GENETIC AND BIOCHEMICAL STUDIES ON GALACTOMANNAN BIOSYNTHESIS IN CLUSTER BEAN

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

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JULY, 2011

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **GENETIC AND BIOCHEMICAL STUDIES ON GALACTOMANNAN BIOSYNTHESIS IN CLUSTER BEAN** in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2008 to July 2011 under the supervision of Prof. G. S. Randhawa, Department of Biotechnology and, Prof. S. K. Tripathi, Department of Water Resources Development and Management , Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

PBhatele

(PRANITA BHATELE)

This is to certify that the above statement made by the candidate is correct to the best of my (our) knowledge.

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Date: 18.07.2011

The Ph. D. Viva-Voce Examination of **Ms. Pranita Bhatele** Research Scholar, has been held on . .13.03.2012

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Signature of External Examiner
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ABSTRACT

Guar pods were collected from different developmental stages by tagging the flowers on the day they open as first day after flowering. Pods from 5th day after flowering to 35th day after flowering were collected in this way. RNA from their seeds was used to study the pattern of expression of four enzymes of the galactomannan biosynthesis pathway i.e. mannan synthase which makes β-1, 4-linked mannan backbone, galactomannan galactosyltransferase which adds galactosyl residues to the mannan backbone, alpha-galactosidase which is known to remove some galactose residues in the end and phoshomannoisomerase which is involved in the conversion of fructose-6-phosphate to mannose-6-phosphate. The expression of mannan synthase and galactosyltransferase was found to increase gradually with increasing developmental stages. Alpha-galactosidase was not found to express in any of the stages suggesting that in cluster bean it may not have any role in regulating the man/gal ratio in the final stages by post-depositional modification. Expression analysis for these enzymes was also done in other tissues like leaf, stem, root and flower bud. None of the enzymes were found to express in these tissues suggesting that they are endosperm specific enzymes involved in galactomannan biosynthesis which occurs only in the walls of the endosperm.

Two marker techniques i.e. EST-SSR and AFLP were taken up to study 12 varieties of cluster bean. Among these 12 selected varieties, five were landraces, five commercial varieties and two wild varieties. Moreover these varieties have been chosen from different clusters formed on the basis of RAPD analysis (Nagesh K. A.; unpublished results).

Twelve varieties were analyzed using 26 SSR primers. Out of these amplification was obtained with 20 primers. Six were found to be polymorphic and the polymorphic bands obtained were analyzed. The polymorphic bands were obtained with the wild variety, *Cyamopsis senegalensis*, in the 180-220 bp range.

For AFLP, standardization of the technique was done with one variety i.e. M-83. Eight *Mse*I and eight *Eco*RI selective primers were chosen and all 64 combinations were used in selective

amplification. Out of these 64 combinations, 8 combinations i.e. M-CAA/E-AAG, M-CAA/E-ACC, M-CAC/E-ACT, M-CAC/E-ACA, M-CAG/E-ACT, M-CAT/E-ACT, M-CAT/E-ACA, M-CTA/E-ACT generating more fragments than others, were chosen after analyzing the explorer gel for further analysis in 12 varieties. All the accessions were then subjected to selective amplification with these primer combinations. Proper bins were obtained and the results were arranged in (1/0) format. Similarity matrix and phylogenetic tree were generated with the help of this data. The binary data obtained by gene mapper software was converted to a similarity matrix using Jaccard's coefficient. A dendrogram was generated by Unweighted Pair-Group Average (UPGMA) method using the similarity matrix by Jaccard's similarity coefficient [51] to determine the relatedness of 13 genotypes analyzed. Using 30% similarity, three clusters were formed. *Cyamopsis senegalensis* clearly out-grouped in both the marker analysis studies used.

ACKNOWLEDGEMENTS

With great pleasure and sense of obligation I express my heartfelt gratitude to my supervisor, **Prof. G. S. Randhawa**, Department of Biotechnology, Indian Institute of Technology, Roorkee, who is not only a teacher and guide to me, but my mentor and well wisher too. I will cherish his ever supporting nature and patience to solve my problems during my training tenure. I take pride in saying that I have successfully completed my thesis under his eminent guidance. In spite of his heavy work commitments and a busy schedule, his persistent encouragement perpetual motivation, everlasting patience and valuable technical inputs in discussion during period of research have benefitted me to an extent, which is beyond expression and he also took pains for imparting expert knowledge for design and development of the thesis.

He has not only trained me in science but in all aspects of life. I have a different perspective of developments in life, now being in his company for these years. I cannot write enough about his contribution to my life given the limited space but I can't help mentioning one of his advices to me. He has taught me to be fearless and jump stairs and not be very wise all the time.

I would also like to thank **Prof. S.K. Tripathi**, Water Resource Development and Management, Indian Institute of Technology, Roorkee, for his constant support and guidance throughout the period of research. He has an indispensible contribution in completion of my thesis.

I wish to acknowledge my deep sense of gratitude for **Prof. Ritu Barthwal**, HOD, Department of Biotechnology, Indian Institute of Technology, Roorkee, for providing all the necessary resources and lab facility.

I would like to give warm compression of thanks to **Dr. Kulvinder S. Gill,** Professor & Vogel Endowed Chair in wheat Breeding and Genetics, Washington State University, USA and **Dr. Kanwarpal S Dhugga**, Scientist, Crop Genetics and Research and Development, Pioneer Hi-Bred International, Johnston, USA, for their valuable help and guidance. Without their contribution the completion of this thesis would have been impossible. I express my deep sense of gratitude to them for sparing time from their busy schedule.

It gives me immense pleasure to acknowledge my heartiest thanks to **Dr. Sunita Dhawan,** Scientist, Central Institute of Medicinal and Aromatic Plants, Lucknow, for providing her lab facilities and guidance for the completion of my work. I greatfully acknowledge her help and support.

I would like to thank CAZRI, Jodhpur, NBPGR, New Delhi and CCS HAU, Hisar for providing guar accessions.

The successful completion of the thesis is generally not an individual effort. It is an outcome of the cumulative effort of the number of persons, each having their own importance to the objective. I would like to express profound thanks to all my labmates Shilpi Kumari, Manisha Choudhary,

Swati Verma, Shalini Pareek, Pallavi Gahlot and Navneet Kaur Sekhon who have assisted me at every stage of work and for maintaining a friendly environment in the lab. A special mention of **Mr. Nagesh K.A.** and **Mr. Umesh Tanwar** is inevitable. I would like to take this opportunity to extend my gratitude to my seniors Dr. Durga Prasad Panigrahi and Dr. Megha Agrawal for their timely advices, guidance and good will which enabled me to start and successfully complete my work.

My husband, **Dr. Gulab Tiwari,** deserves a great deal of thanks for his prompt cooperation. Without his support this work would have remained a dream. The completion of this work is really a return for him. Also I would like to acknowledge the support and understanding of my in-laws for giving me a constant support and bearing with me, excusing me from all my household duties. I am blessed to have such patient and understanding family.

The greatest gift I ever had ...
Came from God, I call him Dad ...

Last, but not least, I express my deep appreciation to my father and an alumnus of University of Roorkee, **Dr. Ram Prakash Bhatele,** for his encouragement. His guiding hand on my shoulder will remain with me forever. A special thanks to my loving mother Mrs. Geeta Bhatele and sisters, Pratibha Nagaich and Priyanka Bhatele for their morale boosting and encouraging words they have always given to me. I thank them for their blessing and prayers.

Finally I am thankful to God for successful completion of my work.

DATE: 18-07-2011

PRANITA BHATELE

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LIST OF ABBREVIATIONS

α	Alpha
%	Percentage
°C	Degree centigrade
M	Molar
mg	Milligram
ml	Milliliter
mM	Millimolar
µg	Microgram
µM	Micromolar
ng	Nanogram
nm	Nanometer
Man	Mannose
Gal	Galactose
UDP	Uridine-di-phosphate
GDP	Guanosine-di-phosphate
ManS	Mannan synthase
M/G	Mannose to galactose
GMGT	Galactomannan galactosyltransferaase
α-gal	alpha- galactosidase
PMI	Phosphomannoisomerase
NSTs	Nucleotide sugar transporters
CesA	Cellulose synthase A
Csl	Cellulose synthase like
EST	Expressed sequence tag
DNA	Deoxyribo nucleic acid
cDNA	Complementary DNA
kb	Kilobase pair
GUS	Beta glucuronidase
SSR	Simple sequence repeats

AFLP	Amplified fragment length polymorphism
RAPD	Random amplified length polymorphism
UPGMA	Unweighted Pair-Group method with arithmetic mean
SCARs	Sequence characterized amplified regions
RFLP	Restriction fragment length polymorphism
DAF	DNA amplification fingerprinting
Nt	Nucleotide
BSA	Bulk segregant analysis
PCR	Polymerase chain reaction
RNase	Ribonuclease
RNA	Ribonucleic acid
ARDRA	Amplified ribosomal DNA restriction analysis
RAP-PCR	RNA fingerprinting by arbitrarily primed PCR
SSCP	Single stranded conformational polymorphism
HCl	Hydrochloric acid
DTT	Dithiotheritol
EDTA	Ethyl diamine tetra acetic acid
CTAB	Cetyl trimethyl ammonium bromide
NaCl	Sodium chloride
TE	Tris-EDTA
LiCl	Lithium chloride
DEPC	Diethyl pyrocarbonate
CCSHAU	Chaudhary Charan Singh Haryana Agricultural University
CAZRI	Central Arid Zone Research Institute
NCBI	National Center for Biotechnology Information
NBPGR	National Bureau of Plant Genetic Resources
PVP	Polyvinyl pyrrolidone
DAF	DNA amplification fingerprinting
Bp	Base pair

Chapter I

Introduction

CHAPTER-1

Cluster bean (guar) *Cyamopsis tetragonoloba* is an important leguminous crop grown in *kharif* season in arid and semi-arid regions of North-Western India. India is the world's largest producer and accounts for 80% of total guar produced in the world [63]. Being drought hardy and deep-rooted it is mainly grown in dry habitats of Rajasthan, Haryana, Gujarat and Punjab and to a limited extent in Uttar Pradesh and Madhya Pradesh. It can also tolerate saline and moderately alkaline soils with pH ranging from 7.5 to 8.0. It prefers warm climates and can also be grown in subtropical areas during summer. It is known for its exceptionally high adaptation towards poor rains, low inputs and less care, soil enrichment properties, multiple uses in cropping systems and in industrial uses. Thus it has become the most favored crop of the arid areas.

The tender pods are used as a vegetable and in the southern part of India they are dehydrated and stored for use. It is principally used as a feed for livestock and poultry. The discovery that guar seed endosperm could be a source of useful industrial gum brought this little known crop, worldwide recognition. It has emerged as an industrial crop, and helps in earning foreign exchange for India. For instance, guar gum export reached 101.09 thousand ton providing foreign exchange worth Rs. 543.80 crore during the year 1997-98. In 1999-2000, 110,000 metric ton guar gum costing Rs. 814 crores was exported to western countries. Thus growing guar is a good opportunity for the farmers and the industrialists as well. Cluster bean is a self-pollinated crop, yet some outcrossing, i.e. 2% has been reported. Thus, lesser heterosis is available. Due to less seeds in each pod, economical commercial hybrid seed production in cluster bean is not possible.

Seeds of cluster bean consist of seed coat (14-17%), endosperm (35-42%) and germ (43-47%) as in Figure 1.1. It has attained an important place in industry because of its galactomannan rich endosperm. Galactomannan is accumulated only in the endosperm and constitutes more than 90%

of this tissue at maturity. The hull and germ portion of guar seed, heavily protein rich, are termed as guar meal.

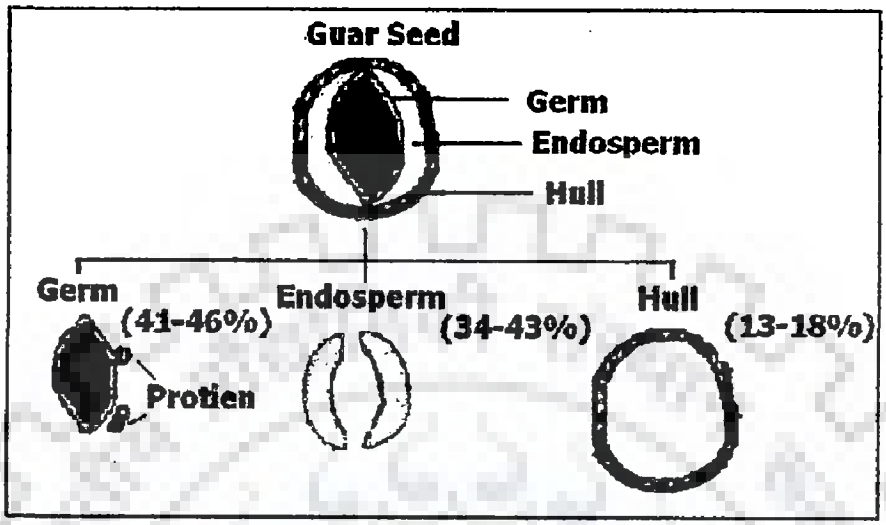

Figure 1.1: Seed structure of guar

Galactomannans are the principal polysaccharide component of the walls of endosperm cells of legume seeds. Guar galactomannan consist of a linear (1- 4)-β- linked D-mannan backbone with single-unit (1-6)-α-linked D-galactopyranosyl side chains (Figure 1.2).

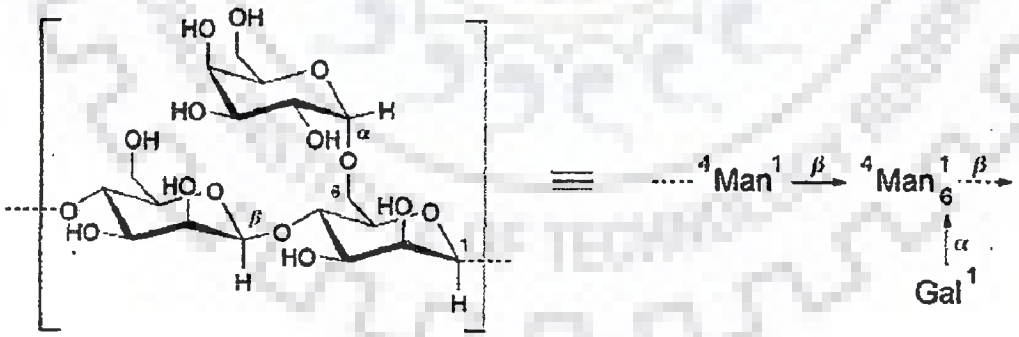

Figure 1.2: Structure of galactomannans

The galactomannan molecule is assembled by the action of two membrane-bound glycosyltransferases. A GDP-mannose-dependent mannan synthase (MS) catalyzes the successive transfer of mannose residues from GDP-mannose to an endogenous (presumably galactomannan)

acceptor, thus elongating the mannan backbone [33]. Another enzyme i.e. galactomannan galactosyltransferase (GMGT) catalyzes the transfer of galactose residues from UDP-galactose to form the galactosyl side chains [96]. Galactose residues can be transferred only to an acceptor mannose residue at or near the growing (non-reducing) end of an elongating backbone chain [32]. The biosynthesis of galactomannans requires a specific functional interaction between MS and GMGT, within which the transfer specificity of the GMGT is important in determining the statistical distribution of galactosyl residues along the mannan backbone and the Man/Gal ratio [32]. Degree of galactosyl substitution on the mannan backbone determines the quality of galactomannan as a gum, e.g. a mannose/galactose ratio (M/G) of 4 in locust bean is superior to a ratio of 2 in guar [29]. The degree of galactosylation of natural galactomannans is also believed to be determined by the action of an α-galactosidase later in the seed development [53]. Therefore there can be two mechanisms by which mannose/galactose ratio may be controlled during galactomannan formation. The biosynthetic control, where the M/G ratio is a consequence of biosynthetic process alone or it can be controlled by the post-depositional modification, where some galactose residues are removed from the primary biosynthetic product by an α-galactosidase activity during late galactomannan deposition. It has been shown that it is possible to use genetic approaches to modify the galactomannan. Further identification of other enzymes and the regulatory factors involved in the biosynthesis of plant polysaccharides would open up the possibilities of biotechnological production of the gums, with the prospect of providing a stable and cost-efficient supply of functional polysaccharides. Continued focus on the identification of novel glycosyltransferases involved in plant polysaccharide biosynthesis is the key to achieve these goals.

So far, cluster bean has received practically little attention by the researchers as far its genetic improvement is concerned. The genes involved in the biosynthesis of galactomannan have been recently discovered and cloned [29]. Therefore the main objective of the present research work was to study the mechanism of galactomannan biosynthesis in guar seed and the genetic diversity present in cluster bean.

Assessment of genetic diversity is a prerequisite in any crop improvement programme. Molecular markers like RAPD, SSR, SCAR, SSCP, AFLP etc. have been used for the identification of cultivars and the genetic relationships among cultivars of other crops including barley [37], *Mentha* [103], *Labtab purpureus* [85], maize [48], dolichos bean [110] etc. Such techniques do not require any sequence information. These technologies help to identify the presence of sequence differences. While they cannot tell what the variant is, they can help to narrow down the range of strategies to be used for detecting it.

