.1.5.8 Linkage group 8 (Linkage group 8A and Linkage group 8B)

Two sub groups LG8A (67 cM) and LG8B (28.2 cM) were considered to be part of linkage group 8 as marker TA127 was mapped in same linkage group 8 in skeleton map of ILC272 x ILC3279 F_2 mapping population (H. K. Buhariwala personal communication). The subgroups remained unlinked due to some missing markers in between them in spite of their placement in the same grouping node. LG8A included TS45, TA159, TA144 and TA25. However, only two markers, TA127 and AGLC29, constituted LG8B.

4.1.5.9 Ungrouped Markers

The markers GA6, TAA169, TA11, CaSTMS10, TS29, AGLC66, TA118, TR5, TA136, CaSTMS25, GA26, TS46, GAA60, TA196 and CaSTMS21 remained ungrouped. Most of these markers showed deviations from the Mendelian ratio of 1:2:1. However, the markers TA118, CaSTMS25 and TA196 followed the normal Mendelian segregation.

The gene-based markers (108 ESTs markers) were attempted for linkage study. However, very low polymorphism between parents was the main bottleneck for their utilization in linkage analysis. Three markers, AGLC11 (arm repeat containing protein) AGLC29 (hypothetical protein) and AGLC66 (probable cystein proteinase), were polymorphic between the parents ICC4991 and ICCV04516. Two of these markers AGLC11 and AGLC29 were mapped on

LG1 and LG8B, respectively. Association of these gene-based markers to disease resistance would have more practical applications in the molecular breeding programs.

4.2 Identification of AB resistance QTLs

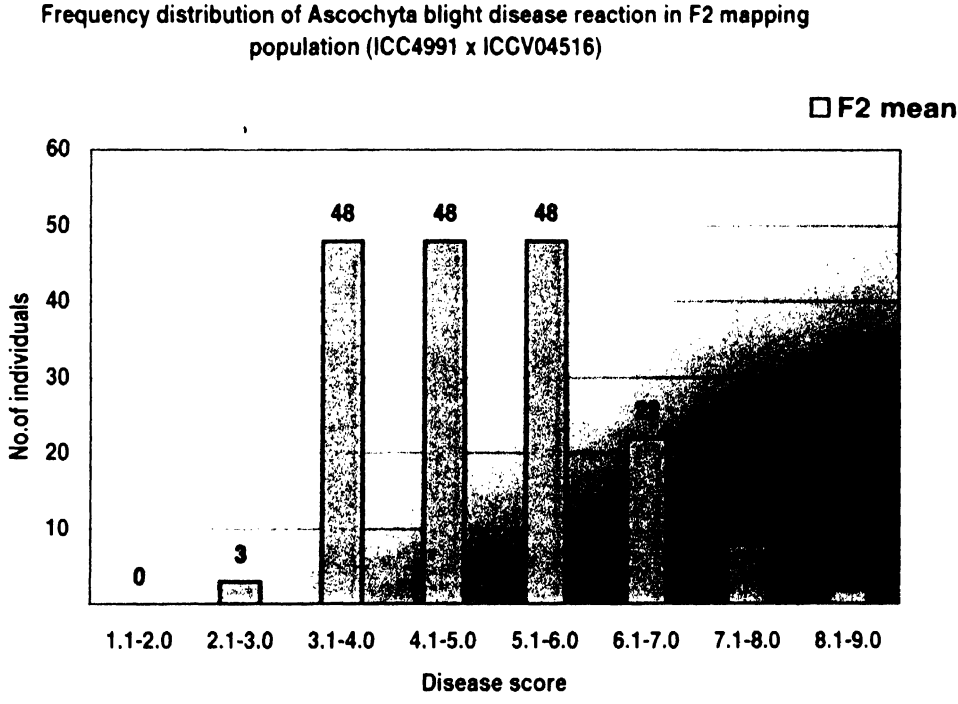
4.2.1 F_2 and $F_{2:3}$ Phenotyping for AB resistance

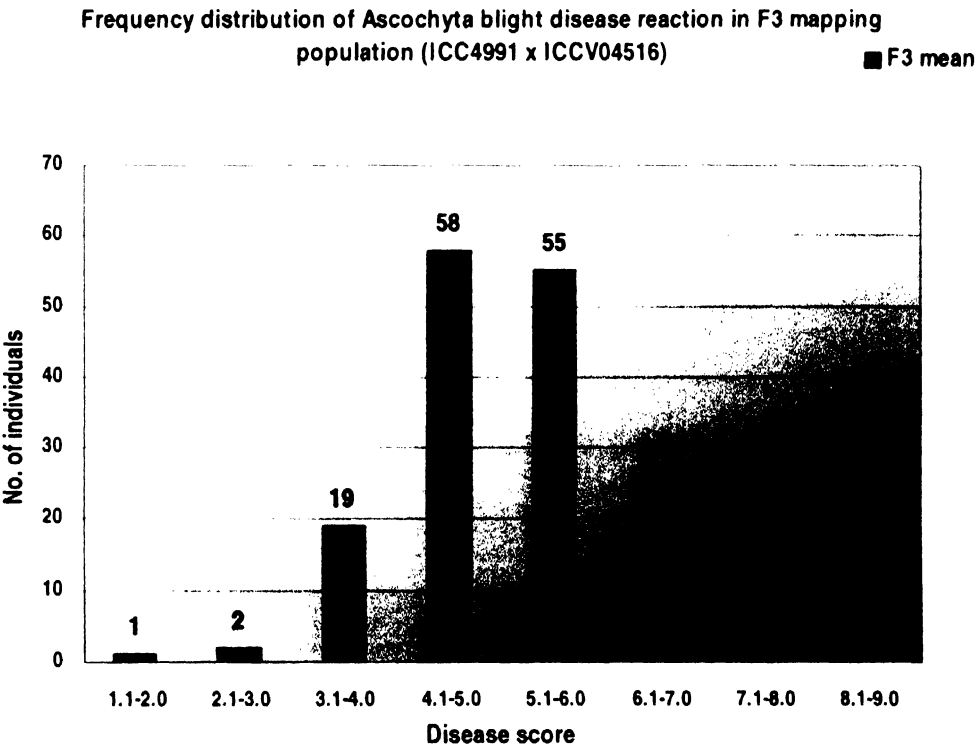
The phenotyping against Ascochyta blight disease was carried out in F_2 as well as $F_{2:3}$ progenies of ICC4991 x ICCV04516. F_2 plants (n=179) were genotyped and screened against AB pathogen by cut twig method using 80 days old plants (Figure 3.2). The destructive seedling screening method was avoided in F_2, so that seeds can be harvested from these plants to obtain next generation. F_3 phenotyping was carried out by seedling screening method (Figure 3.3). Screening was carried out under controlled conditions using a standardized method developed at ICRISAT. The inoculum was a single spore virulent culture of Hissar isolates of *Ascochyta rabiei* at a concentration of 50,000 spores /ml. The disease was scored when the susceptible check completely dried out i.e., on 10 DAI on a 1-9 scale (Figure 3.4). Consistent data from 179 plants was used in both generations for marker association studies and QTL detection. The mean disease reactions are presented in Table 4.4.

4.2.1.1 F_2 phenotypic variation

The resistant cultivar ICCV04516 showed a mean disease reaction of 3.67 while susceptible parent was scored 9. The frequency distribution of the average disease score of three cut twigs, across 179 F_2 plants is presented in Figure 4.8. Immune reaction was absent in the entire population. The pattern of segregation in the F_2 population was found to be continuous depicting quantitative nature of AB resistance. However, large number of plants could be classified into major

Figure 4.8 Frequency distribution of disease scores for Ascochyta blight resistance in F_2 and $F_{2:3}$ populations





categories of moderate resistant class (3.1-5.0) and susceptible class (5.1-7.0). Only three plants were classified as resistant (1.1-3.0) and 10 plants as highly susceptible (7.1-9.0).

4.2.1.2 F_3 phenotypic variation

The frequency distribution of the mean disease score of F_3 plants (n=24) across 179 lines is presented in Figure 4.8. The segregation of the AB disease reaction revealed a continuous quantitative nature. Immune reaction was absent in the entire population. Similar to the F_2 population, majority of the F_3 plants could be classified into categories of moderate resistant class (3.1-5.0) and susceptible class (5.1-7.0) and lesser proportion of AB population in the tails. Descriptive statistics of mean AB disease reactions in the F_2 and $F_{2:3}$ segregating populations is presented in the Table 4.5.

4.2.1.3 Analysis of variance (ANOVA)

The mean AB disease score of eight seedlings for each $F_{2:3}$ progeny in the three replications were subjected to analysis of variance (ANOVA).The ANOVA obtained by completely randomized design is presented in Table 4.6.

The F calculated value was significant at 1% level of significance, suggesting that the genotypes under consideration showed considerable variation of the AB disease reactions. The standard error mean is 0.3013 and standard error of difference is 0.4256. The heritablity (in broad sense) was 0.973.Best Linear Unbiased Predictors (BLUPs) of the random effect were computed in restricted maximum likelihood (REML) Variance Components Analysis from Genstat version 8.0 with replicates as fixed model and genotypes as random effects. The estimated variance is 1.2439 and standard error is 0.1587.The data showed a good variation for the character under study, deduced from the ratio of estimated

variance and standard error. The predicted means (BLUPs), thus obtained were used for QTL detection in F_3 generation.

4.2.2 QTL Mapping

QTL mapping was undertaken with AB disease reactions of F_2 and $F_{2:3}$ mapping populations using QTL cartographer 2.0 which is based on the maximum likelihood algorithm was used for detecting the AB resistance QTLs. Phenotyping for the disease reaction was done at different developmental stages of plants, adult plant (80 days) in F_2 population and seedling stage (14 days) in $F_{2:3}$ population. Therefore detection of genomic regions responsible for AB resistance was carried out using phenotypic data obtained in both F_2 and $F_{2:3}$ generations separately in order to identify adult plant and seedling AB resistance QTLs, respectively. Single marker analysis, and composite interval mapping (CIM) strategies were employed to detect AB resistance QTLs.

4.2.2.1 Adult plant resistance

4.2.2.1.1 Single marker analysis

Simple linear regression was performed to identify markers significantly associated with resistance to AB based on the LRmapqtl program. F-statistics were used to test the significance of the regression (that marker was unlinked to the quantitative trait). A significance level of $P < 0.01$ was used to declare the regression significant. LR is likelihood ratio test was $-2\log(L0/L1)$, where L1 was the likelihood that the marker was associated with the resistance and L0 was the likelihood that the marker was not associated with the resistance. The single marker analysis detected seven SSR markers associated with AB resistance (Table 4.7). Likelihood ratio was highest for TA39 (6.1818) followed by CaSTMS15 (5.3312).

Table 4.4 Mean Ascochyta blight disease reactions in F_2 and F_3 population (ICC4991 x ICCV04516)

S. No.	F_2mean	F_3 mean	S. No.	F_2mean	F_3 mean	S. No.	F_2mean	F_3 mean
1	5.70	5.34	41	5.40	5.46	81	5.30	5.86
2	4.00	4.42	42	6.60	6.39	82	5.00	5.83
3	3.70	5.21	43	7.30	6.18	83	6.00	6.46
4	4.30	4.64	44	7.00	6.46	84	4.00	4.88
5	7.00	7.78	45	5.00	6.39	85	4.00	4.49
6	4.30	4.86	46	4.80	5.30	86	4.00	5.05
7	4.70	5.35	47	6.00	5.23	87	3.70	5.69
8	3.50	4.38	48	5.80	5.55	88	3.17	4.07
9	6.00	6.39	49	7.00	5.15	89	4.00	5.90
10	6.00	7.03	50	7.20	5.93	90	4.70	4.79
11	5.00	5.71	51	7.00	6.00	91	6.00	8.15
12	5.70	5.27	52	6.00	5.98	92	3.83	4.24
13	4.40	4.64	53	6.83	5.30	93	4.33	5.02
14	6.33	5.90	54	6.60	6.85	94	3.17	4.75
15	6.30	6.21	55	6.00	6.18	95	4.00	5.23
16	5.20	5.83	56	5.00	5.64	96	4.70	5.90
17	5.30	4.77	57	5.50	5.97	97	7.00	7.87
18	2.70	3.40	58	4.00	4.53	98	4.00	5.79
19	4.00	4.36	59	3.80	4.48	99	4.00	5.90
20	5.25	5.34	60	5.00	5.86	100	5.30	5.62
21	5.00	5.76	61	5.00	5.37	101	4.70	4.91
22	5.00	6.18	62	4.33	4.69	102	3.50	4.16
23	5.00	5.34	63	5.30	4.95	103	3.67	4.07
24	5.00	6.08	64	6.00	5.81	104	4.40	4.61
25	3.00	4.42	65	5.00	4.77	105	6.00	7.03
26	5.00	6.75	66	5.33	5.90	106	6.19	6.06
27	5.80	7.17	67	5.00	5.09	107	5.70	5.44
28	7.00	6.75	68	4.33	4.53	108	4.60	5.62
29	4.30	4.74	69	4.50	4.24	109	6.00	6.66
30	4.80	4.91	70	4.35	4.53	110	4.33	4.77
31	5.00	6.95	71	4.50	4.60	111	4.80	4.91
32	4.00	4.67	72	4.00	4.24	112	4.00	4.96
33	4.00	4.89	73	5.17	4.98	113	4.00	5.62
34	6.30	5.79	74	3.30	4.21	114	4.30	5.09
35	5.80	6.43	75	5.80	4.98	115	3.00	3.60
36	3.30	3.67	76	7.00	6.04	116	6.00	5.93
37	3.30	2.98	77	6.67	7.03	117	4.30	5.41
38	6.00	5.61	78	5.50	5.41	118	4.00	4.52
39	5.20	5.02	79	4.00	4.04	119	5.50	6.46
40	5.50	5.55	80	4.70	5.15	120	4.00	4.53

Cont...

