Chickpea genomics: BAC library construction, Resistance gene analog (RGA) mapping and tagging double-podded trait

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BY

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CERTIFICATE

Certified that the work incorporated in the thesis **"Chickpea genomics: BAC library construction, Resistance gene analog (RGA) mapping and tagging double-podded trait"** submitted by **Mr. P. N. Rajesh** was carried out by him under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

Date:

P. K. RANJEKAR
(Research Guide)

DECLARATION

I hereby declare that the thesis entitled "**Chickpea genomics: BAC library construction, Resistance gene analog (RGA) mapping and tagging double-podded trait"** submitted for Ph. D degree at University of Pune has not been submitted by me for a degree at any other University.

P. N. RAJESH

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dedicated to my beloved father

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List of abbreviations

AFLP	amplified fragment length polymorphism
AP-PCR	arbitrarily primed polymerase chain reaction
BAC	bacterial artificial chromosome
cDNA	complementary DNA
CHEF	clamped heterogeneous electric field
cM	centimorgan
CTAB	hexadecyl-trimethyl-ammonium bromide
DDRT	differential display reverse transcription
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5’ triphosphate
DTT	dithiothreitol
EST	expressed sequence tag
hr	hour
HMW	high molecular weight
IAA	iso-amyl alcohol
ISSR	inter simple sequence repeat
kb	kilo base
LB	luria-bertani
LMP	low molecular weight
LRR	leucine rich repeat
MAS	marker assisted selection
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
NBS	nucleotide binding site
PAC	P1 derived artificial chromosome
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PHYLIP	phylogeny inference package
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RGAP	resistance gene analog polymorphism
RIL	recombinant inbred line
RNA	ribonucleic acid
rpm	revolution per minute
rRNA	ribosomal RNA
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second
SSR	simple sequence repeat
STMS	sequence tagged microsatellite site
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tris	Tris-hydroxymethyl amino methane
UPGMA	unweighted pair group method, arithmetic mean
YAC	yeast artificial chromosome
µg	microgram
µl	microlitre

Thesis abstract

During the past five years, comprehensive biological and technical resources have been established to explore the various facets of the chickpea genome though this crop has very ancient history of cultivation. Knowledge of the genetic diversity of a species is particularly important since modern breeding practices have narrowed the genetic diversity of cultivated crops. Genetic relationship studies can direct the breeding program which will result in reduced time to generate lines with expected combination. This information has been utilized in hybrid breeding program in various crops like rice and wheat. Characters of single Mendelian inheritance may have great importance in crop breeding. In chickpea, doublepodding is a trait, which plays a role in yield and hence identification of a marker to this trait can be utilized in chickpea breeding program. In addition to the ongoing work on genetic mapping of agronomic traits including disease resistance like fusarium wilt and ascochyta blight and the development of associated markers to them from our lab, I have made an attempt to isolate ascochyta blight resistance genes by constructing a large insert Bacterial Artificial Chromosome (BAC) library. Such large insert libraries have been extensively used to isolate disease resistance genes in various crops like rice, soybean and *Arabidopsis*.

In recent times, isolation of resistance genes by designing primers based on the conservative motifs present in all well characterized resistance genes has been in practice. RGAs are potential markers, which can serve both as candidate genes for cellular recognition and as informative markers for inferring genetic relationships between germplasms. Their utility has been reported in various crops, like maize, rice, *Arabidopsis* and soybean. The concept of differential display is to use a limited number of short arbitrary primers in combination with the anchored oligo-dT primers to systematically amplify and visualize most of the mRNA in a cell. It will be interesting to study the switching on and off of various genes upon pathogen infection and DDRT will be a good approach to study the differential gene expression pattern.

In the past decade, biotechnological tools have evolved to great extent, which

are being utilised to perform the genome analysis in various crops. However, the application of them in chickpea is in infancy state.

My thesis work specifically involves the following aspects of chickpea genome analysis:

- Genetic relationship among various wild species of chickpea using microsatellite based molecular markers
- Tagging of double-podded gene in chickpea using DNA marker approach
- Resistant gene analogue (RGA) mapping for identification of a marker to ascochyta blight resistance gene(s) and application of DDRT to study differential gene expression
- A Bacterial Artificial Chromosome (BAC) library construction in Chickpea

1. Genetic relationships among annual and perennial species of *Cicer* using Inter Simple Sequence Repeat (ISSR) polymorphism

To study the genetic diversity, I screened 23 wild *Cicer* species consisting of 6 annuals and 7 perennials with two accessions of each species wherever possible including 4 cultivated samples. Among 100 ISSRs, 15 primers showed reproducible polymorphic bands and yielded a total of 115 bands which were scored for genetic relationship analysis using the software Taxan. Bootstrap analysis was also performed using Winboot to determine the confidence limits of UPGMA based dendrogram. The latter showed the similarity index values, which ranged from 0.84 to 0.55 with an average value of 0.70.

My results showed no correlation between the genetic distance of the species and their geographical distribution. Species, which were grouped together in various sections using morphological observations, are similar to the cluster developed using ISSR data. Since the genetic relationship analysis based on ISSR data supports the morphological and crossability data, the ISSR markers have proved to be an efficient marker system for such type of studies in chickpea.

2. Identification of an STMS marker for the doublepodding trait in chickpea

In chickpea, “doublepodding” is an agronomically important trait, which is conferred by a single recessive gene and denoted as “*s*”. Earlier reports have indicated that this trait has a positive effect on yield stability using near isogenic lines. Hence identification of a closely linked marker to this trait can be efficiently used in Marker Assisted Selection (MAS) for breeding chickpea genotypes with higher yields.

I procured near isogenic lines, which were developed, for double-podded trait from University of Cordoba, Spain. After screening near isogenic lines, CA2156 (single-podded) and JG62 (double-podded) with 400 RAPDs, 100 ISSRs and 100 STMS markers, I identified one polymorphic STMS marker locus named as TA-80. This marker was analyzed among 102 $F_{5:6}$ RILs developed from a cross between Surutato-77 (single-podded) and JG62 (double-podded) to find the map distance between the marker and the double-pod gene. A 200bp fragment associated with singlepodding while a 240bp fragment associated with doublepodding were identified. Segregation (53:49) for this marker was found to fit the expected 1:1 ratio and TA-80 was linked to double-podded gene at the distance of 4.9cM. Since STMS markers are co-dominant in nature, they are reliable for application in MAS.

3. RGA mapping and differential expression analysis of genes in response to *Ascochyta rabiei* infection

A) RGA mapping

In order to isolate the ascochyta blight resistance gene(s), Resistant Gene Analog Polymorphism (RGAP), which utilizes resistance gene focused primers was adopted. The primers were designed based on the conserved sequences present in all the well-characterized resistance genes isolated from various plant species. When these primers are used in polymerase chain reaction, they amplify the genomic region called as Resistant Gene Analog (RGA). I used 24 pairs of available RGA primers for Bulk Segregant Analysis. Out of 24 pairs and 48 different combinations of RGA primers, only one pair (Pto kin 1 & 2) showed polymorphism which was later run in the mapping Recombinant Inbred Line (F6 generation) population of size 142 to study its segregation. The marker was mapped in the linkage group 5 where blight

resistance QTLs are not present. In chickpea, mapped disease resistant genes like fusarium wilt and ascochyta blight show recessive inheritance pattern and hence, NBS-LRR primers might be unlikely candidates for these genes.

B) DDRT analysis

Since RGA analysis did not yield an informative probe for blight resistance genes, I aimed to characterize the events at the gene expression level upon *ascochyta* pathogen infection. I infected 10 day old Flip 84-92C, a resistant cultivar and PI 489777, a *C. reticulatum* susceptible variety, seedlings with 1 $X10^6$ conidia of a virulent *A. rabiei* strain A20. The RNAs of 1, 2, 3, 7 and 8 days infected seedlings of resistant infected plants, susceptible infected, resistant control and susceptible control were pooled and used for differential display reverse transcription analysis. Some DDRT reactions were tried with RGA primers too. Differentially expressed DDRT products of these samples were cloned in pGEM T vector and sequenced. Two of the clones showed 87% and 86% homology with serine hydroxy methyl transferase and aldolase of pea counterparts respectively.

4. Bacterial Artificial Chromosome (BAC) library construction in chickpea

Of all the large insert libraries, bacterial artificial chromosome (BAC) libraries are being used as the foundation for physical mapping, map-based cloning and sequencing projects. There are many advantages of BAC libraries over YAC and PAC large-insert libraries. Some of these include a large insert cloning capacity of 100-400Kb, a low rate of Chimera formation, high efficiency of long insert cloning and recovery and stable maintenance of the insert. Since the ascochyta blight disease is governed by two major QTLs and a minor QTL and also my long-term objective was to isolate the blight resistance genes, I constructed the *Hind* III BAC library of Flip 84-92C which is a resistant cultivar against this disease.

The high molecular weight DNA of chickpea was isolated using nuclei method and was embedded in agarose plugs. A binary vector pCLD04541 was isolated using CsCl/Ethidium bromide equilibrium centrifugation to avoid any bacterial chromosomal contamination. The high molecular weight DNA agarose plugs were digested with *Hind* III restriction enzyme and run on 1% agarose under pulsed field. The digested

DNAs of the size 100Kb, 150Kb and 200Kb were selected and these DNAs were run on another 1% low melting point agarose under pulsed field for second size selection. After the second size selection, the DNAs in the agarose were treated with agarase enzyme to digest the agarose. After agarase treatment both the size selected chickpea genomic DNA and the *Hind* III digested vector were ligated in the presence of T4 DNA ligase at 16^0C overnight. The ligated DNA and the vector complex were transformed into ElectroMax™ DH10B™ host strain by electroporation method.

The recombinant white colonies and the non-recombinant blue colonies were selected in the presence of X-gal and IPTG using tetracycline as selective antibiotic marker. The recombinant colonies were picked up by Flexys Biorobot and arranged in 384 well plates. Randomly 80 colonies were picked up and the plasmids were digested with *Not*I restriction enzyme and run on pulsed field gel electrophoresis. The library was screened with chloroplast specific probe, which detected negligible amount of organellar DNA contamination.

Based on the size of the inserts and the genome size of chickpea, I calculated that the chickpea BAC library had 3.8times genome coverage and 95% probability of finding any chickpea genomic fragment from the library. This is the first chickpea BAC library constructed so far.

Chapter 1

Review of literature

1.1. Global production of pulses with special reference to chickpea

Legume, pronounced as legyoom or ligyoom, is a common name for any plant of the family leguminosae. The fruit of the leguminous plants, a pod, usually splits along two sides, with the seeds attached along one of the sutures. Figure 1 gives a glimpse of various pulses in the leguminosae family while Table 1 indicates global productivity of some important pulses.

Figure 1. Different pulses of leguminosae family.

Legumes	Production (in million tons)	Area (in million hectares)
Dry beans	18	22.4
Pea	14.53	8.6
Chickpea	8.6	11.2
Lentil	2.9	3.4
Pigeonpea	2.6	4.6
Cowpea	3	12.5

Table 1. World pulse production and area under cultivation (www.icrisat.org)

Although chickpea production stands third in the world, it is the primary pulse crop in South Asia where it is an important source of protein, particularly in

Regions	Countries (No.)	Area (1000 ha)	Production (1000 T)	Yield (kg/ha)
Africa	11	477	316	663
Asia	15	10159	7745	762
N/C/S America	5	147	200	1225
Europe	6	146	86	588
Australia	1	265	241	909
World	38	11194	8587	767

Table 2. World production of chickpea (FAO 1998)

vegetarian diets (Table 2). Further, India, with 71 percent of the global chickpea production, is the world's largest producer of chickpea with 7600 thousand hectares land under cultivation producing 6000 thousand tones.

1.2. Origin and cytology of chickpea

In a report by Vavilov (1926), southwest Asia and the Mediterranean were identified as the two primary centers of origin of chickpea, while Ethiopia as a secondary center of origin. Later, Singh (1997) reported that chickpea most probably originated in a region of present day southeastern Turkey and adjoining areas of Syria. Regarding the origin of kabuli and desi types of chickpea, it is reported that desi originated first followed by kabuli type which was developed by selection and mutation (Singh 1997). There is a linguistic evidence that kabuli type reached India via the Afghan capital Kabul about two centuries ago and acquired the name as "kabuli chana" in Hindi (van der Maesan 1972).

Availability of living material is a major constraint in some of the wild species of *Cicer* to carry out cytological studies and hence such studies have been performed on a limited number of *Cicer* species (van der Maesan 1987). A study on seven annual species of *Cicer* revealed that these species differ from each other in definite karyotypic features (Ohri and Pal 1991). Such a discontinuous pattern of DNA distribution has been observed in various other genera also (Narayan 1982; 1988, Ohri and Khoshoo 1986). In *Cicer,* the nuclear DNA content shows a 1.95 fold variation among the species and *C. judaicum,* with the least DNA amount, exhibits maximum karyotypic asymmetry. Though the DNA content varies between seven species, the cultivars of *C. arietinum* show constant DNA amounts with minor differences and similar karyotypes (excepting minor variation). Most significantly, no differences in the nuclear content have been detected in "desi" and "kabuli" cultivars despite their ancient divergence and disparate cultivars associated with clear-cut phenotypic differences.

1.3. Nutritional, medicinal and soil conditioning properties of chickpea

Among legumes, chickpea, pigeonpea and mungbean are the major pulses that are used in the vegetarian diet in India fulfilling a major requirement of protein. Chickpea, in particular, is the most preferred legume since it can be consumed in various ways and has got good nutritional properties. Figure 2 shows different types

of chickpea seeds (desi and kabuli) which are being grown and consumed all over the world.

Figure 2. Different types of kabuli and desi seeds of chickpea

Raw chickpea seeds contain per 100g: 357 calories, 4.5-15.69% moisture, 14.9-24.6g protein, 0.8-6.4% fat, 2.1-11.7g fiber, 2-4.8g ash, 140-440mg calcium, 190-382mg phosphorous, 5-23.9mg iron, 0-225mg beta-carotene equivalent, 0.21-1.1mg thiamin, 0.12-0.33mg riboflavin and 1.3-2.9mg niacin. Sprouting is said to increase the proportionate amounts of ascorbic acid, niacin, available iron, choline, tocopherol, pantothenic acid, biotin, pyridoxine, inositol and vitamin K. It contains 17% to 24% protein with essential amino acids such as tryptophan, methionine and cysteine. It is a good source of carbohydrates and proteins, together constituting 80% of the total dry seed weight. For the chickpea-growing world as a whole, availability of nutrients from chickpea is calculated to be 12 calories and 0.6g proteins per seed.

Chickpea is the most hypocholesteremic agent where germinated chickpea is reported to be effective in controlling cholesterol level in rats (Geervani 1991). Glandular secretions of the leaves, stems and pods contain of malic and oxalic acids,

which are supposed to lower the blood cholesterol levels. Additional medicinal applications of this legume crop include use for aphrodisiac, bronchitis, catarrh, cutamenia, cholera, constipation, diarrhea, dyspepsia, flatulence, snakebite, sunstroke and warts while seeds are considered to be antibilious in nature (Duke 1981).

Chickpea is better at fixing atmospheric nitrogen than lentil. In India, chickpeas are also grown as a catch crop in sugarcane fields and often as a second crop after rice (van der Maesen 1972). Although usually considered a dry-land crop, chickpeas develop well on rice lands. This legume crop has several agronomic properties that are beneficial to cropping systems with respect to its capacity to increase soil fertility due to its ability to fix nitrogen only in association with *Cicer* specific *Rhizobium* species *Bradyrhizobium,* while the strain which infects lentil and field pea does not work. It also stimulates mycorrhiza populations in the soil to the benefit of other crops in rotation, thus avoiding excessive use of chemical fertilizers and soil acidification. Because of its capability to dissolve calcium phosphates by the root exudates rich in citric acid, this crop is unique in mobilizing phosphorous from sources that are not available to other crops.

1.4. Biotic, abiotic and physiological constraints to chickpea production

In India, after the green revolution, which provided food security, there was a drastic shift in agriculture pattern from sustainable pulse crops to cereal crops like wheat and rice. Table 3 shows the reduction in the area and production of pulses and the increase of wheat cultivation in Haryana state which is a representative state in North India. However, this deficit is compensated by increased production in the states of central and south zone and as a result, the area and overall yields of chickpea have remained the same for decades in the country.

The major problem in chickpea is that its production falls short of demand leading to a decline in its per capita availability from 27 to 17g. This gap is further increasing due to population growth in India and in Asia which are the main

consumers of chickpea. Biotic and abiotic stresses represent the most important factors causing low production of chickpea (Singh et al. 1994). For example, the

Crop		1962	1970	1980	1998
Chickpea	**Area (000ha)**	1562	1046	553	335
	Yield (Kg/ha)	610	676	806	1120
Wheat	**Yield (kg/ha)**	716	1468	2928	3124

Table 3. Progressive reduction of pulse cultivation and increase of wheat production.

number of pathogens affecting chickpea has shown a three fold increase during last 15 years (Nene et al. 1996). The main fungi that affect chickpea are *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, causing the plant to wilt and Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Lab. Ascochyta blight is the most serious disease in North India, Pakistan, the U.S. and the Middle East (sometimes causing l00% losses) (Smithson et al. 1985). Other fungi known to attack chickpea include leaf spot (*Alternaria* sp.), *Ascochyta pisi*, rust (*Uromyces ciceris-arientini*), gray mould (*Botrytis cinera*), powdery mildew (*Leviellula taurica*), *Pythium debar-yanum*, *P. ultimum*, dry root rot (*Rhizoctonia bataticola*), foot rot (*Sclerotium rolfsii*) and wilt (*Verticillium albo-atrum*). Viruses isolated from chickpea include alfalfa mosaic, pea enation mosaic, pea leaf roll, pea streak, bean yellow mosaic, and cucumber mosaic (Duke 1981; Kaiser 1988; Smithson et al. 1985; Van Emden et al. 1988).

Among pests, pod borer (*Helicoverpa armigera*) is the most important pest of chickpea, which feeds on leaves and developing seeds (Smithson et al. 1985). Additional insect pests attacking chickpea include cutworms (*Agrotis* sp.), lesser armyworms (*Spodoptera exigua*) and leaf minor, groundnut aphid (*Aphis craccivora*), pea aphid (*Acyrthsosiphon pisum*), cowpea bean seed beetle (*Callosobruchus maculatus*), and Adzuki bean seed beetle (*C. chinensis*). In general, estimates of

yield losses by individual pests, diseases or weeds range from 5-10 % in temperate regions and 50-100 % in tropical regions (van Emden 1988).

Drought stands to be the number one problem among the abiotic factors in major chickpea growing regions because the crop is grown on residual moisture and is eventually exposed to terminal drought (Johansen et al. 1997). In West Asia and North African countries, low temperature causing freezing injury or death or delayed onset of podding reduces yield tremendously (Singh 1987). Heat and salinity problems are also important following drought and cold stresses (Singh et al. 1994).

Additional insurmountable constraints in all grain legumes including chickpea are three processes namely photorespiration, nitrogen fixation and photosynthetic energy relationships which operate against high grain yields. Photorespiration occurs in the light and consumes about 30% of the products of photosynthesis in all grain legumes. Secondly, the symbiotic relationship between the legume plant and the *Rhizobium* to fix nitrogen diverts the carbohydrates from the plant to the bacteria and reduces potential grain production by about 10%. Lastly, the plant needs more energy to produce a given amount of oil and protein than starch. This energy intensive process ultimately manifests itself in lower yields in grain legumes (Hymowitz 1990). Therefore, taking into account various limitations on chickpea production, better genotypes of this legume crop are being developed through conventional agricultural practices all over the world.

1.5. Role of conventional breeding in chickpea production improvement

Plant breeding has been one of the most successful technologies developed in modern agriculture all over the world because its methods are adaptable to various production schemes, require inexpensive inputs and the products have pervasive social benefits. For most grain crops, yields have increased continuously since the 1930s and nearly 50% of the gains may be attributed to the enhanced genetic potential of the cultivars. Besides increased productivity, plant breeding has contributed to remarkable transformations in quality, growth habits and use and

adaptation. Though few endeavors were less successful, the genetic modifications of crop species achieved through plant breeding have been very positive and important (Lee 1995).

There are enormous resourceful wild germplasms available especially for resistance to important diseases in chickpea. For example, *C. judaicum*, *C. montbrettii* and *C. pinnatifidum* show resistance to ascochyta blight; *C. judaicum* to fusarium wilt; *C. pinnatifidum* and *C. judaicum* to grey mold; and *C. bijugum* to cyst nematode (Singh et al. 1990). ICARDA has identified important wild species like *C. bijugum*, *C. judaicum*, *C. pinnatifidum* and *C. echinospermum* possessing multiple stress resistance (Singh et al. 1994). However, to the best of our knowledge, there are no reports available so far for the successful introgression of these genes into the cultivated chickpea because of the crossability barrier. *C. arietinum* is cross compatible only with *C. reticulatum* and *C. echnospermum*. Singh and Ocampo (1997) observed 28-153% hybrid vigor in the F1s of crosses between *C. arietinum*, *C. echinospermum* and *C. reticulatum* compared to the 75% reported in intraspecific crosses (Singh et al. 1994). This shows that interspecific hybridization, which causes genetic reshuffling, could combine favorable genes and increases the yield.

Among the cultivated species, ICRISAT has identified several hundreds of resistant sources after evaluating 12,000 accessions and has shared them with national programs. For wilt disease, Punjab Agricultural University developed lines GL87078 and GL87079 which showed resistance to the predominant races, namely race 1, 2, 3 and 4 of *Fusarium oxysporum* f. sp. *ciceri*. Similarly for ascochyta blight, ICARDA released 39 cultivars in 12 countries. For Phytophthora resistance, "Barwon" was developed from an intraspecific cross. (Singh et al. 1994). Further, doublepodding is another trait which is reported to be playing a positive role in yield stability in chickpea from a study using near isogenic lines (NILs) developed from an intraspecific cross (Rubio et al. 1998).

To improve the world position of chickpea, the area under chickpea cultivation needs to be expanded as it is tolerant to dry conditions and can be used as fallow replacements in many areas of the arid regions especially in Africa and America,

where area of the crop is small but yield is much higher than in Asia. Introduction of higher input use is yet another approach to increase the chickpea production as practiced in European countries (Jodha and Subba Rao 1986). Selection of various appropriate traits is an important criterion to improve the crop production. In chickpea, variation for growth duration, yield, biomass and disease resistance are recorded. (Muehlbauer and Tullu 1997) (www.hort.purdue.edu). Seed mass is another highly heritable and important yield component in chickpea, which can be used effectively as an indirect selection criterion for improving seed yield (Singh and Paroda 1986). Development of new plant types like erect/upright plant with few branches instead of traditional bushy plant canopy for better light interception and canopy air exchange and; tall plant height for better competition against weeds and suitability for mechanical harvesting can be manipulated to increase the yield potential. Seed cost can be reduced to improve the production, as the costs are very high because of strong demand and short supply. Also, regular application of fertilizers, pesticides and fungicides will improve the yield of this legume crop.

Apart from these conventional approaches, productivity enhancement in chickpea can be achieved by amalgamation of classical breeding techniques and the biotechnological advances especially in developing countries. This will also help to enhance profitability of chickpea, particularly in the major chickpea growing countries where yields are low.

1.6. Genomics and its potential in chickpea improvements

Genomics is a new science that studies genomes at a whole genome level by integrating the five traditional disciplines of genetics with new technology from informatics and automated systems (Figure 3) (Liu 1998). In the past one decade, this field has revolutionizing impact on agricultural research. Some of the important

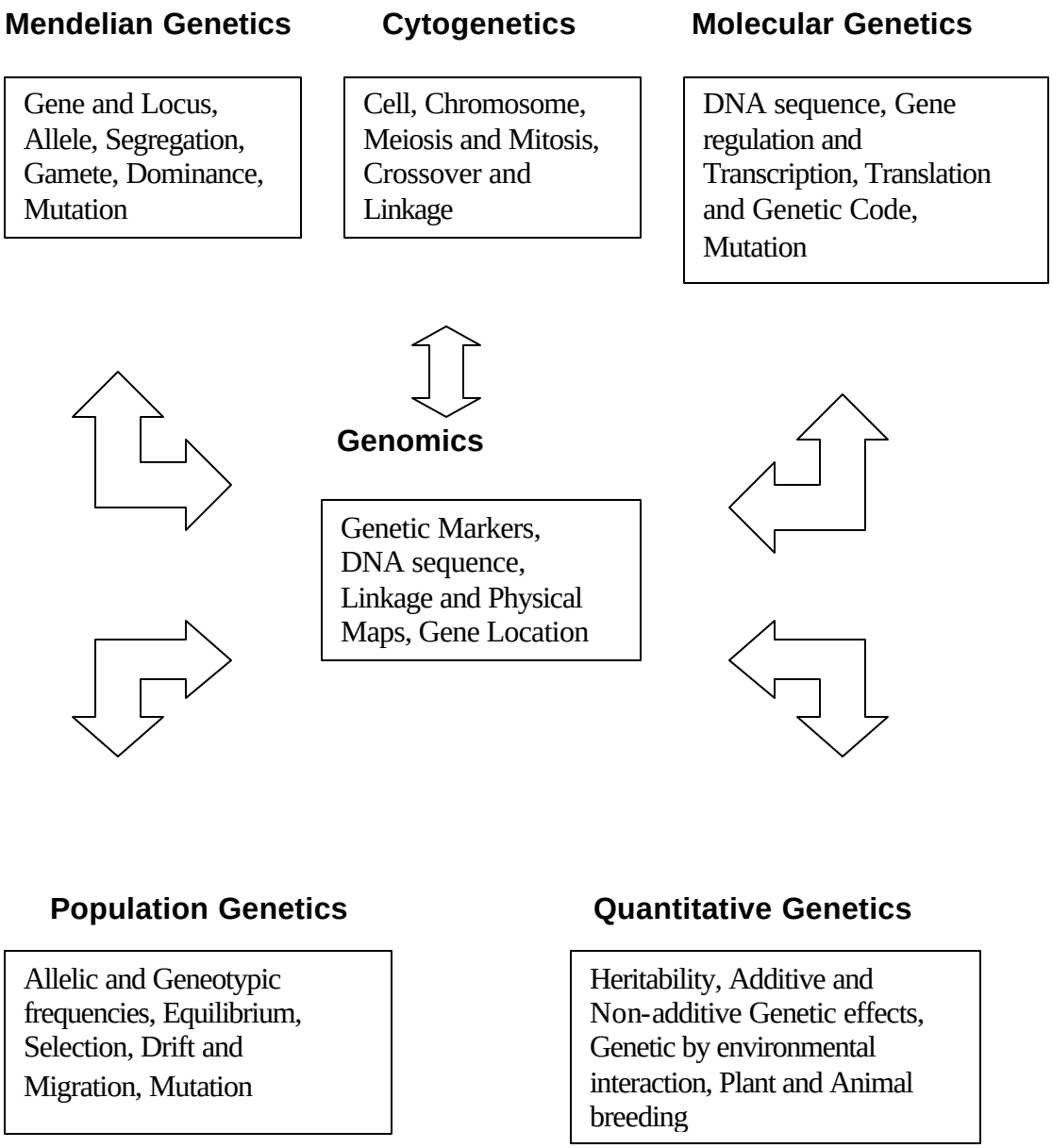


Figure 3. Definition of Genomics (Liu 1998)

milestones include isolation and functional characterization of many agronomically important genes from cereals, potato, cotton and soybean, and sequencing of the complete genome of *Arabidopsis* and rice. These have provided a better

understanding of plant genome evolution, gene function and the level of homology between various plants.