Molecular markers do not have any biological effect; they can be considered as constant landmarks in the genome. They are identifiable DNA sequences, found at specific locations of the genome, and transmitted by the standard laws of inheritance fom one generation to the next. The existence of various molecular techniques and differences in their principles and methodologies require careful consideration in choosing one or more of such marker types [101].

The present study is focused on expression analysis of genes involved in galactomannan biosynthesis during different developmental stages of guar. This would throw light on the factors important in the regulation of M/G ratio of the galactomannan and would help in quality improvement strategies. Diversity study has also been done using SSR and AFLP markers to analyze different varieties of guar for various economically important traits and their relation to its genetic variation. This will help in selection of important varieties for breeding purpose. In

conventional guar breeding, one usually considers the phenotypes of the plant in selection for high yield and quality parameters. These traits have been used for the assessment of genetic diversity in cluster bean but such traits are influenced by environmental factors and developmental stage of the plant. Molecular markers offer several advantages over the conventional breeding tools for selection of diverse parents. The application of the study would highlight the main genetic factors which are important for high quality and quantity of guar gum. The study will lead to marker based selection of crop varieties for production of high value galactomannan than the present gum produced in cluster bean plant.

Guar gum amounts to an export of more than Rs.1000 crore per annum. With the price of gum varying on the quality of galactomannan in its endosperm, genetic studies of galactomannan synthesis and its subsequent application will lead to increase in value of guar gum and there by profit the guar growing regions of India. Genomic studies will help to select diverse varieties with contrasting characters that can be used for breeding purpose.

The main objectives of the present work are as follows:

- To study the expression of four genes i. e. mannan synthase , galactosyltransferase, alpha-galactosidase and phosphomannoisomerase, involved in galactomannan biosynthesis during different stages of guar seed development
- To study the genetic polymorphism present in 12 accessions of guar using SSR markers
- To perform the AFLP analysis using 64 primer combinations to shortlist the primers that work in cluster bean
- To perform the AFLP analysis in 12 accessions of cluster bean using the shortlisted primer combinations

Chapter II

Review of Literature

CHAPTER-2

The literature on galactomannan biosynthesis and other related aspects was reviewed and has been presented under suitable headings.

2.1 Galactomannan

The quality and quantity of the galactomannan vary in different plant species. It has been reported that the degree of galactose substitution of the D-mannan backbone varies within the leguminosae, and is taxonomically important [97, 26] It has been shown that the degree of galactose substitution of the D-mannan backbone is an important factor determining the functionality of galactomannans in mixed-polysaccharide interactions; lower degrees of substitution (consistent with solubility) favouring the interactions [25].

It has been reported that unlike the seeds of other legumes, guar seeds have a large endosperm, accounting for 42% of seed weight [5]. The predominant portion of the endosperm is mucilage or gum, which forms a viscous gel in cold water. Approximately 80–85% of the gum is a galactomannan [47]. The galactomannan is in the form of non-ionic polydisperse rodshaped polymers consisting of about 10,000 residues, which accumulate in the primary cell walls of the endosperm [89].

2.1.1 Structure of galactomannan

Low galactose galactomannans (25–35% galactose substitution) are typical for the more distantly related *Caesalpinoideae* sub-family of the *Leguminosae*, whereas higher degrees of galactose substitution (up to 97% in the tribe *Trifolieae*) are characteristic of the more closely related *Papilionoideae* legume sub-family [97]. Guar galactomannan has a mannose to galactose (M/G) ratio of 1.6 [33]. Pure mannan without galactose is shown to be completely insoluble in water, and increasing galactose substitution increases the solubility of the polymer by allowing it to become

extended [81, 15]. The mechanism of gelation of various polysachharides and mannans was studied and it was concluded that it involves chain-chain associates by aggregation of regularly ordered cellulose or mannan backbones. For galactomannans there is evidence for a correlation between galactomannan structure (i.e. degree of galactose substitution and amount and length of unsubstituted mannose regions) and the ability to form gels at low water activity [25, 24]. A model was proposed where the gel junction zones comprise aggregates of regular unsubstituted regions of mannan backbone, while the more heavily substituted regions of the molecules serve to solubilise the network as depicted in Figure 2.1 [27].

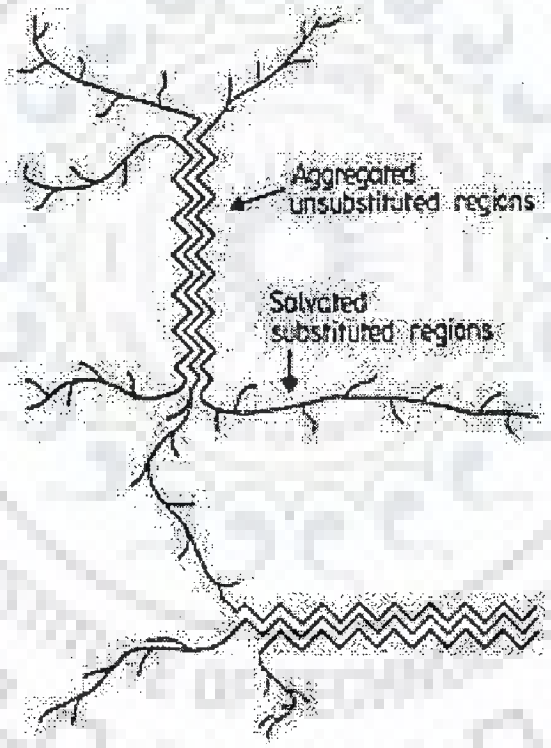

Figure 2.1: Proposed model of galactomannan gel structure [27]

It was concluded by Dea that galactomannans with a larger proportion of longer regions of unsubstituted blocks or sides along the mannan backbone interact best with agars, carrageenans and xanthan as in Figure 2.2.

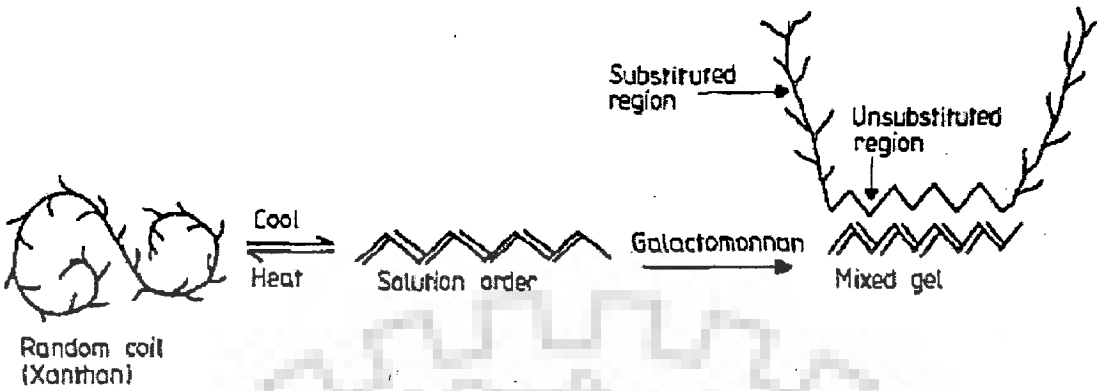

Figure 2.2: Model for the interaction between xanthan and galactomannan [27]

2.1.2 Galactomannan biosynthesis pathway in plants

Galactomannan is made by the combined actions of two enzymes: mannan synthase (ManS), which makes β-1, 4-linked mannan backbone, and α-galactosyltransferase, which adds galactosyl residues to the mannan backbone [33, 96] The mannose:galactose (man:gal) ratio in galactomannans is genetically controlled [28]. The enzyme activities and interactions could be of importance in regulating man:gal ratio. [94] The statistical distribution of galactosyl substituents along the mannan backbone, and the degree of galactose substitution of the primary product of galactomannan biosynthesis is regulated by the specificity of the galactosyltransferase. [96]. It has been concluded that the transfer of D-galactosyl residues from UDP-galactose to galactomannan was absolutely dependent upon the simultaneous transfer of D-mannosyl residues from GDP-mannose as D-Mannan sequences pre-formed *in situ* using the mannosyltransferase in the absence of UDP-galactose could not become galactose-substituted in a subsequent incubation either with UDP galactose alone or with UDP-galactose plus GDP mannose [32]. Edwards *et al.* proposed a model for the interaction of GDP mannose mannosyltransferase and UDP-galactose galactosyltransferase in galactomannan biosynthesis [32]. The degree of galactosylation of natural galactomannans is also believed to be determined by the action of an α-galactosidase later in seed

development [53]. The degree of galactose-substitution of a galactomannan could be regulated either at the level of the biosynthetic process itself or by the selective removal of galactose residues after deposition [94].

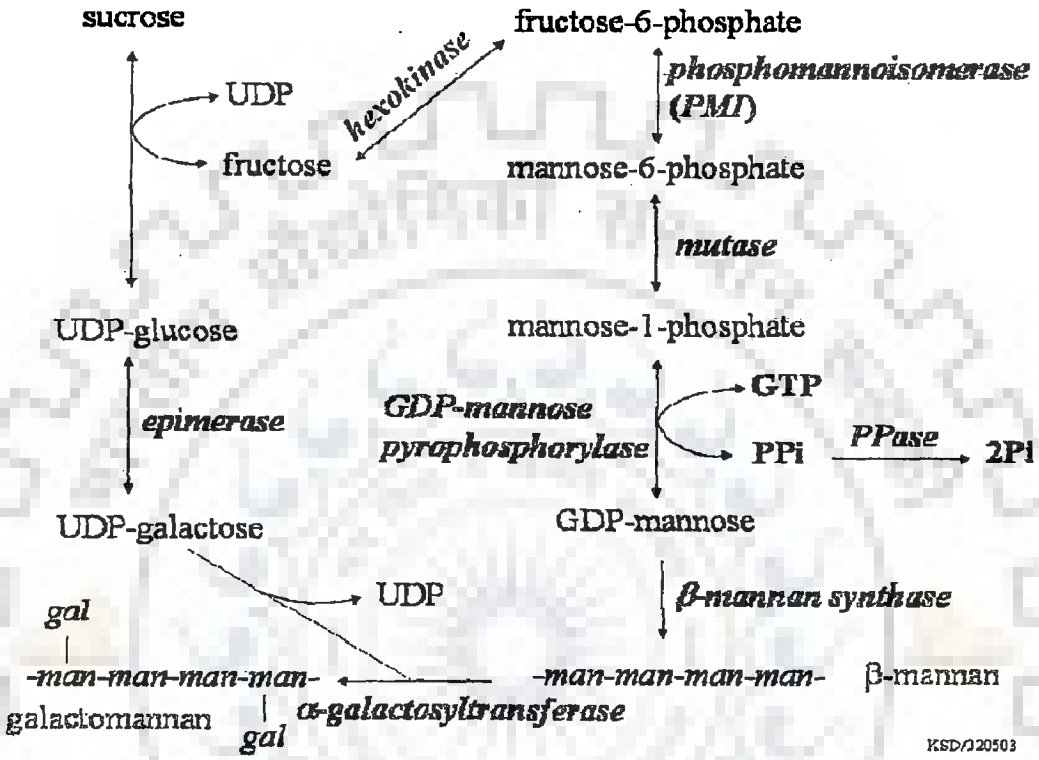

Figure 2.3: Pathway to galactomannan formation in plants [80]

The direct precursors for galactomannan biosynthesis, GDP-D-mannose and UDP-D-galactose, are formed by the actions of GDP mannose phosphorylase (EC 2.7.7.22) and UDP-galactose 4-epimerase (EC 5.1.3.2). *In vitro* experiments have shown that the relative concentrations of these precursors can affect the M/G ratio of the galactomannan polymer [33]. Golgi-localized nucleotide sugar transporters (NSTs) are considered essential for the biosynthesis of wall polysaccharides based on their characteristic transport of a large number of nucleotide sugars to the golgi lumen. The lack of NST mutants in plants has prevented evaluation of this hypothesis in plants [117].

2.1.3 Genes involved in galactomannan biosynthesis

The first plant cellulose synthase gene (CesA) was isolated from developing cotton fibres by random expressed sequence tag (EST) sequences [87]. The cellulose synthase-like (Csl) genes were first identified in the model plant Arabidopsis [98] and rice [45]. A large number of sequences are grouped under the family of cellulose synthase-like based on certain sequence homology [23].

Plant cell wall synthesizing enzymes are integral membrane proteins with single or multiple transmembrane domains. They can be challenging to study biochemically, as they tend to be labile, present in multimeric complexes and encoded by large gene families whose members may have overlapping function [29]. It has been argued that strategies for confirmation would include expression in heterologous systems followed by assays of enzymatic activity or detection of carbohydrate products [88]. This kind of approach was used for mannan synthase (ManS), that makes the β-1,4- mannan backbone of galactomannan, where a cDNA clone for the same was isolated from cDNA library derived from the ESTs. The soybean somatic embryos expressing ManS cDNA contained high levels of mannan synthase activity that localized to golgi. Seeds from the transgenic soybean expressing guar mannan synthase had elevated mannose content [29].

2.1.4 Recent work on improvement of gum quality

Mutagenesis is a powerful tool for creating variation in a crop like guar where exploitable and favorable genetic variability is very meager [6]. In a review article Arora and Pahuja have concluded that lower doses of various mutagens, either alone or in combination, induce much more useful variability than higher doses [6]. However, work on mutation breeding of guar is limited and only a few mutants carrying one or two useful attributes have been obtained so far. Despite having so many applications not much was known about the genes for the enzymes involved in the synthesis of these structural polysaccharides until recently. Fenugreek seed galactomannan is

almost fully substituted by galactose, whereas galactomannan in tobacco seed (Nicotiana tabacum) contains only low levels of galactose. The expression of fenugreek GMGT under a strong constitutive promoter in tobacco resulted in mannan with significantly increased level of galactose substitution [95]. This indicated that the exogenously introduced fenugreek GMGT dominated over the endogenous tobacco GMGT; the exogenous GMGT operated mutually with the endogenous ManS in tobacco, and the result was the synthesis of the fenugreek type of galactomannan. In another approach, the GMGT gene of *Lotus japonicas*, in which native galactomannan is highly substituted with galactose (at a Man/Gal ratio of 1.2-1.3), was down-regulated by sense and antisense galactomannan galactosyltranferase constructs, which resulted in the modification of its galactomannan to one with a lower galactose content at a Man/Gal ratio of 6 [34]. This study clearly showed the potential of modifying low value galactomannan with a high level of galactose substitution to a more valuable galactomannan with low galactose substitution through down regulation of the native GMGT enzyme. An obvious target of such engineering would be guar which is already used for production of mannan. In addition guar is transformable and is an annual crop that is much more easily cultivated than locust bean [34]. To evaluate the potential role of α-galactosidase for the control of the final galactose content, a α-galactosidase gene expressed in immature senna seeds was cloned and transformed into the related high-yielding species guar using a wheat high-molecular-weight glutenin promoter in the vector employed for transformation of guar by *Agrobacterium tumefaciens*-mediated gene transfer. About 30% of the guar transformants produced endosperm with galactomannans where the galactose content was significantly reduced [53].

Development of suitable endosperm- specific promoters for use in guar was desirable for metabolic engineering of the seed gum. Naoumkina *et al.* have isolated an ~1.6 kb guar mannan

synthase (MS) promoter region. It was shown by the quantitative GUS assays that the MS promoter directs GUS expression specifically in endosperm in transgenic alfalfa [79]. Thus, the guar MS promoter could prove generally useful for directing endosperm-specific expression of transgenes in legume species. This promoter can be used for driving high expression of foreign genes in transgenic guar endosperm as well as in other species [79].

Although the genes encoding the enzymes for backbone synthesis (ManS) [29] and side chain addition (GalT) [35] have recently been identified and cloned little is known about the whole pathway of galactomannan biosynthesis and almost nothing is known about its regulation.

2.2 Molecular Markers

Detection and analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants. Since the entire plant kingdom cannot be covered under sequencing projects, molecular markers and there correlation to phenotypes provide us with requisite landmarks for elucidation of genetic variation. The first report about the construction of genetic linkage map in man using restriction fragment length polymorphism was given by Botstein *et al.* (1980) [13].

2.2.1 Random amplified polymorphic DNA

Two reports of RAPD analysis in cluster bean are available. Punia *et. al.* (2009) have optimized PCR conditions using 34 genotypes of guar [90]. Similarly molecular assessment of 32 genotypes was done using 10 random primers by Pathak *et al.* (2010) [84]. Kiss *et al.* (1993) have constructed a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers [58]. It consisted of eight linkage groups representing the haploid chromosome set of the *Medicago* species. Fingerprinting of genomes was done by Welsh and McClelland (1990) using PCR arbitrary primers [115]. Twenty four strains from five species of *Staphylococcus*, eleven strains of

Streptococcus pyogenes and three varieties of *Oryza sativa* were used. In another study, segregating allozymes and DNA polymorphisms were used to construct the preliminary linkage map four faba bean [107]. Two F_2 populations were analyzed and eleven independently assorting linkage groups were identified in this population. Linkage maps for two apple clones were constructed using isozyme anf DNA polymorphisms segregating in a population [46]. Martin *et al.* (1991) described an approach to isolate DNA sequences linked to important plant genes using synthetic primers to amplify random sequences from genomic DNA [72]. They identified markers liked to a *Pseudomonas* resistance gene in tomato using random primers and near-isogenic lines. Paran and Michelmore developed PCR based markers linked to downy mildew resistance genes in lettuce. In this study sequence characterized amplified regions (SCARs) were derived from eight random amplified polymorphic DNA (RAPD) markers. SCAR, RAPD and RFLP markers linked to a dominant gene (Are) conferring resistance to anthracnose were developed in common bean by constructing of near-isogenic lines, in which the *Are* gene was introgressed [1] . RAPD analysis was done in two selected cultivars of betel vine in order to ascertain the relatedness of the two to each other [93]. It was concluded that bands specific to only one of the two types have potential for developing betel vine cultivar- specific probes and SCAR markers.