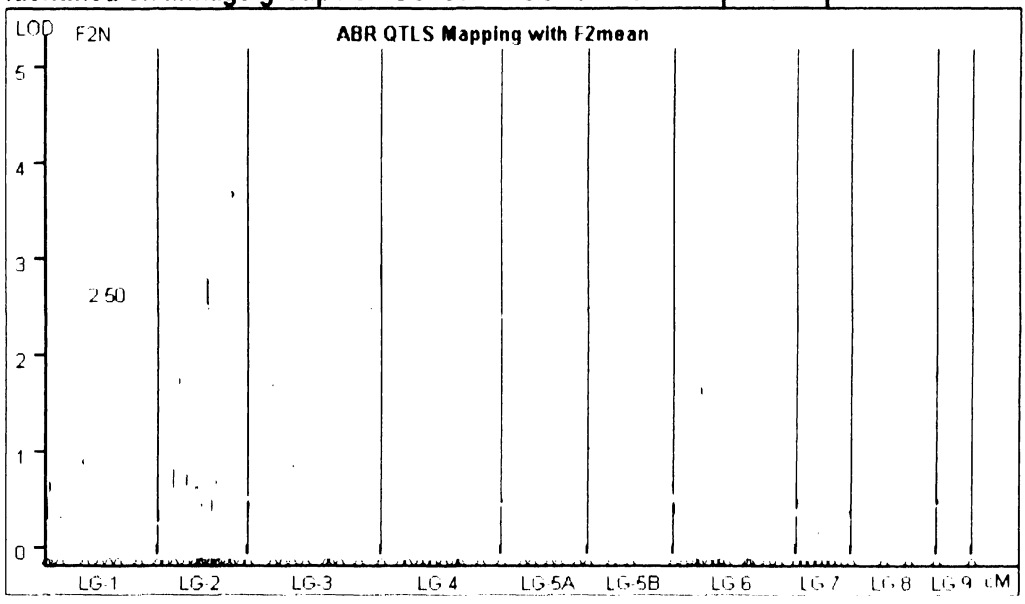
S. No.	F_2mean	F_3 mean	S. No.	F_2mean	F_3 mean
121	5.50	5.62	161	6.00	4.82
122	6.00	6.66	162	8.00	6.84
123	5.33	6.39	163	5.00	4.63
124	4.00	4.77	164	4.33	4.70
125	4.00	4.77	165	8.40	5.46
126	4.50	4.77	166	6.17	5.27
127	6.30	6.96	167	5.16	5.19
128	4.00	3.34	168	4.00	4.21
129	4.00	4.55	169	5.00	4.49
130	5.00	6.62	170	8.50	5.02
131	4.25	4.77	171	4.50	5.22
132	5.60	5.73	172	4.00	4.10
133	4.50	3.75	173	7.33	8.16
134	5.20	5.55	174	7.00	7.65
135	5.20	5.44	175	7.80	3.86
136	3.60	4.36	176	5.70	4.38
137	3.67	3.88	177	4.00	4.34
138	6.00	6.22	178	6.50	5.76
139	5.20	6.89	179	5.17	4.34
140	3.25	3.13			
141	5.30	5.75			
142	4.00	4.17			
143	6.30	6.98			
144	3.67	4.53			
145	7.60	7.31			
146	3.33	3.88			
147	4.00	4.88			
148	4.00	5.29			
149	5.50	6.08			
150	3.83	3.64			
151	4.67	4.77			
152	3.50	3.82			
153	4.00	4.01			
154	4.80	4.89			
155	5.30	5.34			
156	7.60	7.31			
157	4.25	4.98			
158	6.17	5.62			
159	7.70	7.65			
160	7.00	5.90			

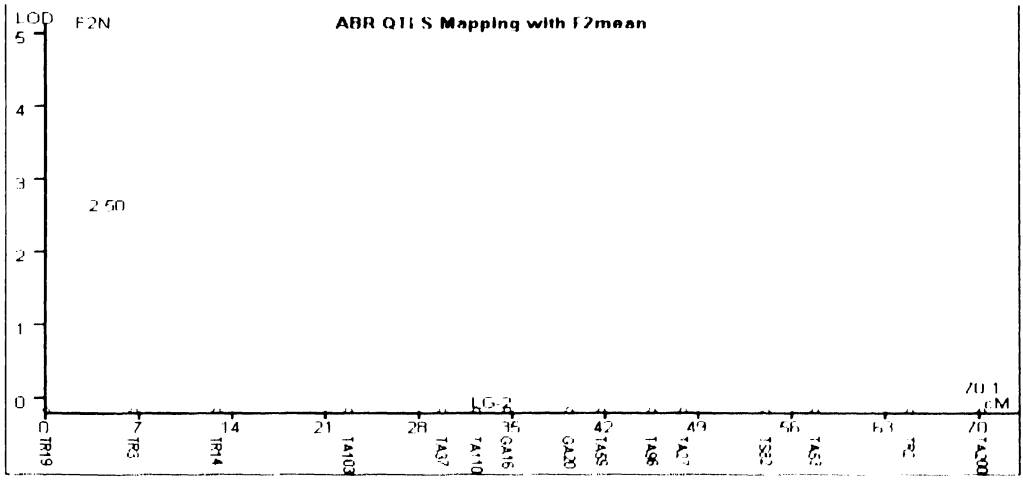
The seven markers identified were further subjected to regression analysis against the F_2 mean ABR data using Genstat version 8.0 to compute the phenotypic variance explained by these markers. The phenotypic variance accounted was as follows: GA20 (1.2%), TA142 (1.5%), TA18 (2.1%), TA21 (1.2%), TA39 (4.2%) and TR58 (1.8%).

4.2.2.1.2 Composite Interval Mapping (CIM)

The CIM method (Jansen and Stam 1994; Zeng 1994) was used to determine the location of QTLs. Cofactors were selected by the program using Model 6 with genetic background controlled by five markers and window size set at 10 cM. Forward and reverse regression analysis was employed for QTL detection. All the linkage groups were scanned at minimum default threshold of LOD 2.4 with 300 permutations (P<0.05%). A QTL peak Figure 4.7 and 4.9 on LG3 detected the presence of a QTL (QTL1) at position 95.11 cM, 9.19 cM away from marker TR58 at a LOD of 2.032 (Table 4.8). The phenotypic variance explained by a single QTL was estimated by the square of the partial correlation coefficient (R^2). Estimates of R^2 value and additive effects for each QTL at its peak LOD position were obtained from the QTL analysis using Zmapqtl program of the QTL Cartographer. The phenotypic variance (R^2) explained was 18.62%. Another putative QTL region was also detected on LG2 Figure 4.9 at loci GA20. Single marker analysis detected a QTL at GA20. However, this could not be confirmed by either IM or CIM strategy.

Figure 4.9 Graphical representation of adult plant AB resistance QTLs identified on linkage groups of ICC4991 x ICCV04516 intraspefic map





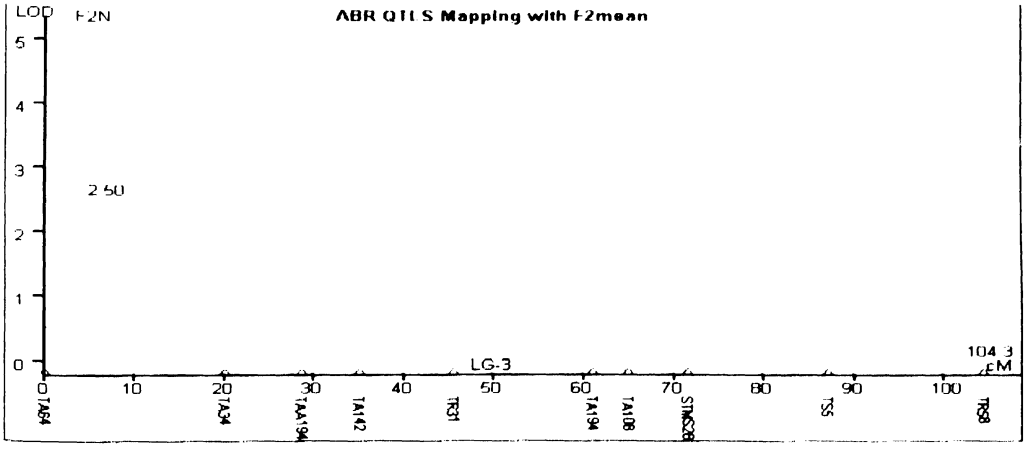


Table 4.5 Descriptive statistics of mean Ascochyta blight disease reaction in the F_2 and $F_{2:3}$ segregating populations of (ICC4991 x ICCV04516)

Population	Sample size	Mean	Range	Standard error	Standard deviation	Coefficient of variance	Average deviation	Skewness	Kurtosis
F_2 (ICC4991 x ICCV04516)	179	5.076	2.7-8.5	0.089	1.197	0.2358	0.969	0.9319	5.866
$F_{2:3}$ (ICC4991 x ICCV04516)	179	5.35	2.98-8.16	0.076	1.0196	0.1907	0.8146	0.4286	3.3057

Table 4.6 Analysis of variance for Ascochyta blight disease reactions in $F_{2:3}$ plants

Source of Variation	Degree of freedom	Sum of squares	Mean sum of Squares	F ratio
Treatment	178	15974.1	89.742	109.8**
Error	358	292.589	0.8172	
Total	536	16266.69		

Table 4.7 Association of genetic loci with Ascochyta blight disease reaction scores based on simple linear regression analysis of F_2 means

S.No.	Loci	Linkage Group	Likelihood ratio (LR)	Pr-value	%Phenotypic variance (R^2) value
1.	GA20	LG2	4.1354	0.0435*	1.2
2.	TA142	LG3	4.0031	0.0469*	1.5
3.	TR58	LG3	3.9707	0.0478*	1.8
4.	TA39	LG5	6.1818	0.0136*	4.2
5.	CaSTM15	LG7	5.3312	0.0219*	0.0
6.	TA21	LG8	4.1187	0.0439*	1.2
7.	TA18	LG8	4.5223	0.0347*	2.1

LR is likelihood ratio test is –2log(L0/L1). Pr is the probability that trait is unlinked to the marker. Significance at 5% level indicated by *.

Table 4.8 Map location and estimated effects of quantitive trait loci providing adult plant resistance to AB in F_2 population based on composite interval mapping

Linkage Group	Marker	Position	LOD	R^2	Additive Effect
LG3	TR58	95.11	2.0322	0.18	0.6725

4.2.2.2 Seedling resistance

4.2.2.2.1 Single marker analysis

The single marker analysis detected nine SSR markers associated with AB resistance (Table 4.9). The nine markers identified were further subjected to multiple regression analysis against the F_3 AB predicted mean data using Genstat version 8.0 to compute the phenotypic variance explained by these markers. The phenotypic variance explained by each marker is given in the Table 4.9. Likelihood Ratio was highest for TA54 (6.87) followed by TA146 (9.33) explaining 4.6% and 6.55% of total phenotypic variance.

Table 4.9 Association of marker loci with Ascochyta blight disease reaction scores based on simple linear regression analysis of F_3 means

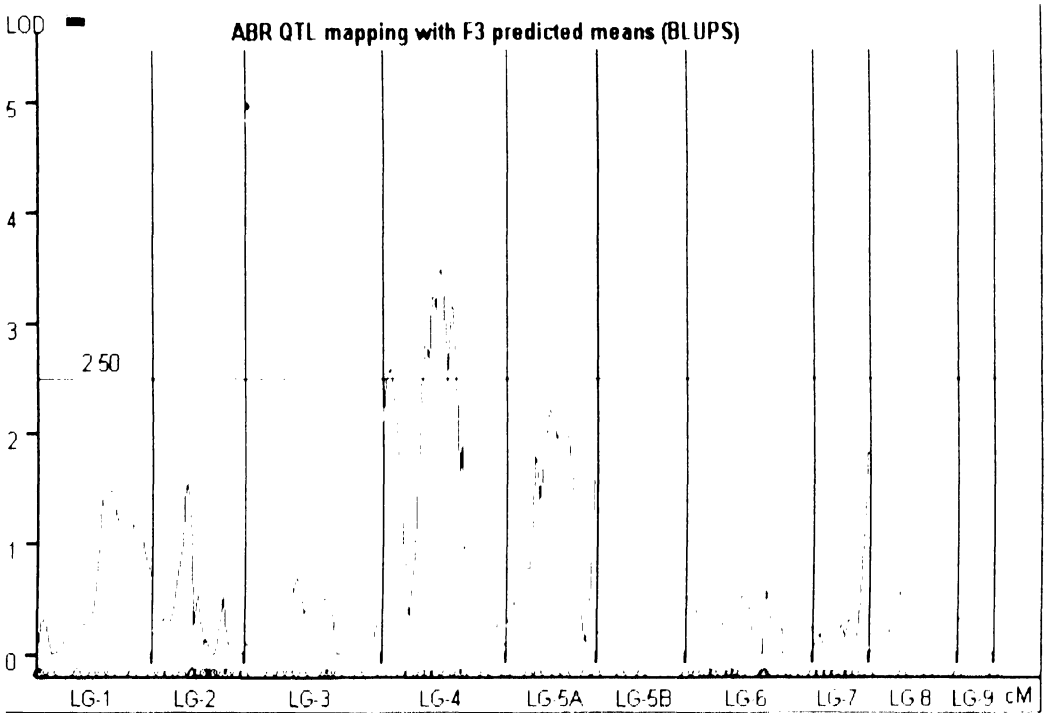
S.No.	Loci	Linkage Group	Likelihood ratio (LR)	Pr-value	%Phenotypic variance (R^2) value
1.	TR43	LG1	3.9870	0.0474*	3.34
2.	TA37	LG2	4.1806	0.0423*	3.75
3.	TA146	LG4	6.8795	0.0092**	6.55
4.	TS54	LG4	9.3392	0.0024**	4.6
5.	TAA170	LG4	4.5078	0.0350*	1.0
6.	TR20	LG4	5.284	0.0225*	2.3
7.	TR55	LG4	3.9826	0.0475*	1.4
8.	GA102	LG5	5.8139	0.0167*	5.32
9.	TA5	LG5	5.256	0.0226*	0

LR is likelihood ratio test is $-2\log(L0/L1)$. Pr is the probability that trait is unlinked to the marker. Significance at 5% and 1% level indicated by * and **.

4.2.2.2.2 Composite Interval Mapping (CIM)

All the linkage groups were scanned at minimum default threshold of LOD 2.4 with 300 permutations ($P<0.05\%$) using Model 6 of CIM of the QTL Cartographer. CIM method confirmed the presence of two QTLs (QTL2 and QTL3) on LG4 with regions covered by SSR markers TA146, TS54, TA2 and TAA170 (Figure 4.7 and Figure 4.10). The location of QTLs on LG4 along with their LOD scores and positions are presented in the Table 4.10.The QTL2 and QTL3 accounted for 7.745% and 9.28% of total phenotypic variance, respectively. Both the QTLs on LG4 together explained a total phenotypic variance of 17.02% for seedling resistance to AB in $F_{2:3}$ populations.

Figure 4.10 Graphical representation of seedling AB resistance QTLs identified on linkage groups of ICC4991 x ICCV04516 intraspefic map with F2:3 means



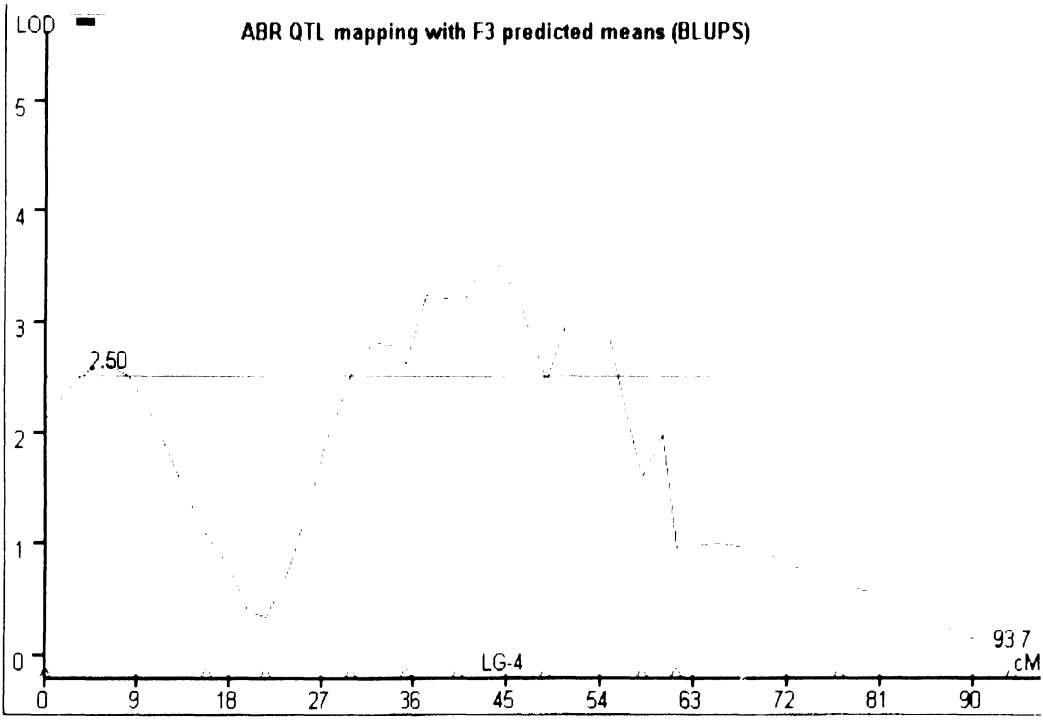


Table 4.10 Map locations and estimated effects of quantitive trait loci providing seedling resistance to AB in population $F_{2:3}$ lines based on composite interval mapping

Linkage Group	Marker	Position	LOD	R^2	Additive Effect
LG4	TS54	40.21	2.6828	0.0774	0.5341
LG4	TA2	50.71	2.15	0.0928	0.5693

4.3 Validation of QTL markers

4.3.1 Validation populations

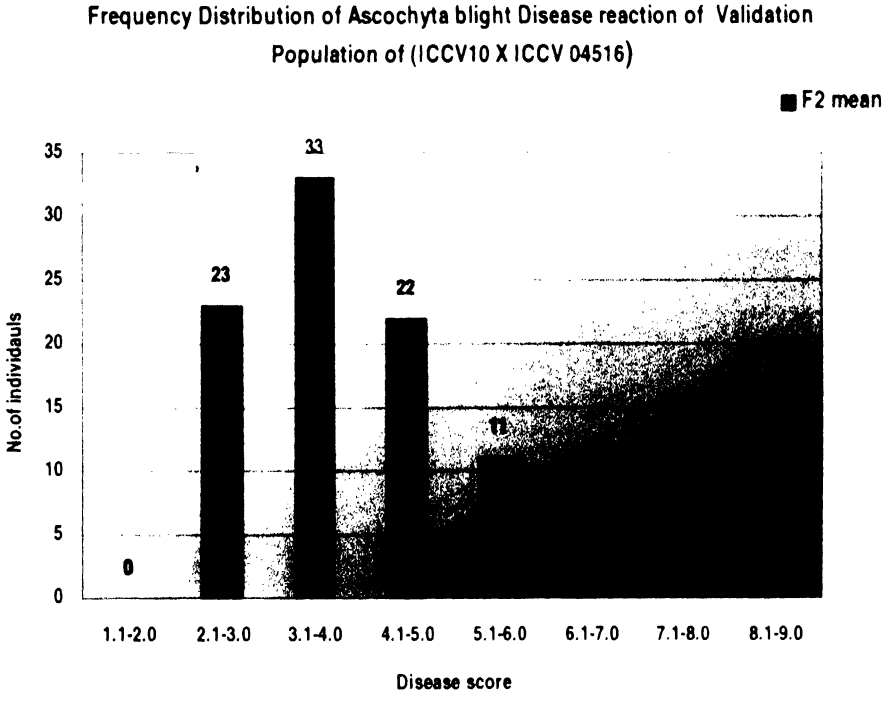
Two validation populations (n=94) were developed by crossing susceptible parents ICCV10 (average disease score of 8.0 to 9.0) and ICCL 87322 (average disease score of 9.0) with the resistant parent ICCV04516 (3.0-4.0) (Figure 3.1). The F_1s were selfed to obtain F_2 populations. DNA was extracted from 10 days old seedlings for the genotyping and the populations were screened for AB resistance at the 14 days old seedling stage.