In the following topics, I have overviewed a few important aspects of plant genomics by giving examples from chickpea research wherever possible and from other important crops where successes have been achieved. This includes genetic diversity using DNA based molecular markers and marker assisted selection especially in chickpea and recent advances in disease resistance and physical mapping using large insert library in general.

1.6.1. Assessment of genetic diversity and its importance in chickpea breeding

The definition of genetic diversity is a statistical concept referring to the variance at individual gene loci (among alleles of a gene), among several loci or gene combinations, between individual plants within plant populations, or between plant populations (Smale and McBride 1996). A detailed knowledge of the genetic relationships among accessions is an important factor for the success of plant breeding programs and for efficient sampling and more informed utilization of available germplasm. The study on genetic diversity of species is emphasized because modern breeding practices have narrowed the genetic diversity of cultivated crops. This reduction in genetic diversity could severely limit future breeding programs for adaptive traits such as resistance to biotic and abiotic stresses and reduce stability of crop yields (Labdi et al. 1996). Pedigree information or morphological characteristics (Tateoka 1962; Moringa 1969; Souza and Sorrels 1989; Smith et al. 1990), qualitative and quantitative traits (Smith et al. 1990; Zhong-hu 1991), biochemical and isozyme markers (Second 1982; Leonardi et al. 1991), seed storage protein analysis (Vairinhos and Murray 1983; Ahmad and Slinkard 1992) and DNA based markers have been exploited to estimate the genetic diversity in various crops.

The main advantages of using molecular markers are that they measure the genetic diversity at DNA level, can account for the effects of selection, are environment-independent, and are available in an enormous number. Further,

invention of PCR technology and its technical simplicity to detect even single-base pair difference facilitated its use in the analysis of phylogenetic relationships, cultivar identification, genetic diversity, parentage determination and marker-assisted selection in several plant genera (Kawchuk et al. 1994; Sharma et al. 1995; Abo-elwafa et al. 1995; Friesen et al. 1997; Wolff and MorganRichards 1998). Although extensive studies were carried on phylogenetic relationship in *Cicer* species using allozymes (Kazan and Muehlbauer 1991, Tayyar and Waines 1996) and seed storage proteins (Ahmad and Slinkard 1992), very few reports are available at the DNA level except RFLP analysis (Patil et al. 1995), RAPD studies (Ahmad 1999), allelic variation studies at a microsatellite locus (Udupa et al. 1999) and STMS analysis (Choumane et al. 2000). Hence, the enormous potential of molecular markers can be further explored in chickpea for genetic relationship studies, genome mapping and indirect selection (marker assisted selection), gene tagging of qualitative and quantitative traits, marker-aided prediction and improvement of hybrid vigor.

1.6.2. Genome mapping and gene tagging followed by molecular breeding

Gaur and Slinkard (1988) developed the first rudimentary linkage map of *Cicer*, with 4 linkage groups consisting of 13 isozyme loci, although the linkage analysis among morphological traits were initiated an year earlier (Muehlbauer and Singh 1987). Three additional linkage groups were then established using both morphological and isozyme loci (Gaur and Slinkard 1990b). With the advent of DNA markers, *Cicer* map was further expanded with 9 morphological, 28 isozyme, 44 RAPD, 9 RFLP and 6 other markers (Simon and Muehlbauer 1997). Currently existing map of *Cicer* consists of 354 various kinds of markers, approximately at an average distance of 6.8cM between markers (Winter et al. 2000), which is much higher than that of rice linkage maps comprising 2000 mapped molecular markers each approximately at every 0.9cM (Causee et al. 1994, Kurata et al. 1994, Shoumara et al. 1994). Many traits have not been mapped in the comprehensive map of *Cicer* except fusarium wilt resistance (Winter et al. 2000). Hence,

development of a high-density map with many morphological traits for *Cicer* must be the first step towards chickpea genomics. Such type of high-density molecular maps make it feasible to identify, map and measure the effect of genes and gene blocks that contribute substantially to quantitative traits. Molecular markers have pronounced applications in indirect selection for linked traits like leaf rust resistance genes in wheat (Feuillet et al. 1997; Schachermayer et al. 1997), fusarium wilt resistance in chickpea (Ratnaparkhe et al. 1998), accelerated backcrossing, analysis and selection of quantitative traits such as blast resistance in rice (Wang et al. 1994), seed protein content in soybean (Diers et al. 1992) and ascochyta blight in chickpea (Santra et al. 2000), identification of hybrids, selection for resistance to pests and pathogens not present in the immediate environment (quarantine traits) and the analysis of alien chromosome segments (Langridge and Chalmers 1998). Marker-assisted breeding programs have been estimated to reduce the time-to-market by 50-70% (Tanksley et al. 1989; Schneider 1997). Isolation of almost twenty R-genes from genetically well-characterized plant species has been facilitated because of molecular markers through map-based cloning over the past five years (Martin 1999). However, the use of markers to study genotype X environment interactions and to genetically dissect complex traits has gained enormous importance recently.

Another application of molecular markers is gene pyramiding which is mostly a resistance breeding procedure where more than one gene is brought together to enhance the resistance life of an otherwise better performing variety against the pathogenic races. Identification of markers for resistance genes can efficiently facilitate pyramiding major genes into a valuable background in less time and make it more cost effective (Tanksley et al. 1989). Gene pyramiding was successfully performed in apple for the scab and mildew resistance (Gianfranceschi et al. 1996; Tartarini et al. 1999; Markussen et al. 1995), in wheat for rust resistant genes (Roelfs et al. 1992; Jiang et al. 1994) and in barley for scald resistance (http://www.regional.org.au/au/abts/1999/raman3.htm).

According to a recent report (Jones 2001), pyramiding of R gene alleles from one species into a single genotype might not provide durable resistance, but will

select pathogens that can tolerate the loss of multiple compatibility factors and may lead to yield penalties. Hence it is suggested that a population which is heterogeneous for different R genes have an advantage over a single cultivar with all R genes. In heterogeneous population, only a specific proportion of the plants can support the growth of any virulent race, so the rate of epidemic will be correspondingly reduced. The intense pathogen pressure of avirulent pathogen races may trigger systemic acquired resistance, which will reduce susceptibility to virulent races. Furthermore, any mutation affecting the virulence of a race that can overcome one R gene which enables it to grow on plants that have another R gene may result in slightly reduced parasite fitness against the first R gene. The "heterogeneous population" approach for developing resistance against pathogens can be attempted in chickpea to defend various fungal pathogens especially like *Fusarium oxysporum* in which different loci confer resistance to different races of this fungus (Tullu et al. 1999; Ratnaparkhe et al. 1998) and also *Ascochyta rabiei* (Santra et al. 2000) by the breeders.

1.6.3. Plant disease resistance genes:

1.6.3.1. Model for resistance mechanism

The understanding of how the plant recognizes a foreign body as a pathogen and responds to it, will have long-term practical applications in agriculture. The first insight into the genetics of plant disease resistance involving the hypersensitive reaction (HR) was by Flor (1956; The complementary genic systems in flax and flax rust). This model proposed a gene-for-gene hypothesis for the genetic interaction between plant and pathogen. It suggests that a dominant gene from the host interacts with a corresponding dominant avirulence gene from the pathogen. An *Avr* gene is from pathogen whose expression causes the pathogen to produce a signal that triggers a strong defense response in a plant with the appropriate resistance gene (R) (Keen 1990, De Wit 1992). However, expressing an *Avr* gene does not stop the pathogen from being virulent on hosts that lack the corresponding R gene. The

interaction between the host resistance gene and the pathogen avirulence gene, elicits a HR, thus providing resistance (Table 4)

Pathogen genotype	Host plant genotype	Host plant genotype
	R1	r1
Avr1	No disease (Plant and pathogen are incompatible)	Disease (Plant and pathogen are compatible)
avr1	Disease (Plant and pathogen are compatible)	Disease (Plant and pathogen are compatible)

Table 4. Flor's gene for gene model.

The current model refined Flor's hypothesis, which states that a ligand produced by the pathogen interacts directly with a corresponding plant receptor, which triggers activation of a defense response (Richter and Ronald 2000). Under this model, pathogen and plant genes involved in this interaction are subject to different evolutionary forces.

1.6.3.2. Classification of plant disease resistance genes

The predicted products of R genes are classified into 6 groups: (Richter and Ronald 2000) as detailed with examples in Table 5.

The maize gene *Hm1* conferring race specific resistance to the fungal pathogen *Cochliobolus carbonum* race1 represents the first resistance gene (Johal and Briggs 1992) which was isolated using a transposon tagging method. It encodes a NADPH-dependent HC toxin produced by the fungus and is classified as the fifth group (Table 5).

The next group includes the tomato *Pto* gene, conferring resistance to *Pseudomonas syringae* pv. *tomato* containing the *avr* gene *avrPto* (Martin et al. 1993, Ronald et al. 1992). *Pto* belongs to a multigene family encoding proteins with

Class	R gene	Plant	Pathogen	Pathogen type	*Avr* gene	Structure	Cellular location	Size (aa)
1	*RPS2* *RPS5* *RPS4*	Arabidopsis	*Pseudomonas syringae* pv. *tomato*	Bacteria	*AvrRpt2* *AvrPpB* *AvrRp4*	LZ-NBS-LRR LZ-NBS-LRR TIR-NBS-LRR	Cytoplasmic	909 889 1217
	RPM1	Arabidopsis	*P. syringae* pv. *maculicola*	Bacteria	*AvrRpm1* *avrB*	LZ-NBS-LRR	Cytoplasmic	926
	Rx	Potato	*Potato virus X*	Virus	Coat protein	LZ-NBS-LRR	Cytoplasmic	937
	Gpa2	Potato	*Globodera pallida*	Nematode	unknown	LZ-NBS-LRR	Cytoplasmic	912
	N	Tobacco	*Tobacco mosaic virus*	Virus	TMV replicase	TIR-NBS-LRR	Cytoplasmic	1144
	L^6	Flax	*Melampsora lini*	Fungus	AL^6	TIR-NBS-LRR	Cytoplasmic	1294
	M	Flax	*M. lini*	Fungus	*AM*	TIR-NBS-LRR	Cytoplasmic	1305
	RPP5 *RPP1* *RPP8*	Arabidopsis	*Peronospora parasitica*	Fungus	*AvrRPP5* *AvrRPP1* *AvrRPP8*	TIR-NBS-LRR TIR-NBS-LRR LZ-NBS-LRR	Cytoplasmic	1361 1189, 1221, 1217 906
	12C-1	Tomato	*Fusarium oxysporum*	Fungus	unknown	NBS-LRR	Cytoplasmic	1220
	Mi	Tomato	*Meloidigyne incognito*	Nematode	unknown	NBS-LRR	Cytoplasmic	1257
	Rp1-D	Maize	*Puccinia sorghi*	Fungus	*AvrRp1D*	NBS-LRR	Cytoplasmic	1292

	Xa1	Rice	*Xanthomonas oryzae* pv. *oryzae*	Bacteria	*AvrXa1*	NBS-LRR with unique LRR repeats 93aa long	Cytoplasmic	1802
	Prf	Tomato	*P. syringae* pv. *tomato*	Bacteria	*AvrPto*	NBS-LRR	Cytoplasmic	1824
2	*Pto*	Tomato	*P. syringae* pv. *tomato*	Bacteria	*AvrPto*	Protein kinase	Cytoplasmic	321
3	*Cf-9* *Cf-4* *Cf-2* *Cf-5*	Tomato	*Cladosporium fulvum*	Fungus	*Avr9* *Avr4* *Avr2* *Avr5*	eLRR-TM eLRR-TM eLRR-TM eLRR-TM	Transmembrane	863 806 1112 968
4	*Xa21*	Rice	*X. oryzae* pv. *oryzae*	Bacteria	unknown	LRR, Protein kinase	Transmembrane	1025
5	*Hm1*	Maize	*Cochliobolus carbonum, race1*	Fungus	None	Toxin reductase	Cytoplasmic	357
6	*Mlo*	Barley	*Erysiphe graminis* f.sp. *hordei*	Fungus	unknown	G-protein coupled receptor	Transmembrane	533
	$Hs1^{pro-1}$	Sugar beet	*Heterodera schachtii*	Nematode	unknown	Controversial	Unknown	282

Table 5. The six classes of plant resistance genes

similarity to serine-threonine protein kinases, suggesting a role for *Pto* in cellular signaling via protein phosphorylation (Figure 4).

The largest group (NBS/LRR) contains disease resistance genes from diverse plant species, such as *Arabidopsis* (*RPS2* and *RPM1*), tobacco (*N*), tomato (*prf*) and flax (*L6*) (Bent et al. 1994, Grant et al. 1995, Salmeron et al. 1996, Staskawicz et al. 1995). The derived amino acid sequence of these genes indicates the presence of leucine-rich repeats (LRR), putative cytoplasmic signaling domains and nucleotide binding sites (NBS). LRRs are believed to mediate protein-protein interactions or

determine specific recognition of ligands by the receptor molecules (Kobe et al. 1994). LRR domains of resistance gene products show similarity to diverse proteins controlling cell-cell communication in development and signaling suggesting that these genes may have evolved through duplication and divergence of common ancestors (Torii et al. 1996, Clark et al. 1997, Li and Chory 1997) (Figure 4).

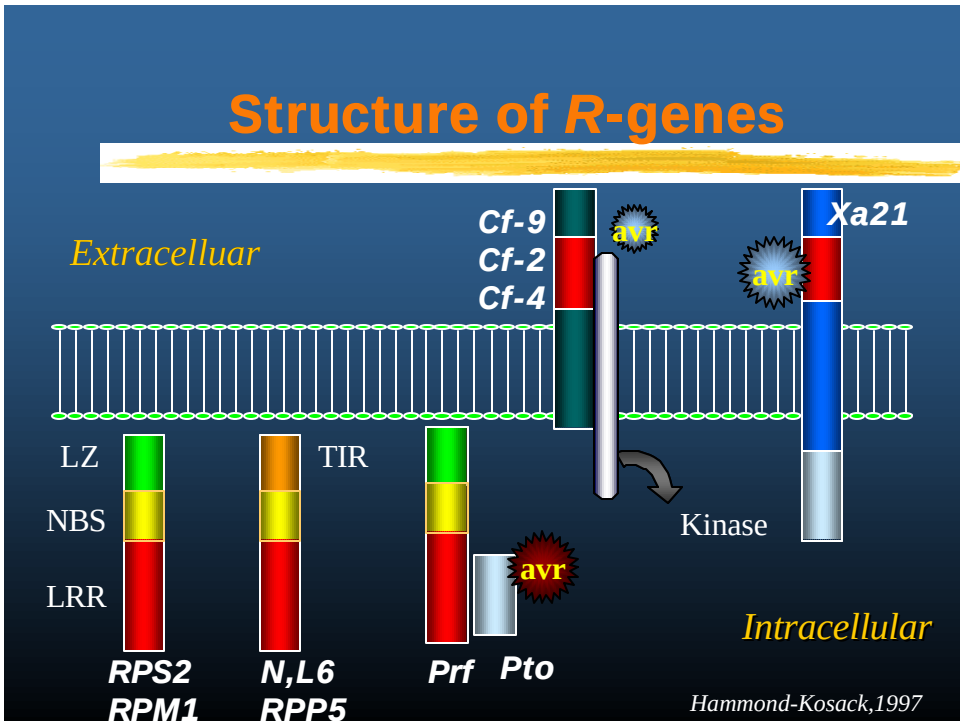


Figure 4. Diagrammatic representation of different types of resistance genes (Hammond-Kosack and Jones 1997)

Yet another class of R genes includes the *cf* genes which encode putative membrane-anchored extracytoplasmic glycoproteins, and show homology to the receptor domain of several receptor-like protein kinases and to members of the LRR family of proteins (Dixon et al. 1996, Jones et al. 1994). A novel class of plant disease R genes encoding a putative receptor kinase (RK) with the presence of LRR motif is represented by the rice gene *Xa*21, conferring resistance to *Xanthomonas*

oryzae pv. *Oryzae (Xoo)*, which was isolated using a map-based cloning strategy (Song et al. 1995) (Figure 4).

The last class of resistance gene, which is *mlo* gene in barley encodes a 60kDa protein anchored in the membrane by seven membrane-spanning helices and a G-protein-linked receptor. Since this gene does not possess any of the common motifs present in usual R-gene, the resistance mechanism of wild-type *Mlo* protein is predicted to attenuate resistance, whereas mutants are derepressed (Rainer et al. 1997). *Hs1*$^{pro-1}$ resistance gene from sugar beet with an unknown *Avr* gene is yet to be analyzed in detail from its structural and functional point of view (Ellis and Jones 1998).

1.6.3.3. Functions of R-genes

R proteins are predicted to fulfill two basic functions: to confer recognition of any *Avr* gene-dependent ligand, and after a recognition event, to activate downstream signaling that leads to rapid induction of various defense responses (Hammond-Kosack and Jones 2000)

Most plant species contain a large number of highly polymorphic disease resistance genes, most of which share common structural motifs (Baker et al. 1997). One such feature is the reiterated LRR motif, which contains leucines or other hydrophobic amino acids at regular intervals within a sequence of about 24 amino acids. Genetic evidence indicates that the beta strand/beta-turn of the LRR (with the consensus sequence xxLxLxx) is a key region in the R-protein and appears to determine its specificity (Figure 5). Studies on Porcin ribonuclease inhibitor crystal structure determined that the conserved leucines (L) in the plant R proteins within this consensus are predicted to occupy the hydrophobic protein core, whereas the other residues (x) form a solvent-exposed surface that can participate in binding other proteins (Hammond-Kosack and Jones 2000).

Most LRR type R proteins also possess a central nucleotide-binding site (NBS) that contains several conserved motifs, the functions of which are not known. Although these R proteins do not appear to have intrinsic kinase activity, they could

bind ATP or GTP and then activate the defense response. Mutations that alter key residues within the proposed NBS destroy R protein function. Some current models view the central NBS as an adaptor region, linking the C-terminal LRR recognition domain to various N-terminal effectors (Hammond-Kosack and Jones 2000).

Some NBS-LRR R proteins possess a putative leucine zipper (LZ) or coiled-coil sequence between the N terminus and the NBS domains. LZs are well known for their roles in homo- and hetero- dimerization of eukaryotic transcription factors as well as facilitating interactions between proteins with other functions. Other NBS-LRR R proteins contain a large N-terminal domain called the Toll/interleukin-1/resistance

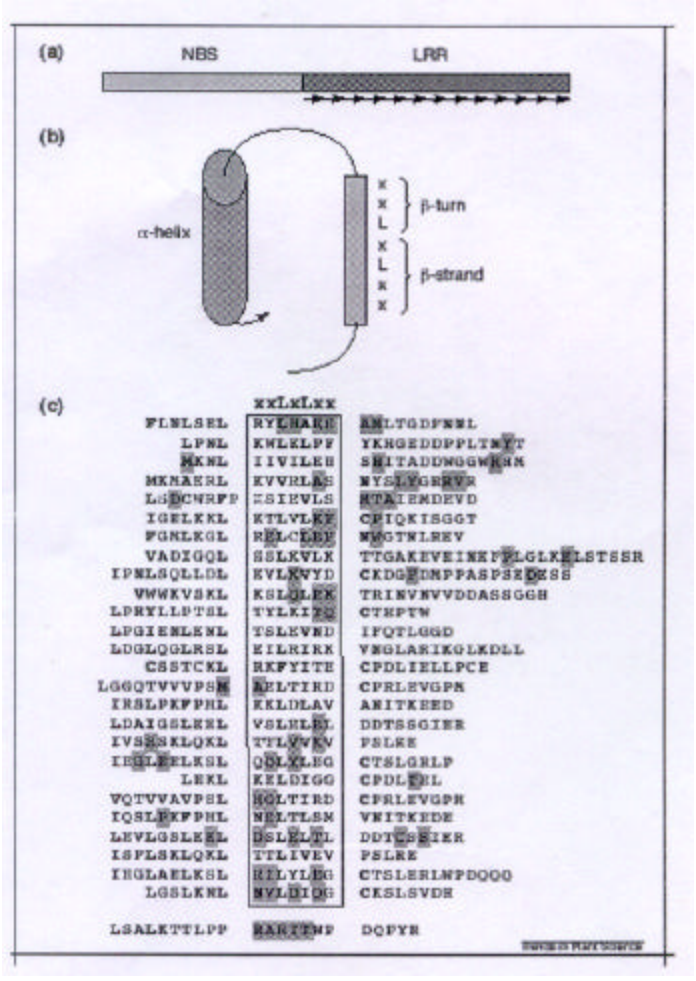


Figure 5. The variable nature of LRRs in plant resistance proteins

a) LRRs occur in extracytoplasmic *cf* class of, *Xa*21 type resistance proteins
b) The structure of an individual LRR unit based on the PR1 protein
c) An alignment of the 26 LRR units of the *L*6 flax rust resistance protein

(TIR) domain, which has some similarity to the cytoplasmic signaling domain of the Drosophila Toll protein, the mammalian interleukin receptor (IL-1R) and a family of mammalian Toll-like receptors, one of which participates in recognition and response to lipopolysaccharides (LPS). Toll, IL-1R and the mammalian Toll homolog all contribute to the immune response. The presence of the TIR domain in several R plant proteins suggests a role for this domain in signaling but not in ligand binding.

1.6.3.4. Durability of R genes

Since virulence is recessive, a mutation in the avirulence gene of the pathogen allows it to become virulent on the host. In contrast, the plant must possess a mechanism that is flexible enough to ensure response to a new pathogen. This boom and bust cycle of disease resistance results, because effective disease control (the boom years) occurs only when the pathogen population consists entirely of races those express the corresponding functional *Avr* gene. Once an *avr* mutant race of pathogens appears, disease control fails (the bust years). Some R genes have provided excellent disease control for more than 15 years. For example *cf*-9 gene mediated resistance to the fungus *Cladosporium fulvum* has remained effective since the early 1980s (Hammond-Kosack and Jones 2000). The genes *Bs*2 and *Xa*21 in pepper and rice, respectively, show resistance to broad spectrum of pathogens. Their durability is because of their ability to recognize '*avr*' proteins secreted by most or all races of *X. campestris* and *X. oryzae,* respectively (Kearney and Staskawicz 1990; Wang et al. 1996).

In certain cases, R-gene program for disease control needs to be abandoned, since the pathogen may overcome the resistance. For example, the causal agent *Phytophthora infestans* for potato late blight overcame the resistance provided by all eleven R-genes that had been introgressed from the wild species into the cultivated potato (Landeo et al. 1995).

In conclusion, the identification of few distinct classes of R genes suggests that plants have evolved only a limited number of mechanisms to defend themselves against microbial attack. Initially, plant breeders preferred to choose resistant plant

germplasm as it provided the possibility of a cheap solution to disease control in crops. But prevalence of "Boom and Bust" cycle of disease control in many crops made it rarely achievable (Agrios 1988). Hence, cloned R genes may provide novel tools for plant breeders to improve the efficiency of plant breeding strategies, via, marker assisted breeding, and by using transformation for accelerating the introgression of useful R genes from related species (Michelmore 1995). Hopefully, a combination of strategies will reduce the requirement for agrochemicals to control crop diseases and will accelerate effective retrieval and deployment of the natural variation in R genes of wild plant species (Hammond-Kosack and Jones 1997).

1.6.4. Technical and technological advancement in genome characterization

The field of genomic research is expanding at an exponential rate and is leading to basic insights into genome organization, evolution and all other related aspects. Since many agronomically important genes are known only by their phenotypes and very little information on their biochemical function is available, map-based cloning has become an efficient and widely used strategy in isolation of such genes (Arondel et al. 1992; Bent et al. 1994; Chang et al. 1993). Our understanding of organismal genetics now extends from phenotypes to their molecular genetic basis and it is now clear that next wave of progress in genetics will hinge on genome physical mapping. A physical map will provide an invaluable and readily accessible system for many detailed genetic studies and isolation of genes of economic and biological importance (Zhang and Wing 1997). Genome sequencing is a powerful tool for large-scale gene discovery, cloning and decoding. It is necessary to determine complete sequences and intron/exon structures of all genes, including those whose functions are not yet known, to map all genes and other sequences to the genome, to reveal the regulatory elements of all genes, to uncover any unexpected sequences and to decipher genome organization and evolution (Zhang and Wu 2001).

1.6.4.1. Bacterial artificial chromosome library construction

In order to fulfill all the above mentioned objectives, one needs a large insert DNA library that can be maintained stably for a long term and can be readily used for genetic and biological studies. The stability of the source library represents the reliability and long term value of the information explored from it. There are basically four types of large insert libraries available. In the order of evolution, cosmids have been used as early as the beginning of plant molecular biology research, Yeast Artificial Chromosome (YAC) library is the second (in late 1980s) followed by Bacterial Artificial Chromosome (BAC) (Shizuya et al. 1992) and P1 derived artificial chromosome (PAC) (Oannour et al. 1994) libraries, which are relatively new types of large insert libraries. Cosmids are used to clone DNA fragments as high as 40kb. The cloning capacity in YACs is limitless and depends on the quality and size of the DNA to be cloned, while DNA fragments as high as 400kb have been cloned in BAC vectors (Shizuya et al. 1992). YAC vector was the only system used for genomic library construction capable of accepting long inserts despite drawbacks such as a high rate of chimera formation, internal deletion (Anderson 1993), low efficiency of insert cloning and insert DNA preparation, till BAC and PAC systems were discovered.