2.2.2 DNA amplification fingerprinting

A modification of the RAPD technique called DNA amplification fingerprinting (DAF) was introduced by Caetano-Anolles and Bassam (1993) where one or more very short (less than or equal to 5 nt) arbitrary oligonucleotides were used to direct the enzymatic amplification of DNA [16]. The spectrum of DAF products were resolved in to detailed and reproducible patterns using polyacrylamide gel electrophoresis and silver staining. The relationship of five species of *Petunia* and ten cultivars of cultivated *Petunia* were investigated using DNA amplification fingerprinting

by Cerny *et al.* (1996) [18]. The results demonstrated the utility of DAF in establishing relationships among closely related species and cultivars of *Petunia* [18]. Men *et al.* (1998) demonstrated genome mapping through a combination of bulk segregant analysis (BSA) with DNA amplification fingerprinting and identified DAF markers close to the symbiosis-ineffective *sym*31 mutation of pea [75]. DNA amplification fingerprinting parameters were optimized in coconut by Manimekalai *et al.* (2004) [70]. Subsequently these results were used in tagging resistance genes for root disease in coconut.

Harvey and Botha (1995) compared two PCR based methods i.e. RAPD and microsatellite primers for the determination of DNA diversity between *Saccharum* varieties [44]. It was suggested that these specific primers would not be suitable for determination of DNA diversity, but could be used more effectively in the development of a methodology for routine, rapid identification of sugarcane varieties [44]. Subtyping of *Streptococcus uberis* by DNA amplification fingerprinting was done by Jayarao *et al.* (1992) and compared with restriction endonuclease fingerprinting [52]. The results suggested that DAF is a useful technique for sutyping of *S. uberis.*

2.2.3 Simple Sequence Repeats (SSR markers)

Analysis of genetic diversity of Tibetan wild barley was done using SSR markers where one hundred and six accessions were analyzed using thirty SSR markers [38]. Based on this study it was concluded that Chinese cultivated barley has evolved from *Hordeum vulgare* ssp. *spontaneum* (HS) via *Hordeum vulgare* var. *lagunculiforne* (HL) to *Hordeum vulgare* ssp. *agriocrithon* (HA). Kubik *et al.* (2009) analyzed the genetic diversity of creeping bentgrass cultivars using thirty SSR markers [62]. Four hundred and nine different alleles were amplified and it was concluded that SSR markers are useful to measure genetic diversity withing creeping bentgrass. Twenty SSR loci distributed uniformly throughout the maize genome were used to assess the genetic diversity of

twenty Albanian local maize populations [48]. In this study SSR marker were shown to be a powerful tool for detection of genetic diversity in maize populations and they can be useful for maize breeders, and also for devising strategies for conserving and managing maize germplasm. Chagne *et al.* (2004) compared cDNA-SSRs and genomic-SSRs in pine [19]. Overall, genomic SSRs performed better in terms of heterozygosity and number of alleles. This study suggests that useful SSR markers can be developed from pine ESTs. First in-depth molecular analysis of cotton cultivars grown in Greece using SSR markers was done by Kalivas *et al.* (2011) using 12 SSR primers in 29 cultivars where an analysis of association of SSR markers with fiber quality traits of 29 cotton cultivars was reported for the first time [54]. A Chinese group analyzed the genetic diversity in maize (*Zea mays* L.) landraces from Wuling mountain region in China based on SSR markers [91]. The obtained results suggested that 180 individual genotypes could construct the maize land-race core collection of Wuling mountain region.

Assesment of genetic diversity of Philippine rice cultivar was done using SSR markers by Lapitan *et al.* (2007) [64]. Twenty four rice cultivars carrying good quality traits were evaluated for genetic diversity using 164 SSR markers. The results indicated that these quality rice cultivars exhibited a higher genetic diversity and therefore very useful for rice breeding programs, especially for genetic mapping studies and eventually for application of marker assisted selection in the programs [64]. Mullan *et al* (2005) developed molecular markers to monitor the introgression of *Lophopyrum. elongatum,* a close relative of wheat, chromosome segments into hexaploid wheat [77]. This study details methodologies for the generation of SSRs for detecting *L. elongatum* loci. Sharopova *et al.* (2002) developed 1051 novel SSR markers for maize from microsatellite enriched libraries and by identification of microsatellite-containing sequences in public and private databases [102]. Three mapping populations were used to derive map positions for 978 of these markers. The maize

research community now has the most detailed and comprehensive SSR marker set of any plant species.

2.2.4 Amplified fragment length polymorphism

A new DNA fingerprinting technique called AFLP was described by Vos et. al. (1995) for the first time where the reliability of the RFLP technique is combined with the power of the PCR technique [111]. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps; restriction of the DNA and ligation of oligonucleotide adapters, selective amplification of sets of restriction fragments, and silver staining [10] of gel followed by analysis of the amplified fragments (Figure 2.4).

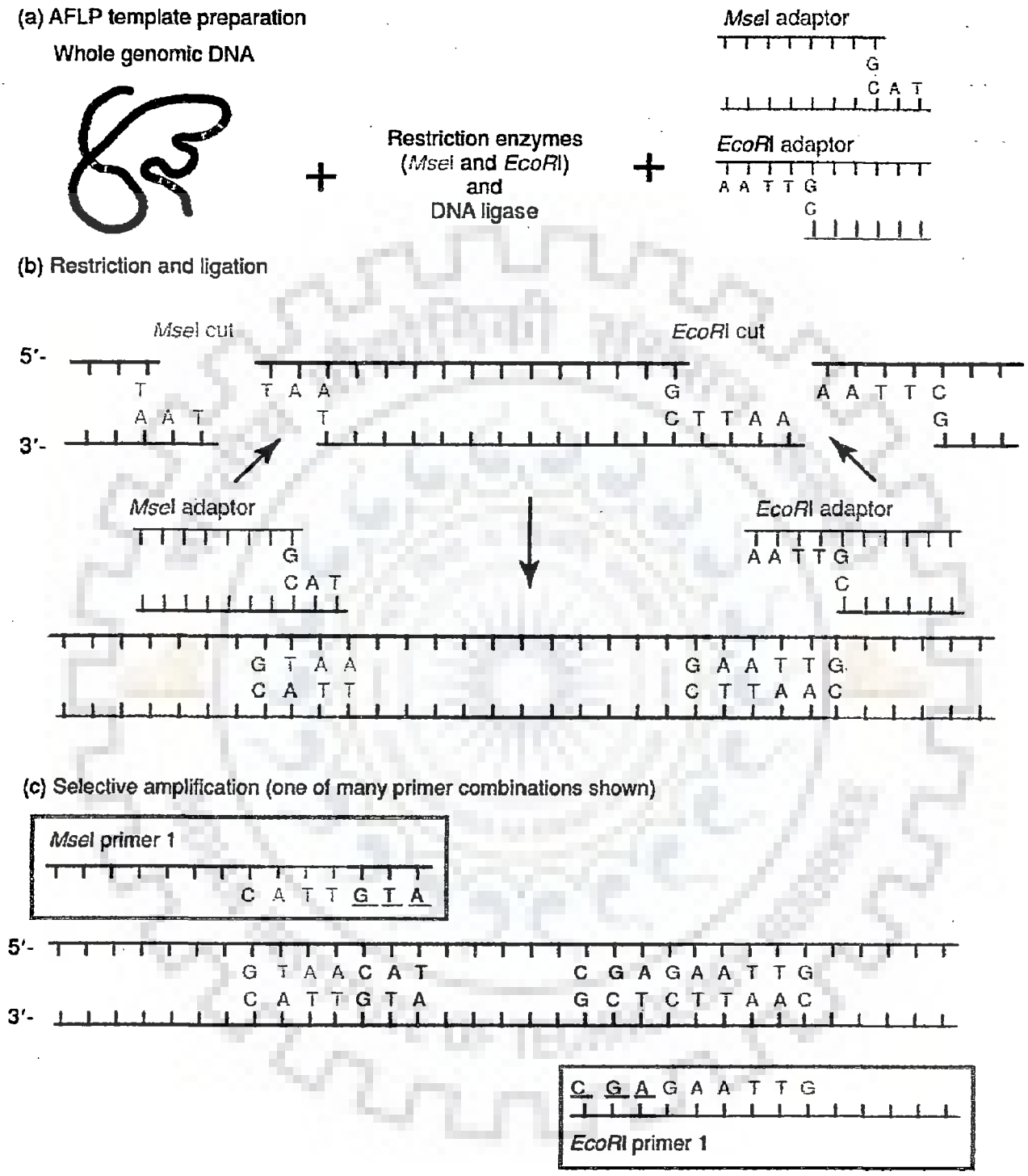

Figure 2.4: Generating amplified fragment length polymorphism (AFLP) markers [76]

Maughan *et al.* (1995) used AFLP technique to assess species diversity in cultivated (*Glycine max*) and wild soybean (*G. soja*) [73]. AFLP data clearly separated G. *max* and *G. soja* accessions. Zhu

et al. (1998) used AFLP markers for the study of biodiversity in rice with 5 primer combinations (118). A protocol for the detection of AFLPs with the non radioactive digoxigenine labeling was given by *Vrieling et a.l* (1997) in which the sensitivity and reliability of the digoxigenine labelled primers in the AFLP technique was of the same order as the sensitivity and reliability of the radioactive assay [112].

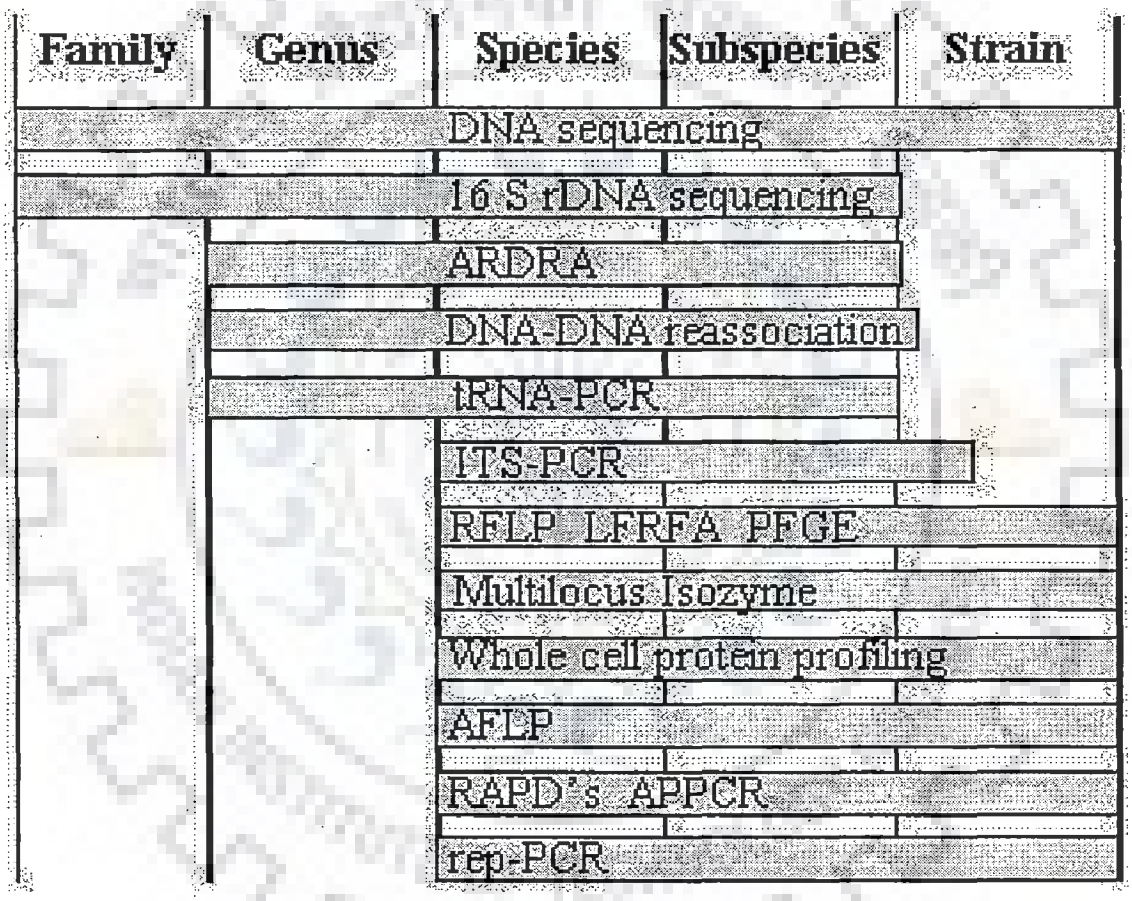

Figure 2.5: Relative resolution of various fingerprinting and DNA techniques [17]

A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) was done by McGregor *et al.* (2000) in tetraploid potato (*Solanum tuberosum* L.) germplasm using 39 potato cultivars [74]. The results show that each of the four techniques can individually identify each cultivar, but that techniques differ in the mean number of profiles generated per primer.

Biochemical and molecular (RAPDs, AFLPs) markers were compared for their efficiency and repeatability in malt fingerprinting by Faccioli *et al.* (1999) [37]. AFLP was reported as an excellent tool for fingerprinting. In order to facilitate reasoned scientific decisions on its management and conservation and to prepare for a selective breeding programme, genetic analysis of seven population of *Moringa oleifera* was performed using AFLP primers by Muluvi *et al.* (1999) [78]. A high level of population differentiation was detected. AFLP markers linked to a major quantitative trait locus controlling scab resistance in wheat was developed by Bai *et al.* (1999) [9].

A study was done by Gerber *et al.* (2000) in which the properties of dominant markers, such as amplified fragment length polymorphism was compared with those of codominant multiallelic markers, such as microsatellites in reconstructing parentage [39]. Loh *et al.* (2000b) conducted a study on genetic variation and relationships within the bamboo subtribe by amplified fragment length polymorphism using eight primer combinations on 15 species of bamboo [68]. Identification of closely related banana cultivars is difficult, particularly when sterile. Amplified fragment length polymorphism (AFLP) analysis using eight primer combinations was carried out by Loh *et al.* (2000a) on 16 banana cultivars [67]. Results showed that AFLP could be used to distinguish the different cultivars by their unique banding patterns. Lucchini (2003) has concluded that AFLP technique could be very useful in a wide range of conservation studies on the basis of results of AFLP analysis in three different conservation projects requiring different level of genetic variability [69]. AFLP technique has also proved to be useful in identifying inter and intra-specific hybids, eg. *Mentha* [103]. Several research groups have utilized AFLP marker technique for the assessment of genetic variation in different plant species. Out of several varieties of *Agave tequilana,* only *A. tequilana* Weber var. ''azul'' is accepted for the production of tequila and discrimination between the different varieties was difficult. The relationships between them at the

genotype level were unclear. Gil-Vega *et al.* (2006) conducted AFLP analysis of *Agave tequilana* varieties as a result of which a significant level of diversity was observed among the varieties [40]. Analysis of genetic diversity in low chill requiring walnut (Juglans regia L.) genotypes was done by Bayazit *et al.* (2007) [11]. In this study, the genetic relatedness of 22 low chill requiring walnut genotypes adapted to the south east Mediterranean region of Turkey was analysed by amplified fragment length polymorphism (AFLP) markers [11]. A relatively low level of genetic variation was found among the genotypes examined by five primer combinations, suggesting that these walnut genotypes selected predominantly for their low chill requirement have relatively narrow genetic base. AFLP and SSR marker analysis of grape rootstocks in Indian grape germplasm was done by Upadhyay *et al.* (2007) [108]. Twenty-one rootstock accessions were analyzed with seven grape microsatellite (SSR) primers and seven AFLP primer combinations. Rootstocks belonging to same species or having common parents were grouped together in the dendrograms generated by both the marker systems. Assessment of genetic relationship was done in *Mentha* species using 11 accessions [57].

A comparative study was done in olive to assess the discriminating capacity of RAPD, AFLP and SSR markers [12]. The comparision was done in terms of their informativeness and efficiency in a study of genetic diversity and relationships among 32 olive cultivars cultivated in Italy and Spain. On the basis of this comparision it was concluded that SSRs have a higher level of polymorphism and a greater information content, as assessed by the expected heterozygosity, than AFLPs and RAPDs. The extent of intra-population genetic variation was evaluated in neem accessions growing in district Kanpur (UP), India, along with two exotic accessions using two PCR-based markers namely, AFLP and SAMPL [105]. Based on this analysis, it was concluded that neem maintains high levels of genetic variation at intra-population level. Koeleman *et al.* (1998) compared thirty-one strains of *Acinetobacter* species by amplified ribosomal DNA restriction

analysis (ARDRA), random amplified polymorphic DNA analysis (RAPD), and amplified fragment length polymorphism (AFLP) fingerprinting. ARDRA showed low discriminatory power for differentiating *Acinetobacter* at the species and strain level [59].