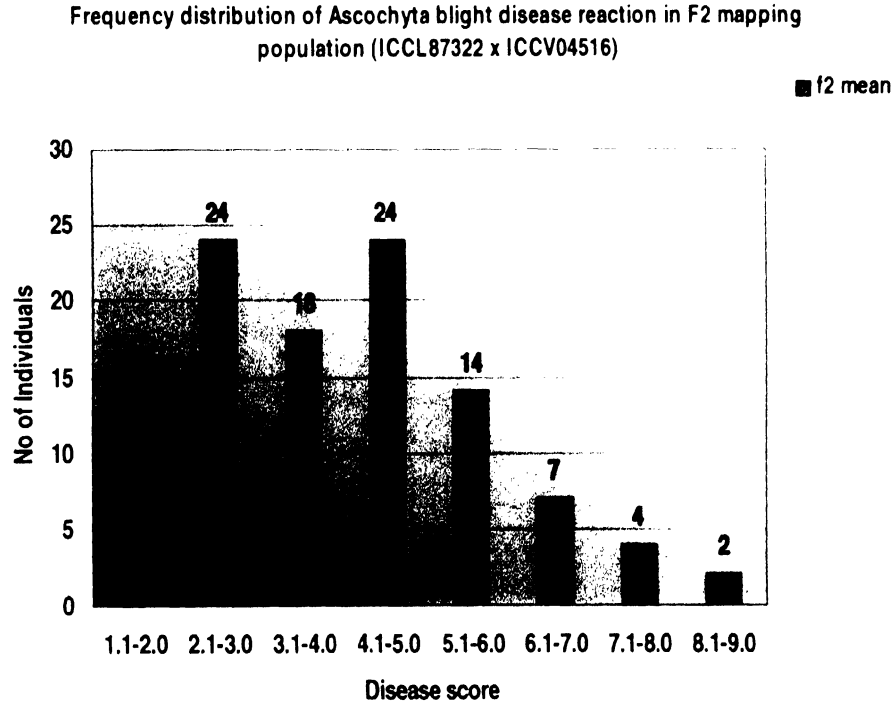
The frequency distribution of the disease score of validation each population was presented in Figure 4.11. The segregation of AB disease reaction revealed a continuous quantitative nature. Immune reaction was absent in the entire population. The mean disease reaction is presented in Table 4.11 Descriptive statistics of mean AB disease reactions in the both segregating populations is presented in the Table 4.12.

Table 4.11 Mean Ascochyta blight disease reactions in F_2 validation populations (ICCV10 x ICCV04516-VP1) and ICCL87322 x ICCV04516-VP2)

S. No.	VP1	VP2	S. No.	VP1	VP2	S. No.	VP1	VP2
1	3	5	38	6	5.5	75	4	3
2	2	4.5	39	3	3	76	4	3
3	3	4.5	40	3	6.5	77	4	3
4	3	5	41	4	7.5	78	2	4.5
5	2	3.5	42	3	4	79	4	5
6	4	6.5	43	3	2.5	80	5	2.5
7	2	4.5	44	3	4.5	81	3	6
8	3	5.5	45	4	4	82	4	5.5
9	2	3	46	6	5	83	4	6
10	3	7	47	2	3	84	4	4.5
11	3	5	48	2	3	85	3	3.5
12	4	8	49	2	3	86	6	4
13	5	7	50	2	4	87	2	3
14	3	5.5	51	3	4	88	5	3.5
15	3	4	52	2	4	89	2	3
16	3	9	53	3	4.5	90	4	4.5
17	4	4.5	54	3	5	91	3	2.5
18	4	7.5	55	3	2.5	92	4	2.5
19	5	6	56	2	3.5	93	4	6
20	4	5	57	3	2	94	2	3.5
21	3	4	58	2	5			
22	3	6.5	59	3	2			
23	5	6	60	5	1.5			
24	5	4.5	61	2	4			
25	4	4.5	62	5	5			
26	2	4.5	63	4	5.5			
27	5	3.5	64	5	3			
28	5	4	65	2	4.5			
29	2	7	66	3	6			
30	4	7.5	67	3	3.5			
31	9	5	68	2	9			
32	2	3	69	3	3			
33	2	5	70	4	6			
34	6	6.5	71	3	2			
35	3	5.5	72	3	2			
36	3	3.5	73	4	6			
37	3	3	74	2	2			

Figure 4.11 Frequency distribution of disease scores for Ascochyta blight in F_2 populations





The earlier reported markers were attempted for validation in these two populations (Table2.5). One of the main limitations was lack of polymorphism for the reported markers between the parents under study (ICCV10, ICL87322 and ICCV045160). The polymorphic markers scored for their segregations in these populations are presented in the Table 4.13. A total of six markers were genotyped across the two populations. The data from the genotyping was further subjected to regression analysis against the F_2 AB disease scores using Genstat version 8.0 to compute the phenotypic variance explained by these markers. The marker TA146 was found significantly associated with the seedling resistance in the F_2 population of ICCV10 x ICCV04516, explaining 18.89% of phenotypic variation followed by TR20 explaining 2.5 % of the phenotypic variation. TA146 was a tightly linked marker to AB QTLs at 0.1 cM (Flandez-Galvez et *al.*, 2003a) and also reported to be within QTL3 detected by Tekeoglu *et al.* (2004). This marker showed significant association to AB resistance marker analysis and further confirmed by CIM in $F_{2:3}$ progenies of ICC4991 x ICCV04516.

The indicative marker TA146 (Table2.4) was validated in the mapping population of ICCV10 x ICCV04516 also. So the validation can be said to have been accomplished across environments, diverse mapping population both intra and interspecific and against a different isolate of the pathogen.

However, markers TA146, GA16 and TS45 did not associate to AB resistance in second validation population of ICC4991 x ICCV04516 (Table 4.13).

Table 4.12 Descriptive statistics of mean AB disease score of AB in validation population of F_2 (ICCV10 X ICCV 04516) and F_2 (ICCL87322 x ICCV04516

Population	Sample size	Mean	Range	Standard Error	Standard Deviation	Coefficient of variation	Average deviation	Skewness	Kurtosis
F_2 (ICCV10 x ICCV 04516)	94	3.4149	2.0-9.0	0.1299	1.247	0.0365	0.9837	1.2666	3.129
F_2 (ICCL87322 x ICCV04516	94	4.5106	1.5-9.0	0.1685	1.618	0.0358	1.278	0.5444	0.0267

Table 4.13 Association of marker loci with Ascochyta blight disease reaction scores based on simple linear regression analysis of F_2 data

Population	Size	Markers	%Phenotypic Variance	SE_m
F_2 (ICCV10 x ICCV 04516)	94	TA146	18.89	1.19
		GA16	0.0	1.25
		TR20	2.5	1.23
F_2(ICCL87322 x ICCV04516)	94	TA146	*	*
		GA16	*	*
		TS45	*	*

* Markers found unassociated to AB resistance.

This study has validated the markers TA146 for AB resistance QTLs in chickpea and thus can be used in pyramiding AB resistance genes from diverse sources for developing cultivars with enhanced resistance to AB.

Chapter V

Discussion

CHAPTER-V

DISCUSSION

In recent years, the use of molecular markers has accelerated plant breeding in a number of areas including disease resistance, insect resistance and improving nutritious quality.(Melchinger, 1990). By utilizing a linkage map as a 'framework', the number and genomic positions of genes conferring quantitative resistance may be determined using QTL analysis (Patterson, 1996 and Young, 1996). The number and position of QTLs for resistance to many plant diseases have been determined using QTL analysis (Michelmore, 1995; Young, 1996 and Mohan *et al.*, 1997).

In chickpea, progress in development of genetic linkage map was initially hampered by low genetic polymorphism (Ahmed and Slinkard, 1992; Udupa *et al.*, 1993 and Labdi *et al.*, 1996). Considerable progress has been made after development of SSR markers (Huttel *et al.*, 1999 and Winter *et al.*, 1999). Interspecific and intraspecific linkage maps have been published (Table 3.2). In spite of the availability of several chickpea maps most of genomic regions harboring genes for important traits are not yet sufficiently saturated with co-dominant markers to apply MAS in plant breeding. Therefore, high density saturated genetic linkage map of chickpea with co-dominant PCR based markers (SSRs, gene based RGAs and ESTs and SNPs) is needed to provide sufficient markers for markers-assisted selection (MAS).

Ascochyta blight (AB) in chickpea is a highly devastating disease in many chickpea producing areas. In the Indian scenario AB is major constraint in the northwestern India. However, identification and molecular mapping of AB

resistance QTLs against an Indian isolate was lacking. A new chickpea cultivar ICCV04516 selected from F_6 progeny bulks of a double cross (C235 x NEC 138-2) x (FLIP 87-4C x ILC4421) developed at ICRISAT was identified as AB resistant against an Indian isolate collected from infected fields of Hissar region of northwestern India. ICCV04516 was utilized for developing a mapping population to construct an intraspecific linkage map, saturated with SSR markers and to detect the QTLs involved in AB resistance against an Indian isolate. The present investigation on "**Molecular mapping of Ascochyta blight resistance in chickpea (*Cicer arietinum L.*)**" was carried out with three major objectives: i) Construction of intraspecific genetic linkage map ii) Detection of the QTL regions responsible for Ascochyta blight resistance and iii) Validation of the markers linked to AB QTLs using different populations.

5.1 Construction of an intraspecific genetic linkage map

5.1.1 Simple Sequence Repeats

Proportion of parental polymorphism detected with SSR markers have been reported to be in a range from 41% to 50% (Flandez-Galvez *et al.*, 2003a; Huttel *et al.*, 1999 and Udupa and Baum, 2003). The PCR amplification conditions, size of polymorphic parental bands and the mode of electrophoretic separation for the polymorphic SSR and EST markers used for the linkage analysis are given in Table 4.1. Optimization of individual component concentrations was done using a modified Cobb and Clarkson (Cobb and Clarkson, 1994) as described by Buhariwalla *et al.* (2005) (Appendix 2.0). The total reaction volume was scaled to 5 µl and achieved in 384 well formats. It

resulted in considerable reduction in PCR costs and time consumed for genotyping.

5.1.2 Expressed Sequence Tags

A set of 108 ESTs was screened for parental polymorphism. The polymorphism detected was very low as only three primer pairs (2.7%) were polymorphic. Gene based markers are ideal markers for mapping disease resistance genes. However, this study detected low polymorphism for ESTs in contrast to higher polymorphism detected by Buhariwalla *et al.* (2005) in their diversity studies of *Cicer* species.

5.1.3 Resistance Gene Analogues

A set of 15 RGAs (JB1 to JB7 and Rga A to H) was screened between the parents. No polymorphism was detected between the two parents used (Figure 4.3b). The candidate gene approach has been particularly useful for the investigation of pest and disease resistance. A large group of plant resistance genes encode cytoplasmic receptor-like proteins that contain Leucine-Rich Repeat (LRR) and Nucleotide-Binding Site (NBS) domains. As a group, these genes have been called Resistance Gene Analogs (RGAs). The high degree of sequence conservation among the NBS-LRR class of resistance genes has permitted the design of degenerate oligonuleotides for use in PCR for gene isolation and subsequent development of molecular markers. The same approach has been used in chickpea where *C. arietinum* RGAs were used to isolate the orthologous alleles from *C. reticulatum* and where alleles were found to cluster into distinct classes, each associated with a known resistance phenotype (Huttel

et al., 2002). The RGAs A to H are reported by Huttel *et al.* (2002) have been used in this study but were monomorphic between the parents.

5.1.4 Cleaved Amplification Polymorphic sites

All the 15 chickpea RGAs and a sub set of 24 ESTs were subjected to CAPSs analysis. Out of these three ESTs, namely AGLC53 with *Aci* I, AGLC59 with *HpyCH*$_4$ V, and AGLC87 with *Hae* III, were found polymorphic. However, the CAPs generated were not used for the linkage analysis due requirement of further standardization of the protocol. The RGAs can be converted to a single copy PCR marker like CAPs (Konieczny and Ausubel, 1993). The CAPs were generated for RGAs and used for genetic mapping of sugarcane mosaic virus resistance in maize (Quint *et al.*, 2002). Recently, Rajesh *et al.* (2005) reported generation of six CAPs and dCAPs markers and fine mapping of QTL for Ascochyta blight resistance in chickpea.

5.1.5 Inheritance of the markers and linkage analysis

Each segregating marker was tested for goodness of fit to the expected 1:2:1 ratio using χ^2 test ($P < 0.05$). Sixty-three markers gave a good fit to the expected segregation ratio. A total of 39 (33.3%) markers in present study deviated from the 1:2:1 ratio in F_2 population. 20.9% showed highly skewed segregation pattern (Appendix V).

Segregation distortion for SSR markers has been reported earlier in chickpea. Thirty nine percent of the markers studied by Winter *et al.* (1999) and 27 % of the markers in the study of Collard *et al.* (2003) were reported to have distorted segregation ratios in interspecific mapping populations. High number of distorted loci in the interspecific population may have been due to recombination

suppression at meiosis caused by considerable degree of non-/ or partial homology between the species *C. arietinum* vs. *C. reticulatum* or *C. echinospermum*. Further segregation distortion would still accumulate in population with progressive selfing of cycles of meiosis undergone in the development of the RILs. A very low proportion of total markers studied revealed segregation distortion in the study of Udupa and Baum (2003) and up to 26.8% in study of Flandez- Galvez (2003a) were reported in their intraspecific mapping populations. However, all markers were used for the linkage analysis in spite of the distorted segregations for few markers.

5.1.6 General features of linkage map

An intraspecific genetic linkage map of Pb-7 (ICC4991) x ICCV04516 is constructed using Kosambi mapping function with SSR and EST markers at a minimum LOD value > 3.0. A total of 84 markers, 63 markers with 1:2:1 Mendelian inheritance and 19 markers with distorted segregation were mapped into eight major and two minor linkage groups. The total map length spanned a distance of 724.4 cM with an average marker density of 8.62 cM. The linkage groups were numbered after comparative study of the previously published intraspecific chickpea maps of Udupa and Baum (2003) and Millan *et al.* (2003). The markers were distributed into ten linkage groups; however, eight major linkage groups were assigned corresponding to the basic chromosome number of chickpea. LG5A and LGB5B are sub groups of LG5. Simialrly LG8A and LG8B are considered to be part of LG8. The markers assigned to LGs were compared with previously published maps (Winter *et al.*, 2000; Millan *et al.*, 2003 and Udupa and Baum, 2003.). The map published by Winter *et al.* (2000) is the most

comprehensive map of chickpea published so far and several workers have used it as reference map for comparison with their maps. Forty-two markers of this map were placed in the same LGs as that of Winter *et al.* (2000).