Theoretically, it is possible to calculate the number of clones required from each of these libraries to be screened to complete the objective of performing a map based walk (Table 6). For example, to assemble a 1Mb overlapping contig, larger the inserts; less are the steps required and more is the probability of completing a contig or walk. If a cosmid library is used with 50kb average insert size, 39 steps would be required and the probability of completing the walk would be almost nil. If a BAC library is used (e. g. 150kb average insert size), it would require 13 steps and would have 90% probability of completing the walk. For the YAC library with an average insert size of 500kb, about three steps would be required to cross the same 1000kb distance and would have a 99% chance of success (Figure 6) (Zhang et al. 1995).

Table 6. Comparative information of different types of libraries for different crops

Scientific name	Common name	Genome size (Mbp/1C)	Cosmids 40kb	BACs 150kb	YACs 500kb
Allium cepa	Onion	15, 290	$1.76X10^6$	$4.7X10^5$	$1.4X10^5$
Arabidopsis thaliana	Arabidopsis	145	$1.7X10^4$	$4.5X10^3$	$1.3X10^3$
Avena sativa	Oat	11,315	$1.3X10^6$	$3.5X10^5$	$1X10^5$
Beta vulgaris ssp. esculenta	Sugar beet	758	$8.7X10^4$	$2.3X10^4$	$7X10^3$
Brassica napus	Canola	1182	$1.36X10^5$	$3.2X10^4$	$1.1X10^4$
Phaseolus vulgaris	Common bean	637	$7.4X10^4$	$2X10^4$	$5.9X10^3$
Oryza sativa ssp. indica & japonica	Rice	431	$5X10^4$	$1.3X10^4$	$4X10^3$
Triticum aestivum	Wheat	15,966	$1.8X10^6$	$4.9X10^5$	$1.5X10^5$
Zea mays	Maize	2504	$2.9X10^5$	$7.7X10^4$	$2.3X10^4$
Nicotiana tabacum	Tobacco	4434	$5.1X10^5$	$1.4X10^5$	$4.1X10^4$
Lycopersicon esculentum	Tomato	953	$1.1X10^5$	$2.9X10^4$	$8.8X10^3$
Gossypium hirsutum	Cotton	2246	$1.8X10^5$	$6.9X10^4$	$2.1X10^4$

Of all large insert libraries, BAC libraries are suitable for most of the large genome applications. They are more 'user friendly' than the YAC and PAC libraries, because they have a reasonably large insert cloning capacity of 100-300kb, a low rate of chimera formation, high efficiency of long insert cloning and recovery and stable maintenance of the insert (Nakamura et al. 1997).

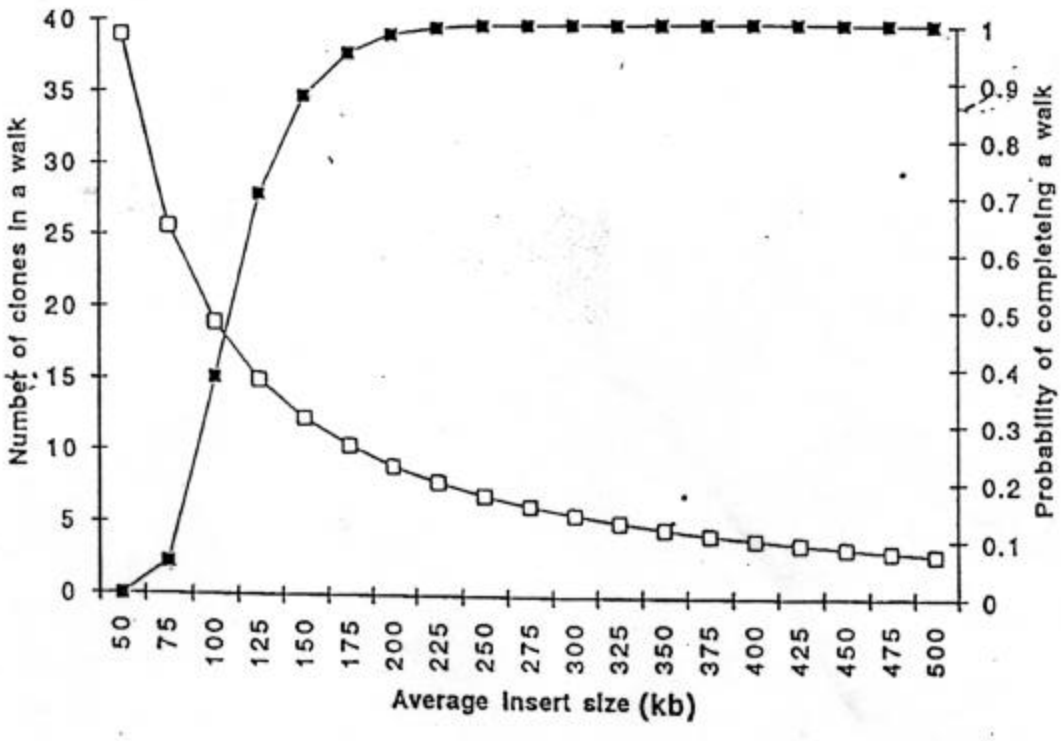


Figure 6. Plot of theoretical number of steps needed to walk 1000kb.

1.6.4.2. Application of BAC library in physical map development

Recently, there have been many reports of construction of BAC libraries from a few plant species (Woo et al. 1994; Wang et al. 1995) as well as from an individual human chromosome (Wang et al. 1994), in order to exploit them for various further applications. Many disease resistance genes have been cloned as shown in Table 5, and about half of them are by positional cloning (Martin et al. 1993; Bent et al. 1994;

Grant et al. 1995). Success of map-based cloning in crop plants depends on the target gene being localized to such a short genetic interval (0.1-0.01cM) that the markers and the target gene are separated from regions rich in repeated sequences Zobrist et al. 2000). Fine mapping to this degree can be accomplished 10-50 fold more efficiently with a physical map that encompasses the whole genome than by marker saturation and chromosome walking or landing (Zhang and Wing 1996). Large insert DNA libraries have allowed the development of physical maps for a few organisms (Burke et al. 1987).

Genes underlying QTL or with related functions such as disease resistance, seem to be organized in clusters (Staskawicz et al. 1995). BAC vectors are designed for cloning large fragments that might contain a gene cluster or an intact locus. Therefore, the ability to introduce BACs into plant cells provides potential for functional genomics, genetic engineering of complex loci and the assembly of several unlinked genes into a single locus (Hamilton 1996).

1.6.4.3. Strategies for genome physical mapping with large-insert clones

i) Iterative hybridization

It is a colony hybridization-based strategy of the whole library, which was developed and first applied to generate high-resolution cosmid- and P1-based maps of fission yeast *Schizosaccharomyces pombe* (Hoheisel et al. 1993). It is also described as sampling "without replacement" / "double end-probe clone limited" hybridization.

ii) Mapped DNA marker-based chromosome landing

This is a hybridization- and/or PCR based strategy for genome physical mapping, depending on the types of DNA markers to be used for landing the large-insert clones of the source library to the target linkage map. This was applied to generate large-insert YAC based physical maps of human (Chumakov et al. 1995), mouse (Nusbaum et al. 1999), rice (Kurata et al. 1997) and *A. thaliana* (Canilleri et al.

1998; Schmidt 1995, Zachgo et al. 1996) and more recently, the BAC-based physical map of the euchromatic portion of the *Drosophila* genome (Hoskins et al. 2000).

iii) Restriction fingerprint analysis

This is based on the hypothesis that the clones derived from the same regions of a genome share similar restriction patterns (a large number of common bands). BAC fingerprinting of an organism can be rapidly performed using Fpase kit developed for large-insert BAC clones (Zhang HB and Tao Q, Invention no. TAMUS 1228). This produces fragments having sufficient overlaps that can help to develop contigs by passing through a set of overlapping nearest neighbors. This strategy was successfully applied to generate the physical maps of *Saccharomyces cerevisiae* (Olson et al. 1986; Riles et al. 1993) and *Caenorhabditis elegans* (Coulson et al. 1986; Hodgkin et al. 1995).

iv) BAC end sequencing and fingerprint analysis

This is a combined BAC end sequencing and restriction fingerprinting strategy. In this method, genome sequencing and physical mapping are performed simultaneously. This has been used in the sequencing projects of *Arabidopsis* (Lin et al. 1999; Mayer et al. 1999), *Drosophila* (Adams et al. 2000) and human (Dumham et al. 1999; Hattori et al. 2000).

1.6.4.4. Application of a physical map for isolation of genes from genomic library

Once the physical map is developed, one can easily investigate genome organization, evolution, gene regulation or gene interaction using contigs from a specific chromosomal region. It can also be used for isolation of genes of interest. A physical map actually consists of continuously overlapping contigs of large insert genomic DNA clones, in which the distance between two DNA markers is measured in kb. Figure 7 shows a general scheme for development of a physical map for any crop.

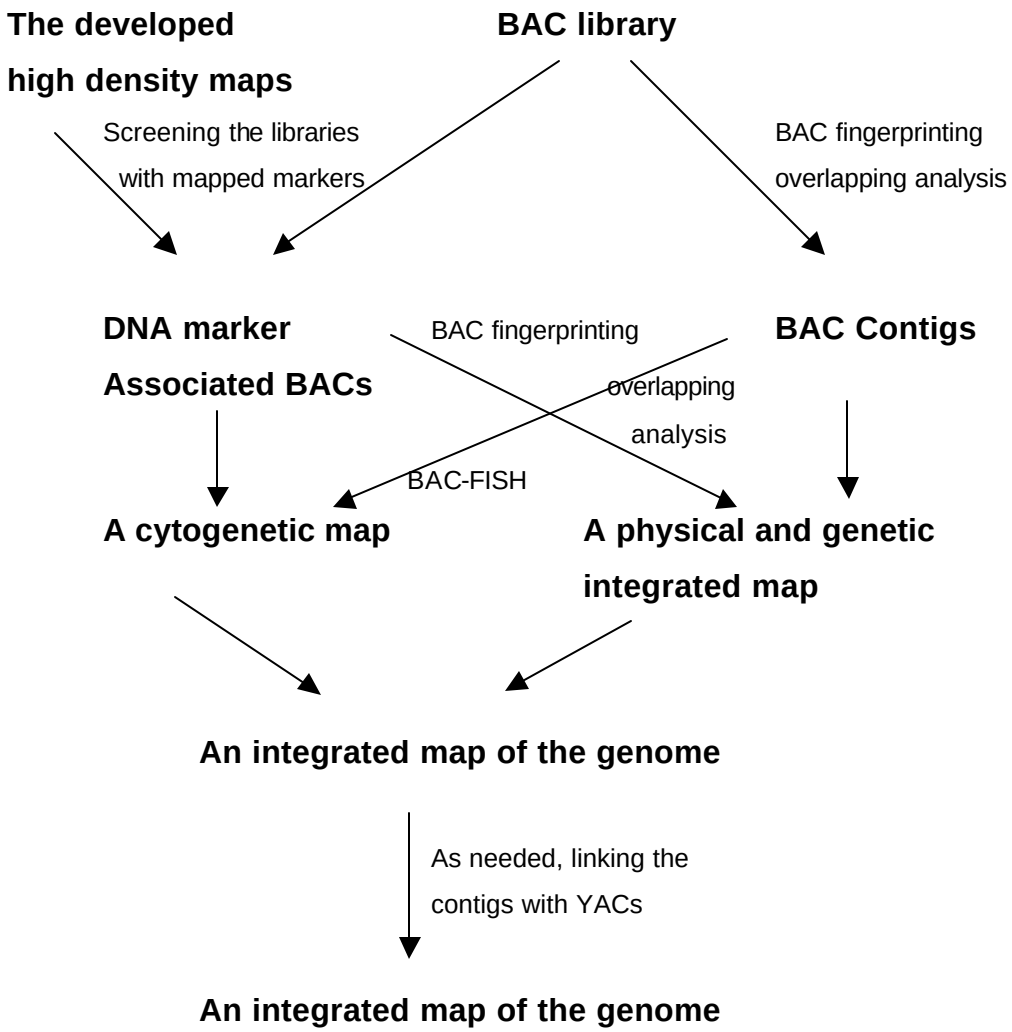


Figure 7. A strategy for physical mapping of any plant genome with BACs.

Two different methods by which a gene can be further isolated using physical map information are as follows:

i) Gene Fishing:

A physical map is extremely useful for plant genetic and biological studies and is a highway for rapid isolation of numerous genes. To isolate the genes from a

chromosomal region of interest, a piece of a contig for that region can be obtained from the BAC library and can be used as a “hook” to fish the genes from that region by “gene fishing” (contig-based cDNA library screening). However, the map-based cloning strategy is time-consuming and unreliable because it requires many technical steps and its success is inversely related to the size and complexity of the genome (Leung et al. 1994; Martin et al. 1992).

ii) Gene golfing

It is a new strategy based on physical maps and is ideally suited for rapidly cloning a large number of genes known only by phenotypes. For example, if a DNA marker has been identified 10cM away from the gene of interest, it is difficult to approach the target gene by the current map-based cloning strategy using this available DNA marker. It is possible that the repeated sequences could block chromosome walking towards the target gene. However, the gene can be readily approached by the gene golfing strategy using a developed physical map. First, the available DNA marker is used to probe the large-insert DNA library used for the development of physical map so as to determine the position of the DNA marker in the physical map. Based on the position of the DNA marker-hybridized clone(s) in the physical map and the physical/genetic distance ratio in the region between the DNA marker and the target gene, a piece of contig containing the target gene can then be directly selected from the library. To verify the relationship between the contig segment and the target gene, single-copy DNA fragments from the contig are isolated and used as probes to perform RFLP mapping against the target gene. If the RFLP mapping indicates that the contig segment does not contain the target gene, a second golf stroke is performed. Now the chances of getting closer to the gene are higher. Additional steps can be taken until the clone containing the target gene is isolated. This method is comparatively easier than the cumbersome map-based cloning strategy (Zhang and Wing 1997)

1.7. Genesis of my thesis

I joined NCL in September 1996 as a CSIR-JRF with a strong motivation of doing Ph. D in plant molecular biology area. At that time, a major program on chickpea in collaboration with Washington State University, Pullman, USA was ongoing with support from the McKnight Foundation, USA. The important achievements in chickpea research such as construction of a genetic linkage map, identification of markers linked to both wilt and blight disease resistance genes and isolation of proteins and genes with potent inhibitory activity against pod borer inspired me to join the chickpea team and undertake further work on chickpea genome with the following objectives:

1. Studies on genetic relationship between annual and perennial species of chickpea that will facilitate the introgression of useful genes into the cultivated *Cicer* for which very few reports are available.
2. Tagging of an agronomically important double-podded trait with molecular markers, which will have a direct application in chickpea marker-assisted breeding.
3. Identification of markers which have tight linkage to ascochyta blight resistance genes that will help to isolate the resistance genes from the genomic library.
4. Analysis of gene expression profiles during the pathogen infection with special reference to *Ascochyta rabiei*.
5. Construction of a large insert library for chickpea – This will play a major role in the isolation of important genes and physical mapping of the *Cicer* genome.

1. 8. Organization of my thesis:

I have organized my thesis into following 4 chapters excluding the first chapter of Review of Literature.

Chapter 2. Genetic relationships among annual and perennial wild species of *Cicer* using Inter Simple Sequence Repeat (ISSR) polymorphism

In this chapter, I have discussed about genetic relationship among 6 annuals and 7 perennials with two accessions of each species wherever possible, including 4 cultivated samples using ISSR markers. These studies will reveal the genetic distance between the species.

Chapter 3. Identification of an STMS marker for the doublepodding trait in chickpea

This chapter encompasses tagging of an agronomically important trait, which plays a role in the crop yield. The identified STMS marker for this double-podded trait can be used in marker assisted selection.

Chapter 4. Resistant Gene Analog mapping and differential expression analysis of genes in response to *Ascochyta rabiei* infection

In this chapter, I have attempted to exploit resistance gene analog (RGA) markers to tag the ascochyta blight resistance genes and have reported functional changes in the host (chickpea) during *ascochyta* pathogen infection using DDRT approach.

Chapter 5. Bacterial Artificial Chromosome (BAC) library construction in chickpea

Here, I have described the construction of a large-insert library for chickpea, which opens new avenue for many applications like isolation of genes, physical mapping and so on. This is the first BAC library constructed in chickpea.

Chapter 2

Genetic relationships among annual and perennial species of Cicer using Inter Simple Sequence Repeat (ISSR) polymorphism

The contents of this chapter have been revised as a full-length paper and resubmitted to Euphytica

2.1. Abstract

Wild *Cicer* germplasm is known to have genes for disease resistance, stress tolerance and other important traits, and hence can be exploited for improving cultivated genotypes. However, only few *Cicer* species are interfertile and it is essential to overcome crossability barriers to utilize the germplasm more effectively. Genetic diversity analysis of *Cicer* species can give important clues in understanding species relationships and may assist in developing and planning breeding strategies. I selected 6 annual and 7 perennial wild species and evaluated the genetic diversity using 15 ISSR primers, where on an average, 6.6 polymorphic bands per primer were observed. Cluster analysis using unweighted pair group method, arithmetic mean (UPGMA) algorithm indicated five groups of the species which was in agreement with the data based on crossability, seed storage protein, isozyme, allozyme and RAPD marker analysis. My results further indicated that species from similar sections in the classification based on morphological similarities, clustered together except *C. nuristanicum* and also suggested that annuals of *Cicer* wilds were not monophyletic in nature. There was no clear correlation between the clusters of the species based on ISSR markers and their geographical distribution.

2.2. Introduction

The genus *Cicer* contains 42 wild species including 9 annuals and 33 perennials with chromosome number of 2n=2x=16 in almost all the species (Labdi et al. 1996). The cultivated *Cicer arietinum* is an economically important crop in India, the Middle East, North Africa and Ethiopia, and is the third most important pulse crop in the world next to *Phaseolus vulgaris* and *Pisum sativum*. In India, over 70% of the world's chickpea crop is produced. The oldest report concerning this species is presented in 5450 BC (Helbaek 1959) and it has been cultivated for at least 7000 years (van der Maesen 1972). It is a self-pollinated crop, with natural cross-pollination ranging between 0-1% (Singh 1987). This nature of *Cicer* and its sexual incompatibility with most of the other wild types in natural interspecific crosses may have led to its presumed narrow genetic base (Sant et al. 1999). The latter has been responsible for the limited unconventional exploitation of wide hybridization for introgression of economically important traits like sturdiness, high seed number per pod, disease resistance and efficient plant type in chickpea leading to reduction in its yield (Saxena and Singh 1987). Moreover, there are constraints of strong post fertilization barrier to interspecific hybridization between many of the wild species in the genus *Cicer* and the lack of complete crossability data. Considering the agro-economic importance of chickpea, it becomes essential to study the genetic variability within and among the *Cicer* species and this data may help the chickpea breeders to exploit the genetic resources for hybridization programs.

Most of the interspecific relationship studies in *Cicer* have been carried out using plant morphology (Robertson et al. 1997), karyotype (Ocampo et al. 1992; Tayyar et al. 1994), crossability data (Ladizinsky and Adler 1976; Pundir and Van der Maesan 1983; Ahmad et al. 1987), RFLP (Patil et al. 1995) as well as seed storage protein analysis (Vairinhos and Murray 1983; Ahmad and Slinkard 1992), allozyme markers (Kazan and Muehlbauer 1991) and more recently RAPD markers (Ahmad 1999). In addition, allelic variation at a microsatellite locus [$(TAA)_n$] (Udupa et al. 1999) and also in Ty1-copia like retrotransposan sequence (Sant et al. 2000) has been utilized to study diversity in *Cicer*. Since annual and perennial wild *Cicer*

germplasm were partially represented in these studies, it would be useful to evaluate additional germplasm systematically, which would ultimately help to gather more information about various important traits (Van der Maesan 1987).

Molecular markers like RFLP, PCR-based RAPD, AFLP, ISSR and SSLP have been extensively used to study the genetic variability in many crop species. Representative examples are pigeonpea (Ratnaparkhe et al. 1995), Amaranthus (Chan and Sun 1997), wheat (Barret and Kidwell 1998; Plaschke et al. 1995), soybean (Thomson et al. 1999) and rice (Aggarwal et al. 1999; Joshi et al. 2000, Davierwala et al. 2001). In chickpea, RFLP was of less use in detecting polymorphism because of its self-pollinating nature, which eventually led to the homogeneity of *Cicer* (Simon and Muehlbauer 1997). RAPDs, however, have revealed more diversity among the annual wild species of *Cicer* (Ahmad 1999). In my laboratory, we have demonstrated the use of another class of molecular markers, the microsatellite markers, for diversity analysis, which were highly polymorphic even within the cultivars of *Cicer arietinum* (Sant et al. 1999). Other laboratories also have shown the utility of these markers in chickpea genome analysis (Sharma et al. 1995; Gotz et al. 1998; Udupa et al. 1999). More recently, the microsatellite sequences have been used as primers in polymerase chain reaction, where repeat motifs are anchored either at 5' or 3' end with one or few specific nucleotides and amplify the sequences between the two microsatellite loci referred to as inter simple sequence repeat (ISSR) markers. In addition, ISSRs can be targeted towards particular sequences, which are reported to be abundant in the genome and can overcome the technical difficulties of RFLP and RAPD. These primers have been successfully used in identifying markers linked to fusarium wilt resistant gene cluster in chickpea (Ratnaparkhe et al. 1998). In this chapter, I report the use of ISSRs for the assessment of genetic diversity in perennial and annual wild species of *Cicer.*

2.3. Materials and methods

2.3.1. DNA procurement and isolation

DNAs of 6 annual and 7 perennial wild species of chickpea (Table 1) were procured from Washington State University, USA. They were isolated from vegetative

buds and leaf tissues of the wild species using the miniprep method of Doyle and Doyle (1987), with little modifications as described by Simon and Muehlbauer (1997). DNAs of *Cicer arietinum* cultivars; Vijay, JG 62 and ICC4958 were isolated by the same protocol at National Chemical Laboratory, Pune, India. One gram of young leaves of the cultivars was ground in liquid nitrogen to a fine powder, which was quickly transferred to a tube containing 7.5ml of ice–cold extraction buffer (0.35M sorbitol, 0.1M Tris-HCl, 5mM EDTA pH 7.5). The tube was briefly shaken and 7.5ml nuclei lysis buffer (2M NaCl, 0.2M Tris-HCl, 50mM EDTA, 2% CTAB, pH 7.5) was then quickly added, followed by 3ml of 5% sarkosyl solution. Sample sets were incubated in 65^0C water bath for 20min and these were allowed to cool and 18ml of chloroform: isoamyl alcohol (24:1) was added to each tube. The tubes were centrifuged at 5000*g* for 15min, further, the aqueous layer was removed and given another chloroform/isoamyl alcohol treatment. The aqueous layer was transferred to a new tube and the DNA was precipitated with double volume of chilled ethanol. The DNA pellet was dried, suspended in 500µl TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8.0) and quantified by mini-gel method (Sambrook et al. 1989)

2.3.2. Oligonucleotide primers, PCR amplification and electrophoresis

A set of 100 primers representing di, tri, tetra and pentamer repeats (UBC set # 9) was procured from the Biotechnology Laboratory, University of British Columbia, Canada. PCR amplifications were performed in 100mM Tris-HCl, pH 8.3; 500mM KCl; 15mM $MgCl_2$; 0.01% (w/v) gelatin, 200mM dNTP, 0.5mM spermidine, 5pM primer and 20ng of genomic DNA, per 20µl reaction volume and 0.8 unit of AmpliTaq DNA polymerase (Perkin Elmer, USA), using a PTC-200 Peltier thermal cycler (MJ Research, USA). After initial denaturation at 94^0C for 4min, 40 cycles of PCR were carried out where each cycle comprised 1min denaturation at 94^0C, 45sec annealing at 54^0C and 2min extension at 72^0C for 5min with a final extension at 72^0C for 5min, at the end of 40 cycles. PCR products were separated on 2% agarose gels, stained with ethidium bromide and scored for the presence or absence of bands.

2.3.3. Data analysis

The genetic relationships between 13 wild species of *Cicer* were studied by means of scorable bands using 15 different ISSR primers. Since ISSRs are dominant, a locus was considered to be polymorphic if the band was present in one lane and absent in the other. The presence or absence of bands was scored as binary code (1 and 0) and simple coefficient was used to calculate similarity for all the possible pairs of wild species. A dendrogram was constructed based on the similarity index matrix using the arithmetic average of unweighted pair group method (UPGMA) (Sneath and Sokal 1973) to illustrate the genetic relationships among the wild species using software package Taxan. Winboot, a software program developed at IRRI, was run for bootstrapping. PHYLIP (Phylogeny Inference Package) format (Felsenstein 1985) was used to determine the confidence limits of UPGMA based dendrogram by carrying out 2000 replications in this program (Nelson and Yap 1996).

2.4. Results

2.4.1. Detection of DNA polymorphism in *Cicer* germplasm

Using ISSRs, I screened 23 *Cicer* species consisting of 6 annuals and 7 perennials with two accessions of each species wherever possible as listed in Table 1 and three local cultivars of *C. arietinum*: Vijay, JG62 and ICC4958 apart from V65R. Among 100 ISSR primers representing di, tri, tetra and penta repeats, 25 primers showed good amplification but no polymorphism while 15 primers revealed reproducible polymorphic patterns. With 15 primers, a total of 115 bands were scored giving an average of 7.6 bands amplified per primer. Among 115 bands, 100 were polymorphic leading to an average of 6.6 polymorphic bands per primer (Table 2). As seen in Table 2, dinucleotide $(GA)_8$ with single nucleotide anchor T/C/A at the 3' end and $(AC)_8$, with dinucleotide anchor YT at 3' end, were found to be the most polymorphic (~ 100%) compared to all others.