DNA polymorphism between two major *japonica* rice cultivars, Nipponbare and Koshihikari, was identified by AFLP and AFLP markers were converted to sequence specific markers by Shirasawa *et al.* (2004) [104]. The procedure of conversion of AFLP-markers to the sequence-specific markers used in this study enables efficient sequence-specific marker production for closely related cultivars. AFLP analysis was used for germplasm identification for *Amaranthus cruentus* L. and *Amaranthus caudatus* L. [21]. An AFLP based linkage map of *Arabidopsis thaliana* was developed by Alonso-Blanco *et al.* (1998) [3]. Paul *et al.* (1997) used AFLP markers and revealed diversity and genetic differentiation among populations of Indian and Kenyan tea [86].

Efficiency of RFLP, RAPD and AFLP markers was compared for the construction of an intra specific map of the tomato genome by Saliba-Colombony *et al.* (2000) [99]. The genetic linkage map was constructed based on an intraspecific cross between two inbred lines of *Lycopersicon esculentum* and *L. esculentum* var. *cerasiforme.* It was observed that RAPD and AFLP markers were not randomly distributed over the genome but were grouped in clusters located around putative centromeric regions [99]. Genetic analysis of drought tolerance was done in maize by Sari-Gorla *et al.* (1999) using molecular markers. The experiment was carried out under two environmental conditions, well-watered and water-stressed, on a maize population of 142 recombinant inbred lines [100]. Levels of genetic variation and patterns of population structure within and among eight wild or cultivated populations of *G. jasminoides* Ellis in China were investigated using 11 amplified fragment length polymorphism (AFLP) markers and it was concluded that there is an association between geographic and genetic distances between populations [43]. Genetic fingerprinting in Dolichos Bean was done using AFLP markers and

morphological traits by Venkatesha *et al.* (2010) [110]. The high number of clusters formed from both morphological and AFLP analysis indicated considerable genetic diversity. The clusters formed based on the AFLP data were not in agreement with the clusters formed based on morphological data found in the study, indicating the presence of discrepancy between the two methods [110]. A phylogenetic and taxonomic study of the genus *Calopogon* was done by Goldman *et al.* (2004) using nuclear internal transcribed spacer (ITS) ribosomal DNA sequences, amplified fragment length polymorphisms (AFLPs), chloroplast DNA restriction fragments, and chromosome counts [41]. Silver stained denaturing polyacrylamide gels (PAGEs) and fluorescent denaturing automated capillary electrophoresis (CE) were used to detect amplified fragment length polymorphism patterns obtained from white-rot fungi belonging to the genus *Trametes* by Dresler-Nurmi *et al.* (2000) [31]. AFLP fingerprinting detected by the fluorescence-based method as well as by silver staining showed a high discriminatory power in differentiating strains of *Trametes.*

Using the AFLP technique highly informative DNA fingerprints were generated from 19 taxa of *Solanum* (potatoes) and three taxa of *Solanum* (tomatoes) by Kardolus *et al.* (1998). It was concluded that AFLP is an efficient and reliable technique to generate biosystematic data and therefore a promising tool for evolutionary studies [55]. Ellis *et al.* (1997) used AFLPs to examine genetic relatedness in barley [36]. Allele specficity of comigrating AFLP markers was used to align genetic maps from different potato genotypes by van der Voort *et al.* (1997). It was concluded that consequently AFLP analysis may replace more laborious locus-specific marker techniques [109].

2.2.5 Organelle microsatellites

2.2.5.1 Chloroplast microsatellite based markers

Genetic discontinuity was revealed by Clark *et al.* (2000) using chloroplast microsatellites in North American *Abies* [20]. Parducci *et al.* (2001) analyzed genetic variation at chloroplast microsatellites in Abies species [83]. In another investigation, nine chloroplasts, paternally inherited simple-sequence repeat (cpSSR) markers were used to describe genetic variation of three closely related species belonging to the *halepensis* complex [14].

2.2.5.2 Mitochondrial microsatellite based markers

Rajendrakumar *et al.* (2007) reported the identification of a mitochondrial repeat specific marker for distinguishing wild abortive type cytoplasmic male sterile rice lines [92].

2.2.6 CAPS Marker

The CAPS markers are codominant and locus specific and have been used to distinguish between plants that are homozygous or heterozygous for alleles [61]. Weiland and Yu (2003) identified a cleaved amplified polymorphic sequence (CAPS) marker associated with root-knot nematode resistance in sugarbeet. Methods to differentiate these coffee species could prove to be beneficial for the detection of either deliberate or accidental adulteration [113]. A study was done by Spaniolas *et al.* (2006) using molecular genetics (PCR-RFLP) approach to differentiate *Arabica* and *Robusta* coffee beans [106]. A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype- specific PCR based markers was developed by Konieczny and Ausubel (1993) [61].

2.2.7 Target region amplified polymorphism

A rapid and efficient PCR-based technique, which uses bioinformatics tools and expressed sequence tag (EST) database information to generate polymorphic markers around targeted

candidate gene sequences, was developed by Hu and Vick (2003) [50]. A wheat intervarietal genetic linkage map based on microsatellite and target region amplified polymorphism markers was constructed by Liu *et al.* (2005) and its utility for detecting quantitative trait loci was demonstrated [66]. Hu *et al.* (2005) used TRAP technique for genotyping of lettuce [49]. Alwala *et al.* (2006) used TRAP technique for assessing genetic diversity in sugarcane germplasm [4].

2.2.8 RNA based molecular markers

RNA based studies will help to develop markers relating to the genes expressing in different conditions of stress or different environmental conditions.

2.2.8.1 cDNA-SSCP

The SSCP analysis of RT-PCR products can be used to evaluate the expression status (presence and relative quantity) of highly similar homologous gene pairs from a polyploid genome. Quantitative analysis of transcript accumulation from genes duplicated by polyploidy using cDNA-SSCP was done by Cronn and Adams (2003) [22].

2.2.8.2 RAP-PCR

The RAP-PCR technique was introduced by Welsh *et al.* (1992) which involves fingerprinting of RNA populations using arbitrarily selected primer at low stringency for first and second strand cDNA synthesis followed by PCR amplification of cDNA population [114].

2.2.8.3 cDNA-AFLP

cDNA-AFLP, coupled with bulked segregant analysis (BSA), was used by Yao *et al.* (2007) to screen genes expressed differently between low- and high-acid apple fruits from hybrids [116]. A novel gene was identified which contributes to lowering the acidity of fruit. A Genome wide cDNA-AFLP analysis of genes rapidly induced by combined sucrose and ABA treatment in rice

cultured cells was conducted by Akihiro *et al.* (2006). 27 genes were identified which were induced by combined sucrose and ABA treatment [2]. Aluminium –regulated genes were identified by cDNA-AFLP in rice by Mao *et al.* (2004) [71]. Differential display of eukaryotic messenger RNA was obtained by Liang and Pardee (1992) using polymerase chain reaction [65]. A detailed protocol for the cDNA-AFLP procedure and an analysis of interactions between its various parameters was given by Bachem *et al.* in 1998 [8]. Gene expression during potato tuber development was analyzed by Bachem *et al.* (1996) and differential gene expression was visualized using RNA fingerprinting based on AFLP [7].

Chapter III

Materials and Methods

CHAPTER-3

3.1 Plant material

Plant material which consisted of guar seeds was collected from various sources. Commercial varieties were obtained from Central Arid Zone Research Institute (CAZRI), Jodhpur. They were sown to obtain seeds for further propagation and experimentation. Landraces of guar were obtained from NBPGR, New Delhi. Wild varieties were collected from CCSHAU, Hisar (Table 3.1). The seeds were grown in Indian Institute of Technology Roorkee, Roorkee and leaf sample and pods were taken for further analysis.

Table 3.1: List of varieties and their location from which they were collected

S. No.	Accession number	Location
1	M-83	CAZRI, Jodhpur
2	RGC-197	CAZRI, Jodhpur
3	RGC-1002	CAZRI, Jodhpur
4	RGC-1003	CAZRI, Jodhpur
5	RGC-1055	CAZRI, Jodhpur
6	RGC-1066	CAZRI, Jodhpur
7	RGC-936	CAZRI, Jodhpur
8	IC-103323	NBPGR, New Delhi
9	IC-113304	NBPGR, New Delhi
10	IC-116751	NBPGR, New Delhi
11	IC-116767	NBPGR, New Delhi
12	IC-116958	NBPGR, New Delhi
13	*C. serrata*	CCSHAU, Hisar
14	*C. senegalensis*	CCSHAU, Hisar

3.2 Extraction and purification of genomic DNA from leaf tissues

Genomic DNA was isolated by modified CTAB method described by Doyle and Doyle (1987), and treated with RNase to eliminate RNA.

3.2.1 DNA extraction and purification reagents

Tris.HCl- 1M (pH 8.0), EDTA- 0.5M (pH 8.0), NaCl- 5M, CTAB-10%, RNase solution (10mg/ml), Chloroform: Isoamyl alcohol- 24:1, Isopropanol and Absolute ethanol

3.2.2 DNA Extraction buffer

The composition of DNA extraction buffer is as described in Table 3.2.

Table 3.2: Composition of the DNA extraction buffer

S.No.	Name of the reagent	Concentration
1	Tris.HCl (pH 8.0)	100 mM
2	EDTA (pH 8.0)	20 mM
3	NaCl	1.4 M
4	CTAB	5%
5	β-mercaptoethanol	0.01%

All the chemicals used were purchased from HiMedia (Molecular biology grade).

3.2.3 Procedure

The leaves were ground in liquid nitrogen using a mortar and pestle. The pulverized leaves were quickly transferred to 1 ml of freshly prepared pre-warmed (65°C) extraction buffer (CTAB, Tris HCl, EDTA, NaCl) and shaken vigorously by inversion to form slurry. The tubes were incubated at 65°C in a water bath for 1 hour with intermittent shaking and swirling. An equal volume of

chloroform: isoamyl alcohol (24:1) was added and mixed properly by inversion for 10 minutes and centrifuged at 8000 rpm for 10 minutes at room temperature to separate the phases. The supernatant was decanted off and transferred to a new tube and precipitated with equal volume of cold isopropanol. It was gently mixed to produce fibrous DNA and incubated at -20°C for 30 minutes. The sample was then centrifuged at 8000 rpm for 10 minutes.

The pellet was washed with 70% ethanol, air dried and re-suspended in 200 µl TE buffer (Tris HCl and EDTA), 5 µl of RNase was added and incubated overnight at room temperature.

The dissolved DNA was extracted with 300 µl of chloroform: isoamyl alcohol (24:1) at 8000 rpm for 10 minutes. The upper aqueous layer was transferred to a fresh 1.5 µl tube and double volume of pre-chilled absolute alcohol was added and incubated at -20°C for 30 minutes followed by centrifugation at 12,000 rpm for 10 minutes. The pellet was air dried and re-suspended in 100 µl of TE buffer.

3.2.4 Quantification of DNA

The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity can also be determined by running the samples on 0.8% agarose gel based on the intensities of band when compared with lambda DNA marker.

3.3 Total RNA extraction from seeds

Total RNA extraction from seeds collected at different developmental stages was done using the hot phenol method described by Kohrer and Domdey (1991) [60].

3.3.1 RNA extraction reagents

Tris.HCl- 1M (pH 8.0), LiCl- 8 M and 2 M, EDTA- 0.5 M (pH 8.0), SDS- 0.5gm, DEPC, Phenol, CH_3COONa- 3 M, Isopropanaol and Absolute ethanol

3.3.2 RNA extraction buffer

DEPC treated water (0.1%) was used for RNA extraction. In 1 litre distilled water 1 ml of DEPC was added and shaken properly to mix. It was kept at room temperature overnight and then autoclaved. This DEPC treated water was used for washing and other purpose during RNA extraction. The composition of 50 ml extraction buffer is as follows:

Table 3.3: Composition of RNA extraction buffer

S.No.	Name of the reagent	Amount
1	1M Tris-HCl (pH 8.0)	5 ml
2	8M LiCl	0.625 ml
3	0.5M EDTA (pH 8.0)	1 ml
4	SDS	0.5 gm
5	DEPC treated water	43.2 ml

Equal volume of phenol (pH 4.7) was added to the extraction buffer (Table 3.3) i.e. 50 ml to make the final concentration of 1:1 and the mixture was heated to 80°C for at least half an hour. The seeds (50-100 mg) were ground in liquid nitrogen and 750 µl of extraction buffer with phenol was added followed by homogenization by vigorous vortexing. Then half volume of chloroform was added to the samples followed by vortexing. The eppendorf tubes were then centrifuged at room temperature for 30 minutes at 4000 rpm and the supernatant was transferred to a fresh tube. This was followed by addition of one-third volume of 8 M lithium chloride to the supernatant. The above solution was precipitated on ice for two hours. After precipitation the tubes were centrifuged at 10,000 rpm for 30 mins at 4°C. Then the supernatant was discarded and RNA pellet was washed with 500 µl of 2 M LiCl and again cetrifuged at 10,000 rpm for 5 minutes at room temperature. RNA pellet was then washed with 500 µl of 70% DEPC treated ethanol followed by centrifugation

at 10,000 rpm for 5 min at room temperature. After centrifugation ethanol was discarded and pellet was air dried. Finally RNA was dissolved in 100 µl of DEPC treated water. Then 50µl of 3M sodium acetate and 1250 µl of 100% ethanol were added at room temperature. RNA was precipitated at –80°C for 20 minutes and centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet was washed with 500 µl of 70% ethanol followed by centrifugation at 10,000 rpm for 5 mins at 4°C. The pellet was air dried and RNA was dissolved in 100 µl of DEPC treated water.

3.3.3 RNA quantification

RNA was quantified using UV visible spectrophotometer. 1ml of DEPC water was used as blank. 2 µl of RNA solution was added to 998 µl of DEPC water and 260nm/280nm and 260nm/230nm readings were taken. RNA was also visualized on 1% agarose gel to see the purity of RNA.

3.3.4 cDNA synthesis

For cDNA synthesis 5µg of RNA was taken in 10µl autoclaved milliQ water and 1µl oligo dT were mixed. The mixture was pulse centrifuged and kept at 65°C for 10 minutes and then quenched on ice immediately. Then 29 µl of reaction mixture was used with the composition as 2.5X RNase inhibitor 0.25µl, 10X DTT 1.0µl, 5X reverse transcriptase buffer 6.0 µl, 50mM $MgCl_2$ 3 µl, 20mM dNTP 10.0µl, reverse transcriptase enzyme 1µl and milliQ water 7.75 µl. The PCR profile for cDNA preparation was 25°C for 10 minutes, 42°C for 1 hour and 95°C for 2 minutes. cDNA was stored at -20°C till further use.

Table 3.4: Primer sequences used for expression analysis

Gene Name	Forward primer	Reverse primer
MS	5'ATGGTTACAAAGCAGGTGCC	5'AAGAGATTAGCTGGACCGCA
GT	5'AACAAGGTGGATTACTGCCG	5'TGCCATTCCAGCAATCATTA
α-Gal	5'GGGTGGAATAGCTGGAATCA	5'TCTTCTGTGGTCATCCCTCC
PMI	5'GCGAGGCTCTACGCGCTCAA	5'AGGACCTGGCACTGCTGGGA
Actin	5'GGCTGGATTTGCTGGAGATGATGC	5'CAATTTCTCGCTCTGCTGAGGTGG

3.3.5 Expression analysis of the genes involved in galactomannan biosynthesis

Amplification of desired genes using the cDNA prepared- For expression analysis cDNA was used as a template using the primer sequences given in Table 3.4. The reaction mixture consisted of 2 µl of 10 X PCR buffer, 1 µl of 50 mM $MgCl_2$, 2 µl of 25 mM dNTP, 1 µl of forward and reverse primers each, 1 µl of *Taq* DNA polymerase, 3 µl of cDNA and 9 µl of autoclaved miliQ water. The PCR profile for MS, GMGT, alpha-Gal and actin was 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, 1 cycle of 72°C for 10 minutes and hold at 4°C. For PMI the PCR profile was 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute, 1 cycle of 72°C for 10 minutes and hold at 4°C.

3.3.6 Gel electrophoresis

Casting the gel- Agarose gel (1%) was prepared by dissolving 1 gm of agarose in 100 ml of 1X TBE buffer. The mixture was heated to dissolve the agarose, cooled to room temperature, 2µl of EtBr was added and the gel was cast.

Loading the sample: 5 µl of PCR product was mixed with 2 µl of 6X loading dye and the mixture was loaded in the wells. The samples were run at 100 V.

Composition of TBE buffer:

Tris- 1.09 gm

Boric acid- 0.556 gm

EDTA- 0.093 gm

Water- 100 ml

3.4 Bioinformatics analysis

Protein sequence of galactosyltransferase enzymes of fenugreek, guar and *Senna* was obtained from NCBI in FASTA format. These sequences were compared by multiple sequence alignment using CLUSTALW program and a dendrogram was generated based on the sequence of the enzyme.