5.1.6.1 Linkage group 1

TA113, TA8, TA203 and TR43 were reported in earlier chickpea maps on the same linkage group (Millan *et al.*, 2003 and Winter *et al.*, 2000). However, the marker TA203 and TR43 were in reverse order and distances between these two markers also deviated. AGLC11 a new EST marker was placed at the distal end of the linkage group for the first time.

5.1.6.2 Linkage group 2

LG2 is the densest group with even distribution of markers. TR19, TA37, TA110, TA96, TA27, TA53, and TA200 SSR markers could be considered as anchor markers for this linkage group. These markers have been reported in the same LG in earlier chickpea maps, (Millan *et al.*, 2003; Udupa and Baum, 2003 and Winter *et al.*, 2000). However, the order of markers was slightly different.

5.1.6.3 Linkage group 3

TA64, TA194, TA142 and CASTMS 28 have been assigned to the same linkage group in earlier studies (Millan *et al.*, 2003; Udupa and Baum, 2003 and Winter *et al.*, 2000). This is the longest linkage group spanning a distance of 104.3 cM. TA64, TA34, TR31 and CASTMS28 had the same marker order as described by Winter *et al.* (2000).

5.1.6.4 Linkage group 4

This linkage group spanned a distance of 93.7 cM. The SSR markers TA72, TA146 TA2 and TR36 have been reported in the same LG by Winter *et al.*

(2000); Millan *et al.* (2003) and Udupa and Baum (2003), therefore could be considered as anchor makers for this linkage group.

5.1.6.5 Linkage group 5 (Linkage group 5A and Linkage group 5B)

This linkage group includes two subgroups LG5A and LG5B. LG5A spanned a distance of 68.1 cM. The markers included TAA57, GA102, TAA104, TAASH, TA5, TS53 and TA3. Only four markers TS43, TA116, TR29 and TR60 were distributed on the second sub group LG5B at average density of 16.75. Udupa and Baum (2003) and Millan *et al.* (2003) assigned TS43 to LG5. TS43 and TR29 were designated on LG 5 by Winter *et al.* (2000). Therefore, LG5B was designated as sub group of LG 5. Though these two sub groups were in the same grouping node in the Join map linkage analysis, these could not be joined due to insufficient linkage. Additional polymorphic markers would be needed for joining these sub groups.

5.1.6.6 Linkage group 6

The markers TA14, CaSTMS15, TR1, TR35, TA80, TA176, TA106, GA9 and GA34 were also assigned into the same linkage group by Winter *et al.* (2000) and Millan *et al.* (2003). The markers order was the same except for the reversal of marker order of TA14 and CaSTMS15 at proximal end of the linkage group.

5.1.6.7 Linkage group 7

The markers TA78, TA21, TAA55, TA180, TA18, TAA59, TA28 and TA117 were placed in this linkage group. Except for the SSR marker TAA55, the rest were assigned to the same LG by Winter *et al.* (2000).

5.1.6.8 Linkage group 8 (Linkage group 8 A and Linkage group 8B)

Two sub groups LG8A (67 cM) and LGB (28.2 cM) were considered to be part of linkage group 8 as marker TA127 was mapped in same linkage group 8 in skeleton map of ILC272 x ILC3279 F_2 mapping population (H.K.Buhariwala personal communication). The subgroups remained unlinked due to some more missing markers in between them in spite of their placement in the same grouping node.

5.1.7 Comparison with *Cicer* linkage maps

GAA47 was assigned to LG 7 in interspecific map of Winter *et al.* (2000) and Millan *et al.* (2003). However, due to close linkage this was assigned to LG1 in our map. Except for this deviation, the distribution of markers to linkage groups is comparable with earlier published maps. The deviation in the marker order in LGs was observed when compared to maps of Millan *et al.* (2003); Udupa and Baum (2003) and Winter *et al.* (2000), which were developed from RIL populations, whereas, the present map was developed from F_2 mapping population. So reversal of markers order may occur in few cases due to slight variation in recovery of recombinants.

A large variation in map length of *Cicer* genome, 550 cM (Simon and Meuhlbauer, 1997) to 2077.9 cM (Winter *et al.*, 2000), has been reported with 9 to 16 LG. The inclusion of different type of marker systems, inclusion of skewed markers, use of different mapping populations (F_2 or RILs), use of different mapping software (which vary in their estimate of map distances), are few reasons which attributed to varied map length of the *Cicer* genome.

Estimated physical size of chickpea genome was 750 Mb (Arumuganathan and Earle, 1991). The genetic distance of 1 cM is equivalent to approximately 1.4 Mbp (1,400 Kbp). It requires at least 107 extensively distributed markers to resolve a marker density of 5 cM which is the upper limit required for marker assisted pyramiding of genes (Winter *et al.*, 1997). SSR markers remain the marker of choice for marker-assisted selection in many breeding programs. An intraspecific linkage map saturated with more markers would be quite useful to mapping and tagging of genes of complex traits like disease or drought resistance and marker assisted selection in breeding programs.

The linkage map published by Winter *et al.* (2000) included 118 SSR into 16LG and was based on an interspecific cross of *C. arietinum* x *C. reticulatum*. The interspecific maps suffer from disadvantage of having little direct application in breeding programs that generally use intraspecific crosses. The markers identified from interspecific crosses may not be polymorphic in intraspecific crosses. Thus a genetic linkage map constructed from an intraspecific mapping population is desirable.

The earlier published intraspecific maps (Table 3.2) include a maximum of 68 SSR markers (68 STMS distributed in 14 LGs of Cho *et al.*, 2002; 52 SSR loci distributed in 8 LGs of Udupa and Baum 2003; and 53 SSR loci in 8LGs of Cho *et al.*, 2004). In the present study, 82 SSR and 2 EST markers were mapped in 10 LGs (8+2). It is a step towards developing a saturated map within the cultivated pool.

Recently Litchenvig *et al.* (2005) has developed 233 SSR from BAC and BIBIC library of *C. arietinum* cv Hadas. The utilization of these markers in

different mapping populations would enhance the saturation of the existing *Cicer* maps. There is a need to develop more SSR markers for creating saturated maps comparable to rice, tomato or soybean. However, combining different published maps into a consensus map is in progress (http:// www.icgc.wsu.edu).

In chickpea the association of all genetic linkage groups to well defined chromosomes has not yet been achieved. Efforts have begun to bridge the gap between the recombination based genetic map and the chromosome based maps. Individual chickpea chromosomes have been successfully sorted by flow cytometry (Vlacilova *et al.*, 2002) and utilized for mapping specific DNA sequences and genes to individual chromosomes. Fluorescent *In-Situ* hybridization (FISH) had resulted in localization of specific genes (coding for various RNA loci), major random repetitive DNA sequences, STMS markers, microsatellites, En/Spm-like transposon sequences, simple sequence repeats, and Arabidopsis-type telomeric sequences on the chickpea chromosomes (Gortner *et al.*, 1998 and Vlacilova *et al.*, 2002). Shortest LG8 identified by STMS GAA46 was associated to smallest chromosome 8 (H) by Vlacilova *et al.* (2002). In the present study GAA46 was monomorphic between the parents ICC4991 and ICCV04516, therefore could not be used in the linkage analysis.

Progress towards developing physical maps of chickpea has been initiated. Rajesh *et al.* (2004) developed bacterial artificial chromosome (BAC) library from FLIP84-92c to facilitate positional cloning of resistance genes (*Foc3* fusarium wilt resistance gene) and physical mapping of LG-2 genomic region where additional R genes against other races of wilt causing pathogen are positioned.

5.2 Identification of AB resistance QTLs

Genetics of Ascochyta blight resistance has been studied earlier and depending upon the resistance source, fungal isolate and scoring method, the resistance has been reported to be controlled by a single dominant or recessive gene, oligogenes or few to several QTLs. (Table 2.2). Santra *et al.* (2000) detected three QTLs in a RIL population developed from an interspecific cross *C. arietinum* (FLIP84-92C) x *C. reticulatum* (PI 599072) and linked them to RAPD markers. Since then several researchers have exploited RILs and F_2 mapping populations and detected QTLs conferring resistance to AB. Tekeoglu *et al.* (2002) added SSR markers and confirmed two QTLs for Ascochyta blight resistance earlier identified by Santra *et al.* (2000). The F_2 interspecific mapping population (*C. arietinum x C. echinospermum)* was used for detecting seedling resistance and adult plant resistance QTLs by Collard *et al.* (2003). Later on intraspecific populations were exploited for detecting AB resistance QTLs. Using an F_2 mapping population, Flandez-Galvez (2003a) reported 7 QTLs significant for blight resistance. Udupa and Baum (2003) attempted to elucidate the genetics of pathotype-specific blight resistance in chickpea using a RIL population. They mapped a major QTL *ar1* for resistance to pathotype I close to GA16 on LG2. Another two QTLs against pathotype II, *ar2a* and *ar2b*, were identified as independent recessive major resistance loci with complementary gene action on LG2 and LG4, respectively. Recently, Cho *et al.* (2004) employed both controlled greenhouse and field conditions to screen an intraspecific RIL mapping population. A total of five QTLs attributing to specific pathotype were detected on the genetic linkage map constructed with 53 STMS markers.

Different methods are applied for assessment of disease severity. Testing under controlled glass-house or growth chamber conditions (Singh *et al.*, 1992; Udupa and Baum, 2003 and Millan *et al.*, 2003) combined with field screening (Cho *et al.*, 2004) would very much help to improve the reproducibility of the result since severity and spread of disease are highly dependent on environmental conditions, especially humidity.

In many chickpea growing regions several patho- and genotypes of the fungus may coexist in the same field or even in the same lesion (Morjane *et al.*, 1994; Jamil *et al.*, 2000 and Peever *et al.*, 2004). Since random mating may occur between different pathotypes of the fungus carrying different mating type alleles (Barve *et al.*, 2003), genetic recombination may contribute to genotypic diversity and provide the fungus with an additional means to adapt to newly introduced resistant germplasm.

5.2.1 QTL mapping

Quantitative trait locus or "QTL" mapping is a means to estimate the locations, numbers, magnitude of phenotypic effects, and modes of gene action, of individual determinants that contribute to the inheritance of continuously variable traits. A wide range of mapping populations, backcross, F_2 selfing, or intercrossing, recombinant inbred lines, near isogenic lines and double haploid population are utilized. F_2 selfing or intercrossing of heterozygous F_1s creates population that segregates in the traditional 1:2:1 ratio, and enjoy the advantage of permitting the genetics to see the consequences of all possible 'dosages' of an allele. This permits estimation of mode of gene action (dominant, recessive, additive or most frequently some where in between). A traditional argument

against the use of F_2 populations in basic genetic studies is the difficulty in distinguishing whether heterozygotes at consecutive marker loci are replacement double parentals or double recombinants, but the implementation of maximum likelihood algorithms in a number of excellent software packages obviates this.

In the present study, detection of AB QTLs was undertaken using F_2 population and $F_{2:3}$ progenies of ICC4991 x ICCV04516 intraspecific cross of *C. arietinum*. Single marker analysis, interval mapping (IM) and composite interval mapping (CIM) strategies were employed to detect AB resistance QTLs using software QTL Cartographer 2.0.

5.2.2.1 Adult plant resistance

A QTL peak Figure 4.7 and 4.9 on LG3 detected the presence of a QTL (QTL1) at position 95.11 cM on the linkage group, 9.19 cM away from marker TR58 at a LOD of 2.03 (Table 4.8). The phenotypic variance (R^2) explained was 18.62%. Another putative QTL region was also detected on LG2 Figure 4.9 at loci GA20.Single marker analysis detected a QTL at GA20. However, this could not be confirmed by either IM or CIM strategy.

Cho *et al.* (2004) reported two QTLs (to be a single gene designated as Ar19), between GA20 and GA16 on LG2A + 6B with LOD score of 3.08 and 2.66, respectively. In this study, single marker analysis identified significant association of GA20 to AB resistance at adult plant stage at a LOD score 4.1354, which explained only 1.2% of total phenotypic variance. Though phenotypic variance explained was low it could be considered significant as it was validating the QTLs identified by Cho *et al.* (2004). However the IM and CIM strategy could not confirm a QTL in the region (Figure 4.9).

5.2.2.2 Seedling resistance

The QTL2 and QTL3 accounted for 7.745 % and 9.28 % of total phenotypic variance, respectively. Both the QTLs on LG4 together explained a total phenotypic variance of 17.02 % for seedling resistance to AB in $F_{2:3}$ populations. Out of the 9 SSR markers detected by single marker analysis, four SSR markers, TA37, TA146, TS54 and TR20, have been reported as indicative markers for AB resistance (Table 2.3). TA37 (LR =4.1806 & r^2 = 0.0375) was also reported as indicative marker for QTL 3 specific to pathotype 1 (Ar21d) on LG2B of 2 week old seedlings (Cho *et al.*, 2004). Flandez-Galvez *et al.* (2003a) identified 7 QTLs conditioning AB resistance in which CLRR inv and TA146 markers flanked QTL 3. The flanking STMS marker TA146 to QTL 3 was placed at interval of 0.1 cM in their map, which was advocated as a ready to use marker for gene pyramiding. The single marker analysis for seedling resistance at LR statistics of 6.87 % and explaining 6.5 % of phenotypic variance was confirmed in this study against the Hissar isolate. Tekeoglu *et al.* (2004) positioned QTL 2 on a LG4, which included 5 SSR markers, TA72s, TA2, TS54, TA146 and GA2. Single marker analysis associated the seedling resistance to the marker TA146. (LR Statistics 6.8795; r^2=6.5%) and TS54 (LR=9.33 and r^2=4.6) in this study confirming two QTLs with indicative markers TS54 at LOD 2.6828 and TA2 at LOD 2.15 together contributing to 17.02% of total phenotypic variance for seedling resistance to AB in $F_{2:3}$ progenies. Thus results of this study validated the QTL 2 positioned in LG4 by Tekeoglu (2004).

The present study identified three QTLs (QTL1, QTL2 and QTL3) Figure 4.7 influencing AB resistance. AB adult plant resistance QTL1 positioned on

LG3 at distance of 9.19 cM away from TR58 marker is reported for the first time against an Indian isolate of the pathogen. QTL2 and QTL3 were positioned of LG-4 with indicative markers TA146, TS54, TA2 and TAA170 were influencing AB seedling resistance against Indian isolate. In the previous studies AB resistance QTLs were reported in the same region of the LG-4 by Tekeoglu *et al.* (2002) and against a pathotype specific *ara2b* by Udupa and Baum (2003).

5.3 Validation of QTL markers

Marker Assisted Selection (MAS) is most useful for traits where phenotypic evaluation is expensive or difficult, particularly for those polygenic traits with low heritablity that are effected by the environment (Nienhuis *et al.*, 1987). MAS offer many advantages, like reducing the number of generations, selection based on genotypes rather than phenotypes and overall lowering of cost. MAS is now routinely used in breeding of major cereals like rice bacterial blight resistance (Ahmadi *et al.* 1992; Huang *et al.*, 1997; Sanchez *et al.*, 2000 and Yoshimura *et al.*, 1995) submergence tolerance (Xu *et al.*, 2004), maize (Ribaut *et al.*, 1997), barley (Laurie *et al.*, 1995 and Thomas *et al.*, 2003). In the legume crops, successful examples of MAS have been reported in soybean for soybean cyst nematode (SCN) resistance (Cregan *et al.*, 1999) and seed weight (Hoeck *et al.*, 2003); in common bean for bacterial blight resistance (Yu *et al.*, 2000); and in lentil for Ascochyta blight resistance (Ta'ran *et al.*, 2003). However, the expectations generated by MAS have yet to be realized.