Figure 1 shows a representative amplification pattern of *Cicer* species using $[(GA)_8C]$ primer with bands in the range of 2.5kb to 0.5kb. A total of 9 loci were amplified with this primer and not a single band was monomorphic in all the species of *Cicer,* thus revealing a high level of polymorphism in *Cicer* genome. Only one band

GROUP	WILD SPECIES	ANNUAL/ PERENNIAL	SECTION	ORIGIN
I	*C. acanthophyllum* W6-11515 & W6-14190	Perennial	Acanthocicer	Tajikistan
	C. macracanthum W6-11177	Perennial	Acanthocicer	Pakistan
II	*C. pungens* W6-17172 & w6 19489	Perennial	Acanthocicer	Afghanistan
	C. nuristanicum W6-15003	Perennial	Polycicer	Pakistan
III	*C. anatolicum* PI 383626 & W6-17512	Perennial	Polycicer	Turkey
	C. microphyllum W6-015003 & w6 19489	Perennial	Polycicer	India
	C. oxyodon CS-11	Perennial	Polycicer	Turkey
IV	*C. arietinum* V65R, Vijay, ICC4958 & JG62	Annual	Monocicer	India
	C. reticulatum 5667	Annual	Monocicer	Turkey
	C. echinospermum 020689-0302 & 527932	Annual	Monocicer	Turkey
V	*C. yamashitae* 510657 & 510664	Annual	Monocicer	Afghanistan
	C. bijugum 5781 & 458550	Annual	Monocicer	Turkey
	C. judaicum 510660	Annual	Monocicer	Lebanon

Table 1. List of *Cicer arietinum* and related wild species used in the present study.

No.	SEQUENCE	NUMBER OF MONOMORPHIC BANDS	TOTAL NUMBER OF BANDS SCORED
1	$(GA)_8T$	1	15
2	$(GA)_8C$	-	9
3	$(GA)_8A$	-	9
4	$(GA)_8YT$	1	4
5	$(AG)_8YT$	4	11
6	$(CT)_8RC$	2	8
7	$(CA)_8T$	-	5
8	$(CA)_8RT$	2	8
9	$(AC)_8YT$	-	9
10	$(GT)_8A$	-	5
11	$(GT)_8YT$	-	7
12	$(TG)_8A$	-	5
13	$(ATG)_6$	2	8
14	$(GAA)_6$	2	6
15	$(GGAGA)_3$	1	6
	TOTAL	15	115

Table 2. List of ISSR primers used in genetic variability analysis

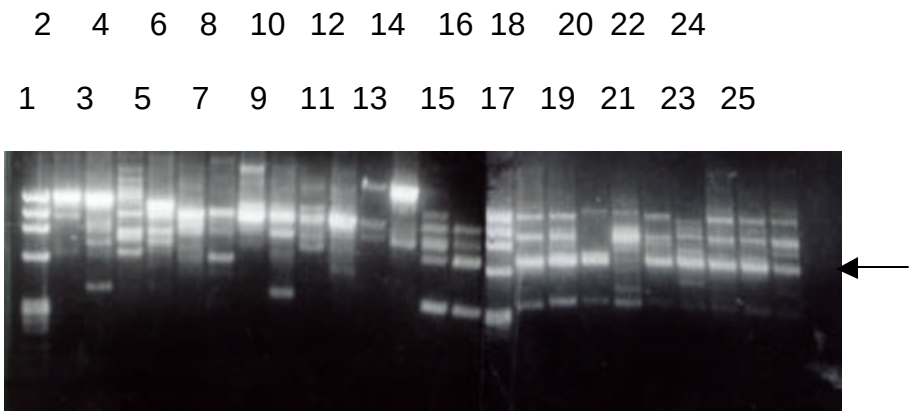


Figure 1. ISSR profile obtained with primer $(GA)_8C$ on a 2% agarose gel

Lanes 1, 16	Marker DNA (ϕX 174 *Hae* III digest)
2, 3	*C. acanthophyllum* (W6-11515 & W6 11513)
4, 5	*C. pungens* (W6 17172 & W6 14190)
6, 7	*C. anatolicum* (PI 383626 & W6 19489)
8	*C. nuristanicum* (W6 11190)
9, 10	*C. microphyllum* (W6 15003 & W6 19489)
11	*C. macracanthum* (W6 11177)
12, 13	*C. yamashitae* (510657 and 510664)
14, 15	*C. bijugum* (5781 & 458550)
17	*C. arietinum* (V65R)
18, 19	*C. echinospermum* (020689-0302 & 527932)
20	*C. oxyodon* (CS-11)
21	*C. reticulatum* (5667)
22	*C. judaicum* (510660)
23, 24, 25	*C. arietinum* (Vijay, JG62 & ICC4958)

A band of 0.8kb shown by an arrow was common to all accessions of *C. bijugum* (lanes 14&15), *C. arietinum* (lanes 17, 23, 24 & 25), *C. echniospermum* (lanes 18 & 19), *C. reticulatum* (lane 21) and *C. judaicum* (lane 22)

of 0.8kb shown by an arrow was present in all the accessions of *C. bijugum* (lanes 14&15), *C. arietinum* (lanes 17, 23, 24&25), *C. echinospermum* (lanes 18&19), *Cicer reticulatum* (lanes 21) and *Cicer judaicum* (lane 22).

2.4.2. Clustering pattern of chickpea genotypes

Using the software package "Taxan", similarity indices were generated based on pairwise comparison of genotypes. This matrix was used as a basis to cluster the genotypes in the form of a dendrogram (Figure 2), which revealed the genetic relationships between the *Cicer* species with similarity index values ranging from 0.8 to 0.53 with an average value of 0.70. Three broad clusters of chickpea genotypes were obtained in the dendrogram at a similarity value of 0.60. Cluster I included accessions of *C. acanthophyllum*, *C. macracanthum, C. pungens, C. nuristanicum, C. arietinum, C. reticulatum* and *C. echinospermum*. Cluster II included *C. yamashitae, C. bijugum and C. judaicum* while cluster III had *C. anatolicum, C. microphyllum and C. oxyodon* accessions. These three major clusters had many subgroups at various levels of similarity values.

C. arietinum cultivars, Vijay, ICC4958, JG62 and V65R and *C. reticulatum* and *C. echniospermum*, the progenitors of cultivated *C. arietinum,* formed a closer cluster at a genetic distance of 0.23 while the accessions of wild perennials *C. acanthophyllum* and *C. pungens* formed two clusters at a genetic distance of 0.29 and 0.31 with *C. macracanthum* and *C. nuristanicum*, respectively. In cluster II, *C. bijugum* and *C. judaicum* showed maximum similarity (0.80), which joined together with *C. yamashitae* at a genetic distance 0.37. In Cluster III, accessions of *C. anatolicum* showed higher similarity with *C. microphyllum* with genetic distance value of 0.34 and later clustering with *C. oxyodon* at a genetic distance 0.39.

Dendrogram is constructed based on the degrees of similarity between a pair of individuals at a time. But bootstrapping is a way to obtain a nonparametric estimate of these confidence limits. It involves repeated sampling with replacement (bootstrapping) of the characters in a matrix of Operational Taxonomic Units (OTUs) X characters to create numerous bootstrap matrices of the same size as the original matrix (Nelson and Yap 1996). Comparing the bootstrap values of all clusters, *C.*

bijugum and *C. judaicum* showed the maximum value of 91, while remaining interspecific clusters showed relatively lower values. Intraspecific similarities possessed higher bootstrap values than the interspecific ones (data not shown).

2.5.Discussion

There have been a number of efforts to transfer agro-economically important genes from wild species into cultivated species through conventional breeding practices. For example, interspecific hybridizations were performed to introgress perennial germplasm to cultivated one in alfalfa (McCoy and Echt 1993), nematode resistance gene in peanut (Garcia et al. 1996), and to study the transferability of simple sequence repeat (SSR) loci of soybean to other legume genera (Peakall et al. 1998). However, such attempts were not successful to develop hybrids between *C. arietinum* and all wild species of *Cicer* except *C. reticulatum* and *C. echinospermum*. A few efforts using plant tissue culture techniques like embryo rescue to develop hybrids between *C. arietinum* and *C. pinnatifidum* were reported (anonymous 1995). High hybrid vigor was obtained in F1 and F2 generations, when *C. arietinum* was crossed with *C. reticulatum* and *C. echinospermum* (Singh and Ocampo 1997) ascertaining the potential of increasing the chickpea yield using interspecific hybridization. However, knowledge of genetic relationships among various wild species is necessary for successful and efficient exploitation of genetic diversity present in the wild species and such information is very poorly available in the genus *Cicer,* especially using molecular markers. In all the previous studies, the genetic relationships were carried out either only among annuals (Ladizinsky and Adler 1976; Labdi et al. 1996; Ahmad 1999) or among annuals with one (Kazan and Muehlbauer 1991) or two perennials (Tayyar and Waines 1996). Ours is the first report where many perennial (7) and annual (6) wild species of chickpea have been used for diversity analysis at DNA level.

In order to study inter and intraspecific relationships in genus *Cicer,* I used 15 ISSR primers representing twelve dinucleotide repeats anchored at 3' end, two unanchored trinucleotide repeats [$(ATG)_6$ and $(GAA)_6$] and one unanchored penta nucleotide repeat [$(GGAGA)_3$]. I did not use the ISSR primers anchored at 5' end as

they lack selective nucleotides at the critical 3' end and impose selection for long simple sequence repeats that bind along the entire length of the primers. Since such a selection pressure is not exerted by the 3' anchored primers, they may detect microsatellites at a higher frequency than 5' anchored primers (Blair et al. 1999). My studies have shown relatively a high level of polymorphism between wild species of chickpea but less polymorphism between the accessions of each wild species used in the study including the cultivated ones. This less polymorphism between the cultivated *Cicer* could be attributed to less than 1% outcrossing reported in cultivated *Cicer arietinum* (Singh et al. 1987; Tuwafe et al. 1988).

2.5.1. Grouping of chickpea based on ISSR data

Earlier studies based on crossability data established 4 groups for 9 annuals of *Cicer.* Group I consisted of *C. arietinum, C. reticulatum* and *C. echinospermum;* group II included *C. bijugum, C. judaicum* and *C. pinnatifidum*; group III comprised only *C. cuneatum* while group IV contained *C. yamashitae* (Ladizinsky and Adler 1976; Ahmad et al. 1987; Ahmad 1988). These groups also resembled the clustering of annual *Cicer* species based on their seed protein profiles (Vairinhos and Murray 1983). Interestingly, the clustering of annual species based on ISSR markers in my studies is in agreement with the clustering based on crossability as well as seed storage protein data except that *C. yamashitae* was added to group II (Figure 2).

2.5.2. Relationship between geographical location and the clusters

C. arietinum cultivars used in the present study were developed in India, *C. reticulatum*, *C. echinospermum* and *C. bijugum* originated from Turkey, while *C. judaicum* originated from Lebanon. Among the perennials, *C. anatolicum* was from Turkey, while *C. microphyllum* originated from India. In my attempt to correlate the origin of chickpea genotypes with their groupings using ISSRs, the dusters showed Turkey originated *C. anatolicum* grouping with *C. microphyllum* (Table 1) from India. Similarly, *C. yamashitae*, *C. bijugum* and *C. judaicum* from Afghan, Turkey and Lebanon, respectively, clustered together. Thus there was no clear correlation

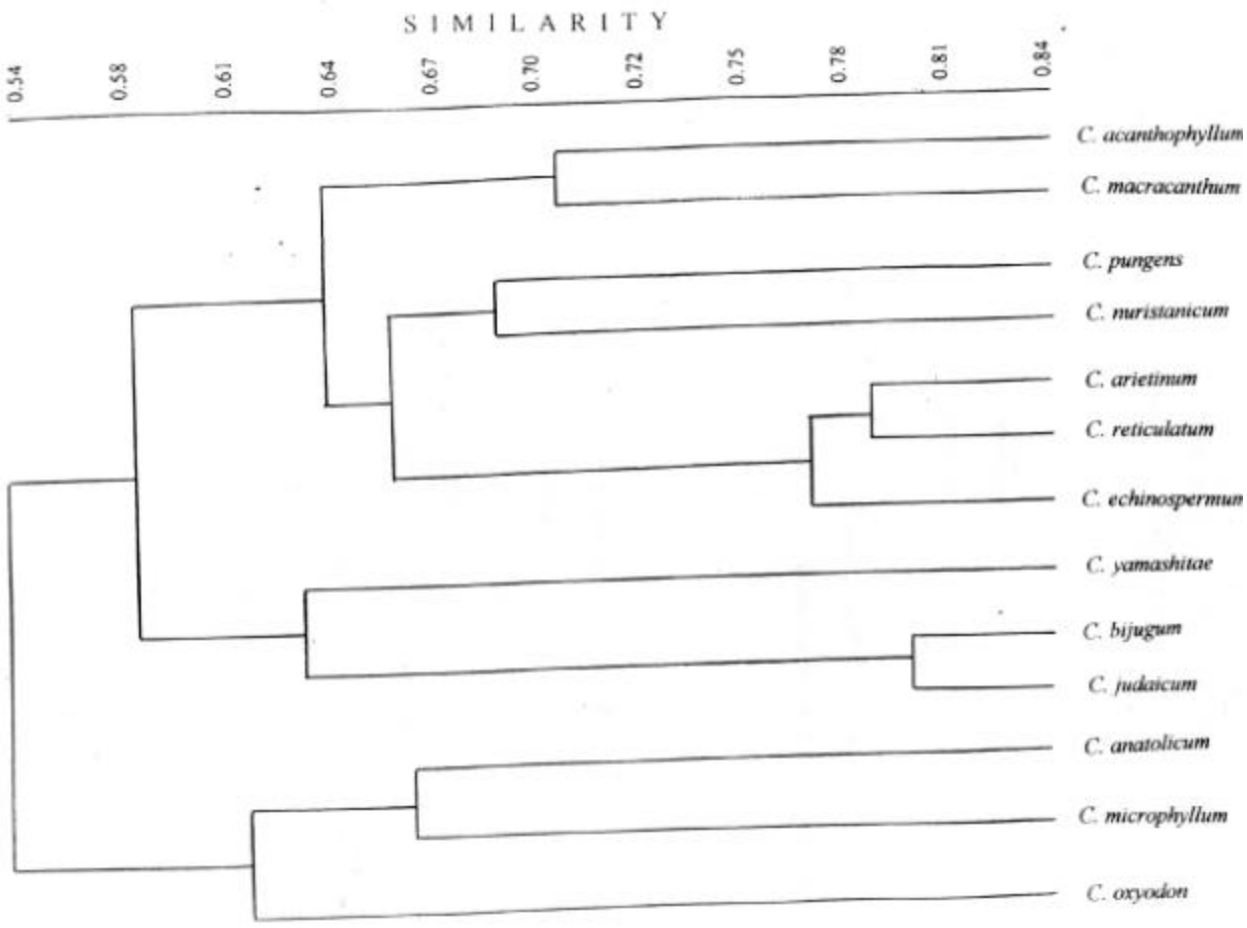


Figure 2. Dendrogram of the annual and perennial *Cicer* species based on the similarity matrix developed using ISSR markers.

between the clusters of the species based on ISSR markers and their geographical distribution.

2.5.3. Morphological and genetic relationship

Depending on morphological features, the genus *Cicer* has been divided into four sections. Monocicer comprises all eight annuals except *C. chorssancium*, Chaemecicer includes one annual, *C. chorssancium* and one perennial, *C. incisum*. Remaining 23 perennials and seven woody perennial species are grouped in sections polycicer and acanthocicer, respectively (Kazan and Muehlbauer 1991). The groups which emerged from the ISSR data in my study were similar to the groups of species in various section based on morphological observation. I also observed that all monocicers were distributed in two groups, IV and V, all polycicers were grouped together in cluster III, while *C. acanthophyllum* and *C. macracanthum* which fall in acanthocicer grouped together in cluster I (Table 1). *C. acanthophyllum* has spiny stipules of 2-6mm length often with a second spinelet of 1-2mm and an incised foliate stipule of 2-6mm at the base of the plant. *C. macrocanthum* has larger and more leaflets and broader calyx teeth and stipules up to 25mm but the areas overlap. Morphological data suggest that *C. macracanthum* resembles quite closely to *C. acanthophyllum* (Saxena and Singh 1987), which is in agreement with my data based on ISSR markers. However, no information is available for correlation of *C. pungens* with *C. nuristanicum* based on other morphological characters.

2.5.4. Study on evolution of annuals

Various molecular markers including chloroplast DNA, RFLP, RAPD, ISSR and STMS have been utilized earlier to analyze the origin and evolution of different plant species including the genus *Oryza* (Ishii et al. 1988; Wang et al. 1992; Ishii et al. 1996; Joshi et al. 2000; Davierwala et al. 2001). Allelic variation at a microsatellite locus like $(GATA)_n$ and $(CAC)_5$ in rice (Gupta et al. 1994) and $(TAA)_n$ in chickpea (Udupa et al. 1999) has given direction to understand the genetic relationship among various species. Yang et al. (1994) have predicted that 28% of allelic diversity has

been lost during the process of cultivar development from landraces in rice. Retrotransposons, specifically Ty1-*copia* rt domain, have also been used to estimate the evolutionary relationships between wheat, rice and maize (Matsuoka et al. 1999). In a recent study from my laboratory, Ty-1 *copia* like retrotransposon sequences has been used to study the phylogenetic relationship among the *Cicer* species (Sant et al. 2000).

There are different theories regarding transition of chickpea wilds from perenniality to annual state. Based on analysis of allozyme variation in 9 annuals and one perennial, it is reported that annual species are monophyletic (Kazan and Muehlbauer 1991). On the contrary, on the basis of isozyme studies using 9 annuals and 2 perennials, it is reported that the annual species are not monophyletic in their origin (Tayyar and Waines 1996). These contrasting conclusions might be due to small size of the sample surveyed and variation in the source of seeds, in the enzyme system studied and in the procedure of sample analysis. My results based on 7 perennials and 6 annuals probably support the view of Tayyar and Waines (1996) in which annual species are not monophyletic. The seven perennials included in my study do not form a single cluster as is clear from Table 1. Here *C. acanthophyllum* and *C. macracanthum* form group I; *C. pungens and C. nuristanicum* form group II while *C. anatolicum, C. microphyllum* and *C.oxyodon* form group III all representing perennials. Secondly, three annuals namely *C. arietinum, C. reticulatum* and *C. echniospermum* form group IV while three more annuals namely *C. yamashitae, C. bijugum* and *C. judaicum* form group V. However, the annuals of group IV in Table 1 cluster with perennials *C. pungens and C. nuristanicum* of group II, while the three annuals of group V cluster with perennials of group I and annuals of group IV and group Ii of perennials. Thus, the dendrogram in Figure 2, suggests that probably all the annual species might not have evolved simultaneously from the perennials. For the precise phylogeny and evolutionary analysis of *Cicer* genus, studies with more perennial and annual species and with large number of accessions of each species are required. However, the scarcity of perennial seeds and their poor growing ability at various locations are the major limitations to carry out this exercise.

In summary, the evolutionary data generated using ISSRs with 6 annuals and 7 perennials of *Cicer* has supported the earlier report that the annuals are not monophyletic. The ISSR data further prove that no correlation exists between the clusters based on ISSR data and the geographical distribution of *Cicer* wild species. Since the genetic relationship analysis based on ISSRs supports the morphological and crossability data, ISSRs prove to be an efficient marker system for such type of studies.

Chapter 3

Identification of an STMS marker for the doublepodding trait in chickpea

The contents of this chapter have been accepted for publication as a full-length paper in Theoretical and Applied Genetics (2001)

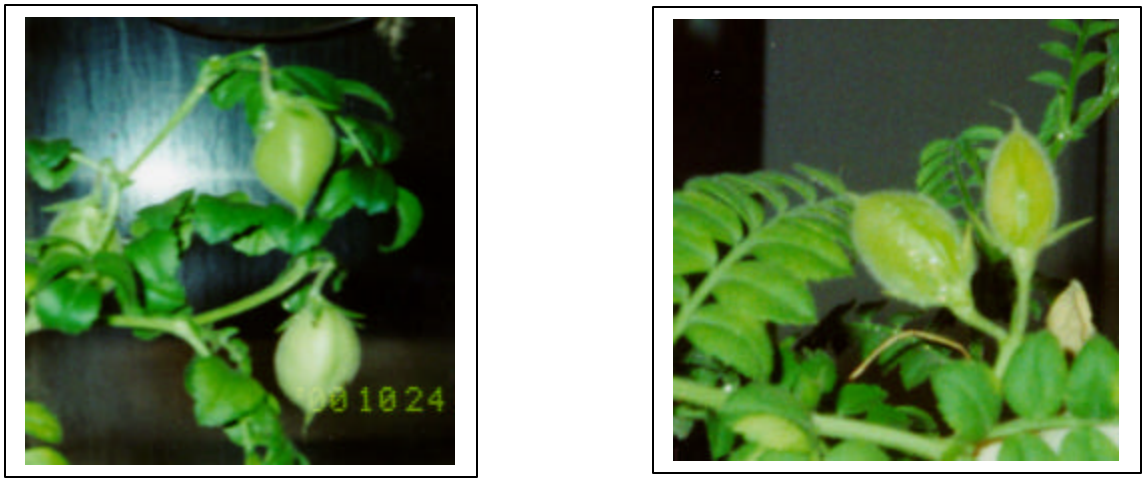
3.1. Abstract

Chickpea is an important food legume crop throughout the world and especially in developing countries. A gene that confers doublepodding, with symbol "*s*", is considered important for breeding higher yielding cultivars. Positive effects of "*s*" on yield stability were found by comparing single- and double-podded near isogenic lines (NILs) derived from a cross of CA-2156 (single-podded) with JG-62 (double-podded). Considering significant effects on seed yield, the NILs were used to identify molecular markers closely linked to "*s*". Sequence Tagged Microsatellite Site (STMS) markers developed for chickpea, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers were used to analyse the NILs. Out of 400 RAPD, 100 STMS and 100 ISSR markers, one STMS marker (TA-80) was polymorphic and was used to evaluate the recombinant inbred line population developed from the cross of Surutato-77 (single-podded) x JG-62 (double-podded) for co-segregation analysis of the locus with "*s*". The results have indicated that the marker and "*s*" are 4.84cM apart and hence, it appears that this marker can be used by breeders for marker assisted selection (MAS) to monitor the incorporation of the doublepodding gene into improved germplasm.

3.2. Introduction

Chickpea is the third most important pulse crop in the world and first in Mediterranean basin and South Asia (FAO 1996). This legume crop is grown in at least 33 countries, including Central and West Asia, Southern Europe, Ethiopia, North Africa, North and South America and Australia (Ladizinsky and Adler 1976; Singh 1997). Although chickpea has high economic value, its world average yield is about 700kg/ha, which is much below its potential of 4000/ha. One of the main reasons for this low yield is its susceptibility to various biotic and abiotic stresses (Singh et al. 1994). Development of cultivars tolerant to such stresses would be an ideal approach to overcome this problem. Another direct approach to fetch higher yields for chickpea is introgression of various yield improving traits into cultivated germplasm.

In chickpea, various parameters such as seed mass, seed yield and seed size contribute to yield (Rao et al. 1994) and doublepodding is one such trait for yield improvement. This trait is conferred by a single recessive gene that has been assigned the gene symbol "*s*" or "*sfl*" (Ahmad 1964; D'Cruz and Tendulkar 1970; Khan and Akhtar 1934; Patil 1966; Singh and Van Rheenen 1989, 1994). Double-podded cultivars have two flowers per node (Figure 1) that usually develop into two



a) Single-podded b) Double-podded

Figure 1. Representative single-podded and double-podded plants of chickpea

pods although the second flower is often irregular and forms pods at variable frequency (Knights 1987). Doublepodding can be used to improve the sink capacity of the chickpea plant, which is generally limiting in the short-duration environments (Onkar Singh and van Rheenan 1994). Previous results have indicated that the doublepodding trait could decrease the seed size in chickpea (Singh 1987; Kumar et al. 2000). However, another study using near isogenic lines has reported that "s" is not linked to genes affecting seed size and has a positive effect on yield stability (Rubio et al. 1998).

There have been many successful attempts to identify markers closely linked to genes for specific traits using near isogenic lines (Martin et al. 1991; Hartl et al. 1993). Sequence Tagged Microsatellite Site (STMS) markers are available in chickpea and are predominantly co-dominant in nature (Huttel et al. 1999). STMS markers have been successfully used in the generation of a high-density marker map of the human genome (Weissenbach et al. 1992) and these markers are available for *Arabidopsis* (Bell and Ecker 1994), maize (Taramino and Tingey 1996), soybean (Akkaya et al. 1995), and wheat (Roder et al. 1995). In chickpea, STMS markers have been reported to be linked to generate a linkage map (Winter et al. 2000). Many genes in wheat, rice and soybean have already been tagged using these markers. Protein content QTLs (Korzun et al. 1998b), stripe rust resistance (*Yr15*) (Fahima et al. 1998) and yellowberry tolerance (Ammiraju et al. 2001) in wheat, waxy gene (Bligh et al. 1995) and bacterial leaf blight resistance (*Xa5*) (Blair and McCouch 1997) in rice and mosaic virus resistance (*Rsv*) (Yu et al. 1994) and days to flowering (Li and Niwa 1996) in soybean are a few examples of utilization of STMS markers for gene tagging. Apart from STMS markers, other markers such as RAPD and ISSR have also been shown to be linked to fusarium wilt resistance (Ratnaparkhe et al. 1999) and ascochyta blight resistance (Santra et al. 2000) in chickpea.

Considering the agronomic importance and monogenic recessive inheritance of the "s "gene, an effort was undertaken to identify DNA markers that are closely linked to this gene which can be used in Marker Assisted Selection (MAS) to increase chickpea productivity and also to determine its genomic location.

3.3. Materials and methods

3.3.1. Development of mapping population and DNA extraction

The near isogenic lines (NILs) were developed by crossing CA-2156, a single-podded Spanish Kabuli type cultivar to JG-62, a double-podded cultivar at University of Cordoba, Cordoba, Spain (Rubio et al. 2000). From F_2 to F_5, single-podded plants in rows that segregated for single and doublepodding were selected. Finally, in segregating F_6 rows, individual single and double-podded plants were selected and their descendents were sown in a single row in order to eliminate the segregating rows as mentioned in Figure 2. The plots that were either uniformly single- or double-podded were harvested in separate bulks and designated as NIL4-1V (single-podded) and NIL4-2V (double-podded).

Earlier the donor parent of double-poddedness (JG-62) was also crossed with Surutato-77 (single-podded) and the offspring (F1) was advanced from F_2 to F_6 by single seed descent to produce 102 F_6 derived RILs useful for identification of molecular marker linked to the doublepodding character.

DNA was extracted from the leaf tissue of the above mentioned plant materials according to Doyle and Doyle (1987).