3.5 SSR Primer analysis

Genomic DNA was extracted from 12 accessions of guar by CTAB method described above. 26 EST-SSR primers, designed using MIcroSAtellite analyzer (not a part of this thesis), were used to analyze these accessions (Table 3.5). The sequences of the primers are given in Table 3.5. The thermal cycling parameters used are given in Table 3.6.

Table 3.5: List of SSR primers used

S.No	Primer name	Forward primer	Reverse Primer
1	IITR N-1	CGAGGCAACAATATCATCTTC	TTTGGGATCGATCAAAGTGA
2	IITR N-2	CTTTCCCTTCCTTGCTTCCT	TGAACCCAAAACACCAAACC
3	IITR N-3	CCACCACTTAGGTCGTGCTT	CAAAAGCAAGGGAAAGGTCA
4	IITR N-4	CTCCCCTTCCACTTTCCTTC	AAGAACAAGATGTTGGGCATTT
5	IITR N-5	TGCAAGTGACAACAGATTTGC	ACGGCATTATCCGCTACAAA
6	IITR N-6	AAGGAAATTGCTCAGGCTCA	CCCAAAGGCCAACATAATTG
7	IITR N-7	AGGAGAAGGCTTGGGATGAC	TTCCACCATGTGAAAAAGCA
8	IITR N-8	CGGAAATCAACATCAACGAA	TGTGACTTGGAAGCCCTTTT
9	IITR N-9	TCAGCCACAATGACACCAGT	CTTCGTCGTCCGACACTACA
10	IITR N-10	ACTATGAATGCCGTGGAAGC	AAAACAAACGCACCACACAA
11	IITR N-11	AGGAGAAGATGCTCCCAACA	TGAGCATCCATGAAAGCAAG
12	IITR N-12	TACCGTGTTGGTGACCTTGA	ATGTCCAAACCCATCTCCAA
13	IITR N-13	CACGAGGATTTCACTCACACA	AGGAGGGAGTTCAGGTTGGT
14	IITR N-14	AAAGAAGAAAGATCCTTGTTTGGA	AAACAAAAATAACCGCTTGATGA
15	IITR N-15	CTTTGCTTCAAAGGGTTTCG	GCTCTGTCATTGTGGGAAAAA
16	IITR N-16	TCGAATTGAAGGCAAAGAGC	GTAGCTGAGTGCCCTGGAAG
17	IITR N-17	CAACCAGAAACATGAAGCACA	CCTGTTATGCCTGCTGAGTG
18	IITR N-18	CGAGGGAAAGAGTTAAGAGAAAA	CCATAGCCAAAAACCCAGAA
19	IITR N-19	CTGCCTCAACAACCTCAACA	CGAGCAAACCAAGGAAGAAG
20	IITR N-20	CCAAAGATCAACAACAACCAGA	CCAACAGTGGCTTGCTTGTA
21	IITR N-21	TCACCGAGTTATGTGGGTTTC	CCCACAACCAAATACCCAAC
22	IITR N-22	GCCGGAGTATCCATCAAAAC	AATCTTGTCAACCCCCTGTG
23	IITR N-23	CATGATTGCGTCAAAACTCG	CCCAGGGGAGGGTTACTAGA
24	IITR N-24	TACCACCCTAGGCCCTAACC	GGCCCCATTTTTATTTTGTTT
25	IITR N-25	TAAGTGCAATTGTGCGAAGC	AAGCACTTAGAGTGTGCTTTGTTG
26	IITR N-26	TAATCCATGTGCCAACAAGC	TCTCCTTGCTCACCCAAAAT

Table 3.6: Thermal cycling parameters used for SSR primers

HOLD	30 CYCLES			HOLD	HOLD
94°C	94°C	55°C	72°C	72°C	4°C
4min	30sec	30sec	30min	10min	(Forever)

3.6 AFLP analysis

3.6.1 DNA Extraction

DNA required for AFLP experiment should be very pure, free from secondary metabolites or gummy polysachharides and in larger amount than that required for PCR. The DNA extraction protocol given by Khanuja *et. al.* (1999) was used [56].

Table 3.7: Composition of the DNA extraction buffer for AFLP experiment

S.No.	Name of the reagent	Concentration
1	Tris HCl (pH 8.0)	100mM
2	EDTA (pH 8.0)	25mM
3	NaCl	1.5M
4	CTAB	2.5%
5	β-mercaptoethanol (added immediately before use)	0.2% (v/v)
6	PVP (added immediately before use)	1% (w/v)

Table 3.8: Composition of high salt TE buffer

S.No.	Name of the reagent	Amount
1	NaCl	1M
2	Tris-Cl (pH 8.0)	10mM
3	EDTA	1mM

3.6.2 Procedure

The leaves were ground in liquid nitrogen using a mortar and pestle. The pulverized leaves were quickly transferred to 1 ml of freshly prepared pre-warmed (65°C) extraction buffer (Table 3.7) and shaken vigorously by inversion to form slurry. The tubes were incubated at 65°C in a water bath for 1 hour with intermittent shaking and swirling. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed properly by inversion for 10 minutes and centrifuged at 8000 rpm for 10 minutes at room temperature to separate the phases. The supernatant was decanted and transferred to a new tube and 50 µl of 5M NaCl was added. DNA was then precipitated with equal volume of isopropanol. It was gently mixed to produce fibrous DNA and incubated at room temperature for 30 minutes. The sample was then centrifuged at 8000 rpm for 10 minutes.

The pellet was washed with 70% ethanol, air dried and re-suspended in 400 µl of high salt TE buffer (Table 3.8) and kept at room temperature overnight. The dissolved DNA was extracted with 400 µl of chloroform: isoamyl alcohol (24:1) at 8000 rpm for 10 minutes. The upper aqueous layer was transferred to a fresh 1.5 µl tube and double volume of pre-chilled absolute alcohol was added and incubated at -20°C for 30 minutes followed by centrifugation at 12,000 rpm for 10 minutes. The pellet was air dried and re-suspended in 50 µl of nuclease free water.

3.6.3 Restriction digestion of genomic DNA

To check the purity of DNA, restriction digestion was done using the genomic DNA, the composition of the reaction is given in Table 3.9

Table 3.9: Composition of restriction digestion reaction

S.No.	Components	Amount
1	10X Restiction enzyme buffer	2 µl
2	EcoR1 (20U/µl)	0.25 µl
3	DNA	500ng
4	Sterile water	X µl
5	Total	10 µl

The reaction mixture was kept at 37°C for 2 hours and the product was visualized on 1.5% agarose gel.

3.6.4 Restiction Ligation reaction

The enzyme master mix was formed as described in Table 3.10. Restriction ligation reaction was set up using the enzyme master mix (Table 3.11)

Table 3.10: Composition of enzyme master mix

S.No.	Components	1X
1	T4 DNA ligase buffer (10X)	0.1µl
2	NaCl (0.5M)	0.1 µl
3	BSA (1mg/ml)	0.05 µl
4	Mse1 (10U/ µl)	0.1 µl

5	EcoR1 (20U/µl)	0.25 µl
6	T4 DNA ligase	0.1 µl
7	Sterlie water	0.30 µl

Table 3.11: Composition of restriction ligation reaction

S.No.	**Components**	**1X**
1	T4 DNA ligase buffer (10X)	1µl
2	NaCl (0.5M)	1 µl
3	BSA (1mg/ml)	0.5 µl
4	Mse1 adaptor	1 µl
5	EcoR1 adaptor	1µl
6	Enzyme master mix	1 µl
7	DNA (500ng)	5.5µl
8	Total	11 µl

500 ng of DNA was added in 5.5 µl volume and 5.5 µl restiction ligation mixture for each DNA sample. This mixture was kept at room temperature overnight and was then diluted by adding 189 µl of freshly prepared TE buffer to give appropriate concentration for subsequent pre-selective amplification.

3.6.5 Preselective amplification

The restricted and ligated DNA was used as a template for preselective amplification. The reaction mixture was formed as in Table3.12. The cycling parametes used for pre-selective amplification are given in Table 3.13.

Table 3.12: Composition of pre-selective amplification reaction mixture

S.No.	Components	Amount
1	Diluted DNA prepared by restriction ligation	4 µl
2	AFLP preselective primer pairs	1 µl
3	AFLP core mix	15 µl
4	Total	20 µl

Table 3.13: Thermal cycler parameters for preselective amplification

HOLD	20 CYCLES			HOLD	HOLD
72°C 2min	94°C 20sec	56°C 30sec	72°C 2min	60°C 30min	4°C (Forever)

The preselective product was visualized on 1.5% agarose gel by loading 10 µl of the product. The preselective amplification product was diluted by adding 190 µl of TE to be used as a template for selective amplification.

3.6.6 Selective amplification

The product of the preselective amplification was used as a template for selective amplification. The composition of the reaction mixture and cycling parameters used are given in Table 3.14 and Table 3.16. The primer combinations formed are illustrated in Table 3.15. After the selective amplification the product was mixed with ROX size standard and formamide as in Table 3.17 and loaded on ABI sequencer after denaturation.

Table 3.14: Composition of selective amplification reaction

S.No.	Components	Amount
1	AFLP core mix	7.5 µl
2	Diluted preselective product	3 µl
3	Selective Mse1 primer	0.5µl
4	Selective EcoR1 primer (labelled)	0.25 µl each
5	Total	11.75 µl

Table 3.15: Plate design for selective amplification reaction

EcoR1 ↓ / Mse1 →	CAA	CAC	CAG	CAT	CTA	CTC	CTG	CTT
ACT+AAC+AAG	1	4	7	10	13	16	19	22
ACA+AGG+ACC	2	5	8	11	14	17	20	23
AGC+ACG	3	6	9	12	15	18	21	24

Table 3.16: Thermal cycler parameters for selective amplification reaction

HOLD	CYCLE			Number of Cycles
94°C 2min	94°C 20sec	66°C 30sec	72°C 2min	1
-	94°C 20sec	65°C 30sec	72°C 2min	1
-	94°C 20sec	64°C 30sec	72°C 2min	1
-	94°C 20sec	63°C 30sec	72°C 2min	1
-	94°C 20sec	62°C 30sec	72°C 2min	1
-	94°C 20sec	61°C 30sec	72°C 2min	1
-	94°C 20sec	60°C 30sec	72°C 2min	1
-	94°C 20sec	59°C 30sec	72°C 2min	1
-	94°C 20sec	58°C 30sec	72°C 2min	1
-	94°C 20sec	57°C 30sec	72°C 2min	1
-	94 °C 20sec	56°C 30sec	72°C 2min	20
60°C 30min	-	-	-	1
4°C Forever	-	--	-	1

The selective PCR product was run on ABI 3130xl Genetic analyzer

Table 3.17: Sample preparation for ABI 3130xl Genetic analyzer

S.No.	Components	Amount
1	ROX dye	0.25 µl
2	HiDi Formamide	9.25 µl
3	Selective amplification product	1 µl

The samples were denatured at 95°C for 5 minutes and immediately chilled on ice. Then the plate was loaded on ABI 3130xl Genetic analyser. The raw data obtained was analyzed by gene mapper software which gave the results in the binary form. A dendrogram was generated by PAST program [42], Unweighted Pair-Group Average (UPGMA) method using the similarity matrix by Jaccard's similarity coefficient [51].

Chapter IV

Results

CHAPTER-4

4.1 Quantitative expression studies on genes involved in galactomannan biosynthetic pathway

For the study of gene expression guar pods were collected from various developmental stages viz. 5, 10, 15, 20, 25, 30, and 35 days after flowering (DAF) from M-83. Total RNA was extracted from their seeds by hot phenol method using LiCl to precipitate the RNA. Total RNA was also extracted from leaves, stem, root, hypocotyl and flower bud as described in Section 3.3.3. 100 mg of each tissue was taken for RNA extraction. Two bands corresponding to 28s and 18s were seen in all the sample lanes. (Figure 4.1)

The total RNA was quantified using a spectrophotometer by measuring its OD at 260nm (Table 4.1).

Table 4.1: Concentration of total RNA extracted from different samples

S.No	Samples	RNA Concentration (µg/µl)	260/280	260/230
1	5 DAF	2.2	1.9	2.82
2	10 DAF	2.0	1.81	2.98
3	15 DAF	3.9	1.47	1.63
4	20 DAF	0.6	1.36	1.54
5	25 DAF	1.6	1.89	2.96
6	30 DAF	0.9	1.52	2.21
7	35 DAF	2.0	1.47	1.63
8	Flower bud	0.9	1.9	1.9
9	Leaves	2.0	1.86	2.24
10	Stem	1.5	1.76	2.62
11	Root	0.5	1.82	2.34
12	Hypocotyl	-0.9	-	-

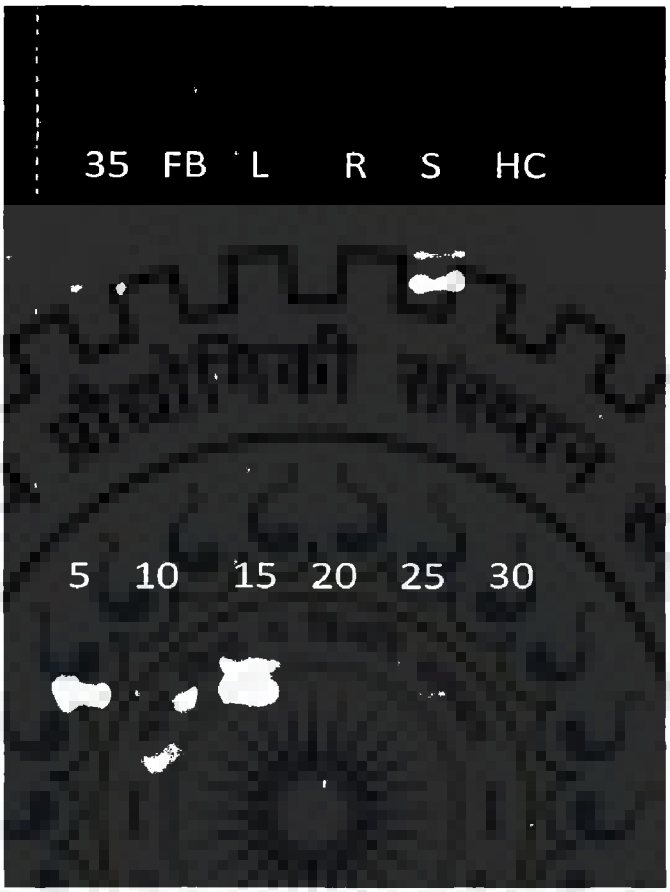

Figure 4.1: Total RNA extracted from different developmental stages [5, 10, 15, 20, 25, 30 and 35 days after flowering, FB (flower bud), L (leaves), R (root), S (stem) and HC (hypocotyl)] as visualized on agarose gel.

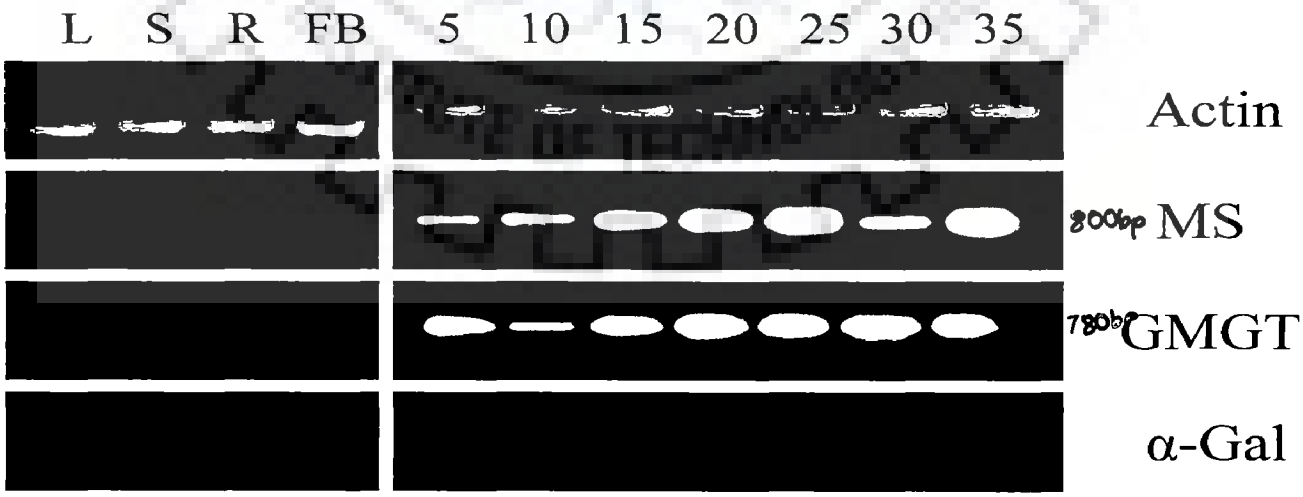

Figure 4.2: Expression analysis of various genes in different developmental stages of guar seed.

5 µg of RNA from each sample was used to make cDNA using oligo dT primer. Expression levels of four genes involved in galactomannan biosynthesis [Mannan synthase (MS), galactomannan galactosyltransferase (GMGT), alpha-galactosidase (α-Gal) and phosphomannoisomerase (PMI)] were studied by amplifying these genes using the cDNA prepared. The expression of actin gene was also monitored as endogenous control in all the samples.