The efficiency of MAS or MAB depends on the size of population, the number of markers used, the distance between loci and the genomic region containing the desired quantitative trait loci (QTL), and the experimental design

used. Successful use of MAS requires tightly linked markers to QTLs of interest and the validation of linkage relations across populations and environments. Validation of QTL markers is critical precursor to routine use in applied breeding programs. At least four levels of validation can be envisaged using a different population from the same cross, a half-sib population, a population from one or more closely related parental genotypes and a population from distantly related parental genotypes. Phenotyping in a number of different environments to simultaneously detect environmental (E) effects and QTL x E interactions for the putative QTL..

5.3.1 Validation of QTL markers in mapping population (ICC4991 x ICCV04516)

Sixteen SSR markers reported from earlier AB QTL mapping studies (Table 3.2) were initially screened with the parents ICC4991 and ICCV04516. Eleven SSR markers, TA2, TS54, TA146, TS45, TA28, GA16, TA72, TR20, TA37, TA200 and GA20, were found polymorphic between the parents and were assigned to their respective LGs (Table 4.2). The QTL analysis identified the markers GA20, TA37, TA146, TS54, TR20 and TA2 associated to AB seedling or adult plant resistance.

Single marker analysis revealed association of GA20 SSR marker with AB resistance (adult plant resistance), which was earlier reported to be, associated with resistance against pathotype I (Cho *et al.*, 2004). Single marker analysis detected four SSR loci, TA37, TA146, TS54 and TR20, associated with seedling resistance in $F_{2:3}$ progenies of ICC4991 x ICCV04516. TA37 was reported as an indicative marker for QTL 3 specific to phenotype I (Cho *et al.*, 2004). TA146 and TS54 were identified in genomic region harboring AB

resistance QTL3 (Tekeoglu *et al.*, 2004). Udupa and Baum (2003) reported TR20 as an indicative marker for AB resistance QTL specific for *ar2b* pathotype on LG2. AB resistance QTLs flanking TS54, TA2 were also confirmed by CIM in the same $F_{2:3}$ progenies of ICC4991 x ICCV04516 in our studies. Thus, these results validated AB resistance QTL2 detected by Tekeoglu *et al.*, (2004) in their interspecific mapping population and as well as AB resistance QTL5 of Flandez-Galvez *et al.* (2003 a) in their intraspecific populations.

5.3.2 Validation of QTL markers in validation population (ICCV10 x ICCV04516 and ICL87322 x ICCV04516)

The earlier reported markers (Table2.5) were attempted for validations in the two validation populations. One of the main limitations was lack of polymorphism for the reported markers between the parents under study (ICCV10, ICL87322 and ICCV045160). The polymorphic markers scored for their segregations in the two populations are presented in the Table 4.12. The marker TA146 was found significantly associated with the seedling resistance in the F_2 population of ICCV10 x ICCV 04516, explaining 18.89% of phenotypic variation followed by TR20 explaining 2.5 % of the phenotypic variation. The TA146 is a tightly linked marker to AB QTLs at 0.1 cM (Flandez-Galvez *et al.*, 2003a) and also reported to be within QTL 3 detected by Tekeoglu *et al.* (2004). However, in the second validation population (F_2 of ICCL87322 x ICCV04516), none of the markers were validated. The possible reason for no association to trait might be small size of population (n = 94).

So the validation can be said to have been accomplished across environments, using diverse mapping populations both intra and interspecific and against a different isolate (Indian isolate) of the pathogen. The AB QTLs thus

validated are candidate QTLs for MAS and MAB. Successful examples have been cited in literature employing marker assisted pyramiding of disease resistance QTLs to achieve durable and broader resistance against different races of pathogens, e.g., bacterial blight resistance in rice (Huang *et al.*, 1997 and Sanchez *et al.*, 2000) and Ascochyta blight resistance in lentil (Tar'an *et al.*, 2003). This study has validated the markers linked to AB resistance QTLs in chickpea and these can be further used in pyramiding AB resistance genes from diverse sources for developing cultivars with enhanced resistance to AB.

In chickpea a saturated intraspecific genetic linkage map based on co-dominant PCR based markers (SSRs) and gene based markers (ESTs) is essentially required. In the present study an intraspecific molecular map using a cross between Pb7 (ICC4991) x ICCV04516 chickpea cultivars was constructed using 82 SSR and 2 EST markers. Two ESTs AGLC 11 and AGLC 29 were mapped on the chickpea genome for the first time. The average marker density of the constructed map was 8.62 cM, spanning a total distance of 724.4 cM, further saturation of this map with more number of co-dominant markers is required. Ascochyta blight is a major biotic constraint in the northwestern regions of India. However, studies pertaining to mapping of AB resistance QTLs against an Indian isolate of the pathogen had not been done before. Three QTLs (QTL1, QTL2 and QTL3) which confer resistance to AB in chickpea were identified using an Indian isolate from Hissar and mapped on the intraspecific map of Pb7 (ICC4991) x ICCV04516 constructed for the first time. Though molecular markers were identified for AB resistance QTLs in chickpea (Table 2.4), attempts for validating these markers using an Indian isolate was lacking. For the first time, the markers

linked to AB resistance have been validated across different populations under controlled conditions. A set of SSR markers linked to different QTLs conferring resistance for AB in chickpea have been identified and validated during the present study. These markers can be routinely used for enhancing resistance to AB and practicing MAS and MAB in chickpea breeding programs.

Chapter VI

Summary, Conclusions & Suggestions for Future Work

CHAPTER-VI

SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 Summary

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n=2x=16), cool season edible food legume valued for good source of seed protein (20-23%). The average yield is only 750 kg ha^{-1}, which has stagnated over the past three decades due to abiotic and biotic constraints that limit the productivity. Ascochyta blight (AB) caused by *Ascochyta rabiei* (Pass.) Labr. is the most serious and devastating disease, sometimes causing total crop failure. Developing chickpea varieties with high level of resistance to AB has been challenging because of non-availability of high level of resistance in the germplasm, conditioning of resistance by several quantitative trait loci (QTLs), and high variability in pathogen. Molecular markers linked to major QTLs of AB resistance can greatly facilitate pyramiding of resistance genes and significantly reduce the time required in developing of a crop variety. In spite of the availability of several molecular genetic linkage maps of chickpea most of genomic region harboring genes for important traits are not yet sufficiently saturated with co-dominant markers to apply MAS in plant breeding. Therefore, developing high density saturated genetic map of chickpea and identification and mapping AB resistance QTLs and validation of the reported markers for utilization in MAS were attempted in this study.

An intraspecific linkage map of chickpea genome was constructed based on simple sequence repeats (SSR) and expressed sequence tags (EST) markers

QTL markers were validated across environments, diverse mapping populations derived from intra and interspecific crosses, and against a different isolate of the pathogen. The AB QTLs thus validated are candidate QTLs for MAS and MAB.

5.2 Conclusions

- The parents of the newly constructed intraspecific mapping population i.e., Pb-7 (ICC4991) and ICCV 04516 showed polymorphism for 41.37% of SSR, 2.7% of EST markers but not for the RGA markers studied.
- A new intraspecific genetic linkage map of Pb-7 (ICC4991) x ICCV04516 is constructed using Kosambi mapping function at minimum LOD score of 3.0. The total map length spanned a distance of 724.4 cM with an average marker density of 8.62 cM. 82 SSR markers and 2 EST markers were distributed into ten linkage groups (8 major and 2 minor groups).
- QTL associated with adult plant resistance of AB was identified on LG3, 9.19 cM away from the SSR marker TR58 at a LOD score of 2.03 explaining total phenotypic variance of 18.62% (R^2 0.1862).
- Six SSR markers, GA20, TA142, TA18, TA21, TA39 and TR58, were found to be associated with adult plant resistance of AB using the single marker analysis.
- Two QTLs associated with AB resistance at seedling stage were placed on LG4 with regions covered by SSR markers TA146, TS54 TA2 and TAA170, both together explained the total phenotypic variance of 17.02%

- Nine SSR markers, *viz.*, TR43, TA37, TA146, TS54, TAA170, TR20, TR55, GA102 and TA5 were found to be associated with AB resistance at seedling stage using the single marker analysis.
- The SSR markers, TA37, TA146, TS54 and TR20 were found to be associated with AB resistance at seedling stage in $F_{2:3}$ mapping population of ICC4991 x ICCV04516, this validated the earlier reports by Cho *et al.* (2004), Flandez-Galvez *et al.* (2003b), Tekeoglu *et al.* (2004) and Udupa and Baum (2003).
- The marker TA146 associated with QTLs of AB resistance at seedling stage identified in the $F_{2:3}$ mapping population of ICC4991 x ICCV04516, was also found significantly associated in the F_2 validation population of cross between ICCV10 x ICCV04516.
- The markers associated with QTLs for AB resistance have been validated across environments, diverse mapping populations (both intra and interspecific) and against an Indian isolate of AB. These markers are suitable for routine application of MAS and MAB in chickpea breeding programs.

5.3 Suggestions for future work

- The intraspecific genetic linkage map of the chickpea needs to be saturated with more number of biallelic markers like SSR, SNPs and gene based markers like ESTs and RGAs. Further, saturation of the map with gene-based markers will improve understanding of the disease resistance mechanism.

- Utilization of a new set of 233 SSR markers developed by Lichtenzveig *et al.* (2005) will enable saturation of the existing map.
- The monomorphic RGAs and ESTs can be subjected CAPs analysis, to generate polymorphism useful for disease resistance mapping.
- The QTLs (QTL1, QTL2 and QTL3) detected in this study should be validated under field-conditions and also using various breeding populations for routine use of MAS or MAB.
- The present study involved detection of Ascochyta blight resistance against an Indian isolate (Hissar isolate) of the fungus. Studies pertaining to other virulent races or pathotypes can be initiated.
- The markers GA20, TA37 of LG2 and TA146, TS54, TA2, TAA170 and TR20 of LG4 should be brought to regular practice for MAS and MAB to enhance AB resistance in chickpea.

"MOLECULAR MAPPING OF ASCOCHYTA BLIGHT RESISTANCE IN CHICKPEA (*Cicer arietinum* L.)"

Pratibha Ramakuri

ABSTRACT

Chickpea (*Cicer arietinum* L.) a self-pollinated diploid (2n=2x=16) cool season food legume of the family Fabaceae, is grown in about 45 countries and mainly used for human consumption. The average yield of chickpea is discouragingly low (750 kg ha^{-1}), which has stagnated over the past three decades due to abiotic and biotic constraints that limit the productivity. Several abiotic and biotic constraints limit chickpea productivity. Ascochyta blight (AB) caused by *Ascochyta rabiei* (Pass.) Labr. is the most serious disease globally. Developing chickpea varieties with high level of resistance to AB has been challenging because of i) non-availability of high level of resistance in the germplasm, ii) conditioning of resistance by several quantitative trait loci (QTLs), and iii) high degree variability in pathogen. Molecular markers linked to major QTLs conferring AB resistance can greatly accelerate the breeding for development of resistant chickpea cultivars and can facilitate pyramiding of resistance genes in short time for the development of AB resistant / tolerant crop varieties. In spite of the availability of several chickpea maps most of genomic region harboring genes for important traits, are not yet sufficiently saturated with co-dominant markers, to routinely apply MAS in breeding programs. Therefore, developing an intraspecific, high density saturated genetic map of chickpea, identification and mapping of QTLs for AB resistance and validation of the reported markers linked to QTLs conferring AB resistance for utilization in MAS, have been chosen as the major objectives of this study.

An intraspecific linkage map of chickpea genome was constructed based on Simple Sequence Repeats (SSR) and Expressed Sequence Tags (EST) markers using F_2 population derived from a cross between ICC4491 x ICCV04516. A total of 84 markers (82 SSRs and two ESTs) were mapped into ten linkage groups at a LOD score of 3.0 using Joinmap 3.0 software. The total map length spanned a distance of 724.4 cM with an average marker density of 8.62 cM. A cut twig method of screening of AB disease was employed to screen an F_2

mapping population of ICC4991 x ICCV04516 at 80 days after sowing whereas seedling screening method was used at 14 days after sowing for screening of the $F_{2:3}$ families (24 plants in each line). The scoring for disease was scored on a 1-9 scale (1 = resistant, 9 = susceptible), when the susceptible check was completely dried out i.e., on 10 DAI. Single marker analysis, interval mapping and composite interval mapping methods were employed for QTL detection using QTL Cartographer Version 2.0. Three QTLs conferring resistance to AB, QTL1 on LG3 and QTL2 and QTL3 were mapped on LG4 were mapped on the linkage map constructed. QTL1 was positioned at 95.11 cM on LG3 close to TR58 at a LOD of 2.03 explaining phenotypic variance (R^2) 18.62% as detected by CIM method. IM allowed mapping of QTL2 and QTL3 on LG4 with regions covered by SSR markers TA146, TS54, TA2 and TAA170. The QTL2 and QTL3 accounted for 7.74% and 9.28% of total phenotypic variance, respectively, and together explained a total phenotypic variance of 17.02% for seedling resistance to AB in $F_{2:3}$ population.

Attempts of validating the earlier reported QTLs gave interesting results. The marker TA146 detected was associated to seedling resistance in the $F_{2:3}$ mapping population of ICC4991 x ICCV04516 was found significantly associated with the seedling resistance in a validation population of ICCV10 x ICCV04516 developed using the same resistant parent, explaining 18.89 percent of phenotypic variance. The validation studies of reported AB resistance QTL markers in F_2 and $F_{2:3}$ mapping population of ICC4991 x ICCV04516 confirmed the earlier reports. The marker GA20, TA37 on LG2 and TA146, TS54, TA2, TAA170 and TR20 on LG4 are the candidate markers for employing MAS and MAB for Ascochyta disease resistance in chickpea. Therefore, in chickpea the markers have been validated across environments, using diverse intraspecific mapping populations and using a different isolate of the pathogen. The AB resistance QTLs markers thus validated are candidate QTLs markers for MAS and MAB.

Date: 20.XII.2005

Place: Raipur

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APPENDIX I

Weather data during the crop growth period (October 8 – March 4, 2004/05).