3.3.2. PCR amplification and electrophoresis

One hundred STMS primers reported from chickpea genome (Winter et al. 1999) were synthesized by Gibco-BRL (USA). PCR amplification using these primers was performed in 10mM Tris-HCl pH 8.3, 50mM KCl, 0.1% Triton X-100, 2.5mM $MgCl_2$, 0.2µM dNTP, 4.5µM of forward and reverse STMS primers, 1 unit of Taq polymerase (Promega, USA) and approximately 20-40ng of genomic DNA per 20µl reaction in the Gene Amp PCR system 9700 (Perkin Elmer, USA). After initial denaturation at 94^0C for 2min, 35 cycles of PCR were carried out, where each cycle comprised 20sec denaturation at 94^0C, 50sec annealing at 55^0C and 2min extension at 60^0C for 50sec. PCR products were separated on 6% denaturing

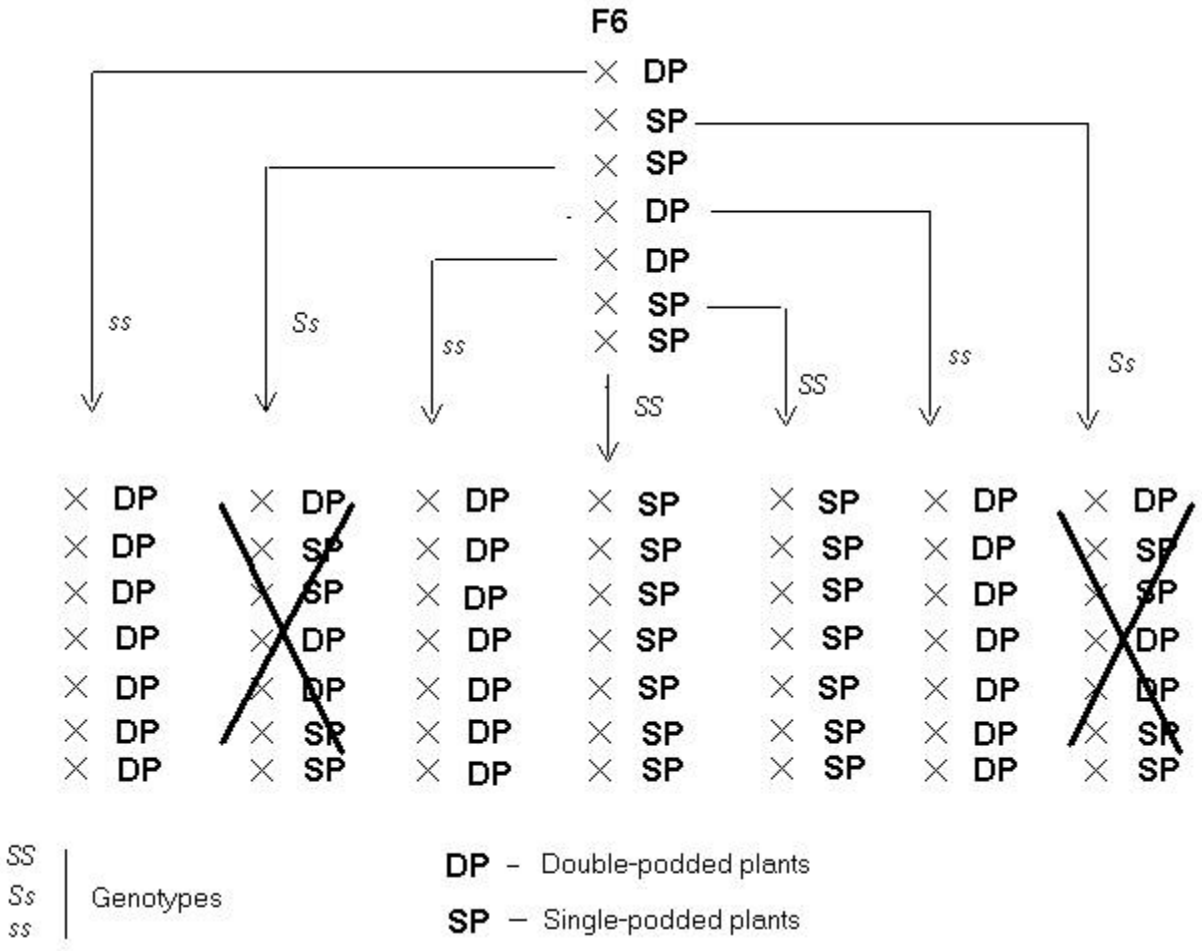


Figure 2. A Scheme for NIL development

polyacrylamide gels and stained using a commercially available silver staining protocol of #Q4132 (Promega, USA). Since STMS markers are locus specific, one band per pair of primer was observed and scored in parental lines and in the RIL progeny.

Random primers such as RAPD (UBC set #2-5) and ISSRs (UBC set #9) were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada. For RAPD, after initial denaturation of the template at 94^0C for 20sec, the primers were annealed at 36^0C for 1min followed by elongation at 72^0C for 1min and this cycle was repeated 40 times.

In ISSR reactions, there were 35 cycles of 1min denaturation at 94^0C, 50^0C annealing for 1min and 2min extension at 72^0C after the initial denaturation at 96^0C for 2min. The amplified products of RAPD and ISSR primers were run on 2% agarose gels and stained with ethidium bromide.

3.3.3. Linkage analysis

To identify a polymorphic band, NILs were screened with RAPD, STMS and ISSR markers. Use of such NILs alongwith their original parents for marker analysis would reveal polymorphic markers with more probability of linkage to doublepodding. The primers giving polymorphism were then used to evaluate the available RILs. Chi-square test ($P<0.05$) was performed for the bands polymorphic in the RIL population for goodness of fit to the expected 1:1 ratio. The Kosambi mapping function was used to determine cM distance between the marker locus and the gene (Kosambi 1994). Linkage analysis was performed at the LOD score of 3.0 using MAPMAKER 3.0 (Lander et al. 1987).

3.4. Results and discussion

3.4.1. An STMS marker TA-80 is linked to doublepodding

I screened 400 RAPD, 100 STMS and 100 ISSR primers between the pair of near isogenic lines (NILs) along with the parents to identify a polymorphic primer. One STMS primer (TA-80) was polymorphic between the pair of NILs and its forward-

reverse sequences were TA-80F: CGAATTTTTACATCCGTAATG and TA-80R: AATCAATCCATTTTGCATTC.

After identifying one polymorphic STMS primer out of a total of 600 primers, I then determined the segregation of TA-80 in the $F_{5:6}$ population of 102 RILs. Figure 3 reveals a silver stained gel showing segregation of TA-80 in the RIL population. Banding patterns for the NILs, CA-2156 and JG-62 (the parents crossed to develop NILs) and Surutato-77 and JG-62 (the parental lines used for developing RILs) (Figure 2, Lanes numbered 1-6) indicate polymorphism for TA-80 depicting a band with a fragment size of 200bp associated with the singlepodding character, while a band of 240bp was associated with doublepodding character.

The $F_{5:6}$ RILs were grown in the greenhouse and the phenotypic expression of "*s*" gene was monitored since plants started flowering. I observed the doublepodding in $F_{4:5}$ RILs also, but did not find significant difference in the expression of this trait between these two successive generations. Segregation (53:49) for the TA-80 marker fit the expected 1:1 ratio. Table 1 shows the statistics of the RIL population

Table 1. Segregation of an STMS (TA-80) marker and "*s*", the gene for doublepodding.

Locus	Mat	Het	Pat	X	N	Map	SE	Low	High	LOD
TA-80	53	0	49					4.0	16.7	
				9	102	4.84	1.69	(95% confidence)		17.5
s	56	0	46					3.1	19.6	
								(99% confidence)		

Mat – maternal, Het – heterozygous, Pat – paternal, X – recombinant, N – number of RILs, Map – map distance in cM, SE – standard error

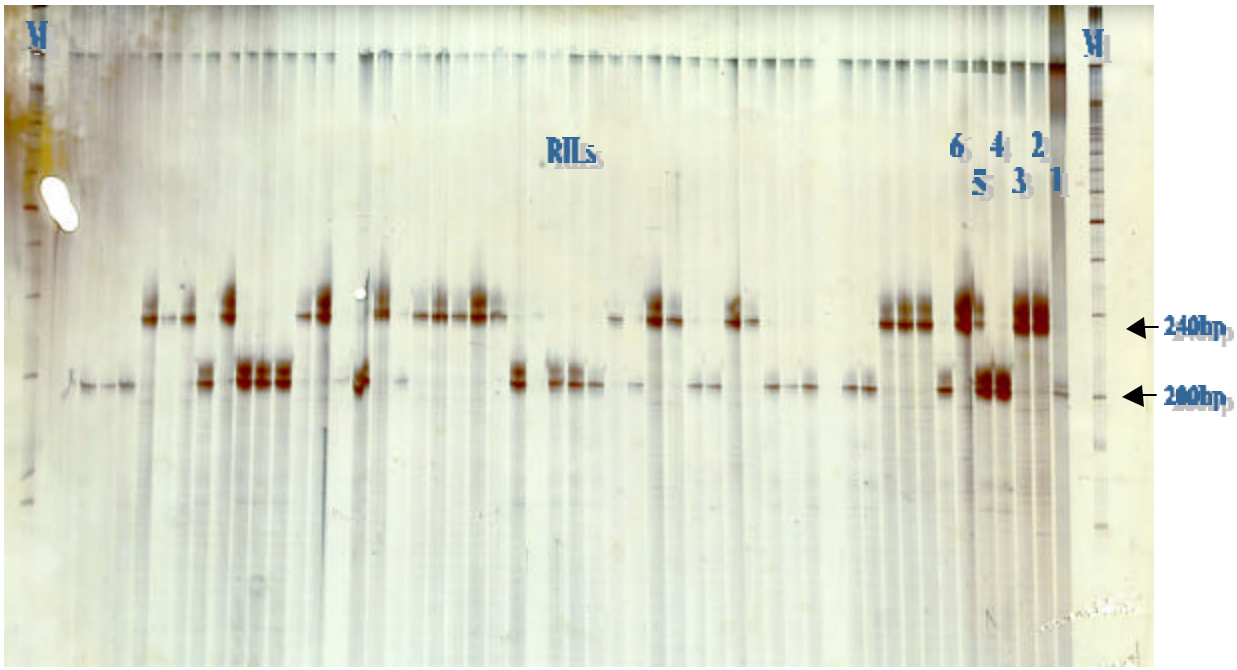


Figure 3. PCR-amplified STMS pattern on 6% denaturing polyacrylamide gel.
1- Single-podded NIL; 2-Double-podded NIL; 3- Double-podded JG-62;
4- Single-podded CA 2156; 5- Single-podded Surutato 77; 6- Double-podded JG-62;
RILs- Recombinant Inbred Lines; M- 50bp ladder marker; Arrows indicate the bands associated with single- (200bp) and double-poddedness (240bp)

analysis, which indicates that this marker is linked to the double-podded gene, "s", at the distance of 4.84cM. Such a close linkage of the marker with the gene of interest was expected since it was polymorphic between the two NILs.

TA-80 was earlier mapped to linkage group 6 of the linkage map of *C. arietinum* (ICC 4958) and *C. reticulatum* (PI 489777), an interspecific cross, which was the most extensive and comprehensive linkage map available for chickpea (Winter et al. 2000). Hence, the gene controlling the double-podded trait could directly be positioned on the linkage group 6 of the linkage map of *Cicer,* even though I studied the linkage between TA-80 and the double-podded trait on an intraspecific cross.

The STMS markers are designed as locus-specific PCR primer pairs based on the sequence information of repeat-flanking regions (Winter et al. 1999). These markers have superiority over other molecular markers because they are locus-specific, co-dominant, PCR-based and have a high polymorphic information content and the potential for non-radioactive detection (Mansfield et al. 1994). The TA-80, an STMS marker can be used not only for identification of the allelic state of the marker locus itself, but also to get information about the allelic state of the 's' locus and hence, will be effective to eliminate linkage drag during marker-assisted backcrossing.

3.4.2. Role of doublepodding in yield improvement

There have been some reports in the past indicating the positive effect of doublepodding on chickpea crop yields (Sheldrake et al. 1978; Onkar Singh et al. 1989, 1994). For example, Sheldrake et al. (1978) obtained 6-11% higher yield in double-podded plants as compared to single-podded plants of the same genotype, in which the second flower had been removed. On the other hand, Knights (1987) reported that it had no effect on yield in diverse genetic backgrounds. Kumar et al. (2000) mentioned that the background genetic constitution played a role in the expressivity of the "s" allele. In order to study the effects of doublepodding on yield, it was suggested that true isogenic lines should be developed and be evaluated in

diverse environments (Knights 1987). Onkar Singh (1989) reported that as the growth cycle became shorter, the double-podded trait contributed positively towards stabilizing grain yield by virtue of a rapidly forming sink capacity. Similar result was obtained by Rubio et al. (1998) using near isogenic lines (NILs) developed for the doublepodding trait.

In my work, the NILs were tested at five locations over two years and the results revealed that the double-podded near isogenic lines had more yield stability when compared to single-podded lines. Though this trait has unstable penetrance and variable expressivity, it showed stability to the seed yield (Kumar et al. 2000). These results indicated that the double-podded trait conferred more yield stability than the single-podded trait. Therefore, a tightly linked marker to this gene can be utilized to exploit the agronomic importance of this trait. In conclusion, I have identified TA-80 as a reliable STMS marker that can be used in Marker Assisted Selection, which would allow plant breeders to perform indirect selection of the double-podded trait prior to phenotypic expression. This is the first report where a molecular marker linked to an agronomically important trait in chickpea has been identified using both NILs and RILs.

Chapter 4

RGA mapping and differential expression analysis of genes in response to Ascochyta rabiei infection

Part of this chapter has been communicated to Euphytica

4.1. Abstract

A) Molecular mapping and characterization of RGA locus RGAPtokin1-2_{171} in chickpea

In order to identify a tightly linked marker for blight resistance genes, a recent approach of resistance gene analog polymorphism (RGAP) was used. Bulk Segregant Analysis (BSA) was performed with these RGA primers on a resistant bulk and a susceptible bulk along with the parents. Of all the available RGAs and their 48 different combinations, only one RGA showed the expected pattern of polymorphism during BSA. This marker was evaluated in the $F_{7:8}$ population of 142 RILs from an interspecific cross of *C. arietinum* (FLIP84-92C) x *C. reticulatum* (PI 599072). The polymorphic RGA was mapped to linkage group 5 where, surprisingly, none of the previously reported QTLs for blight resistance were present.

B) Differential expression analysis of genes in response to *Ascochyta rabiei* infection

In an attempt to study the gene expression profile during Ascochyta infection in chickpea, DDRT approach was deployed where many RNAs can be simultaneously analysed. The arbitrary primers were used to amplify different RNAs expressed at different time intervals after infection with the fungus, A*scochyta rabiei,* in FLIP 84-92C, a resistant cultivar, and the expression pattern was compared with PI 489777, a susceptible chickpea variety. The differentially expressed genes were extracted, cloned and sequenced. Upon homology search, one of the clones showed 87% identity with Serine hydroxy methyl transferase of Pea. Secondly, in order to detect the differential expression of R genes, I substituted arbitrary primers with 10 RGA primers keeping the same anchored primers at Poly A tail end of mRNA. Of all the primers, only Pto kin and PtoFen primers showed amplification and one of the cDNAs amplified by Pto kin showed 88% homology with aldolase of Pea.

A) Molecular mapping and characterization of RGA locus RGAPtokin1-2_{171} in chickpea

4.2.Introduction

Many plant disease resistance genes, with both qualitative and quantitative effects, have been identified and mapped in different crops using various molecular markers. Though some of the markers show close linkage to R-genes in terms of genetic distance in cM, their actual physical distance in kb may not show a tight linkage, which limits the scope of map-based cloning of these genes. In order to identify tightly linked markers to a gene of interest, targeted homology-based approach can be an ideal choice. Although transposon tagging and map-based cloning are the methods employed to isolate resistance genes, PCR strategies that amplify conserved motifs have led to the identification of several NBS-LRR resistance gene homologues (Leister et al. 1998). Recent reports of successful utilization of these RGA markers to identify a closely linked marker to resistance genes in various crops (Potato: Leister et al. 1996; Lettuce: Shen et al. 1998; Soybean: Hayes and Saghai Maroof 2000) prompted us to explore the potential of these markers in chickpea.

Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. is a serious disease with potential to cause 100% yield loss. Figure 1 shows the blight affected field and the symptoms of this disease in plant. As shown by us earlier, resistance to blight is conferred by two major QTLs and a minor QTL (Santra et al. 2000; Tekeoglu et al. 2000a). Though blight resistance is mapped and markers are identified for this trait, the genetic distance between the QTLs and the markers is high, which limits both marker assisted selection and map-based cloning to isolate these genes. I, therefore, used the RGA approach with an assumption that at least one of the segregating RGAs may correspond to the blight disease resistance locus in chickpea. Once mapped, use of this RGA as a probe to identify RGA-containing BAC clone from large-insert library is a good starting point for positional cloning of R-gene-loci. In this

Figure 1a) A blight affected field

Figure 1b. Symptoms of blight in chickpea

chapter, I have undertaken the work of molecular mapping and characterization of RGA locus in chickpea.

4.3. Materials and methods

4.3.1. Plant material and DNA extraction

The 142 $F_{7:8}$ RILs from the interspecific cross of the cultivated chickpea line FLIP 84-92C (resistant) X *C. reticulatum* PI 599072 (susceptible) were used. This population was derived from a single F_1 plant and advanced by single seed descent from the F_2 to the F_7. DNA was extracted from the leaf tissue of each RIL and the parents according to Doyle and Doyle (1987) as described in Chapter 2.

4.3.2. PCR reactions

PCR amplifications were performed in a total volume of 15µl in 100mM Tris-HCl, pH 8.3; 500mM KCl; 0.01% (w/v) gelatin, 5mM $MgCl_2$; 0.2mM dNTP (each), 2.4 ng/µl of both forward and reverse primers, 40ng of chickpea DNA and 0.4U Taq polymerase (Perkin Elmer, USA). Reactions were layered with 10µl of mineral oil. Amplification conditions were, initial denaturation at 94^0C for 5min, followed by 45 cycles of 94^0C for 1min, 45^0C for 1min and 72^0C for 2min, then a final extension of 72^0C for 7min. PCR products were separated on 6% denaturing polyacrylamide gels that were stained using a commercially available silver staining protocol (Promega #Q4132).

4.3.3. Cloning and sequencing of polymorphic fragment

The polymorphic band identified by BSA was extracted from the 6% polyacrylamide gel and reamplified using the same primer pair. The amplified single band was run on 1% agarose gel and the DNA was extracted from the gel using amicon ultrafree-DA filter unit following the manufacturer's instructions (Millipore, USA). All the PCR products were cloned using pGEM-T easy vector system (Promega, USA) and the presence of insert was confirmed by cutting, using *EcoR*I and checking for its insert size. Automated nucleotide sequencing was performed

using the ABI373 sequenator (Applied Biosystems, USA) and homology search was carried out using BLAST (www.ncbi.nlm.nih.gov/BLAST).

4.3.4. Linkage analysis

For data analysis, we considered heterozygotes as missing data. Segregation of marker loci was tested for goodness of fit to the expected Mendelian ratio of 1:1 using the Chi square ($P<0.05$), while linkage analysis was performed using Mapmaker/Exp 3.0 (Lander et al. 1987). Linkage groups were established at a constant LOD score of 4.0 and a recombination value of 0.25 by two point analysis using the "group" command. This marker was analyzed for establishing linkage along with other previously scored marker data. The most likely order of loci within a group was determined using a multipoint "Compare" command and these orders were verified using the "ripple" command. The Kosambi mapping function was used to determine cM distances between markers (Kosambi 1994). Double crossovers were checked by "double crossover" command in Map Manager QTb (version 2.8) (Manly 1998).

4.4. Results and discussion

4.4.1. Segregation of RGAPtokin1-2_{171} in chickpea RIL population

Total 24 pairs of available RGA primers and their 48 different combinations were used to analyze larger fragments of the template (Chen et al. 1998). The list of primer sequences used for analysis is as shown in Table 1. Data on reactions of individual RILs to infection by *Ascochyta rabiei* was available from the raw data used by Tekeoglu et al. (2000). Bulk segregant analysis (BSA) was performed using the above mentioned RGA primer pairs with the resistant (FLIP 84-92C) and susceptible parent (PI 599072) as controls and bulks of ten resistant RILs and ten susceptible RILs. Out of 72 combinations attempted, only one RGA primer pair (Pto kin 1&2) revealed polymorphism in the parents as well as in the bulks, wherein a band of 171bp (RGAPtokin1-2_{171}) was amplified from FLIP 84-92C. Such a low level of polymorphism in *Cicer* has also been observed using various other marker systems

XLRR For: 5’-CCGTTGGACAGGAAGGAG-3’
XLRR Rev: 5’-CCCATAGACCGGACTGTT-3’

CLRR For: 5’-TTTTCGTGTTCAACGACG-3’
CLRR Rev: 5’-TAACGTCTATCGACTTCT-3’

RLRR For: 5’-CGCAACCACTAGAGTAAC-3’
RLRR Rev: 5’-ACACTGGTCCATGAGGTT-3’

NLRR For: 5’-TAGGGCCTCTTGCATCGT-3’
NLRR Rev: 5’-TATAAAAAGTGCCGGACT-3’

NPLOOP: 5’-TCAATTAATGTTTGAGTTATTGTA-3’
Nkin2: 5’-GTAACTAAGGATAGA-3’

Pto kin1: 5’-GCATTGGAACAAGGTGAA-3’
Pto kin2: 5’-AGGGGGACCACCACGTAG-3’

Pto kin3: 5’-TAGTTCGGACGTTTACAT-3’
Pto kin4: 5’-AGTGTCTTGTAGGGTATC-3’

LM637: 5’-ARIGCTARIGGIARICC-3’ **
LM638: 5’-GGIGGIGTIGGIAAIACIAC-3’*

NBS-F1: 5’-GGAATGGGNGGNGTNGGNAARAC-3’
NBS-R1: 5’-YCTAGTTGTRAYDATDAYYYTRC-3’

RLK-For: 5’-GAYGTNAARCCIGARAA-3’
RLK-Rev: 5’-TCYGGYGCRATRTANCCNGGITGICC-3

S1: 5’-GGTGGGGTTGGGAAGACAACG-3’
AS1: 5’-CAACGCTAGTGGCAATCC-3’

S2: 5’-GGIGGIGTIGGIAAIACIAC-3’*
AS3: 5’-IAGIGCIAGIGGIAGICC-3’

PtoFen-S: 5’-ATGGGAAGCAAGTATTCAAGGC-3’
PtoFen-AS: 5’-TTGGCACAAAATTCTCATCAAGC-3’

XLRR-INV1: 5’-TTGTCAGGCCAGATACCC-3’
XLRR-INV2: 5’-GAGGAAGGACAGGTTGCC-3’

CLRR-INV1:	5’-GCAGCAACTTGTGC-3’
CLRR-INV2:	5’-TCTTCAGCTATCTGC-3’
NLRR-INV1:	5’-TGCTACGTTCTCCGGG-3’
NLRR-INV2:	5’-TCAGGCCGTGAAAAATAT-3’
Pto-kin1IN:	5’-AAGTGGAACAAGGTTACG-3’
Pto-kin2IN:	5’-GATGCACCACCAGGGGG-3’
S1-INV:	5’-GCAACAGAAGGGTTGGGGTGG-3’
AS1-INV:	5’-CCTAACGGTGATCGCAAC-3’
S2-INV:	5’-CAICAIAAIGGITGIGGIGG-3’
AS3-INV:	5’-CCIGAIGGIGAICGIG-3’
wlrk-S:	5'-GAAAGATGAGTAAATTACTTG-3'
wlrk-AS:	5'-TGAGGGTCAGGCATGCAG-3'
Cre3Ploop:	5'-GCGGGTCTGGGAAATCTACC-3'
Cre3-k3:	5'-CTGCAGTAAGCAAAGCAACG-3'
Cre3LR-F:	5'-CACACACTCGTCAGTCTGCC-3'
Cre3LR-R:	5'-CAGGAGCCAAAAATACGTAAG-3'
Xa1NBS-F:	5'-GGCAATGGAGGGATAGG-3'
Xa1NBS-R:	5' -CTCTGTATACGAGTTGTC-3'
Xa1LR-F:	5'-CTCACTCTCCTGAGAAAATTAC-3'
Xa1LR-R:	5'-GAGATTGCCAAGCAATTGC-3'

* Codes for mixed bases: Y = C/T, N = A/G/C/T, R = A/G, D = A/G/T, H = A/C/T

** LMS638 and S2 are identical.

Table 1. List of RGA primers used in our study

(Winter et al. 2000). This polymorphic primer was next used to evaluate the $F_{7:8}$ RILs of 142 lines and the representative electrophoretic profile of the amplified products on 6% polyacrylamide gel is as shown in Figure 1. Six of the RILs were heterozygous for this RGA locus (RGAPtokin1-2_{171}) and the segregation pattern was distorted (P>0.05) and was in favor of *C. reticulatum*. This can be attributed to abnormal meiosis which is a common phenomenon in interspecific crosses (Lambie and Roeder 1986).

The RGA marker was further mapped to linkage group 5 using Mapmaker/Exp 3.0 (Figure 2). By comparative mapping, this was further positioned to linkage group 3 of the integrated linkage map of *Cicer* developed from *C. arietinum* (ICC 4958) and *C. reticulatum* (PI 489777), an interspecific cross, which is the most extensive and comprehensive linkage map for chickpea available to-date (Winter et al. 2000).