The expression of mannan synthase was found to increase gradually with increase in the days after flowering, with maximum expression on 35th DAF. Galactosyltransferase was also found to follow a similar pattern with more expression on the later stages of development. Alpha- galactosidase and phosphomannoisomerase did not show any expression in any of the developmental stages. None of the four genes were found to express in stem, root, flower bud or leaf tissues; whereas housekeeping gene, actin, was present in all the samples. (Figure 4.2)

4.2 Bioinformatic analysis

Protein sequence (FASTA format) of galactosyltransferase from the three gum producing crops was taken from NCBI.

```
>gi|5702018|emb|CAB52246.1| alpha galactosyltransferase [Trigonella foenum-
graecum]
ATKFGSKNKSSPWLSNGCIFLLGAMSALLMIWGLNSFIAPIPNSNPKFNSFTTKLKSLNFTTNTNFAGPD
LLHDPSDKTFYDDPETCYTMMDKPMKNWDEKRKEWLFHHPSFAAGATEKILVITGSQPTKCDNPIGDHLL
LRFYKNKVDYCRIHNHDIIYNNALLHPKMDSYWAKYPMVRAAMLAHPEVEWIWWVDSDAIFTDMEFKLPL
WRYKDHNLVIHGWEELVKTEHSWTGLNAGVFLMRNCQWSLDFMDVWASMGPNSPEYEKWGERLRETFKTK
VVRDSDDQTALAYLIAMGEDKWTKKIYMENEYYFEGYWLEISKMYDKMGERYDEIEKRVEGLRRRHAEKV
SERYGEMREEYVKNLGDMRRPFITHFTGCQPCNGHHNPIYAADDCWNGMERALNFADNQVLRKFGFIHPN
LLDKSVSPLPFGYPAASP

>gi|62700759|emb|CAI79403.1| galactomannan galactosyltransferase [Senna
occidentalis]
MAKSVRNKSSLWFSDGCLFLGGAFSALLLVWGFWSFIAPIPITDPNFDSVSTKLKTLKNPRTVPSTVICS
TSAAETTTVPNLRHDPPEATFYDDPETSYTLDKPMKNWDEKRQEWLNRHPSFSAGAKSRILLVTGSQPTP
CKNPIGDHLLLRFFKNKVDYCRLHGYDIFYNNALLQPKMHTYWAKYPVVRAAMMAHPEAEWIWWVDSDAL
FTDMEFKLPLNRYKNHNLIVHGWPTLIHEAKSWTGLNAGVFLIRNCQWSLDFMDVWASMGPQTPSYEKWG
EKLRTTFKDKAFPESDDQTGLAYLIAVEKEKWADRIYLESEYYFEGYWKEIVETYENITDKYHEVERKVR
SLRRRHAEKVSESYGAVREPYVMVAGSGRGSWRRPFITHFTGCQPCSGNHNAMYSPDACWNGMNKALIFA
DNQVLRKFGYVHPDLQDNSVSPIPFDYPA
```

>gi|62700755|emb|CAI79402.1| galactomannan galactosyltransferase [Cyamopsis tetragonoloba]
MAKFGSRNKSPKWISNGCCFLLGAFTALLLLWGLCSFIIPIPNTDPKLNSVATSLRSLNFPKNPAATLPP
NLQHDPPDTTFYDDPETSYTMDKPMKNWDEKRKEWLLHHPSFGAAARDKILLVTGSQPKRCHNPIGDHLL
LRFFKNKVDYCRLHNYDIIYNNALLHPKMNSYWAKYPVIRAAMMAHPEVEWVWWVDSDAVFTDMEFKLPL
KRYKNHNLVVHGWEGLVRLNHSWTGLNAGVFLIRNCQWSLEFMDVWVSMGPQTPEYEKWGERLRETFKDK
VLPDSDDQTALAYLIATDNKDTWREKIFLESEYYFEGYWLEIVKTYENISERYDEVERKVEGLRRRHAEK
VSEKYGAMREEYLKDNKRRPFITHFTGCQPCNGHHNPAYNANDCWNGMERALNFADNQILRTYGYHRQNL
LDKSVSPLPFGYPAA

Protein sequence of galactosyltransferase from fenugreek (Man/Gal-1.1), *Senna* (Man/Gal-3.3) and cluster bean (Man/Gal-1.6) was compared by CLUSTALW (multiple sequence alignment program) and a dendrogram was generated (Figure 4.3).

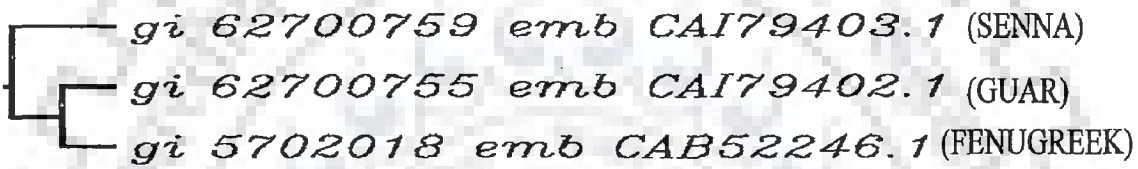

Figure 4.3: Dendrogram depicting relationship between three gum producing crops on the basis of galactosyltranferase enzyme

4.3 SSR analysis

Genomic DNA from 12 varieties of cluster bean was used for SSR analysis. 26 SSR primers, designed using MIcroSAtellite analyzer (not a part of this thesis), were used out of which 20 primers gave proper amplification. Only six of these 20 primers showed polymorphic bands. The list of primers showing polymorphic bands is shown in Table 4.2. The polymorphic bands obtained are shown in Figure 4.4-4.9. Out of the 12 varieties used polymorphism was obtained only in *Cyamopsis senegalensis*, which is a wild variety.

Table 4.2: List of SSR primers showing polymorphism

S.No.	Primer name	Polymorphism	Size of the polymorphic band
1	IITR N-1	_	_
2	IITR N-2	_	_
3	IITR N-3	_	_
4	IITR N-4	_	_
5	IITR N-5	_	_
6	IITR N-6	_	_
7	IITR N-7	_	_
8	IITR N-8	Did not amplify	_
9	IITR N-9	+	180bp
10	IITR N-10	_	_
11	IITR N-11	_	_
12	IITR N-12	_	_
13	IITR N-13	+	More than 250bp
14	IITR N-14	Did not amplify	_
15	IITR N-15	_	_
16	IITR N-16	_	_
17	IITR N-17	+	160bp
18	IITR N-18	Did not amplify	_
19	IITR N-19	+	190bp
20	IITR N-20	Did not amplify	_
21	IITR N-21	+	190bp
22	IITR N-22	_	_
23	IITR N-23	+	220bp
24	IITR N-24	_	_
25	IITR N-25	Did not amplify	_
26	IITR N-26	Did not amplify	_