Year	Std Week	Rain (in mm)'	Evap (in mm)	Max Temp (in°C)	Min Temp (in°C)	Rel Humidity1 at 07:17 (in%)	Rel Humidity2 at 14:17 (in%)	Wind Velocity (in Kmph)	Solar Radiation (in mj/ m^2)	Bright Sunshine (in Hrs)
2004	41	11.4	27.3	31.57	21.19	96.00	57.00	3.34	16.62	6.44
2004	42	14.69	31.29	29.45	15.62	90.28	43.71	2.08	20.54	9.20
2004	43	1.39	25.6	29.68	19.37	94.42	56.14	4.34	14.92	6.71
2004	44	0	34.6	29.04	18.14	86.85	51.71	8.05	14.94	5.65
2004	45	0	31.89	29	17.97	85.57	42.85	5.70	14.82	6.31
2004	46	0	29.6	30.85	17.44	93.00	44.57	5.04	15.31	8.12
2004	47	0	38.1	29.92	12.11	87.57	29.14	4.85	18.02	10.24
2004	48	0	38	28.91	11.51	83.57	28.00	5.20	17.44	10.18
2004	49	0	31.39	28.68	10	88.71	31.57	2.11	17.08	10.08
2004	50	0	29.4	28.92	11.11	95.42	35.71	1.51	16.22	9.71
2004	51	0	29.69	30.65	11.21	92.28	31.42	2.17	16.50	10.05
2004	52	0	34.69	28.8	12.69	96.37	43.87	4.60	15.61	9.40
2005	1	0	34.89	29.61	15.54	94.28	45.28	6.62	14.85	8.37
2005	2	0	30.8	30.21	12.19	90.71	41.85	3.52	16.95	9.87
2005	3	0	35.89	29.74	14.11	86.71	45.28	5.78	17.13	9.78
2005	4	2	34.89	30.41	19	95.00	44.42	8.91	14.25	7.48
2005	5	31	33.19	27.92	16.34	92.71	54.42	8.17	14.84	6.94
2005	6	0	43.9	31.11	15.31	88.85	32.28	7.12	18.45	9.64
2005	7	0	44.9	34.85	14.64	73.42	20.57	4.38	20.62	10.67
2005	8	0	50.3	34.24	17.71	81.42	23.57	6.81	19.3	9.97
2005	9	0	62.2	34.94	17.6	69.00	21.71	6.75	19.71	9.98

APPENDIX –II

SSR primer sequences

SSR Primer Name	Forward Primer	Reverse Primer
TA1	TGAAATATGGAATGATTACTGAGTGAC	TATTGAAATAGGTCAGGCTTATAAAAA
TA4	CGAATTTTTCAGAAACACAATGTC	TTAGTATTGATTATTATGTATTGCGCC
TA13	TAAGTTAAGGGACCAACGAA	CAAGTTGGAGTCAAACCAAT
TA18	AAAATAATCTCCACTTCACAAATTTTC	ATAAGTGCGTTATTAGTTTGGTCTTGT
TA21	GTACCTCGAAGATGTAGCCGATA	TTTTCCATTTAGAGTAGGATCTTCTTG
TA25	AGTTTAATTGGCTGGTTCTAAGATAAC	AGGATGATCTTTAATAAATCAGAATGA
TA27	GATAAAATCATTATTGGGTGTCCTTT	TTCAAATAATCTTTCATCAGTCAAATG
TA28	TAATTGATCATACTCTCACTATCTGCC	TGGGAATGAATATATTTTTGAAGTAAA
TA30	TCATTAAAATTCTATTGTCCTGTCCTT	ATCGTTTTTCTAAACTAAATTGTGCAT
TA36	TTTAATATTTTACCTTATTAGGAATTGAGA	TTCAACTTAAGACATGAAATTTGTTTTT
TA37	ACTTACATGAATTATCTTTCTTGGTCC	CGTATTCAAATAATCTTTCATCAGTCA
TA39	TTAGCGTGGCTAACTTTATTTGC	ATAAATATCCAATTCTGGTAGTTGACG
TA43	GGTTGTGTTCTCCAGATTTT	AAGAGTTGTTGGAGAGCAA
TA44	ACCGAAATGGAAACAAATAA	ACAAAACTGGGGGACTAAAT
TA45	ATGCGTATAAAACCCAGAGA	TGTTTTTATTGGATTTTCAGTTTCA
TA47	TTTTTATAGGTGTCTTTTTGTTGTCTTT	TCTGAATAGGAAATAAGAAAGGTAGGTT
TA53	GGAGAAAATGGTAGTTTAAAGAGTACTAA	AAAAATATGAAGACTAACTTTGCATTTA
TA66	TGAAATCTGCATATGAAAAATATGAAT	GGTATCGATAAGCTTGATCTAAAAAGA
TA72	GAAAGATTTAAAAGATTTTCCACGTTA	TTAGAAGCATATTGTTGGGATAAGAGT
TA46	TTTATTGCAATAAAACTCATTTCTTATC	TTCTTTTTGTGTGAAAAAAAAATATAGTGA
TA76s	TCCTCTTCTTCGATATCATCA	CCATTCTATCTTTGGTGCTT
TA87	AAGGGTCAACTCTAAGATCAATTAGAA	AATCTGTCTGCACCAATACTTAACA
TA93	TTTCTCACACAAATAACAAATTAACTGA	TCAACATTAATTAACTACTATGATCTGTCA
TA96	TGTTTTGGAGAAGAGTGATTC	TGTGCATGCAAATTCTTACT
TA104	TGACACCCTAAACCCTAAAA	AATTCATTTGTGTCATTGGC
TA108	AAACCATTATCGAGTTGGATATAAAGA	TTTCTAAGTGTTCTTTTCTTAGAGTGTGA
TA110	ACACTATAGGTATAGGCATTTAGGCAA	TTCTTTATAAATATCAGACCGGAAAGA
TA125	TTGAAATTGAACTGTAACAGAACATAAA	TAGATAGGTGATCACAAGAAGAGAATG
TA130	TCTTTCTTTGCTTCCAATGT	GTAAATCCCACGAGAAATCAA
TA135	TGGTTGGAAATTGATGTTTT	GTGGTGTGAGCATAATTCAA
TA136	AGATCATTGCAGAGAGTAATATTGGTT	TGCTGTGTGACCTATACAATACAAAA
TA140	TTTTGGCATGTTGTAGTAATCATATTT	TGAAATGAAAAAGAAAAGGAAAAAGTA
TA141	AAAAATTGTCTCACAGACCAAAAA	AATTAATTTGTTGTTGAAGAGGGAGT
TA142	TGTTAACATTCCCTAATATCAATAACTT	TTCCACAATGTTGTATGTTTTGTAAG
TA144	TATTTTAATCCGGTGAATATTACCTTT	GTGGAGTCACTATCAACAATCATACAT
TA146	CTAAGTTTAATATGTTAGTCCTTAAATTAT	ACGAACGCAACATTAATTTTATATT
TA167	TGTGTCTACAGAAAGAAATTAGATTGA	AATAATTTTTGCGGAGATGACAA
TA179	CAGAAGACGCAGTTTGAATAACTT	CGAGAGAGAGAAAGGAAGAAGAG
TA186	ACAAAATTCTAAAAGTTCCTTCTACCA	GTTGTTAGTCGAATAATTGAGAAAAAGA

TA194	TTTTTGGCTTATTAGACTGACTT	TTGCCATAAAATACAAAATCC
TA196	TCTTTTTAAATTTCATTATGAAAATACAAATTATA	CCTCGGGAGAGGTAAATGTAATTTC
TA198	ATCGAGATAAAATTCAAAAGTTGTGTT	ATTAGACGATTCTCCATAACTGTGAGT
TA206	GTCCCACTTCCACTTATAAAGGTT	TAACGTATCTTGCAGATTTCAAATAAA
TR3	GAAGTATCAGTATCACGTGTAATTCGT	CTTACGGAGAACATGAACATCAA
TR7	GCATTATTCACCATTTGGAT	TGTGATAATTTTCTAAGTGTTTT
TR8	AGTTAAAGTCTTATTCGGTCAAAAACA	AAATACCAGGTTCATTGGAAGTAATC
TR19	TCAGTATCACGTGTAATTCGT	CATGAACATCAAGTTCTCCA
TR31	CTTAATCGCACATTTACTCTAAAATCA	ATCCATTAAAACACGGTTACCTATAAT
TR32	TTATTTTAACAWCTTCCTCTTATTGTCC	AAAACGGGTTTGATGTTTGATG
TR33	TCTGATTTAATTTCCTATCATTAGTTGC	ATTTTTGTCGGGGAGTACATAATA
TR35	ACTTTGGTTTAACATTTTCGGTAGTTA	AGTATCAACGTCATGTGTAACTCGTAT
TR40	AAGTGAAATATGTCATCCTTATTACTAACT	AGGAAACTGTGTTTCGTCTTTTTATT
TR43	AGGACGAAACTATTCAAGGTAAGTAGA	AATTGAGATGGTATTAAATGGATAACG
TR44	TTAATATTCAAAAACTCTCTTGTGCAAT	TTTACAACAGCGCTTGTATTTAGTAAG
TR45	CCCATACCTTTATTATTTGGCAAC	AGTGGAACCCACCAATTTTACTA
TR55	TTACTCAACCATAATAATAATAATAAT	CTCTTCAATCTTCACTTATTCAT
TR56	TTGATTCTCTCACGTGTAATTC	ATTTTGATTACCGTTGTGGT
TR58	CTCTATATTTGTTTGTTTTTCGTTTTG	TAAAATGTGTAGGGTGCAGAATAAATA
TR59	AAAAGGAACCTCAAGTGACA	GAAAATGAGGGAGTGAGATG
TR60	TGAGTCAAAACAAAGAACTTG	CTACCGGAAATTTCATTGAC
TS5	GTTGAATAGTACTTTCCCACTTGAGTC	TGAGACTAAAAATCATATATTCCCCCC
TS10	TGACCCACACAAAAAGAAA	TGGTTTTAGTCCCTCTAAGGT
TS11	GAGAGACCAAAACTGTCGAA	TCTATTTTAAATCAAGCAATCAA
TS12	CTTAAATAATAAAATCCTAAATAAT	TAATCATATGAGAATCTTAGAATATCAC
TS16	ATTGTTTTGCAAGGACTTCTGATA	AAAAACCCCTTTTTAATTTCAACTTT
TS19	TTTCTTTTGTTAGAGTTAAAAAAAATT	TCTCATGTTTTGCTTTTTATTATTATTA
TS23	CACCTAATTTTGTCCGACTT	CAAACAAGAACCGAAAACAC
TS24	GTAGAAAGAAAACTGACATGGTTGAG	GCCTAACCCAATAATACCTTCTTTT
TS29	AACATTCATGAACCTACCTCAACTTA	CCATATATGAGTACACTACCTCTCGG
TS35	GGTCAACATGCATAAGTAATAGCAATA	ACTTTCGCGATTCAGCTAAAATA
TS39	ACAAATCAATATACAACACATCACTCC	CATCAAAATAAATTAACAACATAATGG
TS43	AAGTTTGGTCATAACACACATTCAATA	TAAATTCACAAACTCAATTTATTGGC
TS45	TGACACAAAATTGTCTCTTGT	TGTTCTTAACGTAACTAACCTAA
TS46	GTTGATATTTTTGTGTGTGCGTAG	TAATTACTTGCAAAAATAAATGGACAC
TS47	GTTAATATTTTTCCGCTTCGT	TCAAATTGTGTTAAAAATCAAAGTGTT
TS52	ACATAATCGAGTTAAAGAAAAACATAT	TCAAAGTGGTAGTTTGATAAAGAACTA
TS53	GATCNTTCCAAAAGTTCATTTNTATAAT	TTAAAGAACTGATACATTCCGATTATTT
TS54	TACAAGTTAAAAATGAATAAATATTAATA	GAAATTTAGAGAGTCAAGCTTTAC
TS57	TCAATTTATAATCATAGAGAATCNGAGA	CCTAAAACAAATAAAATCTTAAATAATA
TS58	GATTTTTATGACCATCAATTCATTTCT	CAATTTTGTCCGATTTTTACTTTTTAT
TS72	CAAACAATCACTAAAAGTATTTGCTCT	AAAAATTGATGGACAAGTGTTATTATG
TS74	TTACTTCCTTCACATGGGCTTAG	AGATTTGTTGGGTGGACTCATT

TS79	GCTCATGTGTTAAATGAAAAATCTAAA	ACGGCTCAAATACAATTGATAAAA
TS82	CAAAGACATAATCGAGTTAAAGAAAAA	TGGTTAGCTAGAAAATTCAAGGG
TS83	AAAAATCAGAGCCAACCAAAAA	AAGTAGGAGGCTAAATTATGGAAAAGT
TS84	TTATAACAGCTTCCTTCTATTTGTTTTG	AAGGCAAAAGTTTTTATCCCTTAATAG
TS104	TCAAGATTGATATTGATTAGATAAAAGC	CTTTATTTACCACTTGCACAACACTAA
TAA55	GGAACAACAACAACTCAAATG	TGCTATTAAGTGTGACCAGCAAA
TAA57	ATCAAAGAAAGAAACACTTGTTCA	TGGTTGGATACAAAAGACTGGA
TAA58	CATTGCTTAAGAACCAAAATGG	CAATTTTACATCGACGTGTGC
TAA59	GCAGGAAAGACTCCAGCAAC	TGGATTAATCGTTTTGCTCATC
TAA60	TCATGCTTGTTGGTTAGCTAGAAA	CAAAGACATAATCGAGTTAAAGAAAA
TAA61	GGTGAAAGACAAGTTAATAAATCAATG	CACCTAGGCATAAAAATGGATCA
T54II	GCGCTTTGCCGATAGATACT	AAAGGGAACAAAAGCTGGAG
CaSTMS4	AATATATGAATTGGTTCAGACATC	AAACAAATAATAGAAAATTATGCTCC
CaSTMS5	TACAAACTTTTAAGTTCATAAGTTTGA	AACTTCTCGAATTAGTAAATTAAGTTG
CaSTMS6	TCTATCTTCCATTATTTCTTGTTAAGT	TAATTTACATTCTGACTACTTAATCCA
CaSTMS7	GAGGATTCGGATTCAGAT	AAAATCTTGGAAGTGATTGAG
CaSTMS8	GGACTAGAGGCAGAAGCT	AGCATACAAATAAATAATAATGCATG
CaSTMS2	ATTTTACTTTACTACTTTTTTCCTTTC	AATAAATGGAGTGTAAATTTCATGTA
CaSTMS9	CTTCTATATACATAGTCCTACCTACAC	ACCTCATAAAGCTGTTAAAG
CaSTMS10	ATAACAAAAAGATATCTCATCGACTA	AACAATATACAATAAATAACCAAGT
CaSTMS11	GTATCTACTTGTAATATTCTCTTCTCT	ATATCATAAACCCCCCAC
CaSTMS12	GTATTTGTTACTGCATATACTTAATTA	TATTTACTAGGTAAATCCTATTTATTG
CaSTMS13	TATGTTAAAAGAGAAAGAAGAAGTGAT	TTTTATTAGTTGTCGAAATGTATATCA
CaSTMS14	TTGTGTTTCTCCTAATATTCTATTAGC	GAATATGAATAACGTTACA
CaSTMS15	CTTGTGAATTCATATTTACTTATAGAT	ATCCGTAATTTAAGGTAGGTTAAAATA
CaSTMS16	ATCTTAGAATATCTCTTATTA	ATTACAAAGGACTCAACA
CaSTMS19	TGAAGCTGGGGGTTCCTTG	TCAATTGAGTCGCGACGAGAG
CaSTMS20	CTTNTCGTCATCATCGTTTTG	CACCCTACTTTTTTCCACCAC
CaSTMS21	CTACAGTCTTTTGTTCTTCTAGCTT	ATATTTTTTAAGAGGCTTTTGGTAG
CaSTMS22	CTCTTCCTCCTCGAGATC	ATAGATACAATACTCTGTGAGTTGG
CaSTMS23	GATGAAGATAAAAGCATAATTAAGG	TTTCTTCTTCTATGATACACACACT
CaSTMS24	AAAGACAGGTTTTAATCCAAAA	CTAATCTTTCTTCTTCTTTTGTCAT
CaSTMS25	TACACTACTGCTATTGATATGTGGT	GACAATGCCTTTTTCCTT
CaSTMS28	CCCTTCTAGTGATATTTTG	AAATGTGTTTTATGGAATAAGTCAT
TAA170	TATAGAGTGAGAAGAAGCAAAGAGGAG	TATTTGCATCAATGTTCTGTAGTGTTT
TA2	AAATGGAAGAAGAATAAAAACGAAAC	TTCCATTCTTTATTATCCATATCACTACA
TA3	AATCTCAAAATTCCCCAAAT	ATCGAGGAGAGAAGAACCAT
TA5	ATCATTTCAATTTCCTCAACTATGAAT	TCGTTAACACGTAATTTCAAGTAAAGAT
TA8	AAAATTTGCACCCACAAAATATG	CTGAAAATTATGGCAGGGAAAC
TA11	CATGCCATAAACTCAATACAATACAAC	TTCATTGAGGACAATGTGTAATTTAAG
TA14	TGACTTGCTATTTAGGGAACA	TGGCTAAAGACAATTAAAGTT
TA20	ATTTTCTTTATCCGCTGCAAAT	TTAAATACTGCCTTCGATCCGT
TA22	TCTCCAACCCTTTAGATTGA	TCGTGTTTACTGAATGTGGA
TA34	AAGAGTTGTTCCCTTTCTTTT	CCATTATCATTCTTGTTTTCAA