4.4.2. Significance of RGA marker analysis

The nuclear content of chickpea is 738Mbp/1C (Arumuganathan and Earle 1991) and according to this information 1cM of the genetic map corresponds to about 630kbp, which is more than the average insert size cloned in a BAC library. Therefore, in order to isolate ascochyta blight resistant gene from the chickpea BAC library by map based cloning, it is necessary to have a marker tightly linked to these genes and this can be achieved by using a resistant gene focused marker system. I used the primers that were designed based on the conserved sequences present in all the characterized resistant genes isolated from various plant species. When these primers are used in polymerase chain reaction, they amplify the genomic regions, which are called Resistant Gene Analogs (RGA). There are many reports available which explain the use of RGA markers tightly linked to disease resistance genes in various crops (Kanazin et al. 1996; Hayes and Saghai Maroof 2000). These markers have also been mapped using molecular linkage maps in potato (Leister et al. 1996), soybean (Kanazin et al. 1996), rice, wheat and barley (Leister et al. 1998; Seah et al. 1998) and *A. thaliana* (Aarts et al. 1998). In all these cases, some RGAs showed close genetic linkage with known R genes (Aarts et al. 1998), indicating how mapping

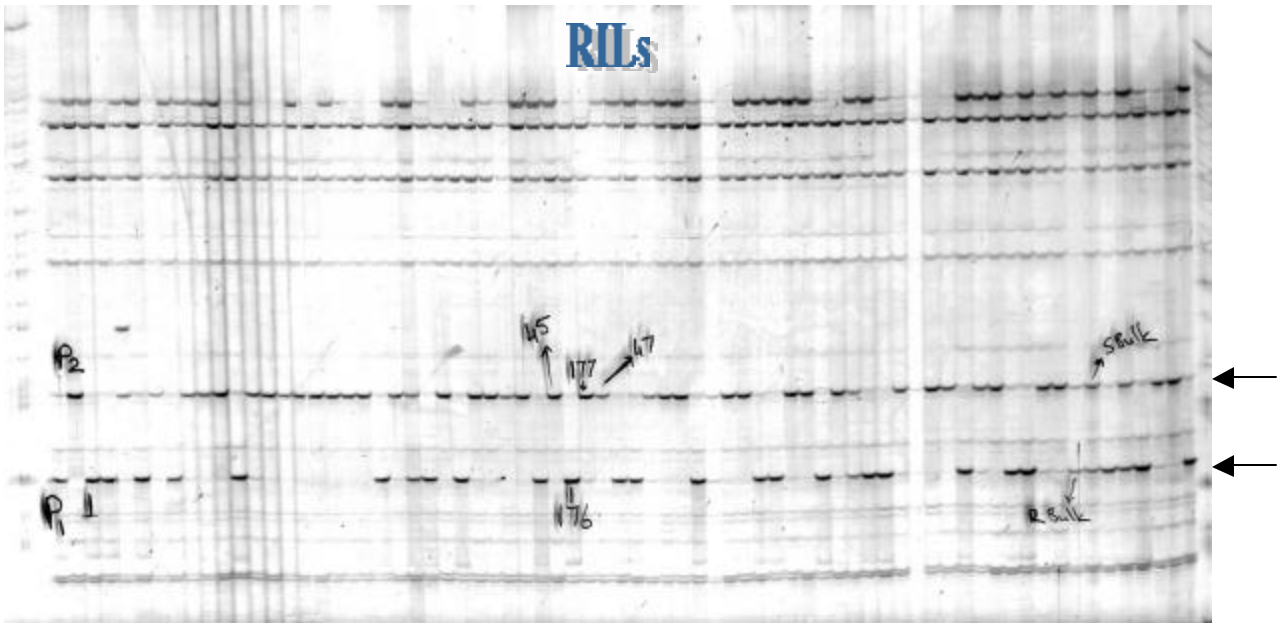


Figure 1. PCR amplified RGA pattern on silver stained 6% polyacrylamide gel
P1: FLIP 84-92C; P2: PI 599072; RILs: Recombinant inbred lines;
Arrows indicate the segregation of the locus in RILs

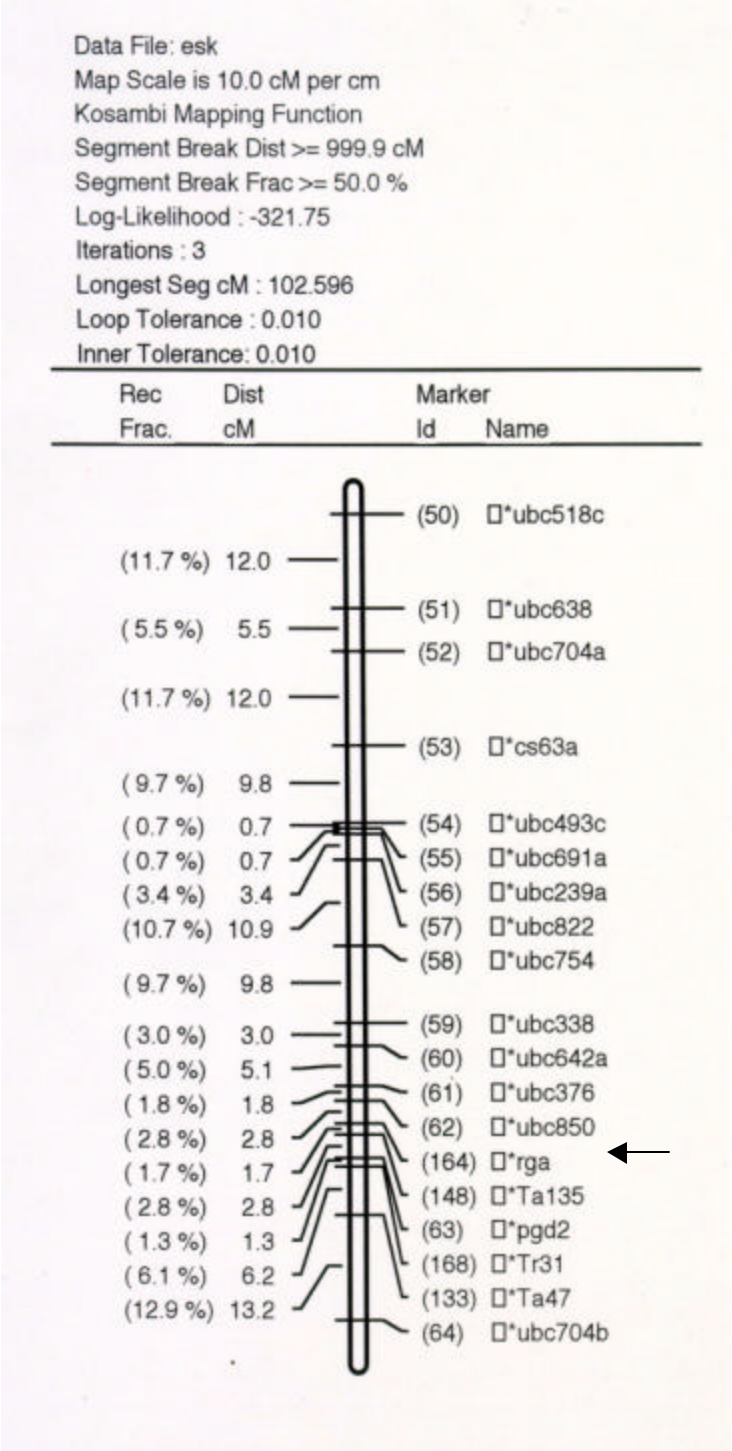


Figure 2. Linkage group 5 where RGA marker was mapped.

The arrow shows the position of the RGAPtokin1-2_{171} in the linkage group; *indicates the segregation distortion of this marker.

of RGAs would facilitate the localization of disease or pest resistance genes in plants. In some cases, the RGA sequences themselves may represent as candidate R genes. For example, the *A. thaliana* RGA proved to be a part of the cloned *RPP5* gene (Aarts et al. 1998). Although not all amplified products using these RGA primers are functional disease resistance genes, they contain elements involved in signal transduction pathway in plants (Chen et al. 1998). Thus the amplified products may represent candidate disease resistance genes or other important signal transduction processes.

As indicated earlier, the segregating RGAPtokin1-2_{171} is mapped in the linkage group 5, where blight resistance QTLs are not reported (Santra et al. 2000; Tekeoglu et al. 2000). The following hypotheses are the possible explanations: Firstly, it has been reported that most of the R-genes that have been characterized in the NBS-LRR class show dominantly inherited disease resistance. In pea, for example, most of the disease resistance genes like *er-1*, *sbm-1* and *mo* show recessive inheritance and the RGAs of NBS-LRR type used do not exhibit linkage to these genes (Timmerman et al. 2000). Similarly in chickpea, mapped disease resistance genes like fusarium wilt and ascochyta blight have recessive inheritance pattern (Tekeoglu et al. 2000b; Santra et al. 2000). Hence, NBS-LRR primers might be unlikely candidates for these genes. No resistance genes, which are genetically found to be recessive have been isolated and characterized to date. Secondly, since I have used RGA primers, which are not only specific to NBS-LRR regions but also to other conserved regions like P-loop and kinase-domain. there could be a resistance gene apart from NBS-LRR type or a modifier gene which plays a role in blight resistance on linkage group 5. This possibility cannot be ruled out because RGA primers can amplify the regions, which are not only a part of R genes, but also the elements, which are involved in signal transduction. Earlier, Singh and Reddy (1989) have interpreted that there could be additional minor genes based on their observation that plants show different degrees of resistance to ascochyta blight at different locations in chickpea. This has been later observed by Tekeoglu et al. (2000) for the same disease suggesting that there could possibly be many minor modifying genes besides the major QTLs. In *Mimulus guttatus* Fischer ex DC, it has been found that a single

major gene controls the presence or absence of tolerance, whereas modifier genes control the degree of expression of the tolerance (Tilstone and Macnair 1997). The role of minor genes in genetic variation has also been reported for several agronomically important traits in *Silene vulgaris*, wheat and soybean (Schat and Ten Bookum 1992; Sourdille et al. 1996; Rebetzke et al. 1998).

4.4.3. Presence of ankyrin protein repeat in RGAPtokin1-2$_{171}$

The cloned RGA fragment in chickpea on sequence analysis and homology search showed partial homology (5' ^{128}ccttgg…….tttggct^{153} 3') with ankyrin protein repeat. One of the inducing factors of salicylic acid signaling pathway in plants is the ankyrin protein repeat containing NPR1 protein (Klessig et al. 2000). It is well known that systemic acquired resistance (SAR) induction requires the signal molecule salicylic acid (SA), which accumulates in plants prior to the onset of it (Dong 1998). Expression of the defense genes PR1, BG2 and PR5 in response to SA treatment requires a gene NPR1 which encodes a novel protein that contains ankyrin repeats that are often involved in protein-protein interactions and is transported to the nucleus in the presence of SA (Glazebrook 1999). In this analysis, I could amplify a DNA fragment of size 171bp with the presence of ankyrin repeats of 26bp, which segregated in the population (Figure 1). In order to generate more information regarding the nature of this RGA fragment, it will be necessary to isolate the complete gene from the BAC library using this fragment as a probe and characterize the particular BAC clone.

In summary, I have been able to identify a RGA marker using primers Pto kin1&2 in chickpea and locate it on the linkage group 5, which has not been earlier reported to have ascochyta blight resistance QTLs. However, further cloning and sequencing of this marker has revealed the presence of an ankyrin repeat involved in protein-protein interaction. This is the first RGA marker reported so far in chickpea linkage map.

B) Differential expression analysis of genes in response to *Ascochyta rabiei* infection

4.5. Introduction

Plants and fungi have evolved together and developed defense mechanisms that have ensured their mutual survival. In order to survive the attack by pathogens, plants deploy defensive responses soon after infection or exposure to them. These plant responses, if not all, are due to changes in the transcriptional activity of the corresponding genes. Among many diseases that affect chickpea, ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. has the potential to cause 100% yield loss (Nene 1984). My aim was to analyze chickpea-*Ascochyta rabiei* interaction and eventually to isolate resistance genes from the BAC library. In order to study the genes with up- and/or down-regulated expression during this fungal pathogen infection, a comparative analysis of the total expression pattern of the resistant and susceptible cultivars of chickpea upon infection as against the uninfected control plants could represent as one of the first essential steps.

In the past, differentially expressed genes have been identified by subtractive hybridizations, differential plaque hybridizations or protein gel differences, followed by micro-sequencing or using antibodies to clone cDNAs (Baldwin et al. 1999). As large sequence databases become available for plants, the number of genes to be monitored becomes too large for traditional analyses such as northern blots. Ideally, an expression assay covering all genes in the plant cell will reveal how these patterns change during different developmental stages. The first RNA profiling techniques are "differential display" by Liang and Pardee (1992) and "arbitrarily primed (AP) PCR" by Welsh et al. (1992). The main difference between these two techniques is that the former uses one anchored oligo-dT primer and one arbitrary primer, whereas the latter is not anchored to 3' end. The technique "differential display" has been used to isolate a large number of plant genes differentially expressed during development (Li and Gray 1997), nodule formation (Heidstra et al. 1997; Szczyglowski et al. 1997; Curioni et al. 2000) and root formation (Butler and Gallagher 1998). Other applications include to understand gene response to hormones (Van der Knapp et al.

1997; Zegzouti et al. 1997), environmental stresses (Sharma and Davis 1995; Kadvrzhanova et al. 1998) and defense (Benito et al. 1996; Horvath and Chua 1996; Hermsmeier et al. 1998) and, most recently, to identify hybrid specific expression (Ni et al. 2000).

To understand the plant response to *Ascochyta*, it is important to identify genes and to assess their individual contribution to resistance mechanism. In this chapter, I have reported about the isolation of two cDNA clones, which are ascochyta stress responsive using DDRT approach.

4.6. Materials and methods

4.6.1. Construction of mist chamber

The mist chamber, measuring 66cm height, 121.5cm width and 95cm depth, was constructed with a covering of 6mil clear polyethylene (Figure 1a). The mist control system was known as "Automatic Misting System" (Phytotronics, USA) and had the tork brand timers.

4.6.2. Fungal material and the infection of plant material

I infected 10 days old Flip 84-92C, a *Cicer arietinum* resistant cultivar and PI 489777, a *C. reticulatum* susceptible variety seedlings with 1 $X10^6$ conidia of a virulent strain A20, a single spore culture, in the mist chamber, which maintained humidity by generating mist at the time interval of every 5min for 10sec (Figure 1b). This strain of *A. rabiei* was isolated from Genesee, Idaho, USA. Single conidial isolates were produced and stored on filter papers at -20^0C. These filter paper pieces were transferred to potato dextrose medium (250g of boiled potato extract, 40g of dextrose in 1000ml of water and autoclaved) to facilitate the growth of the pathogen. After 7-10 days of growth, the medium was filtered through 3 layers of filter cloth to separate mycelia and the conidia. The filtrate containing the conidia was counted under hemocytometer before spraying onto the plants. The control plants were sprayed with water outside the mist chamber. Leaf samples were collected from control as well as from infected seedlings on 1, 2, 3, 7and 8 days after infection as the plants started showing disease symptoms after 7^{th} day.

Figure 1a. A view of the mist chamber

Figure 1b. Resistant and susceptible seedlings in the mist chamber after inoculation

4.6.3. Total RNA isolation

The purity of RNA is an important factor in gene analysis and gene expression studies. In order to inhibit ribonucleases from the reagents and glassware used for RNA isolation, the deionized water and all the glassware were treated with 0.1% diethylpyrocarbonate (DEPC), autoclaved and baked at 160^0C overnight. The total RNAs were extracted from all the samples separately using RNeasy Kit from Qiagen as per the manufacturer's instructions.

4.6.4. Reverse transcription

A kit, RNAimage, involving components for reverse transcription as well as PCR amplification was obtained from GenHunter, USA. Reverse transcription was done in the total volume of 20µl containing 0.2µg of freshly diluted RNA, 125mM Tris-HCl, pH 8.3, 188mM KCl, 7.5mM $MgCl_2$, 25mM DTT, 250µM dNTP and 2µM of H-T_{11}M (where M may be G, A or C). The thermocycler was programmed to 65^0C for 5min, 37^0C for 60min and 75^0C for 5min. After the tubes reached to 37^0C for 10min, the thermocycler was stopped and 1µl MMLV reverse transcriptase (100units) was added to each tube and reverse transcription was continued.

4.6.5. PCR amplification of cDNA and electrophoresis

In a total volume of 20µl, the PCR mixture contained 2µl of reverse transcription product, 100mM Tris-HCl, pH 8.4, 500mM KCl, 15mM $MgCl_2$, 0.01% gelatin, 25µM of dNTP, 2µM of arbitrary primer, 2µM of H-T_{11}M and 1U of Taq polymerase. The reaction was incubated at 94^0C for 30sec, 40^0C for 2min and 72^0C for 30sec for 40 cycles followed by 72^0C for 5min.

Three microlitres of DDRT products were run on 6% polyacrylamide gel and were silver stained (Promega #Q4132). The differentially expressed bands were extracted from the gel, reamplified using the same primers and ran on 1% agarose gel.

4.6.6. Cloning and sequencing of the cDNA fragment

The differentially expressed cDNA fragments eluted from the agarose gel were cloned into the pGEM-T easy plasmid vector (Promega, USA). The recombinant plasmids were used to transform the competent *E. coli* ElectroMax™ DH10B™ cells by electroporation. And the transformants were selected on blue/white screening. Plasmid DNA was prepared and analyzed by restriction digestion with *EcoR*I for the insert size determination. After the PEG purification of these plasmids, sequencing of the cloned cDNA fragments was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA) using the dideoxy sequencing method with T7 universal primer. The homology search was carried out using BLAST (www.ncbi.nlm.nih.gov/BLAST).

4. 7. Results and discussion

The RNAs of all the resistant infected, susceptible infected, resistant control and susceptible control plants were pooled together and were used for differential display reverse transcription analysis. Pooling of RNAs will represent the cumulative RNA population, which are differentially expressed at a given time and will enable to detect those RNAs, which are expressed in very low levels through their amplification.

Figure 2 reveals the quality of total RNA isolated from infected and uninfected seedlings of resistant and susceptible cultivars. The sharp bands of 25S, 23S, 18S and 16S rRNAs and mRNA on 1.2% agarose gel show no degradation of the total RNA and hence, these RNAs were used for DDRT analysis.

In my experiment, I modified the classical DDRT method by excluding radioactive dNTP in the PCR and visualization by autoradiography. Instead, I ran cold PCR reaction and the products were visualized by employing silver staining method. In addition, few reactions were attempted with 10 RGA primers in place of arbitrary primers. Figure 3 and 4 are representative silver stained gel pictures of DDRT products where arrows indicate the differentially displayed products from control and infected resistant and susceptible plants.

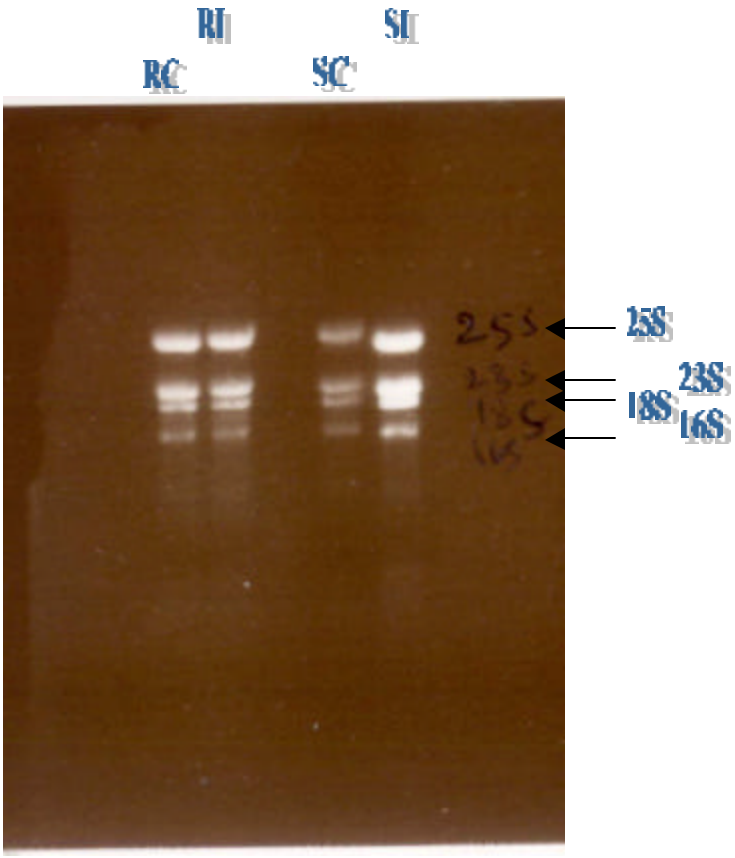


Figure 2. Total RNA gel indicating bands of 25S, 23S, 18S, 16S rRNAs and fast moving mRNA (not clearly visible); RC- Resistant control; RI- Resistant infected; SC- Susceptible control; SI- Susceptible infected

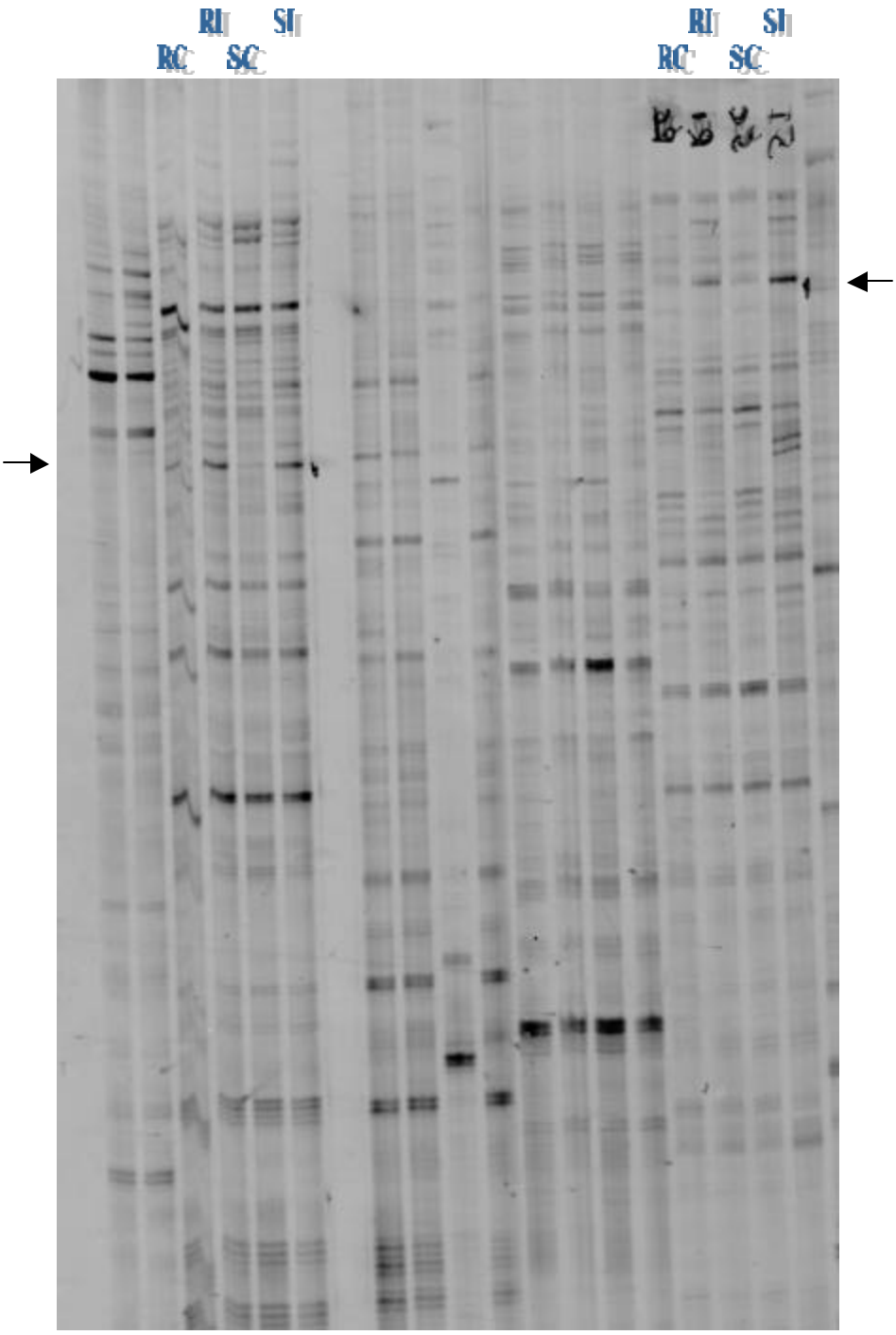


Figure 3. A representative silver stained gel picture of DDRT products using H-AP 6 and H-T(11) G and H-AP 7 and H-T(11)G primers; Arrows indicate differentially expressed partial cDNAs; RC- Resistant control; RI- Resistant infected; SC- Susceptible control; SI- Susceptible infected

A total of 17 differentially expressed products were extracted from the gels and reamplified using the same set of primers before cloning and characterizing them. Of all, only one band was present in resistant plants and was absent in susceptible ones. Remaining other bands showed their presence in control plants also but the differential expression was observed either in resistant infected or in susceptible infected samples. Tentative identity of one of the up-regulated clones was established by sequencing the partial cDNA clones where it showed 87% homology with serine hydroxy methyl transferase of Pea (Figure 5). This band was obtained with H-T(11) A and H-AP26 and its nucleotide sequence was deposited with Genbank (AF416481)

In order to detect expression of resistance genes upon infection, I used RGA primers, which were designed based on the conserved motifs present in previously reported cloned and characterized resistance genes apart from arbitrary primers. The ten RGA primers used for the analysis were Xa1-NBS, XLRR, NPLoop, Pto kin1, PtoFen, NLRR-INV, CLRR, Pto kin1-INV, CLRR-INV and S2. Interestingly, only two of them namely, Pto kin1 and PtoFen showed amplification while others did not amplify. This could be due to the absence of conserved motifs like LRR or NBS at the expressed level upon infection, which resulted in no amplification with NBS or LRR specific primers. It would be worth trying with other NBS or LRR related as well as other RGA primers from the Table 1 to detect the presence of other motifs in the expressed genes. Figure 4 shows the differential display of the Pto kin RGA primer in combination with H-T(11)A. One of the differentially expressed clones, as shown by the arrow, revealed 88% homology with aldolase of Pea (Figure 6). This nucleotide sequence has been deposited with Genbank (AF416480). Apart from these two, sequence analysis and database searches revealed no significant homology to any known sequence for other differentially expressed bands. In order to confirm the induction of these two partial cDNA fragments, which showed homology to serine hydroxy methyl transferase and aldolase, northern blotting needs to be performed using them as probes.