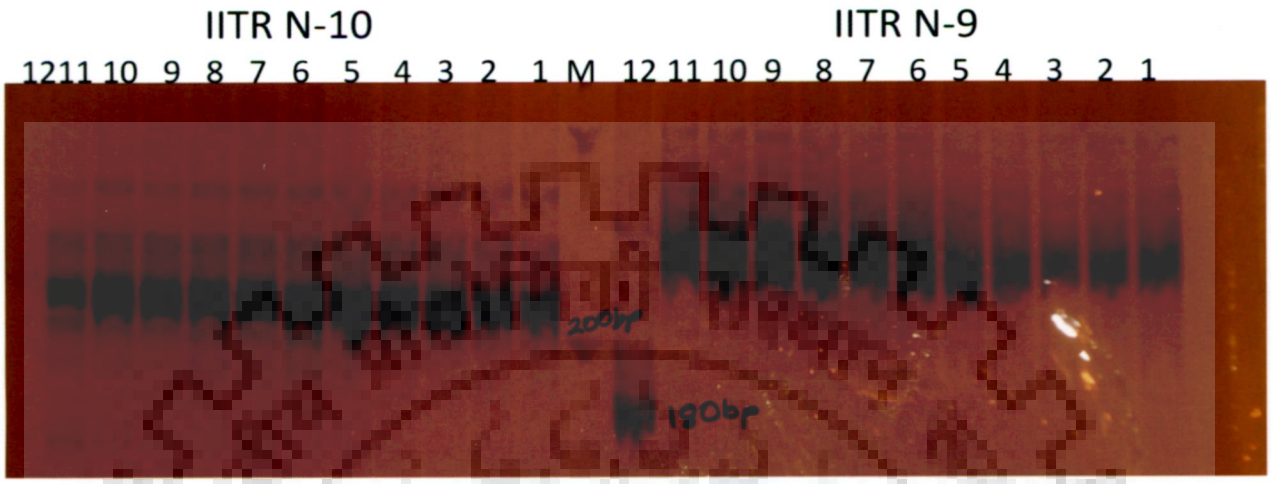

Figure 4.4: Amplification of 12 varieties using IITR N-9 and IITR N-10 primers

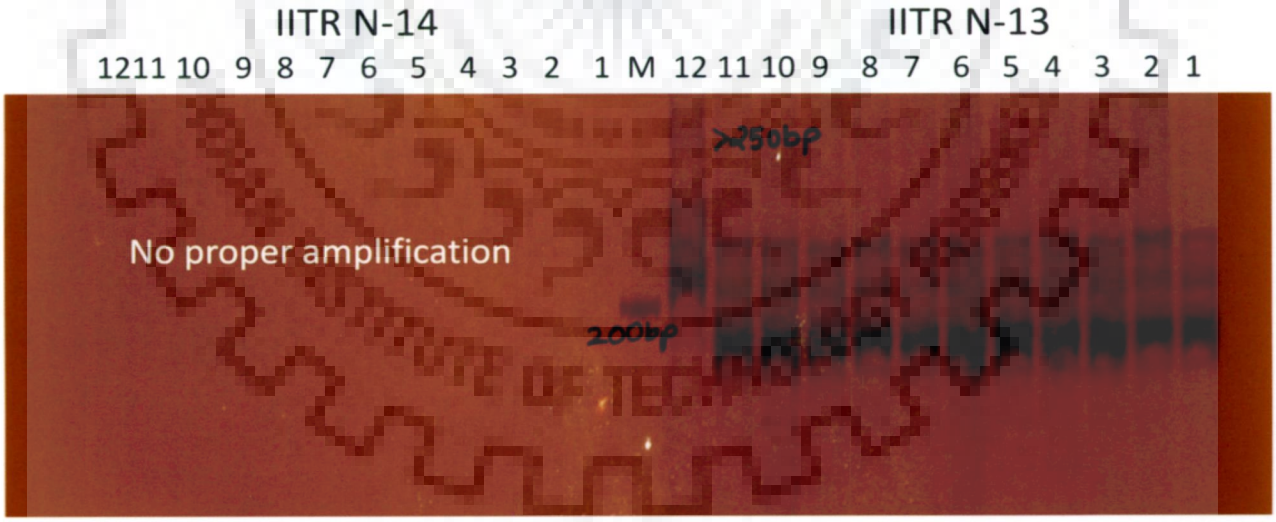

Figure 4.5: Amplification of 12 varieties using IITR N-13 and IITR N-14 primers

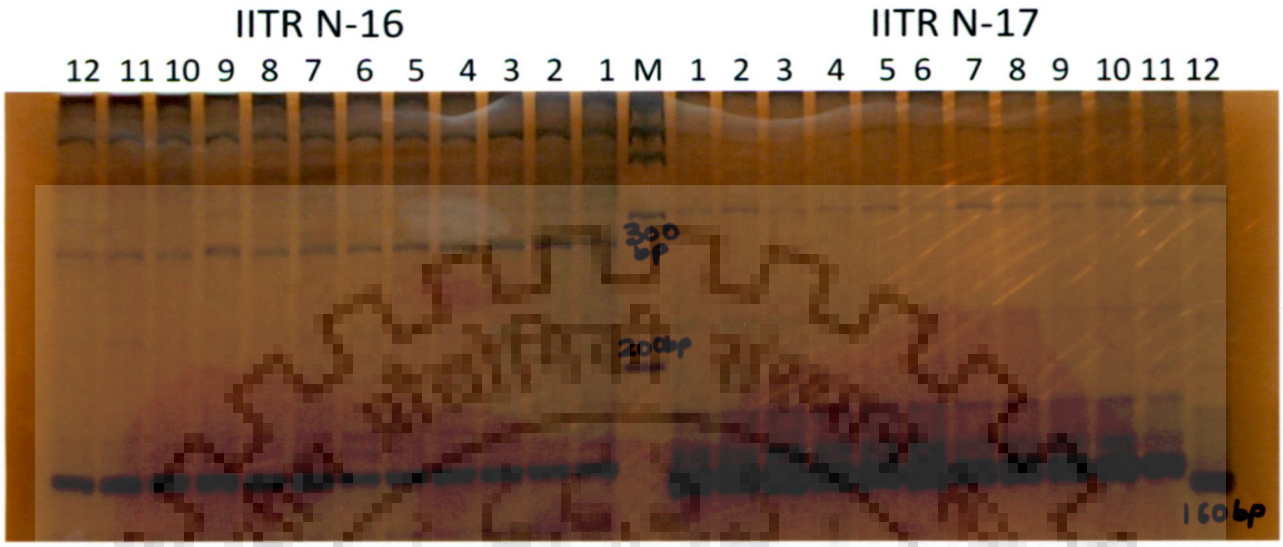

Figure 4.6: Amplification of 12 varieties using IITR N-16 and IITR N-17 primers

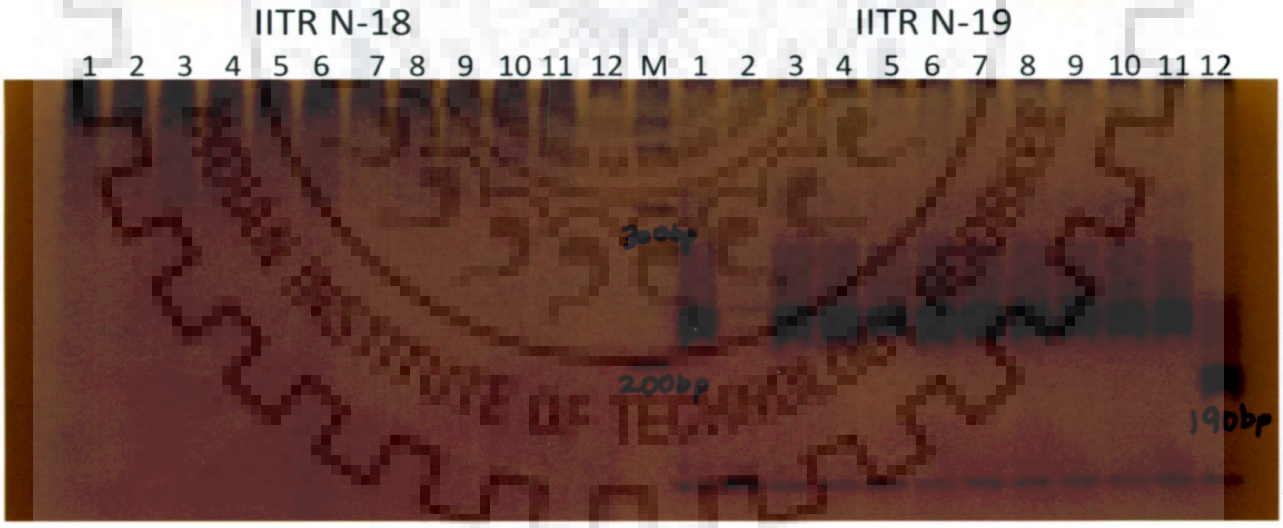

Figure 4.7: Amplification of 12 varieties using IITR N-18 and IITR N-19 primers

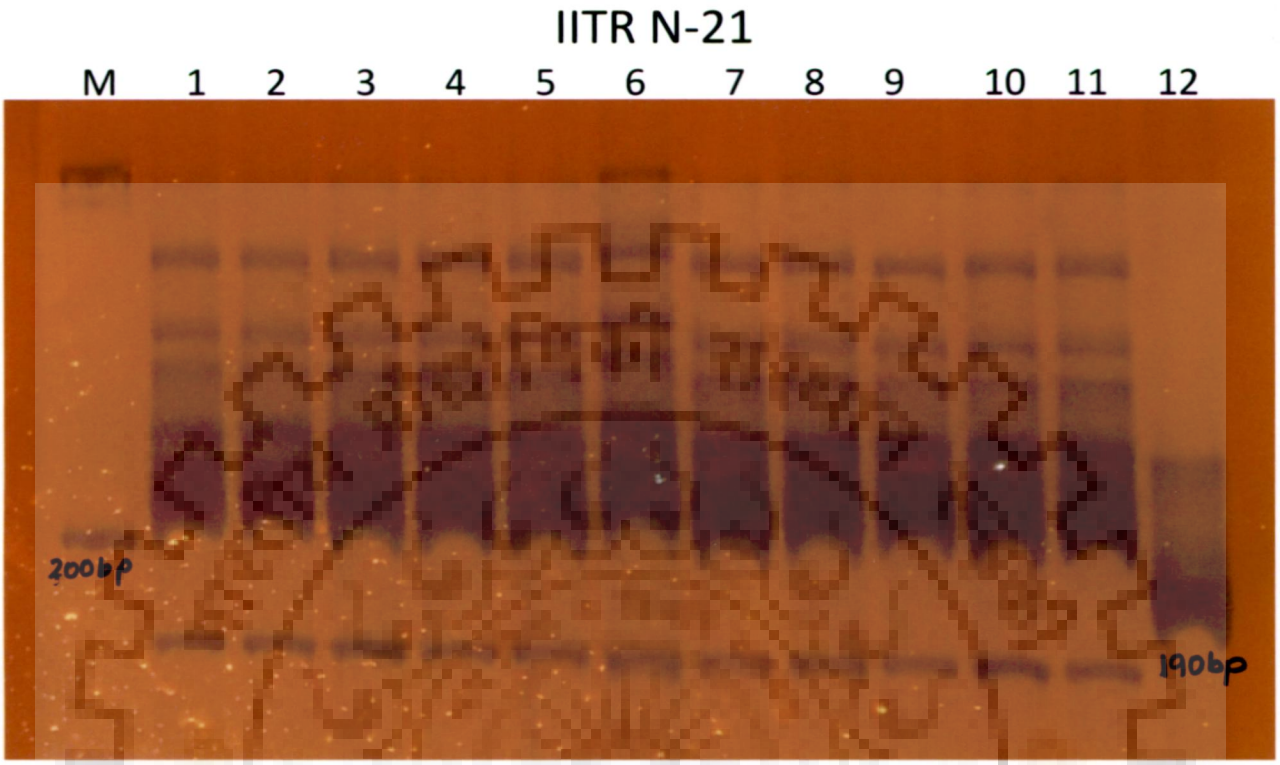

Figure 4.8: Amplification of 12 varieties using IITR N-21

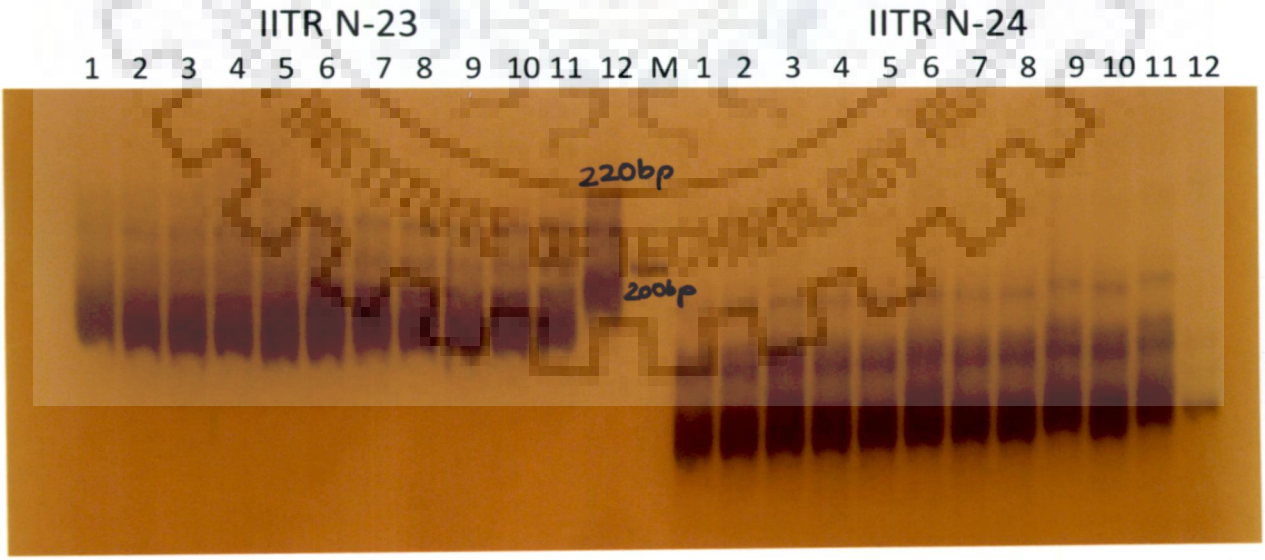

Figure 4.9: Amplification of 12 varieties using IITR N-23 and IITR N-24 primers

4.4 Amplified fragment length polymorphism

4.4.1 Standardization of the AFLP protocol using two varieties

Genomic DNA was extracted from the leaves of two guar varieties i.e. M-83 and RGC-936. The DNA was visualized on 0.8% agarose gel (Figure 4.10).

The purity of DNA and that whether or not it restricts with restriction enzyme, was checked by cutting 500ng of genomic DNA with *Eco*RI. The resticted product was checked on 1.5 % agarose gel. The DNA was properly cut by the restriction enzyme (Figure 4.11).

Restriction and ligation was done simultaneously using 500 ng of genomic DNA by incubating it with the two restriction enzymes i.e. *Eco*RI and *Mse*I, *Eco*RI and *Mse*I adaptors and T4 DNA ligase. The restriction-ligation reaction prepared the template for pre-selective amplification. The product of pre-selective amplification was visualized on 1.2% agarose gel. A very light smear in the range 100-1500 bp was visible (Figure 4.12).

Figure 4.10: Genomic DNA extracted from 2 varieties of guar

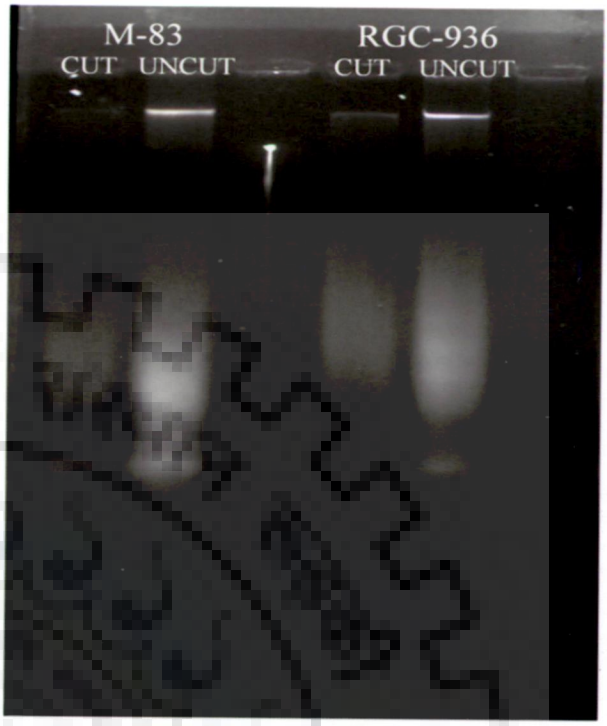

Figure 4.11: Restriction digestion of genomic DNA with EcoRI

Figure 4.12: Pre-selective amplification product

Selective amplification reaction was carried out using the diluted pre-selective amplification product as template. Multiplexing of 64 combinations was done in 24 tubes. The number of bins obtained in each combination is shown in Table 4.3.

Table 4.3: Number of bins obtained in each primer combination

Primer Combination	Dye	No. of bins	Total bins	MseI Primer	EcoRI Primer
1	B	2		CAA	ACT
	G	34	37		AAG
	Y	1			AAC
2	B	1		CAA	ACA
	G	6	20		AGG
	Y	13			ACC
4	B	25		CAC	ACT
	G	5	30		AAG
	Y	0			AAC
5	B	14		CAC	ACA
	G	10	35		AGG
	Y	11			ACC
7	B	18		CAG	ACT
	G	3	22		AAG
	Y	1			AAC
10	B	28		CAT	ACT
	G	7	35		AAG
	Y	0			AAC
11	B	5		CAT	ACA
	G	9	23		AGG
	Y	9			ACC
13	B	18		CTA	ACT
	G	12	30		AAG
	Y	0			AAC

The primer combinations that gave more than 20 bins were selected for analyzing the polymorphism in all the 12 varieties (Table 4.4).

Table 4.4: Primer combinations selected for analyzing the polymorphism

MseI→ / EcoRI↓	CAA	CAC	CAG	CAT	CTA	CTC	CTG	CTT
ACT	Ø	Ø	Ø	Ø	Ø			
AAC	Ø	Ø	Ø	Ø	Ø			
AAG	Ø	Ø	Ø	Ø	Ø			
ACA	Ø	Ø		Ø				
AGG	Ø	Ø		Ø				
ACC	Ø	Ø		Ø				
AGC								
ACG								

Twelve guar accessions were selected randomly, out of which five were commercial varieties, five landraces and two wild varieties. Genomic DNA was extracted from young leaves of these twelve varieties for AFLP analysis and visualized on 0.8% agarose gel (Figure 4.13).

Figure 4.13: Genomic DNA of 12 guar accessions

To check the purity of DNA it was cut by restriction enzyme EcoRI. The restricted product was visualized on 1.5% agarose gel (Figure 4.14).

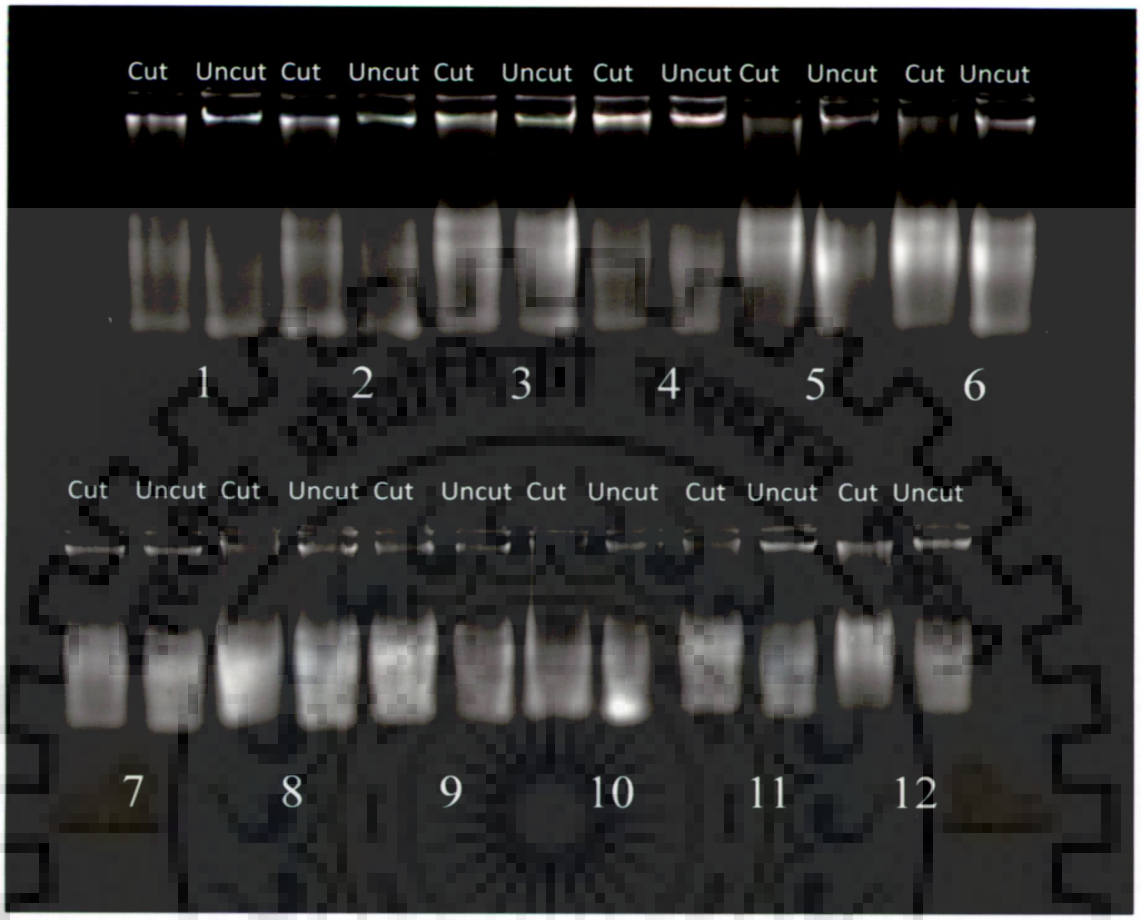

Figure 4.14: Restriction digestion of genomic DNA with EcoRI

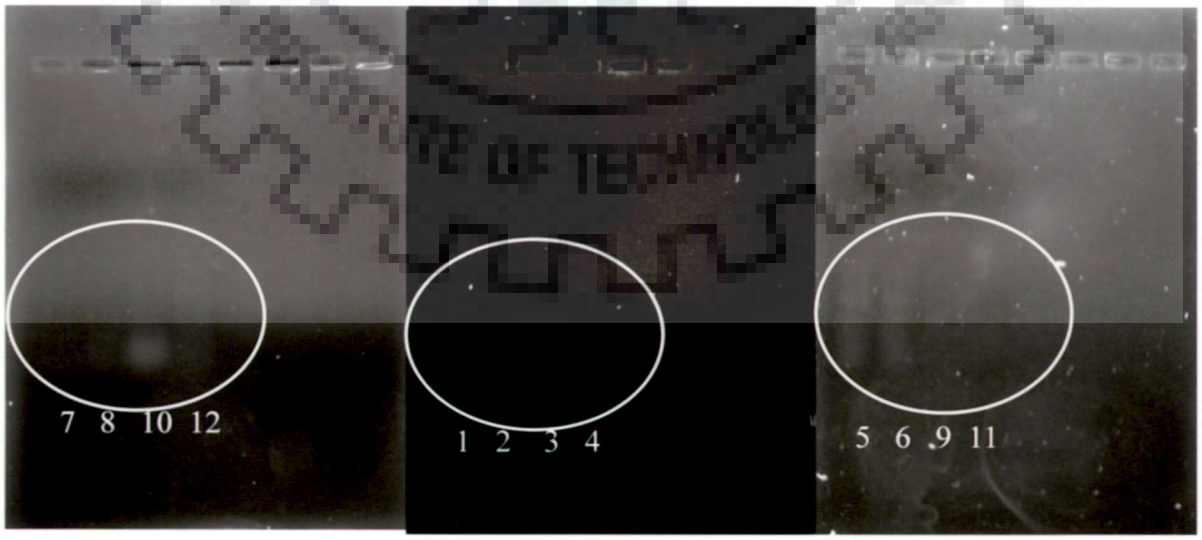

Figure 4.15: Pre-selective amplification product

To carry out the AFLP analysis, restriction ligation reaction was set up for all the samples taking 500 ng of genomic DNA. The restricted ligated product was used as a template for pre-selective amplification. The product of pre-selective amplification was visualized by loading a part of it on 1.2% agarose gel. A smear was seen in the 100-1500 bp range (Figure 4.15).

The pre-selective amplification product was diluted by adding 189 µl of $T_{10}E_1$ buffer which was then used as a template for selective amplification. All the samples were amplified using the following shortlisted primers. The combinations of primers used are shown in Table 4.5.

Table 4.5: Primers used for selective amplification

MseI selective primer	CAA	CAA	CAC	CAC	CAG	CAT	CAT	CTA
EcoRI selective primer	ACT	ACA	ACT	ACA	ACT	ACT	ACA	ACT
	AAC	AGG	AAC	AGG	AAC	AAC	AGG	AAC
	AAG	ACC	AAG	ACC	AAG	AAG	ACC	AAG

The product of selective amplification was loaded on 6% polyacrylamide gel on an ABI Prism 3130xl DNA sequencer. The peaks generated and bins obtained are shown in Figure 4.16 and 4.17.