TA42	ATATCGAAATAAATAACAACAGGATGG	TAGTTGATACTTGGATGATAACCAAAA
TA59	ATCTAAAGAGAAATCAAAATTGTCGAA	GCAAATGTGAAGCATGTATAGATAAAG
TA64	ATATATCGTAACTCATTAATCATCCGC	AAATTGTTGTCATCAAATGGAAAATA
TA71	CGATTTAACACAAAACACAAA	CCTATCCATTGTCATCTCGT
TA78	CGGTAAATAAGTTTCCCTCC	CATCGTGAATATTGAAGGGT
TA80	CGAATTTTTACATCCGTAATG	AATCAATCCATTTTGCATTC
TA89	ATCCTTCACGCTTATTTAGTTTTTACA	CAAGTAAAAGAGTCACTAGACCTCACA
TA103	TGAAATATCTAATGTTGCAATTAGGAC	TATGGATCACATCAAAGAAATAAAAT
TA106	CGGATGGACTCAACTTTATC	TGTCTGCATGTTGATCTGTT
TA113	TCTGCAAAAACTATTACGTTAATACCA	TTGTGTGTAATGGATTGAGTATCTCTT
TA114	TCCATNTAGAGTAGGATNTTNTTGGA	TGATACATGAGTTATTCAAGACCCTAA
TA116	AATTCAATGACGAATTTTTATAAGGG	AAAAAGAAAAGGGAAAAGTAGGTTTTA
TA117	GAAAATCCCAAATTTTTCTTCTTCT	AACCTTATTTAAGAATATGAGAAACACA
TA127	AAATTGTAAGACTCTCATTTTTCTTTATT	TCAAATTAACTACATCATGTCACACAC
TA180	CATCGTGAATATTGAAGGGT	CGGTAAATAAGTTTCCCTCC
TA200	TTTCTCCTCTACTATTATGATCACCAG	TTGAGAGGGTTAGAACTCATTATGTTT
TA203	ATAAAGGTTTGATCCCCATT	TGTGCATTCAGATACATGCT
TA176	ATTTGGCTTAAACCCTCTTC	TTTATGCTTCCTCTTCTTCG
TR1	CGTATGATTTTGCCGTCTAT	ACCTCAAGTTCTCCGAAAGT
TR2	GGCTTAGAGTTCAAAGAGAGAA	AACCAAGATTGGAAGTTGTG
TR20	ACCTGCTTGTTTAGCACAAT	CCGCATAGCAATTTATCTTC
TR24	AACAACTTCCTCTTATTTTCCA	CAGTAAAAATCAGCCCAAAC
TR26	TCATCGCAGATGATGTAGAA	TTGAACCTCAAGTTCTCTGG
TR29	GCCCACTGAAAAATAAAAAG	ATTTGAACCTCAAGTTCTCG
TAA107	ATAACCACCAAACATACTAATGCCATA	ATTCATAATTCAGGACGCAATAGTTAC
TAA137	CATGATTTCCAACTAAATCTTGAAAGT	TCTTGTTTCGTTTAAACAATTTCTTCT
TAA169	CTCAACTTTTCATCTCTTCCACTACTC	CTATATTACTTCCAATTTTACCCTTCG
TAA194	AACGGTTATCTATAATTAATTGTGCAAG	AATCTTGTCAACCGCATTAATAATTT
TAASH	GGTAGACGCAAAAGAGTGGG	GCCACATTGACCAGGAATG
GA2	TGCATTGGAAATACAGCATGA	AATTTTGGTTCGCCACAAAC
GA4	TTGCGTGTCAATCTCATTGG	TCAACACCCCTAACTCGGAC
GA6	ATTTTTCTCCGGTGTTGCAC	AAACGACAGAGAGTGGCGAT
GA8	GCTCTAAAGGGAAGGCGATT	AACCACCAAAGTTCCCCAG
GA9	GAACGGATTGGATGAAGCAT	GTGCAAACAACCCTTTTTGG
GA11	GTTGAGCAACAAAGCCACAA	TTCTTGTCTGGTTGTGTGAGC
GA13	GGGCTCATTTACAGGTTACA	TCAAAGATAATATAAAAGGATGAA
GA14	AACTAACCTGTCACTGGATCTCAA	TCCCTCTTTGACTCTCTCGC
GA16	CACCTCGTACCATGGTTTCTG	TAAATTTCATCCTCTCCGGC
GA17	TAGTCCGTTGTCATCCTCCC	CGTTGTGGCCAGAGAGAGA
GA20	TATGCACCACACCTCGTACC	TGACGGAATTCGTGATGTGT
GA21	CCCCAGGTGAATTCCTCATA	CTCAACCTTTGTTCAGCAACAC
GA22	ATGAGTATCAAGCCAACCTGA	GTCCCAACAATTTCTTACATGC
GA24	TTGCCAAAACCAATAACTCTG	TCCCTTTTACACAAGGCCAG

GA26	GATGCTCAAGACATCTGCCA	TCATACTCAACAAATTCATTTCCC
GA31	TATAGAAGAAAAAGCCGCCG	AACCTATTATTTCTTCAACCATTATCA
GA33	CAAGCACAATCTTCGTCCAA	CTCTCCATTTGCCTCCTTCA
GA34	CCTTTGCATGTATGTGGCAT	CCGTTTATAAAGGATGTAZGAGAC
GA102	CAGAGAACCACATGTTTTAGTTGAA	AGTTTTGATGCGTGCCATTT
GA105	TGAGGAAACACAAAACGACG	ATGCCAGGATTAACAGCACC
GA108	GTTTGTGATGGAGGAAGCGT	GCCGCATAGCATTGGTAAGT
GA117	TTATGGGGGATAGCAAACGA	TCATGGTTCTTGGTCTGGCT
GA119	TGAACAAAGAAAAACCCGTTC	TGGCAATTTGTCTGAGATGC
GAA129b*	TTTTGCACTTCAACGTCACC	TGATTTTCACCTTTTATTCACAAAT
GA129a*	GGACATGGTGCTTCGAAAAT	CCCAACACCCTCACTTCATC
GA137	GGGGGAAGATATGTTGGGTT	GATCCAACGGGAACAAAGAC
GAA39	GCATTGCGAACAAGTGTTAGAT	TTCCTTGAAGATGATGAGAAATACA
GAA40	TTGACGCAGAGAACTCTCAA	ATTGGTGTGATGGGTGGATT
GAA41	TATTCCATCATGCCAGCAAA	ATAGGGCAGAAACTGGAGCA
GAA42	CGCTTCAGTGTAGATATTATTCAAACA	TCTCTCTTTCTCTTCAACACGC
GAA43	TGATCGGAGAGAGAGGAGGA	CGTTGATCCACTGCGATAGT
GAA44	AGCAAGCCCATGATTTTCTC	ATGACATTCCAATCGGCTTC
GAA45	TTGGGATCCATTTCATCCAT	GCCTGGAAGTCACACACTTG
GAA46	TCTCCTGTGAATGAACCGAA	CTGAGCAACAAAATCAGCCA
GAA47	CACTCCTCATGCCAACTCCT	AAAATGGAATAGTCGTATGGGG
GAA50	TTCGTTCCCATCAACATTCA	CCCTCCCGTATTCATACCAA
GAA51	CCAAAACGATTTCCCTTCAA	TCTGTTTTTGCCATCAAGCA
GAA54	TGGACGAGGAATAAAGAGAGAA	TGGAGGCAGTTCACAGTTTG
GAA58	CATGATGCAACATCTCACCA	TGATTATGCTGTTTTGGGGG
GAA60	TTGGTTTGCAAATTGTTCTTC	AAGTCCATTGAAGTGTCGCC
TAA104	CCCCTAAATTAACAACATAATGG	CGGCTTATGAATTTTTATCATTTACAG

APPENDIX –III

EST primers sequences

AGLC_1F	AACATCATCAAGGTCTCCTGGGTA	AGLC_1R	GGTGATGAAGTTACTGATGGTGGA
AGLC_2F	TGTCAGACTGAGCTGTGTATGAGA	AGLC_2R	TTGCCCGTATGGTTATGTTAGGAA
AGLC_3F	TGCTCTGCCCCATCTGAGGA	AGLC_3R	ATCACATGGTGGTGCTGGTCA
AGLC_4F	TTCTCAGACTTCAATCCTAGCA	AGLC_4R	TTGGTCCAACTTATGACTTCCA
AGLC_5F	CGGCCGAGTACAATTTCTTCCA	AGLC_5R	ATTTGCTGATGATTGCGTTCCA
AGLC_6F	GTCGTGAAAAGCCTTGGACGA	AGLC_6R	ATCAACCTTTCAATATCGCGCAGA
AGLC_7F	CAAACTCCTCAATAGCAGGCACA	AGLC_7R	GCTGTATCGGAGAGTGGTCAGA
AGLC_8F	GACCCCCAAAAATGAAAAAGCA	AGLC_8R	TTGCCCATACATTCTTCACCCAA
AGLC_9F	ACTCCTGTAGTGGCATATCTTCGA	AGLC_9R	TGGTCCATTTATGCCGCTGGTA
AGLC_10F	ACTAGTCCTGCAGGTTTAAACGA	AGLC_10R	CCTCTTCCCTCAATTTTCCTCACA
AGLC_11F	ACCCTTTCGGTTGCAGCTGA	AGLC_11R	TGTTCGGATGATTGAGGCAGGA
AGLC_12F	GGCTCCCTCCTGCAAATCCA	AGLC_12R	GAAGTAATTCAGGTAAGTGGCGAA
AGLC_13F	CAACTCTAAGGTGTTTAGGTGGTA	AGLC_13R	ATCCAAAACAGCTCATTGCTCA
AGLC_14F	GCAGCAACTATTTACACTGGTA	AGLC_14R	CTCTCTGGGAGAAAGCTCGGAA
AGLC_15F	ACTGATCAAGGTCTCTTCTAGACA	AGLC_15R	CCCAACAAACTGGACAAAGCAGA
AGLC_16F	GAGTACTTGCCAACTAGCTTAGGA	AGLC_16R	TTGGATATAACAGATGACGGGGAA
AGLC_17F	CACAGCATTATGGCCAACAGCA	AGLC_17R	TGTCAGGGGTTTTGACAAATCTCA
AGLC_18F	CGTTTGGGCTGACAGTTTGGA	AGLC_18R	GCCATGACATCGGATATGATAGCA
AGLC_19F	GCATCCTTCCCACTTCTTTGCA	AGLC_19R	GAATGGACTCGGATGTCTTAAGCA
AGLC_20F	AATGGTGATTCGTCAGTCGCCTA	AGLC_20R	CTGTCTGAAGAAAGTGAACGAA
AGLC_21F	CTCCTGTAGTGGCATATCTTCGAA	AGLC_21R	TGGTCCATTTATGCCGCTGGTA
AGLC_22F	TGCAGCTTGTCCGGATGCA	AGLC_22R	TAGGTCCGAGAGGCATCAGAGA
AGLC_23F	CCAAGGGATCAACATAACGATCCA	AGLC_23R	GCAAAGAAGCATTTCAAGCCAA
AGLC_24F	ACTAGTCCTGCAGGTTTAAACGA	AGLC_24R	GTGACAGTATTTTGGAGGAGTCA
AGLC_25F	TAGTCCTGCAGGTTTAAACGA	AGLC_25R	GGTTGCAGCATTGCTCGA
AGLC_26F	CAAGTGCCACAACTCTAAATCCAA	AGLC_26R	CATCTTCCAATGTGAATGACCCAA
AGLC_27F	CAAATTTCTGTTCTTCCACCCCAA	AGLC_27R	GGCGATCTTCGAGTCCATCGA
AGLC_28F	GCTAAACCTTAGAGCAATGACTCA	AGLC_28R	CCTTGCTTGTGCCTTATCTTCCA
AGLC_29F	TCTTCAACACCTCCATCTAACCTA	AGLC_29R	GACATGAAACCAAAGCATCACA
AGLC_30F	TCTCTGAAACACTCTAGCAAGTGA	AGLC_30R	CGGCTTTGGGGAACGAAGGA
AGLC-53F	CACTCTCCGTTCCGGTTCCA	AGLC-53R	CTGTCCATGCCCTTGTCCA
AGLC-54F	ACCAACAATCTCCCTCTTCCCTA	AGLC-54R	GCGAGGTACACTTTTCCCCAA
AGLC-55F	CAGGTCGCGTTGTTGCA	AGLC-55R	GGCCGAGGTACACTTTTCCA
AGLC-56F	GGTCGCGTTGTTGCAAAGCA	AGLC-56R	GTTGTGTGAGAGAACGCACAGA
AGLC-57F	TTCATCTGGCACTAGCATATCTGA	AGLC-57R	CGACAATTCTTGCTTCAACAACCA
AGLC-58F	TAATCATCGGTCATGAGTCTGTCA	AGLC-58R	CAAAATCGAAGATCTGCATCTGCA
AGLC-59F	GCCGAGGTCAGTAGGAGAGA	AGLC-59R	CTTGCTTACGGATCTGGTCCAA
AGLC-60F	CATGTTTTCTACCCTCACAATGCA	AGLC-60R	TACTCACTTGTTGTTCCAGACA
AGLC-61F	TTCGATCCTCCGACCCCGAA	AGLC-61R	TTCGCTAGATCTGGATACTTCTCA

AGLC-62F	CAGGTCCGCGTTGTTGCAA	AGLC-62R	GGAAGAGTGAGATTGTTGCGTGA
AGLC-63F	CATGATTGGAACTTGAGTCGTA	AGLC-63R	TCAGTTGCTTCCCTTTTTCTGGTA
AGLC-64F	TCTTCTTCTTCTTCTTCAGCCACA	AGLC-64R	GTGGATTGGGAAATGTGAATGTCA
AGLC-65F	GCAGGTCGCGTTGTTAGCA	AGLC-65R	ATTACTATGCTTCCTTCTCCTCCA
AGLC-66F	CCACAAAGGACGACAACAACGA	AGLC-66R	CCCAACACGAACCACACGA
AGLC-67F	ATCCATCACAACCCTCAACTCA	AGLC-67R	CTCCGTCAACCTTTCCGCAA
AGLC-68F	TGTTGTCTCGCCAATTCAAAGCA	AGLC-68R	CGTTTGGTGGCATTCCTGCA
AGLC-69F	GGTCGCGTTGTTGCAAAGCA	AGLC-69R	TGCTTCCTTCTCCTCCATTACCAA
AGLC-70F	CCGAGGTCTTGCCATTGGTA	AGLC-70R	CAGATTCGTTATTGCCTTCCCGTA
AGLC-71F	CGCCATCGTTACTTTCTCTTACCA	AGLC-71R	AGTGCAGGGCACCAATCACA
AGLC-72F	TTTAATTACGCGGTTTCCACGA	AGLC-72R	GAAGACTTGAGACATGGGCACA
AGLC-73F	GATTTGCTTGGTGATGATGCTGA	AGLC-73R	CCTCGTGGTCCACCATAGCTA
AGLC-74F	CGTGGGATTGAAAAAGTTGCTA	AGLC-74R	CACTACCAGCCAAAGCACTCA
AGLC-75F	CAACAACAACCTATCCGAACCTCA	AGLC-75R	ACTATCCCTAACCTTCCATCACCA
AGLC-76F	CATGAGTGGTAGTGGGAGTGGA	AGLC-76R	GTTCGTTTGAGTCGTTTACTGGAA
AGLC-77F	CTAGACAGGAATGTTGTCTAGAGA	AGLC-77R	GAGATTGGGGGATGACAAACACA
AGLC-78F	TCAACAACGCTACCCGATCCAA	AGLC-78R	TTCTCAAGAGCACCACAAAAGAGA
AGLC-79F	CGGCGGCTATATTGGTTTTGCA	AGLC-79R	TCCTAAACCCCACTTATCTCCCTA
AGLC-80F	TCCATCTTTGAGTTGGCATTACCA	AGLC-80R	CGCGGTCGAAAGAACGCAA
AGLC-81F	CTTCAAGTTCTTCGTTTGACGCAA	AGLC-81R	CCTTTCTCCCACAACCTCTCCA
AGLC-82F	TTTGTGATGGTCCTGCTCTCTCA	AGLC-82R	ACCGCTTCAGGATCAACTCGA
AGLC-83F	TCTTCCGATCCTAAGAAAGAGCAA	AGLC-83R	ACCAATATGGAGAGCACCAGTCA
AGLC-84F	CCACCTTCCATCTCCAATTCCAA	AGLC-84R	GACTGAATCGGAGAAGGTTTCTCA
AGLC-85F	CCAGCTTCTAATGTAGGTCTGCA	AGLC-85R	CAGCAGCAGCAGAGAGAGCA
AGLC-86F	TAATCCCCAAACAGGTTACACTGA	AGLC-86R	AGGGCAAGCCAAGGAAATCCA
AGLC-87F	TTGGTGCGATGGCAGCA	AGLC-87R	ACAATCATCGGCGGGCAGA
AGLC-88F	ACTTGGGCGTTCAAAAATCTCA	AGLC-88R	CCATTACGATCAAAGAGCTCAGGA
AGLC-89F	CTTCAATCGCACAAGAGTAAACGA	AGLC-89R	ATCCATCCTTAAGCTGTAAGAGCA
AGLC-90F	CTAGAGTCTGTGAGCTGTAATCCA	AGLC-90R	TACTCACTTGTTGTTCCAGACA
AGLC-91F	GCAGGTCGCAGTTGTTGCA	AGLC-91R	ATCGTTGAACCTGTAGTGTGA
AGLC-92F	CAGGTCGCGTTGTTGCA	AGLC-92R	GAAATTGAGGGAAGAGGGAGA
AGLC-93F	GTCCGAGCTGTGGATAGGGAA	AGLC-93R	GTTCCGCCTTCAATCCATGGAA
AGLC-94F	CCAACTTCCCTCATTCTTATTCCA	AGLC-94R	ACCAATTCCAAATTTCCAGCTCGA
AGLC-95F	GACTAGTCCTGCAGGTTTAAACGA	AGLC-95R	TAACATGGGTCTCTGCTTCTCTCA
AGLC-96F	TCCATATGGCTGAAGAACCCCAA	AGLC-96R	TTCTGAGGTTCAGGTAGTTCGGAA
AGLC-97F	ACTAGTCCTGCAGGTTTAAACGA	AGLC-97R	CCTCTTCCCTCAATTTTCCTCACA
AGLC-98F	CTCTTTCTTTCCCTCTAGTTTCCA	AGLC-98R	CGGCGAACTCGTGTTTGCTA
AGLC-99F	AACATGGGTCTGTGCTTCTCTCA	AGLC-99R	CAGCTATGTCCATGATTACGCCAA
AGLC-100F	CGACTCCCTCATCACCTCCA	AGLC-100R	CTTTGGGTCTCTGTTGTTGCTGA
AGLC-101F	TGTCCAAAATTGGGATCAGAGA	AGLC-101R	AGAACGACTTCAGCAGCAGCA
AGLC-102F	GGTAGGTCGCGTTGTTGCA	AGLC-102R	GAGATTGTTGGTGAGAGAAGCA