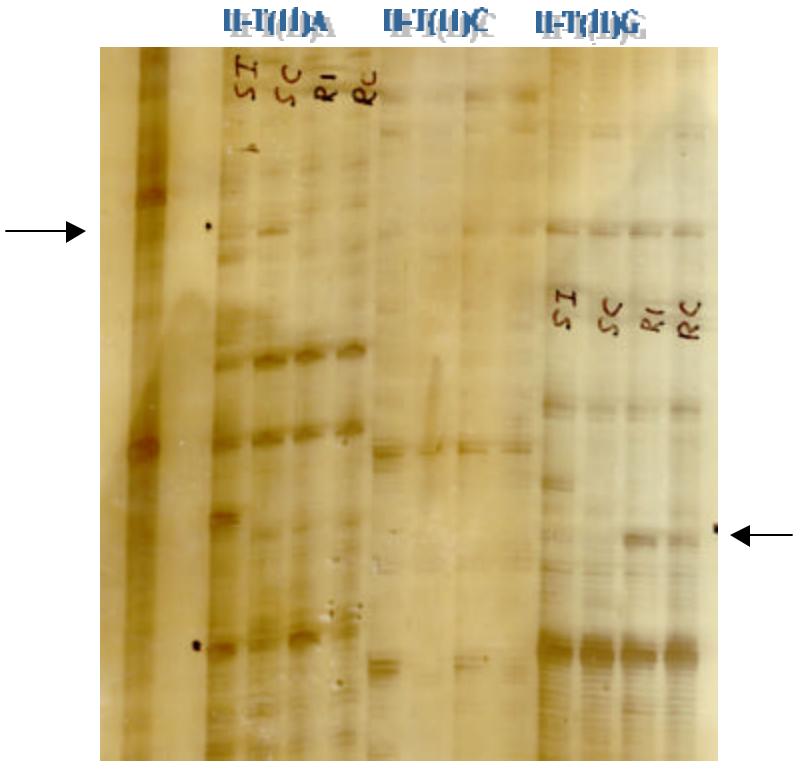


Figure 4. DDRT profile using Pto kin RGA primer.

RC- Resistant control; RI- Resistant infected; SC- Susceptible control; SI- Susceptible infected; H-T(11)N represents different anchored poly T primers where N stands for A, C and G

4.7.1. Limitations of differential display approach

Although DDRT technique has advantage of analyzing the mRNA population as a whole, it has a few inherent drawbacks, which are mentioned below (Baldwin et al. 1999).

First, since the annealing of the arbitrary primers to the cDNA is done at 40^0C, the number of false positives generated by this technique is high (Sung and Denmen 1997). Also the band difference may not always reflect the real difference in the gene expression and the same was observed in my study also. Though I tried 40 arbitrary primers, which statistically covered 80% of the expressed genes at a given time, only two cDNA clones showed homology with the reported genes.

Secondly, the fragments generated include only several hundred bases from the 3'-end. Sequence from this region is often insufficient to identify a gene,

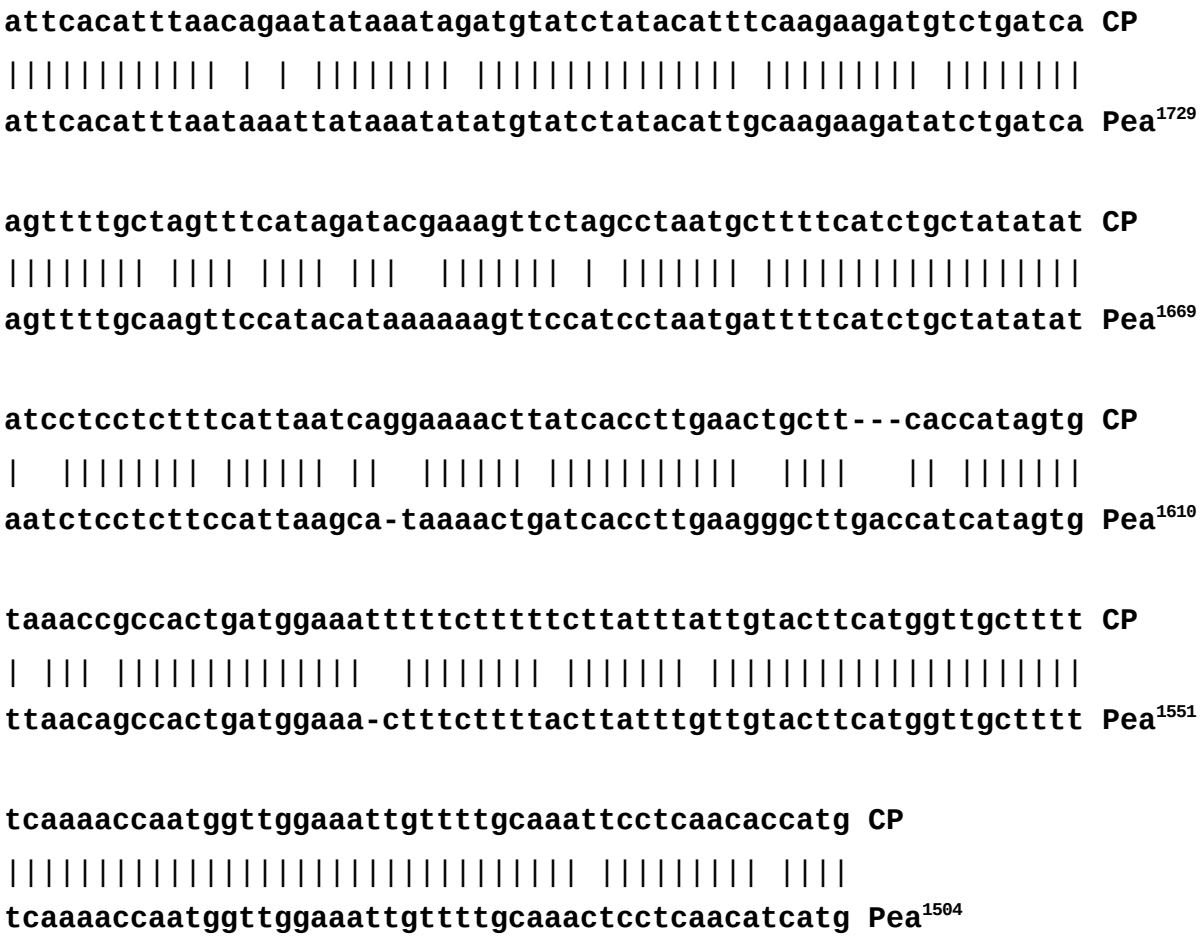


Figure 5. A DDRT product showing homology to Serine hydroxy methyl transferase gene of pea (Genbank AF416481).

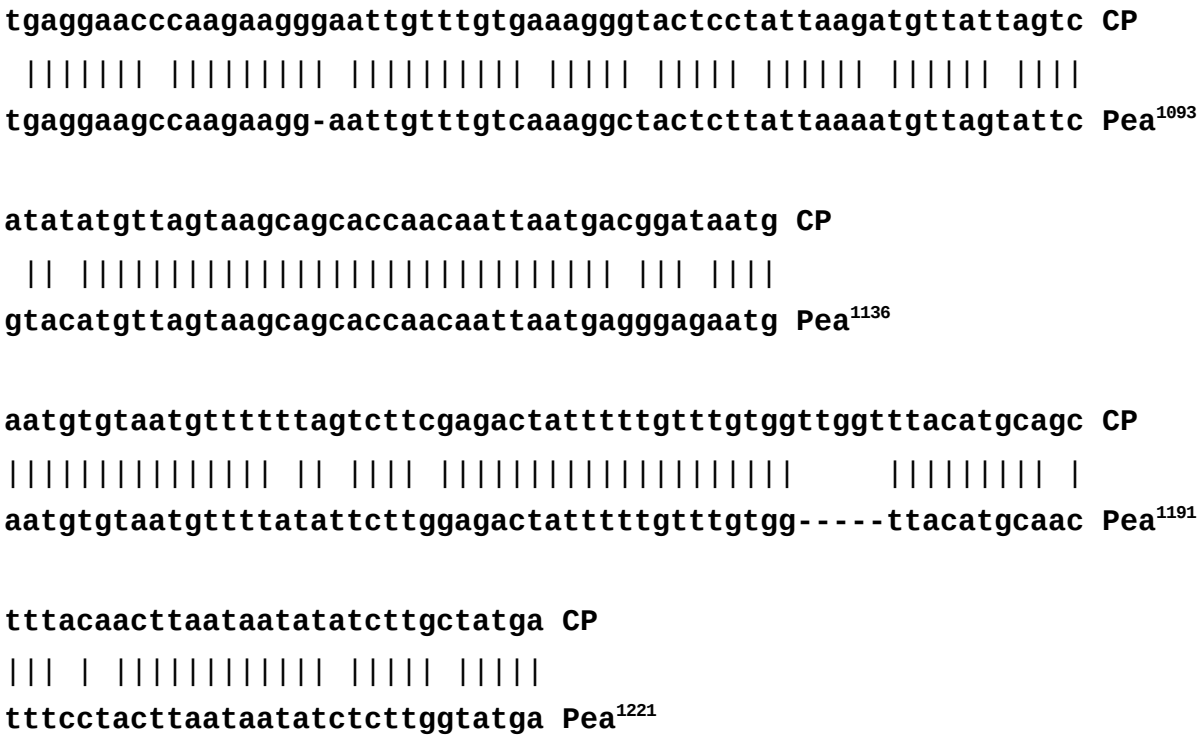


Figure 6. A partial RGA cDNA clone showing the homology to Aldolase gene of Pea. (Genbank AF416480)

especially, when using a database from an unrelated organism. To overcome this problem, a longer cDNA clone needs to be isolated to study the differentially expressed gene.

The third problem is cloning of the isolated fragment after excising from the sequencing gel. It has been reported that several species of cDNA present in a band lead to a mixed population of candidates after reamplification and cloning. Hence it is suggested to sequence more than one clone per band.

Non-specific amplification of traces of DNA in infected RNA samples is also expected. To detect them, a control reaction committing the reverse transcriptase skip must be run along with the infected RNA samples. Though, at times, PCR products could be observed in these control reactions, they, generally, will not co-migrate with the other samples.

Another common problem is that the total RNAs isolated, especially from the infected seedlings may have contamination of RNAs from the pathogen also as during infection, genes from both, the host and pathogen, are involved. To identify that, DDRT-PCR has to be run and the patterns of non-infected plants as well as the fungus need to be compared.

In spite of these problems and difficulties associated with DDRT approach, it is a whole-sum approach to identify genes expressed differentially and I could isolate two partial genes showing homology to serine hydroxy methyl transferase and aldolase of pea. Further studies to confirm their role in blight resistance in chickpea need to be undertaken.

Chapter 5

Bacterial Artificial Chromosome (BAC) library construction in chickpea

The contents of this paper have been communicated to International Chickpea Newsletter

5.1. Introduction

One essential tool for characterizing genomes is the availability of yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) library containing large genomic DNA inserts (Yu et al. 2000). These libraries have been exploited for the development of detailed genetical and physical maps of major crops and positional cloning of genes of interest. BAC libraries are preferred over YAC libraries due to various reasons such as BAC DNA is easy to purify in contrast to YAC DNA purification, BAC clones are stable in the host strains, the ease of using the physical maps constructed with BACs in genome research and low level of chimerism. Since 1994, BAC libraries are available for many crops such as sorghum (Woo et al. 1994), *Arabidopsis* (Choi et al. 1995), apple (Vinatzer et al. 1998), wheat (Moullet et al. 1999), soybean (Meksem et al. 2000) and pea (Coyne et al. 2000) and they have been utilized successfully for many applications. Considering the enormous utilization of BAC library in genome characterization and our interest in chickpea genomics, it was thought to be worthwhile to have such a library for this legume crop which can be used not only by our own laboratory but also by other chickpea researchers in international community.

In my effort to identify ascochyta blight resistant genes in chickpea, two major QTLs and a minor QTL have been mapped in the chickpea genome using mapping populations developed from a cross between FLIP 84-92C (resistant) and PI 599072 (susceptible) cultivars (Santra et al. 2000). These loci in the genome provide information that can be effectively used for eventual isolation and cloning of resistance genes. To facilitate this objective, I constructed a large insert Bacterial Artificial Chromosome (BAC) library from FLIP 84-92C, a resistant cultivar. The methodologies described in this chapter contain the rationale behind each step, the pros and cons of each step and the results obtained and hence no separate subheadings such as results and discussion are included.

5.2. Materials and methods

5.2.1. Pre-treatment of the plant material

Ascochyta blight resistant cultivar FLIP 84-92C was used for the *Hind*III BAC library. The seeds obtained from National Plant Germplasm System, USA, were grown in a growth chamber in soiless mixture for 14 days, with a cycle of 16hr light and 8hr dark at 22^0C. Two weeks after germination, seedlings were grown in continuous dark for 3 days to reduce the carbohydrate content of the leaves.

5.2.2. High Molecular Weight DNA isolation

High molecular weight (HMW) DNA and its quality are a prerequisite for BAC library construction. As it is known, plant cells have rigid cell walls and hence it is more of a challenge to isolate HMW DNA from plants than from mammalian cells. There are two methods available to isolate HMW DNA from plants namely protoplast method and nuclei method. Figure 1 gives the flow chart of these two methodologies. To isolate HMW DNA, protoplast or nuclei must first be embedded in agarose plugs or microbeads. The agarose acts as a solid yet porous matrix, which allows the diffusion of various reagents for DNA purification and subsequent manipulations while preventing the DNA from being sheared (Schwartz and Cantor 1984).

Although the protoplast method yields megabase-size DNA of high quality, the process is costly and labor intensive. For example, young leaves have to be manually feathered with a razor blade before being incubated for 4-5hr with cell wall degrading enzymes (Woo et al 1995), rubbing carborundum on both sides of the leaves with a paintbrush, 50 strokes/side before 4-5hr incubation with cellulysin (Ganal and Tanksley 1989) and so on. The process is time consuming and takes around 7-9hr before embedding in agarose. However, recently developed nuclei method works well for several divergent plant taxa (Zhang et al. 1995) and hence, I adopted the nuclei method for its simplicity and cost-effectiveness to isolate high molecular weight DNA from chickpea.

Nuclei were isolated from 25g of leaves and embedded in 12ml of 0.5% (w/v) low melting point agarose plugs. The frozen tissue was ground into a powder in liquid nitrogen and transferred into an ice cold beaker containing 200ml of 1X

Homogenization Buffer (10X HB= 0.1 M Trizma base, 0.8M KCl, 0.1M EDTA, 10mM Spermidine, 10mM Spermine, pH 9.4) + 300µl β-mercaptoethanol and the contents were allowed to mix well. The slurry was filtered using miracloth and

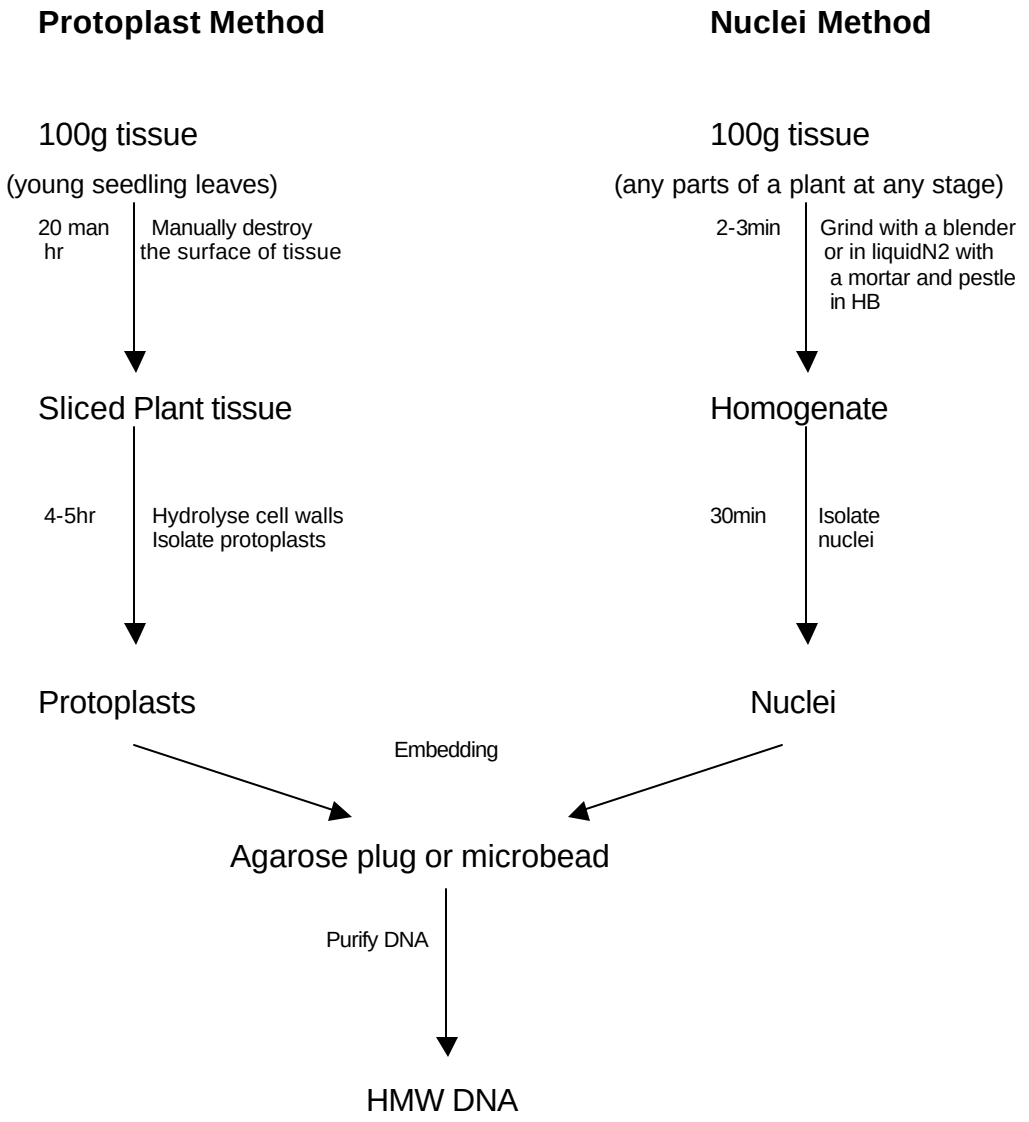


Figure 1. Two different methods to isolate high molecular weight DNA

centrifuged in a swing bucket rotor at 3,600*g* at 4^0C for 20min (Eppendorff, USA). The supernatant was discarded and the pellet was mixed with 1ml of ice-cold wash buffer (1X HB + 0.5% Triton X-100 with no β-mercaptoethanol). 20% Triton X-100 stock was prepared by mixing Triton X-100 in 1X HB without β-mercaptoethanol and

was stored at 4^0C. Further, the pellets were suspended in 30ml of this buffer gently using paintbrush. The nuclei were resuspended and washed twice with the wash buffer. After the final wash, the pellet was suspended in 1ml of 1X HB without β-mercaptoethanol with a paintbrush.

5.2.3. Embedding the nuclei in agarose plugs

One- percent low melting point (LMP) agarose was prepared in 1X HB without β-mercaptoethanol and was stored in a 45^0C water bath. The nuclei were prewarmed at 45^0C and mixed with the equal volume of prewarmed 1% LMP agarose using cut-off pipet tip. The mixture was aliquotted into ice cold plug molds on ice. After the agarose was completely solidified, the plugs were transferred into 5-10 volumes of lysis buffer [0.5M EDTA, pH 9.0-9.3, 1% sodium lauryl sarcosine and 0.1% proteinase K (this was added just before transferring the plugs to lysis buffer)]. These plugs were incubated for 24-48hr at 50^0C with gentle shaking. Later, plugs were washed once with 0.5M EDTA, pH 9.0-9.3 for 1hr at 50^0C, once with 0.05M EDTA, pH 8.0 for 1hr on ice and finally stored in 0.05M EDTA, pH 8.0 at 4^0C.

5.2.4. History of the vector

The BAC vectors are derived from the *E. Coli* "F" factor plasmid, which contains genes for strict copy number control and unidirectional DNA replication. I used the BAC vector pCLDO4541 (V41) for the large insert library construction. This vector was developed by Dr. J. D. G. Jones and generously provided by Dr. H. Zhang (Texas A&M University, USA). Map of this vector is shown in Figure 2. The vector pCLD04541 is 29kb in size and derived from the plasmid vector pRK290 of 20kb, which is a derivative of a native plasmid RK2 (Ditta et al. 1980). RK2 belongs to P1 incompatibility group and has a size of 56Kb. The genes oriV, trfA and trfB constitute the replicons of RK2 and its derivative pRK290, which exist at 5-8 copies/chromosomal equivalent in *E. coli*. It is a binary vector for *Agrobacterium* mediated plant transformation and has been shown to be capable of stable maintenance of large plant DNA (Moullet et al. 1999).

5.2.5. Vector preparation

The V41 was isolated using Qiagen Large Construct Kit followed by cesium chloride/ethidium bromide equilibrium centrifugation at 90,000rpm for 3hr at 20^0C. After the isolation, the vector DNA was digested to completion with *Hind*III restriction enzyme overnight at 37^0C and assayed by gel electrophoresis. After a chloroform/isoamyl alcohol extraction and an ethanol precipitation, the vector was dephosphorylated with 0.25U of calf intestinal alkaline phosphatase (CIAP) (BRL, USA) per picomole of DNA for 30min at 37^0C. CIAP was heat-inactivated

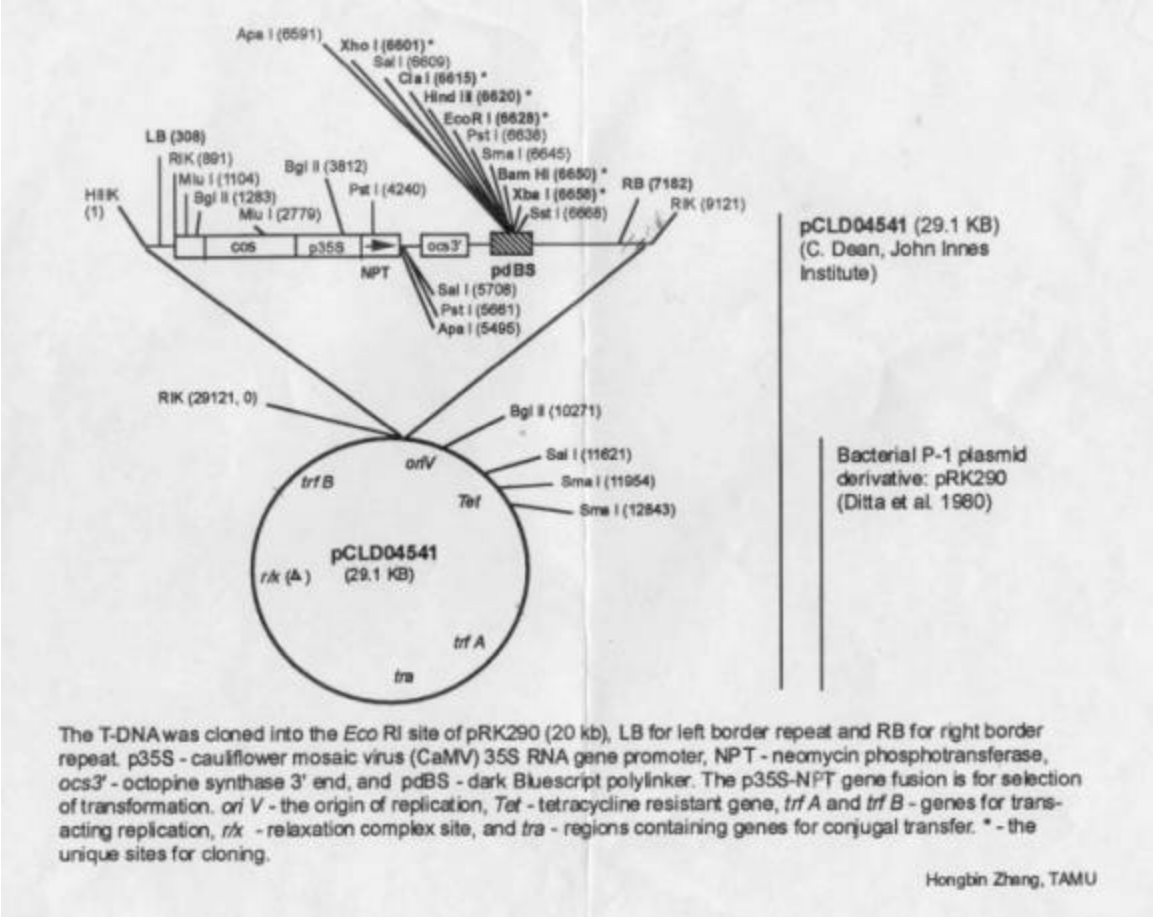


Figure 2. Map of the vector V41 (Zhang HB, TAMU, USA)

at 75^0C for 10min in the presence of 100mM NaCl, 1mM EDTA and 0.25% SDS. The DNA extracted with chloroform: IAA and precipitated with ethanol was resuspended

in TE, pH 8.0 at a concentration of 20ng/µl and directly used in the ligation reaction. The efficiency of dephosphorylation was tested by self-ligation of the BAC vector. Self-ligation of non-dephosphorylated BAC vector was used as a control. Only those preparations in which dephosphorylation resulted in more than 98% reduction in the number of transformants were used for construction of the BAC library. The cloning efficiency of vector preparation was tested by ligation with Lambda *Hind*III; only those preparations that resulted in a minimum of 50% white colonies were used for library construction.

5.2.6. Partial digestion and size selection of HMW DNA:

Agarose plug (each plug contained about 1µg of DNA) was cut into 12 pieces, the amount of the enzyme *Hind*III (Gibco-BRL) added to each plug ranged from 0.1-2 units per µg of DNA and the digestion time was extended to 20min at 37^0C. Each digestion was carried out in a 200µl total volume and the reactions were stopped by adding 20µl of ice cold 0.5M EDTA, pH 8, on ice. Partially digested DNA was fractionated on a 1% LMP agarose gel in 0.5X TBE (45mM Tris-borate, pH 8, 1mM EDTA) by PFGE at 6V/cm, 95sec switch time for 19hr at 11^0C (CHEF DRIII system, Bio Rad, USA). DNA fractions ranging from 100kb to 200kb were excised from the gel and directly used for the second size selection in 0.8% LMP agarose using the following conditions: 4V/cm, 5sec switch time, 9hr, 11^0C, 0.5X TBE). The agarose slices were equilibrated for 1hr, on ice, in 10mM Tris-HCl, pH 7.5, 1mM EDTA and 40mM NaCl with one change of buffer after 30min, melted at 68^0C for 5min and digested with 1U of agarase (Epicenter, USA) per 100mg LMP agarose for 30min at 45^0C. The enzyme was heat-inactivated at 70^0C for 5min and the DNA solution used directly for the ligation reaction (Figure 3). Woo et al. (1994) have found that it is essential to perform two such size selections to increase the average insert size of BACs, due to trapping of small DNA fragments after the first size selection.