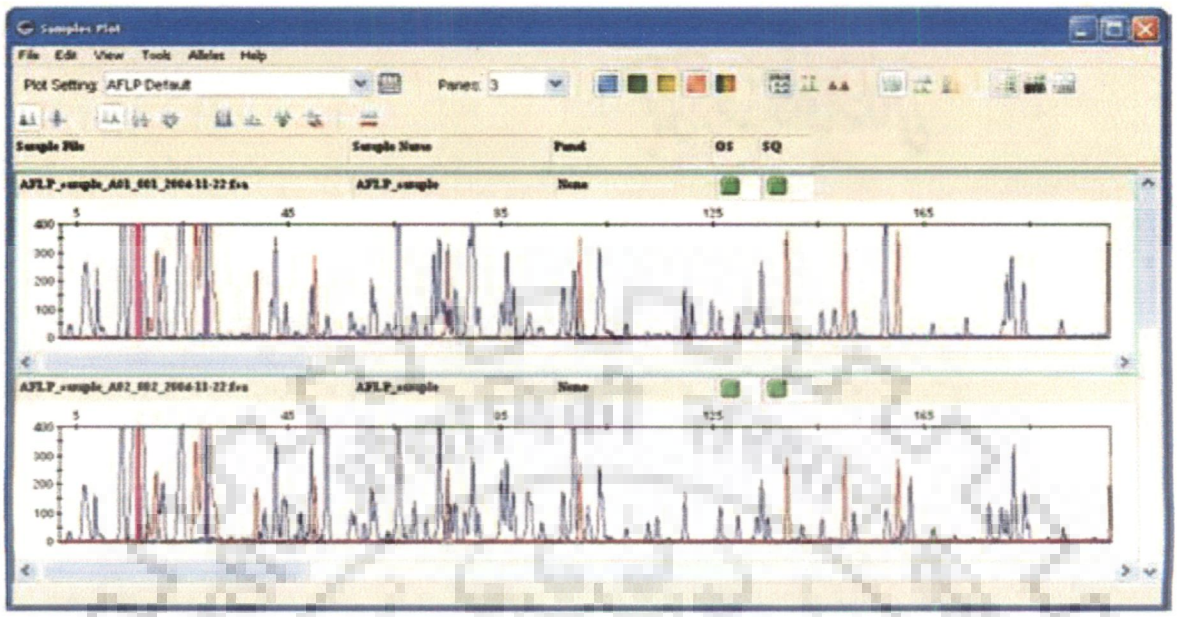

Figure 4.16: Peaks of DNA samples generated by ABI sequencer as visualized in gene mapper

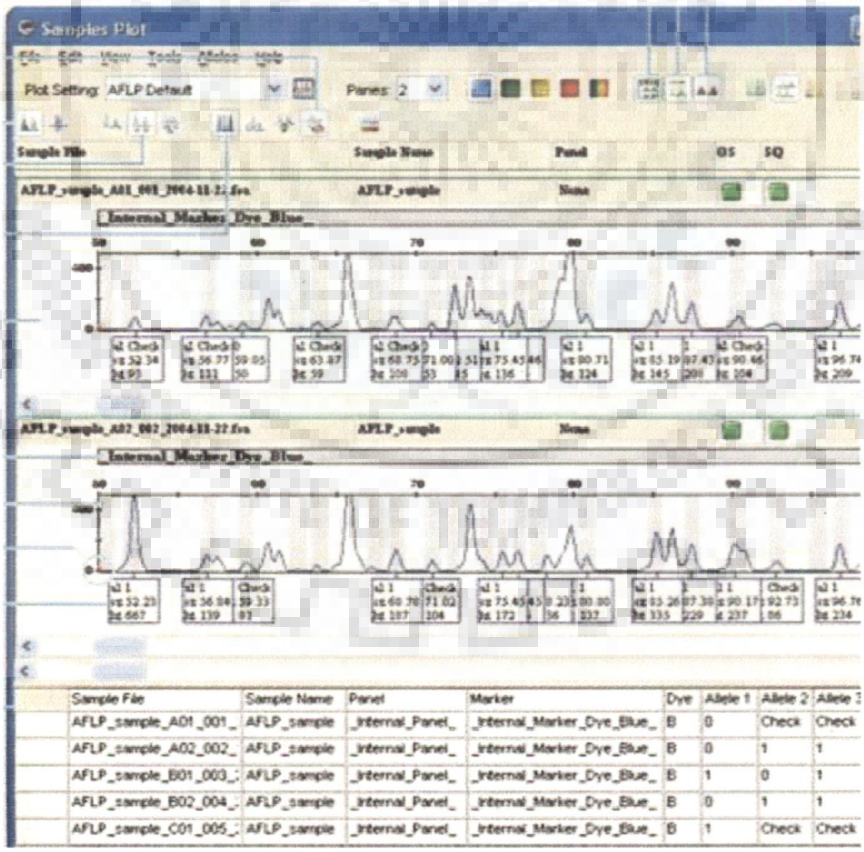

Figure 4.17: Bins of DNA samples obtained by ABI sequencer as visualized in gene mapper

Table 4.6. Similarity matrix generated using Jaccards similarity

	M 83	RGC 1066	IC 103323	IC 116767	C. serrata	RGC 197	RGC 1002	RGC 1003	RGC 1055	IC 113304	IC 116751	IC 116958	C. senegalensis
M 83	1.00	0.17	0.14	0.14	0.15	0.27	0.20	0.24	0.21	0.01	0.03	0.00	0.01
RGC 1066	0.17	1.00	0.39	0.20	0.26	0.23	0.32	0.27	0.33	0.01	0.04	0.00	0.00
IC 103323	0.14	0.39	1.00	0.34	0.37	0.25	0.37	0.34	0.34	0.02	0.05	0.00	0.00
IC 116767	0.14	0.20	0.34	1.00	0.50	0.07	0.10	0.11	0.19	0.03	0.03	0.00	0.02
C. serrata	0.15	0.26	0.37	0.50	1.00	0.17	0.25	0.22	0.29	0.02	0.04	0.00	0.01
RGC 197	0.27	0.23	0.25	0.07	0.17	1.00	0.44	0.56	0.39	0.01	0.02	0.00	0.00
RGC 1002	0.20	0.32	0.37	0.10	0.25	0.44	1.00	0.46	0.44	0.02	0.04	0.00	0.00
RGC 1003	0.24	0.27	0.34	0.11	0.22	0.56	0.46	1.00	0.40	0.01	0.03	0.00	0.00
RGC 1055	0.21	0.33	0.34	0.19	0.29	0.39	0.44	0.40	1.00	0.01	0.03	0.00	0.01
IC 113304	0.01	0.01	0.02	0.03	0.02	0.01	0.02	0.01	0.01	1.00	0.17	0.00	0.00
IC 116751	0.03	0.04	0.05	0.03	0.04	0.02	0.04	0.03	0.03	0.17	1.00	0.00	0.00
IC 116958	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
C. senegalensis	0.01	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	1.00

A dendrogram was generated by Unweighted Pair-Group Average (UPGMA) method (Figure4.18) using the similarity matrix (Figure 4.6) by Jaccard's similarity coefficient [51] to determine the relatedness of 13 genotypes analyzed.

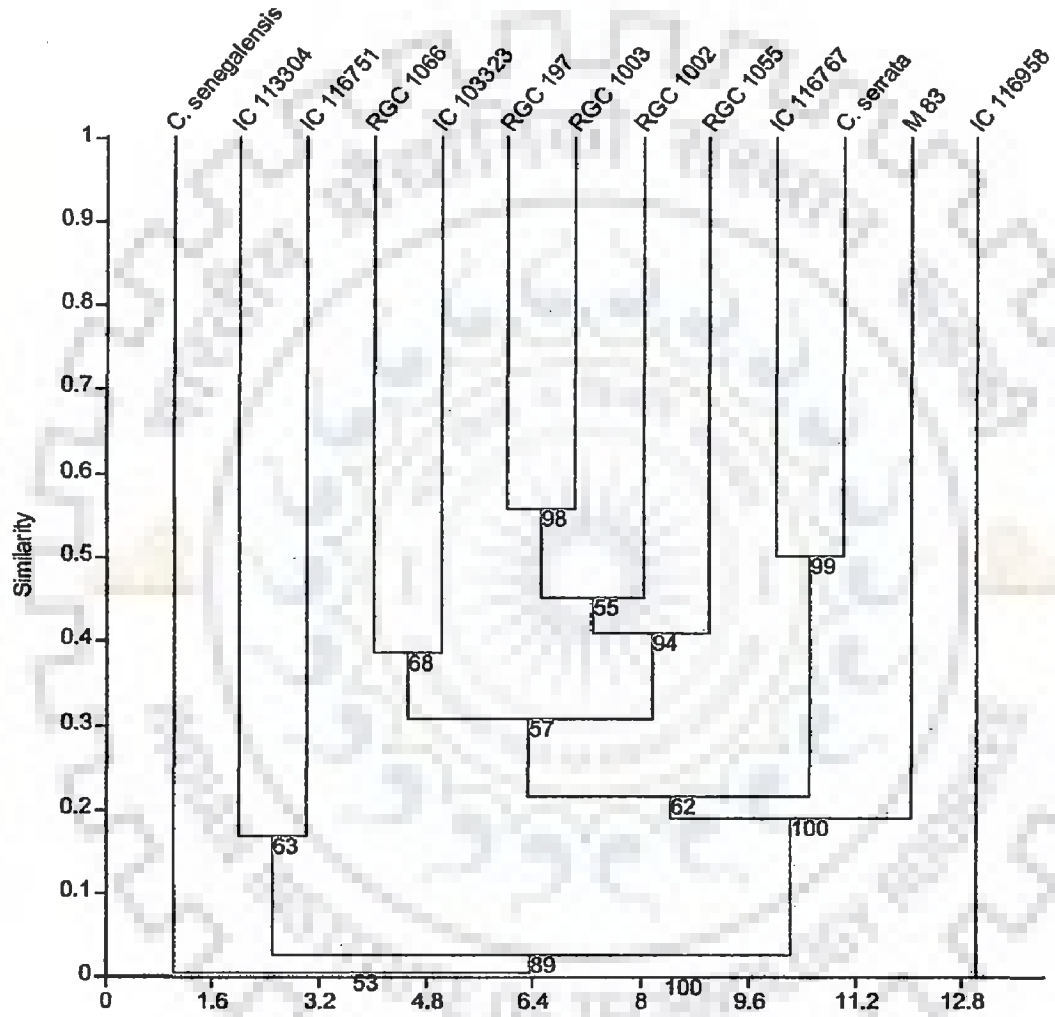

Figure 4.18: Dendrogram generated on the basis of AFLP analysis

Using 30% similarity, 3 major clusters were formed. RGC-1066, which is a commercial variety, and IC- 103323, a landrace, formed one cluster. The other cluster consisted only of commercial varieties i.e. RGC-197, RGC-1003, RGC-1002 and RGC-1055. Third cluster comprised of one

commercial variety, IC-116767 and a wild variety, *Cyamopsis serrata.* Two landraces i.e. IC-113304 and IC-116751 formed a sub-group. Three out-groups were formed comprising of M-83, IC-116958 and *Cyamopsis senegalensis* (Figure 4.18).

Table 4.7: List of number of polymorphic and unique bands obtained with each primer combination

S. No.	Primer combination	Number of polymorphic bands	Number of unique bands
1	E-AAG/M-CAA	36	20
2	E-ACT/M-CAA	25	7
3	E-AAC/M-CAA	21	5
4	E-AGG/M-CAA	22	1
5	E-ACA/M-CAA	11	1
6	E-ACC/M-CAA	17	7
7	E-AAG/M-CAC	11	7
8	E-ACT/M-CAC	28	6
9	E-ACT/M-CAC	11	1
10	E-AGG/M-CAC	21	8
11	E-ACA/M-CAC	20	7
12	E-ACC/M-CAC	24	4
13	E-AAG/M-CAG	6	5
14	E-ACT/M-CAG	29	21
15	E-AAC/M-CAG	13	5
16	E-AAG/M-CAT	5	3
17	E-ACT/M-CAT	19	7
18	E-AAC/M-CAT	21	5
19	E-AGG/M-CAT	14	3
20	E-ACA/M-CAT	30	9
21	E-ACC/M-CAT	36	12
22	E-AAG/M-CTA	9	9
23	E-ACT/M-CTA	22	6
24	E-AAC/M-CTA	1	1
	Total	457	160

A total of 457 polymorphic and 160 unique bands were obtained. E-AAG/M-CAA and E-ACC/M-CAT primer combinations gave maximum number of polymorphic bands i.e. 36 and maximum unique bands were obtained using E-AAG/M-CAA and E-ACT/M-CAG primer combinations. (Table 4.7)

Chapter V

Discussion

CHAPTER-5

Guar (*Cyamopsis tetragonoloba*) is an important leguminous plant which contains a large amount of industrially important gum in its endosperm as a storage polysachharide. Guar gum is mainly composed of galactomannan, a polysachharide containing mannan chain substituted by single galactose residues as a side chain. The resulting M/G ratio is an important aspect of such industrially important gums. The M/G ratio is different in different legumes such as guar has aM/G ratio of 1.6, fenugreek 1.1 and locust bean gum 4. This difference in M/G ratio has made the researchers curious about the mechanism of galactomannan biosynthesis. Until recently much work was not done in this area but after the discovery of some of the enzymes involved in the galactomannan biosynthesis pathway and other developments in legume genomics, the discoveries in the direction have taken up speed. In the present work, attempts were made in the same direction and developing seeds were studied at various stages. Also there is a need to develop some important markers in guar linked to specific traits that would help to screen a large number of varieties for important traits and also contribute to conventional breeding programmes.

5.1 Enzyme expression analysis

Total RNA extracted from the seeds collected at different stages of development was used to study the pattern of expression of 4 enzymes of the galactomannan biosynthesis pathway i.e. mannan synthase which makes β-1, 4-linked mannan backbone, galactomannan galactosyltransferase which adds galactosyl residues to the mannan backbone, alpha-galactosidase which is known to remove some galactose residues in the end and phoshomannoisomerase which is involved in the conversion of fructose-6-phosphate to mannose-6-phosphate. The expression of mannan synthase and galactosyltransferase was found to increase gradually with increasing developmental stages. This finding supported the hypothesis given by Edwards *et al.* (1989) that these two enzymes work

together in coordination. The transfer of D-galactosyl residues from UDP-galactose to galactomannan is absolutely dependent upon the simultaneous transfer of D-mannosyl residues from GDP-mannose. However, mannan synthase can form the mannan chain alone without the involvement of the other enzyme galactosyltransferase. Similar results were obtained by Dhugga *et al.* (2004) and Naoumkina *et al.* (2007). Alpha-galactosidase was not found to express in any of the stages suggesting that in cluster bean it may not have any role in regulating the man/gal ratio in the final stages by post-depositional modification but the genetic regulation of final M/G ratio resides in the biosynthetic process alone. Expression analysis for these enzymes was also done in other tissues like leaf, stem, root and flower bud. None of the enzymes was found to express in these tissues suggesting that they are endosperm specific enzymes involved in galactomannan biosynthesis which occurs only in the walls of the endosperm. No expression of phosphomannoismerase was found. This was in accordance with the results of Naoumkina *et al.* (2007) where EST libraries were screened and no ESTs corresponding to phosphomannoisomerase was found. These results suggested that specificity of galactosyltransferase can be the deciding factor for the Man/gal ratio. Therefore the protein sequence of galactosyltransferase from three species i.e. fenugreek (M/G-1.1), senna (M/G-3.3) and cluster bean (M/G-1.6) was compared by multiple sequence alignment and a dendrogram was generated. Clearly the two species, fenugreek and cluster bean, having almost similar man/gal ratio were shown to be closely linked on the basis of galactosyltransferase enzyme. Enzyme sequences for other genes involved in galactomannan biosynthesis were not available for similar analysis.

Other factors like availability of the substrate, especially galactose residues, should be the important factor in regulating the final man/gal ratio of guar galactomannans. For further studies more genes like galactose transporter genes, galactose epimerase gene etc. should be cloned first.

It is important to mention here that total RNA extraction from guar seed was difficult in the later stages of seed development i.e. 30-40 days after flowering. This may be attributed to the large amount of polysachharides present in its endosperm. A well known and frequently used method for RNA extraction using Trizol reagent was applied but satisfactory amount of RNA was not obtained. Finally hot phenol method was tried which gave fairly enough amount of RNA for the studies done. Therefore it is important that research must be done in this direction and a proper protocol for RNA extraction should be devised.

5.2 SSR analysis

In the present work molecular marker studies were conducted to undersand the genomics of guar Detection and analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants. Very few such studies have been done in cluster bean. In this report two marker techniques i.e. EST-SSR and AFLP were used to study 12 varieties of cluster bean. Among these 12 selected varieties, 5 were landraces, 5 commercial varieties and 2 wild varieties. Moreover these varieties have been chosen from different clusters formed on the basis of RAPD analysis (Nagesh K.A. unpublished results).

Twelve varieties were analyzed using 26 SSR primers. Out of this amplification was obtained with 20 primers, 6 were found to be polymorphic. The polymorphic bands were obtained in the wild variety, *Cyamopsis senegalensis.* All the bands were between 180-220 bp. More number of SSR primers can be used to analyze a population and develop markers to open more and more doors to understand the genomics of cluster bean. Clearly the wild variety *Cyamopsis senegalensis* out-grouped all the other varieties. With more number of primers, it is possible to develop some important markers for guar. Linkage maps can be generated using a population developed by crossing as in maize.

5.3 AFLP analysis

The 12 varieties used in SSR analysis were also used for another marker technique i.e. amplified fragment length polymorphism. For AFLP, standardization of the technique was done with one variety i.e. M-83. Eight MseI and eight EcoRI selective primers were chosen and all 64 combinations were used in selective amplification. Out of these 64 combinations, 8 combinations i.e. M-CAA/E-AAG, M-CAA/E-ACC, M-CAC/E-ACT, M-CAC/E-ACA, M-CAG/E-ACT, M-CAT/E-ACT, M-CAT/E-ACA, M-CTA/E-ACT which generated more fragments than others, were chosen after analyzing the explorer gel for further analysis in 12 varieties. All the accessions were then subjected to selective amplification with these primer combinations. The binary data obtained by using gene mapper software was converted to a similarity matrix and a dendrogram was generated which formed 3 clusters and 3-4 varieties out-grouped these clusters.

The main objective of the study using the marker systems was to assess the level of genetic diversity present in different varieties of cluster bean. Analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants. The high number of clusters from AFLP analysis indicates considerable genetic diversity in the accessions used for the study. Similar diversity was found in an AFLP analysis of dolichos bean [110]. Varieties from different classes i.e. landraces and commercial varieties were grouped in same clusters although one cluster comprising only of commercial varieties was also formed. Pattern of clustering was not according to the location from which they were collected. Similar conclusions were drawn by Patil *et al.* (2009) when genetic diversity in field bean was analyzed [85].

Thus it can be finally concluded that genes of the galactomannan biosynthesis pathway and the mannose and galactose transporter genes should be cloned. More and more groups are now focusing their attention on gum producing crops and the molecular mechanisms involved in

various pathway. Recently Naoumkina *et al.* (2007) developed a cDNA library of guar ESTs from developing seeds and cloned a mannan synthase promoter gene .

Marker analysis of guar and related crops i.e. legumes was also done by some other groups [110,85].

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