AGLC-103F	TTATCATGTTTGCAACATACTCCA	AGLC-103R	GGGTCTCTGCTTCTGTCACCA
AGLC-104F	CTTCACCTCTACTGCTGCTACTACTC	AGLC-104R	GAGAAACTCAGACCCATGTTAATG
AGLC-105F	GCAAAGCATCCTTCACCTCT	AGLC-105R	CCTCCAGTGTGTGTGAGATTG
AGLC-106F	CCGCTGTGTGTTGCAAAG	AGLC-106R	GAGCACTACTAGCATTACACTCAGTAA
AGLC-107F	CTGTTGCAAAGCATCCTTCA	AGLC-107R	TGTTGGTGAGAGAAGCAGGA
AGLC-108F	GCAAAGCATCCTTCACCTCT	AGLC-108R	TCCCTCCCACTTATATGTATGC

APPENDIX IV

PCR OPTIMIZATION PROTOCOL

1. Working solutions were prepared with the following concentrations.

Primer (pm/µl)	10
dNTP (mM)	2
Taq polymerase (U/µl)	0.5
Buffer (x)	10
$MgCl_2$ (mM)	10
DNA (ng/µl)	5

2.The components were varied as given below.

		B	
Primer (pmoles)	0.2	0.3	0.5
DNA (ng)	5	10	15
$MgCl_2$ (mM)	1.0	1.5	2.0
dNTP (mM)	0.1	0.15	0.2
Taq polymerase (units)	0.2	0.3	0.5

3. The combinations were adapted as five protocols

µl/Reaction (Total Reaction Volume = 10 µl)

	Primer	DNA	$MgCl_2$	dNTP	Taq polymerase	Buffer	Water
Protocol 1	0.2 (A)	1.0 (A)	1.0 (A)	0.5 (A)	0.4 (A)	1.0	5.9
Protocol 2	0.2 (A)	2.0 (B)	2.0 (C)	1.0 (C)	0.4 (A)	1.0	3.4
Protocol 3	0.3 (B)	1.0 (A)	1.5 (B)	1.0 (C)	1.0 (C)	1.0	4.2
Protocol 4	0.3 (B)	2.0 (B)	2.0 (C)	0.6 (A)	0.6 (B)	1.0	3.6
Protocol 5	0.5 (C)	2.0 (B)	1.0 (A)	1.0 (C)	1.0 (C)	1.0	3.5

4.Three touch down temperature profiles were used according to T_m value of the primer.

55 - 45°C			60 - 55°C			65 - 60°C		
Temp °C	Duration	Cycles	Temp °C	Duration	Cycles	Temp °C	Duration	Cycles
95	3 min		95	3 min		95	3 min	
94	20 sec	} 10	94	20 sec	} 5	94	20 sec	} 5
55*	20 sec		60*	20 sec		65*	20 sec	
72	30 sec		72	30 sec		72	30 sec	
94	20 sec	} 30	94	20 sec	} 30	94	20 sec	} 30
48 (T_a)	20 sec		56 (T_a)	20 sec		59 (T_a)	20 sec	
72	30 sec		72	30 sec		72	30 sec	
72	20 sec		72	20 sec		72	20 sec	
4	∞		4	∞		4	∞	

*1°C temperature reduction for each cycle.

[1] Annealing temperature should be approximately 5 ^{0} lower than T_m value.

APPENDIX V

Segregation ratios and X^2 values of the total markers used for genetic mapping

S.No.	Locus	a	h	b	c	d	-	X2	Df	Signif.	Classes
1	TA108	62	116	91	0	0	13	11.3	2	****	[a:h:b]
2	TA180	62	141	74	0	0	5	1.1	2	-	[a:h:b]
3	TA113	73	144	56	0	0	9	2.9	2	-	[a:h:b]
4	TA2	75	121	76	0	0	10	3.3	2	-	[a:h:b]
5	TA200	66	151	62	0	0	3	2	2	-	[a:h:b]
6	TA5	66	138	71	0	0	7	0.2	2	-	[a:h:b]
7	TA110	75	131	60	0	0	16	1.8	2	-	[a:h:b]
8	TA78	67	145	68	0	0	2	0.4	2	-	[a:h:b]
9	TA59	72	144	65	0	0	1	0.5	2	-	[a:h:b]
10	TA64	53	160	66	0	0	3	7.2	2	**	[a:h:b]
11	TA117	56	147	74	0	0	5	3.4	2	-	[a:h:b]
12	TA127	56	147	77	0	0	2	3.9	2	-	[a:h:b]
13	TA28	64	139	72	0	0	7	0.5	2	-	[a:h:b]
14	TA53	69	134	73	0	0	6	0.3	2	-	[a:h:b]
15	TR1	72	138	72	0	0	0	0.1	2	-	[a:h:b]
16	TR2	92	123	64	0	0	3	9.5	2	***	[a:h:b]
17	GA16	55	158	50	0	0	19	10.9	2	****	[a:h:b]
19	TA71	80	140	57	0	0	5	3.9	2	-	[a:h:b]
20	TA96	76	137	67	0	0	2	0.7	2	-	[a:h:b]
21	STMS15	64	154	64	0	0	0	2.4	2	-	[a:h:b]
22	TA176	65	141	74	0	0	2	0.6	2	-	[a:h:b]
23	TAA104	71	114	96	0	0	1	14.4	2	*****	[a:h:b]
25	TA186	58	155	63	0	0	6	4.4	2	-	[a:h:b]
26	TA106	88	128	65	0	0	1	6	2	*	[a:h:b]
27	TA14	65	156	60	0	0	1	3.6	2	-	[a:h:b]
29	TA18	68	156	55	0	0	3	5.1	2	*	[a:h:b]
30	TAASH	68	137	72	0	0	5	0.1	2	-	[a:h:b]
31	TA203	67	150	58	0	0	7	2.9	2	-	[a:h:b]
32	TA146	60	159	62	0	0	1	4.9	2	*	[a:h:b]
33	TA80	59	151	71	0	0	1	2.6	2	-	[a:h:b]
34	TAA170	69	142	71	0	0	0	0	2	-	[a:h:b]
35	TS54	61	143	77	0	0	1	1.9	2	-	[a:h:b]
37	TS82	63	149	68	0	0	2	1.3	2	-	[a:h:b]
38	STMS10	54	163	61	0	0	4	8.6	2	**	[a:h:b]
39	STMS28	69	132	73	0	0	8	0.5	2	-	[a:h:b]
40	TR35	69	139	73	0	0	1	0.1	2	-	[a:h:b]

S.No.	Locus	a	h	b	c	d	-	X2	Df	Signif.	Classes
41	TA27	72	147	63	0	0	0	1.1	2	-	[a:h:b]
42	TA120	66	153	62	0	0	1	2.3	2	-	[a:h:b]
43	TA103	75	131	72	0	0	4	1	2	-	[a:h:b]
44	TAA59	51	165	65	0	0	1	9.9	2	***	[a:h:b]
45	TA132	45	174	60	0	0	3	18.7	2	*******	[a:h:b]
46	TA194	62	147	67	0	0	6	1.4	2	-	[a:h:b]
47	TR19	65	159	48	0	0	10	9.9	2	***	[a:h:b]
48	TR31	58	140	81	0	0	3	3.8	2	-	[a:h:b]
49	TR43	63	155	64	0	0	0	2.8	2	-	[a:h:b]
50	TA142	57	150	67	0	0	8	3.2	2	-	[a:h:b]
51	TA34	49	156	76	0	0	1	8.6	2	**	[a:h:b]
52	TS29	48	148	80	0	0	6	8.9	2	**	[a:h:b]
53	TA39	61	125	76	0	0	20	2.3	2	-	[a:h:b]
54	TAA57	69	137	68	0	0	8	0	2	-	[a:h:b]
55	GAA44	65	158	58	0	0	1	4.7	2	*	[a:h:b]
56	GA102	62	157	63	0	0	0	3.6	2	-	[a:h:b]
57	AGLC29	73	134	69	0	0	6	0.3	2	-	[a:h:b]
58	AGLC11	60	157	64	0	0	1	4	2	-	[a:h:b]
59	AGLC66	77	147	54	0	0	4	4.7	2	*	[a:h:b]
60	TA43	73	151	43	0	0	15	11.3	2	****	[a:h:b]
61	TA22	64	136	79	0	0	3	1.8	2	-	[a:h:b]
62	TS5	74	115	72	0	0	21	3.7	2	-	[a:h:b]
63	TS24	77	143	60	0	0	2	2.2	2	-	[a:h:b]
64	TA21	60	146	60	0	0	16	2.5	2	-	[a:h:b]
65	TS53	63	136	73	0	0	10	0.7	2	-	[a:h:b]
66	TA118	58	129	81	0	0	14	4.3	2	-	[a:h:b]
67	TS71	63	154	63	0	0	2	2.8	2	-	[a:h:b]
68	TR5	66	154	52	0	0	10	6.2	2	**	[a:h:b]
69	TAA137	59	136	77	0	0	10	2.4	2	-	[a:h:b]
70	GA9	89	127	65	0	0	1	6.7	2	**	[a:h:b]
72	TR20	60	182	34	0	0	6	33	2	*******	[a:h:b]
73	TA8	66	108	62	0	0	46	1.8	2	-	[a:h:b]
74	GA34	85	129	46	0	0	22	11.7	2	****	[a:h:b]
75	STMS25	73	146	55	0	0	8	3.5	2	-	[a:h:b]
76	TR8	74	126	65	0	0	17	1.3	2	-	[a:h:b]
77	TR40	67	132	71	0	0	12	0.3	2	-	[a:h:b]
78	TS43	53	68	##	0	0	11	136.7	2	*******	[a:h:b]
79	TS45	48	138	85	0	0	11	10.2	2	***	[a:h:b]
80	GA26	89	122	46	0	0	25	15.1	2	*****	[a:h:b]

S.No.	Locus	a	h	b	c	d	-	X2	Df	Signif.	Classes
81	TA25	59	45	##	0	0	19	189.8	2	*******	[a:h:b]
82	TR60	72	120	69	0	0	21	1.8	2	-	[a:h:b]
83	TR14	80	130	63	0	0	9	2.7	2	-	[a:h:b]
84	TR3	80	116	58	0	0	28	5.7	2	*	[a:h:b]
85	TAA194	54	128	78	0	0	22	4.5	2	-	[a:h:b]
86	TA159	62	120	75	0	0	25	2.4	2	-	[a:h:b]
87	TS36	109	88	60	0	0	25	44.2	2	*******	[a:h:b]
88	TR55	53	135	79	0	0	15	5.1	2	*	[a:h:b]
89	TR29	60	129	67	0	0	26	0.4	2	-	[a:h:b]
90	TS84	53	116	63	0	0	50	0.9	2	-	[a:h:b]
91	TA144	49	110	##	0	0	22	26.9	2	*******	[a:h:b]
92	GA20	57	149	66	0	0	10	3.1	2	-	[a:h:b]
93	TA116	55	138	68	0	0	21	2.2	2	-	[a:h:b]
94	TS46	63	131	35	0	0	53	11.6	2	****	[a:h:b]
95	GAA60	74	117	78	0	0	13	4.7	2	*	[a:h:b]
96	TAA55	59	148	64	0	0	11	2.5	2	-	[a:h:b]
97	TA37	68	148	59	0	0	7	2.2	2	-	[a:h:b]
98	TR58	18	185	72	0	0	7	54	2	*******	[a:h:b]
99	TA196	58	146	71	0	0	7	2.3	2	-	[a:h:b]
100	TA72	65	143	68	0	0	6	0.4	2	-	[a:h:b]
101	STMS21	61	163	46	0	0	12	13.3	2	****	[a:h:b]

APPENDIX VI

Map text of the linkage groups obtained from JOINMAP

Nr	Locus	Group	Position	Nr	Locus	Group	Position
1	TR8	1	0	43	TR20	4	61.316
2	TA113	1	3.434	44	TS36	4	76.957
3	TA8	1	10.863	45	TR55	4	93.727
4	TA203	1	29.874	46	TAA137	5A	0
5	TR43	1	41.928	47	GA102	5A	17.593
6	TA43	1	48.916	48	TAA104	5A	25.01
7	TS71	1	55.426	49	TAASH	5A	38.125
8	GAA44	1	72.142	50	TA5	5A	44.859
9	AGLC11	1	87.046	51	TS53	5A	60.291
10	TR19	2	0	52	TA39	5A	68.141
11	TR3	2	6.596	53	TS43	5B	0
12	TR14	2	12.785	54	TA116	5B	18.593
13	TA103	2	22.748	55	TR29	5B	33.909
14	TA37	2	29.677	56	TR60	5B	67.035
15	TA110	2	32.243	57	TA14	6	0
16	GA16	2	34.58	58	STMS15	6	8.067
17	GA20	2	39.291	59	TR1	6	17.651
18	TA59	2	41.682	60	TR35	6	21.676
19	TA96	2	45.352	61	TA120	6	28.403
20	TA27	2	47.819	62	TA80	6	33.467
21	TS82	2	54.018	63	TA176	6	37.001
22	TA53	2	57.555	64	TA22	6	42.267
23	TR2	2	64.726	65	TS84	6	57.925
24	TA200	2	70.147	66	TS24	6	59.907
25	TA64	3	0	67	TR40	6	72.252
26	TA34	3	19.971	68	TA106	6	72.472
27	TAA194	3	28.632	69	GA9	6	82.107
28	TA142	3	34.982	70	GA34	6	95.738
29	TR31	3	45.467	71	TA78	7	0
30	TA194	3	60.905	72	TA21	7	5.812
31	TA108	3	64.979	73	TAA55	7	12.508
32	STMS28	3	71.608	74	TA180	7	17.204
33	TS5	3	87.145	75	TA18	7	22.333
34	TR58	3	104.327	76	TAA59	7	27.616
35	TAA57	4	0	77	TA28	7	33.169
36	TA132	4	15.764	78	TA117	7	43.302
37	TA72	4	21.52	79	TS45	8A	0
38	TA186	4	29.851	80	TA159	8A	26.233
39	TA146	4	35.174	81	TA144	8A	45.526
40	TS54	4	40.178	82	TA25	8A	67.015
41	TA2	4	48.7	83	TA127	8B	0
42	TAA170	4	58.01	84	AGLC29	8B	28.192