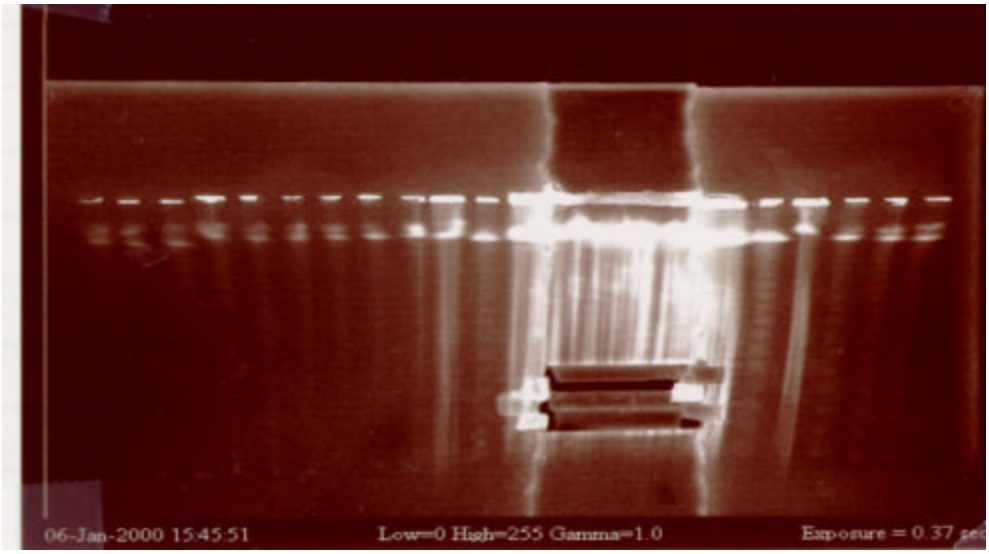


Figure 3a. First size selection

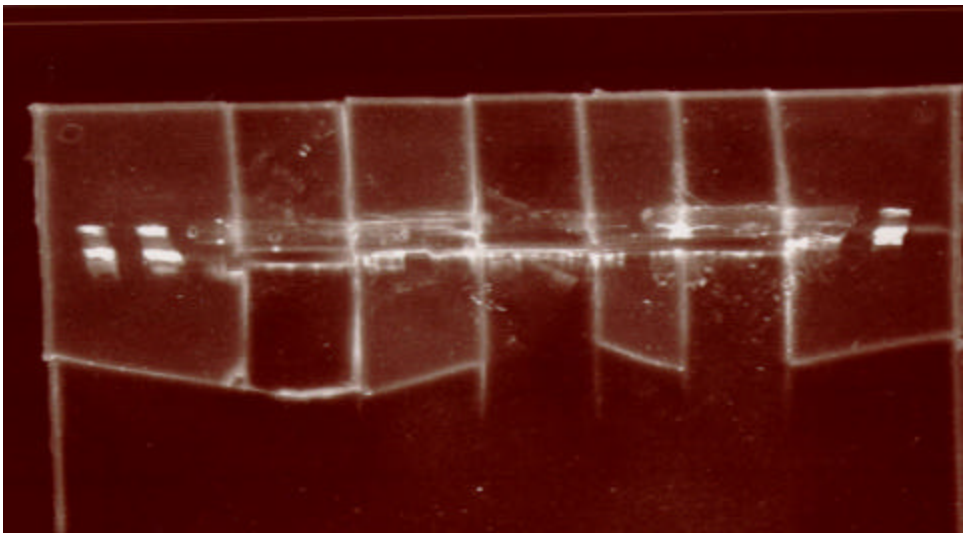


Figure 3b. Second size selection

5.2.7. Ligation and transformation

The ligation was carried out in a 100-150µl volume in which about 60-120ng of *Hind*III partially digested and size selected chickpea DNA was ligated to

200-400ng of vector (molar ratio of about 1:4 with vector excess) with 6U of T4 DNA ligase (Gibco BRL, USA) and incubated overnight at 16^0C.

E. coli cells divide faster and it is easier to isolate DNA from this bacterial strain and to transform. For library construction, the transformation efficiency of the host cell is critical. The BAC library construction involved electroporation to introduce the ligated DNA into *E. coli*. The transformation efficiency of the commercially available electrocompetent cells guarantees at least 10^{10} transformants/µg with control plasmid (pUC19). Because of this feature, the amount of size selected DNA required to make a complete BAC library is less than that needed for YAC library. Since the ligated plasmids are circular, they can be transformed more efficiently than YACs and probably are more stable than a linear YAC.

The vector I used for chickpea BAC library construction is a binary vector, since it has got the important components necessary for *Agrobacterium* mediated transformation. The report which explains that *A. tumefaciens* is capable of transferring 160kb of the Ti plasmid DNA unidirectionally into a plant, *Kalanchoe tubiflorea* (Miranda et al. 1992) and also the fact that chickpea is a dicotyledonous legume crop prompted me to use the binary vector. The average insert size of chickpea genomic DNA in this BAC library was 120kb which is well within the range of the insert size which can be transformed through *Agrobacterium*.

The recombinant vectors were introduced into ElectroMAX™ DH10B™ cells (Gibco-BRL, USA) by electroporation using a Cell Porator and Voltage Booster system (Gibco-BRL, USA). A 1.5µl aliquot of ligation mixture was added to 18µl of electrocompetent *E. coli* cells for a single electroporation. The Cell Porator settings were 350V, 330µF capacitance, low ohms impedance and fast charge rate and the Voltage Booster setting was 4kohms resistance. After electroporation, the cells were transferred to 1ml SOC solution (2% bacto tryptone, 0.5% bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose, pH 7.0) and incubated at 37^0C with shaking at 200rpm for 1hr. The cells were spread on LB plates containing tetracycline (15µg/ml), X-gal (30µg/ml) and isopropylthio-β-D-galactoside (20µg/ml), grown at 37^0C for 20hr and stored at 4^0C. White colonies containing chickpea DNA inserts were transferred to 384-well microtiter plates

(Medos, Australia) containing 50µl of LB-freezing buffer (9:1)(36mM K_2HPO_4, 13.2mM KH_2PO_4, 1.7mM citrate, 0.4mM $MgSO_4$, 6.8mM $(NH_4)_2SO_4$, 4.4% glycerol, 15µg /ml tetracycline, LB) per well with the help of Flexy's Bio robot (Figure 4), incubated at 37^0C for 24hr and stored at -80^0C.

5.2.8. Limitations in transformation

Apart from insert size, efficiency of transformation (cfu/µg of DNA) is affected by many factors such as buffer components, temperature (Antonov et al. 1993) and the electroporation conditions determined by users including voltage gradient, resistance and capacitance (Tekle et al. 1991). In addition, the genetic background of host cells (Hanahan et al. 1991), post-pulse treatment (Dower et al. 1988), the topological form (Neumann et al. 1982) and treatment of DNA samples (Kobori et al. 1993) are some other factors, which contribute to the efficiency observed. These problems can be overcome by tRNA assisted ethanol precipitation, which substantially increased the number of recombinant clones after transformation (Zhu and Dean 1999). The proposed mechanism for this increased efficiency is tRNA precipitation may alter or stabilize the topological form of the ligated DNA molecules. Also the size of the inserts derived from large insert ligation remains unaffected.

The previous results in other systems have shown that recombinant BACs larger than 350kb have not been recovered. Since *E. coli* can replicate its own genome of about 4Mb, it is unlikely that it cannot replicate a 1Mb BAC. One possible reason for this problem is that there may be a limit to the size of a molecule that can be delivered by electroporation.

5.2.9. Isolation of recombinant DNA and insert analysis:

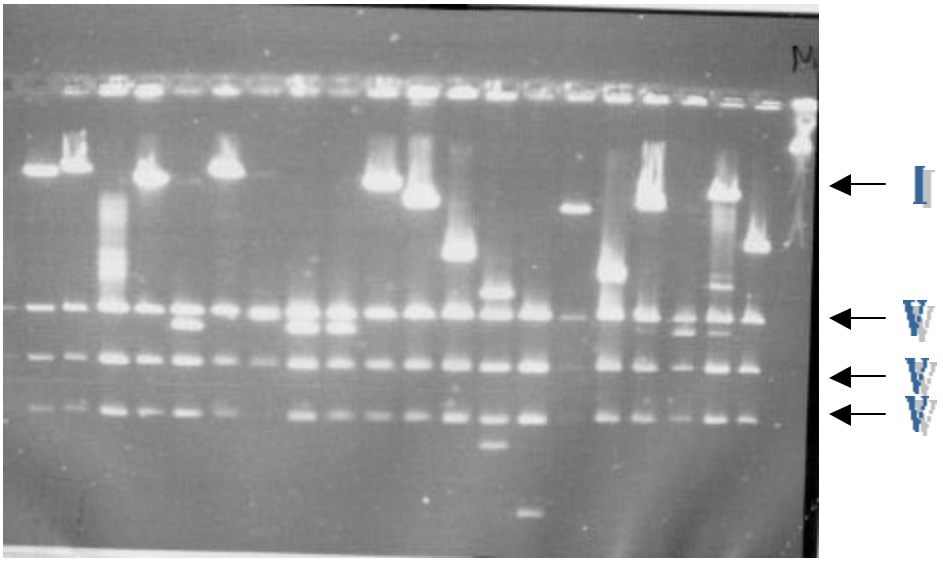
Clones were streaked onto LB plates containing 15µg/ml tetracycline. One hundred and ten random single colonies were picked up and grown in 5ml LB with antibiotic (15µg/ml tetracycline) at 37^0C for 20hr. The alkaline lysis method (Sambrook et al. 1989) was used for preparing the plasmid DNA and samples were digested with *Not*I (NEB, USA) overnight and then loaded on a 0.8% agarose gel.

Figure 4: A picture of Flexy's Bio robot

The gel was subjected to PFGE overnight at 370V in 0.5X TBE at 16^0C using a 4-90sec discontinuous ramped switch time. The average insert size of the BAC library was estimated to be 120kb by comparison with a lambda mid-range size standard (NEB, USA) electrophoresed parallely in the same gel (Figure 5).

5.2.10. Filter preparation

High density filters were made by replicating twenty four 384-well microtiter plates onto single prewet Hybond N+ filters (8X12cm) (Amersham, USA) placed on LB/agar plate containing 15µg/ml tetracycline using Flexy's Bio robot. Each sample was double spotted for confirmation of the hybridization. Filters were incubated overnight on the same medium. The fixation of plasmid DNA to the filter was performed according to the manufacturer's recommendations.



V -Vector; I - Insert

Figure 5. Insert size determination

5.2.11. Quantitation of chloroplast DNA contamination in the BAC library

Since the BAC library was constructed from leaf DNA, clones containing chloroplast DNA were expected to be present in the BAC library. In order to estimate the percentage of BAC clones containing chloroplast DNA, the filters representing the entire chickpea BAC library were hybridized with the barley

Kfp-231 chloroplast DNA probe which is RuBpcarboxylase large sub-unit gene. The result indicated that negligible amount of the clones contained that chloroplast gene (Figure 6).

5.2.12. Statistics of the library

The chickpea BAC library contained 23,780 clones. It represented 3.8 times the genome which was calculated based on the insert size estimated and

$$N = \ln (1-P) / \ln (1-I/GS)$$

Where, N= Number of clones

P= Probability of finding any genomic fragment in the library

I= Average insert size of the clone

and GS= Genome size in Mbp

the genome size of chickpea. Based on the above mentioned formula, it was calculated that our chickpea BAC library has approximately 95% probability of finding any chickpea genomic fragment.

5. 3. Applications of BAC library

This is the first chickpea BAC library constructed so far and can have many applications in chickpea genomics. It can be used in the analysis of the arrangement and development of microsatellites in chickpea (Springer et al. 1994), to study the structure and also organization of multigene families and for cloning disease resistance genes. Genes underlying QTL or with related functions such as disease resistance are many a times organized in clusters (Staskawicz et al. 1995). Ascochyta blight resistance being governed by QTLs, library in BAC vector, which can clone large fragments and may contain a gene cluster (Meksem et al. 2000), is the first step towards isolation of these genes.

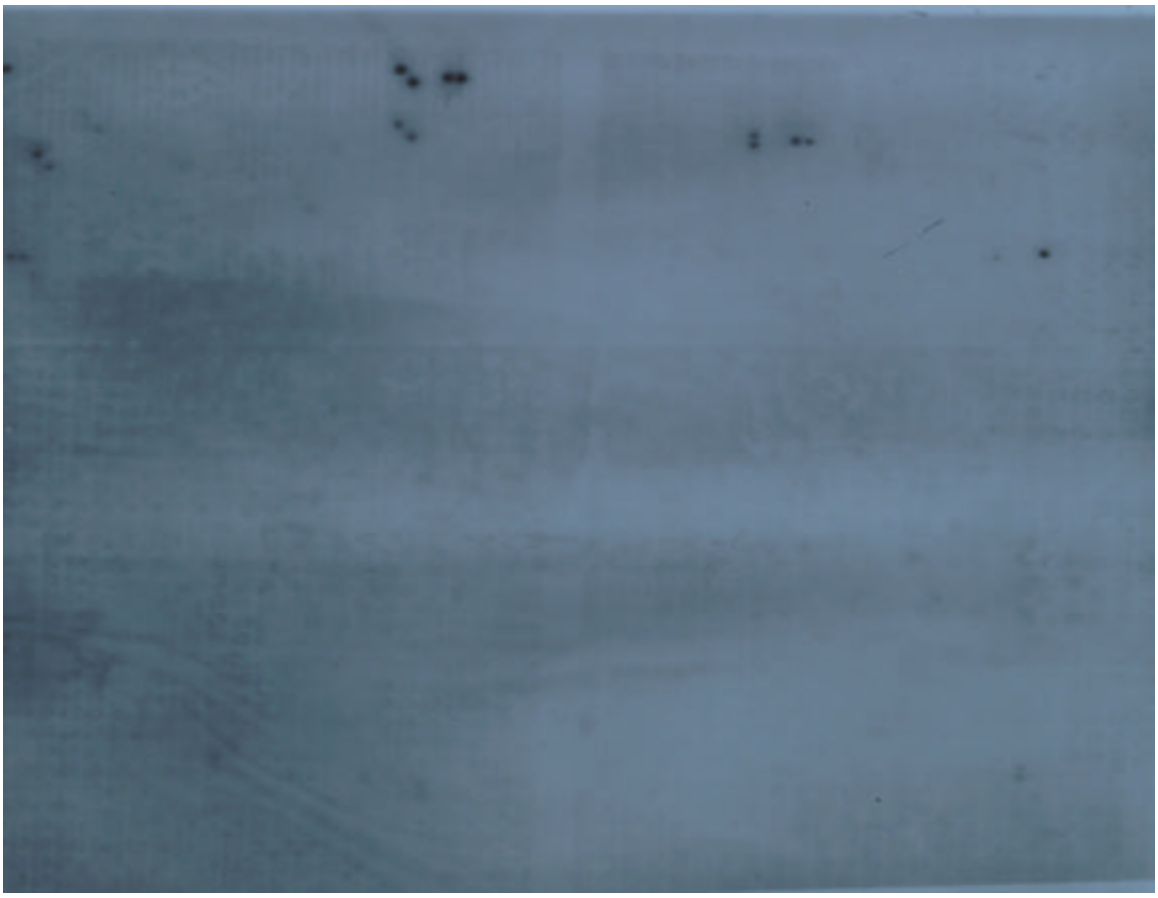


Figure 6. Detection of chloroplast contamination in the chickpea BAC library

This large insert library will make large-scale physical maps of chickpea genomic regions easier to construct. Once generated, the physical map will provide virtually unlimited number of DNA markers from any chromosomal region for gene tagging, gene manipulation and genetic studies of many agronomically important traits. It will also provide an on-line framework for studies in genome molecular structure, genome organization and evolution, gene regulation and gene interaction. The identification, isolation, characterization and manipulation of genes will become far more feasible than ever before. Therefore, the physical map will become central to all types of genetic and molecular inquiry and manipulation including genome analysis, gene cloning and crop genetic improvement (Zhang and Wing 1997).

Future Perspectives

Two plant families of wide importance to world agriculture are the Poaceae (cereals and grasses) and the Fabaceae. In Fabaceae, the legumes constitute about 650 genera and 18,000 species. Further, legumes have been classified as cool season food legumes which include pea, (*Pisum sativum* L.), lentil (*Lens culinaris* Medik.), faba bean (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), and grasspea (*Lathyrus sativus* L.) and warm season pulses which contain major grain legumes such as soybean (*Glycine max* L.), peanut (*Arachis hypogea* L), common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* L.). As the dusk approaches to study the genetic mapping and genome characterization for cereals, it is the golden time for the members of the Fabaceae, especially legumes. There are many areas, which are untouched by the researchers in legumes except few representative species. As far as chickpea is concerned, the term "genomics" is a new field of research since several opportunities need to be exploited and expedited. Also its smaller genome size (740Mb), shorter lifecycle, less complex genome with no ploidy level, may make this pulse crop as a model for legumes along with *Lotus japonicus* and *Medicago truncatula*. Further studies on this crop will not only help to identify the enormous untapped potential of chickpea but also to understand legume biology. Another aspect of using chickpea for such technologically advanced studies is its growing popularity because of high market demand, well adaptation of this pulse crop to plains region and its rotational benefits (http://weeds.montana.edu). Some of the prospective areas of research in case of chickpea are discussed below.

Molecular dissection of QTLs to facilitate Map-based cloning

Many of the agronomically important traits are quantitative in nature. They are complex and are affected by environmental variations. Hence it is difficult to perform precision linkage mapping of these QTLs. Fine mapping of a QTL as a single Mendelian factor is required to make it feasible to delimit a candidate genomic region for the QTL. Fine mapping of QTLs using backcross-derived lines has been reported in various crops like maize (Dorweller et al. 1993; Vladutu et al. 1999), tomato (Paterson et al. 1990; Albert et al. 1996) and rice (Yamamoto et al. 1998; 2000).

In case of ascochyta blight, which is governed by QTLs in chickpea, such type of analysis is required. Once the region is mapped as a single Mendelian factor, positional cloning of the genes at these QTLs will be easier. In order to map these genes precisely, it is necessary to increase the population size enough to minimize the size of candidate genomic region. Moreover, genetically proven recessive resistance pattern of this disease is another complex issue, as the mechanism of this pattern is not explained yet. It may have totally different gene composition and may not fit in common class of disease resistance genes. Hence, the production of region specific markers to blight QTLs is a crucial factor in the fine-scale mapping of these genes. In chapter 4, I have reported the attempts made using the RGAs, which are resistance gene specific markers. It will be interesting to map RGAs on chickpea linkage map, which are lacking at present.

Functional genomics

A variety of methods for global analyses of gene expression combined with predictions from DNA-sequence data are greatly increasing our ability to make inferences on gene and protein function (Lemieux et al. 1998; Baldwin et al. 1999; Eisen 1998). Genes that have altered expression in compatible and incompatible plant-pathogen interactions have been targeted for characterization, because these analyses will provide comprehensive data on expression profiles, both for genes already implicated in plant-pathogen interactions as well as for many genes that were not previously known to be involved in resistance or susceptibility. Comparison of the regulatory regions of groups of co-regulated genes will indicate potential regulatory sequences and the regulatory networks that control their expression (Rushton and Sornssich 1998; Brazma et al. 1998; Thieffry 1999).

In my attempt to explore the functional genomics during plant–pathogen interactions especially with respect to *ascochyta* fungus infection, I could identify two genes, which are differentially expressed using DDRT approach. There are different approaches like serial analysis of gene expression (Velculescu et al. 1995), electronic northerns and microarrays to study the functional changes. The advantage of arrays is that they give quantitative information on the abundance of hundreds or

thousands (depending on the array design) of specific genes simultaneously. Microarrays are invaluable at providing a global view of gene expression changes.

Recent rapid progress in genetic technology and the availability of various automated genetic analysis instruments have made it possible to perform large-scale isolation and partial sequencing of anonymous cDNA clones. These sequences can be used not only as expressed sequence tags (ESTs) on RFLP linkage maps (Kurata et al. 1997) but also as effective probes to screen BAC library for the construction of physical maps of chromosomes (Umehara et al. 1995). A non-redundant set of 2057 ESTs from the *L. japonicus-Mesorhizobium loti* interaction and 9698 non-redundant ESTs from whole *L. japonicus* plant are available in the public database (Michael Udvardi, Max Planck Institute, Golm, Germany and Satoshi Tabata, The Kasuza Institute, Chiba, Japan). Similarly, in another model legume plant *M. truncatula* 14,000 of root specific (CNRA-INRA, Toulouse, France) and 17,000 of pathogen interaction specific ESTs (Kate VandenBosch and Maria Harrison, Texas A&M University, USA) are available. In case of soybean, more than 125,000 ESTs have been deposited into dbEST (www.ncbi.nlm.nih.gov). From this, it is clear that model legumes also offer unique opportunities for the dissection of interactions with pathogens and for the analysis of development. These studies are the beginning of the large-scale analysis at the gene expression level in legumes, which can be extended to chickpea, an example for cool season food legume crop, and will advance our understanding of legume biology.

Physical map development and sequencing of chickpea

The BAC library of chickpea can be used for physical map development and large-scale genome sequencing. Successful development of a BAC based physical map of high genome coverage depends on several factors, including the insert sizes and genome representation of the source library, the size and complexity of target genomes, methodologies and availability of other related sources (Zhang and Wu 2001). The reliability of a physical map will be much higher if genetic and cytogenetic maps, which can minimize the mapping errors from chimeric clones and/or DNA fragment duplications, guide its construction. Since the distribution of restriction sites

is uneven in the genome, it will be necessary to construct BAC libraries with different restriction enzymes to fill the gaps in the genome during the development of physical maps. Even though a whole genome could be completely sequenced without a physical map, it is necessary to develop a clone-based physical map for subcloning and analysis of a long-range region of the genome. Using the sequence and structure information obtained from the genome sequencing projects and taking the genome-wide functional analysis approach will lead to the understanding of fundamental biological systems in plants by simply transferring information on the structure of one plant species to another using their nucleotide sequences as a common language (Tabata and Caboche 2001). Dr. Walter Gilbert envisioned speculating that the genome sequencing for most organisms will be completed in the near future in 1993 in his following note:

"The new paradigm, now emerging, is that all genes will be known (in the sense of being resident in databases available electronically), and that the starting point of a biological investigation will be theoretical."

Comparative genome analysis among legumes:

Only a finite number of chromosomal rearrangements have occurred during the evolution of angiosperm plants. Significant blocks of genetic material may, therefore, be syntenic among genomes of related species (Michelmore 2000). Comparative genomic efforts have focused on three species in legumes namely *Glycine max* (soybean), *Lotus japonicus* and *Medicago truncatula* out of which the latter two are considered as model species. Genetics, physiology and agricultural traits of these genera have been studied extensively. Among cool season food legumes, it has already been shown that nearly 40% of the lentil map arrangements can be found in pea while this number may not be as high in chickpea (Weeden et al. 1992). Comparing the maps of pea, chickpea and lentil has revealed at least five genomic regions that resemble each other (Simon and Muehlbauer 1997). With the information of using conserved portions of the genome of pea, which is much better defined than that of its relatives such as chickpea, genomic arrangements can be predicted for other genomes. It is through comparative genomics that researchers will

deduce the mechanisms and pathways by which plant genes and genomes have diverged to give the diversity of form, function and adaptation that now characterize the world's flora (Ku et al. 2000)

In summary, the grain legumes exhibit an enormous amount of variation and this variation is silently awaiting commercial exploitation. Recent advances in yield increase of wheat, rice, and maize have raised hopes that similar results may be possible with the grain legumes by merging classical plant breeding techniques and newer genetic engineering approaches.

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Curriculum vitae

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Publications

1) Peter Winter, Ana Maria Benko Iseppon, Bruno Huttel, Milind Ratnaparkhe, Abebe Tullu, Gabriella Sonnante, Theo Pfaff, Mucella Tekeoglu, Dipak Santra, V. J. Sant, **P. N. Rajesh**, Gunter Kahl and Fred J. Muehlbauer. A linkage map of the chickpea (*Cicer arietinum* L.) genome based on recombinant inbred lines from a *C. arietinum* x *C. reticulatum* cross: Localization of resistance genes for fusarium wilt races 4 and 5. Theoretical and Applied Genetics (2000) 101: 1155-1163
2) **P. N. Rajesh**, A. Tullu, J. Gil, V. S Gupta, P. K. Ranjekar and F. J. Muehlbauer. Identification of an STMS marker for the doublepodding gene in Chickpea. (accepted in Theoretical and Applied Genetics)
3) **P. N. Rajesh**, V. J. Sant, V. S. Gupta, Fred. J. Muehlbauer and P. K. Ranjekar. Genetic relationships among annual and perennial wild species of *Cicer* using ISSR polymorphism. (submitted revised version to Euphytica)

4) **P. N. Rajesh**, M. Tekeoglu, V. S. Gupta, P. K. Ranjekar and F. J. Muehlbauer. Molecular mapping and characterization of RGAPtokin1-2_{171} in chickpea. (communicated to Euphytica)
5) **P. N. Rajesh**, K. Meksem, C. J. Coyne, D. Lightfoot and F. J. Muehlbauer. Construction of BAC library in Chickpea (Communicated to Intl. Chickpea Newsletter)
6) M. Tekeoglu, **P. N. Rajesh** and F. J. Muehlbauer. Addition of sequence tagged microsatellite sites and a resistant gene analog locus to the chickpea genetic map. (communicated to TAG)

Presentations

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3) Clarice J. Coyne, Mucella Tekeoglu, Seungho Cho, **P. N. Rajesh**, Dipak Santra, Meksem Khalid, Eric Storlie, David Lightfoot and Fred J. Muehlbauer. Construction of a large insert library to facilitate cloning resistance genes in Chickpea. PAG VIII, San Diego, CA, USA, January 2000
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5) **P. N. Rajesh**, V. J. Sant. A. U. Deshpande, V. S. Gupta, M. N. Sainani, R. B. Deshmukh, F. J. Muehlbauer and P. K. Ranjekar. Chickpea genome mapping using molecular markers. Oral presentation at First National Plant Breeding Congress, Coimbatore, TamilNadu, India, July 1998

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