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Legumes in the Omic Era

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Chapter 11
Towards Enriching Genomic Resources in Legumes

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Abstract Food legumes, mainly comprising dry beans, dry peas, soybean, chickpea, pigeonpea, groundnut, greengram, blackgram, cowpea, lentil and lathyrus, have considerable area under cultivation globally and these are important constituents of cereal-based vegetarian diets. Keeping in view their tremendous importance for diversification and intensification of contemporary agriculture, systematic efforts towards their genetic improvement have been undertaken with classical breeding tools, lately complemented by the use of genomic tools. These genomic tools provide comprehensive information on genes involved in biochemical pathways leading upto nutritional compounds and can be used to understand the genetics of traits of interest and consequently, helping in marker assisted breeding. During the last two decades powerful genetic and genomic tools such as establishment of genetic and physical maps, expressed sequence tags, bioinformatic tools, genome-wide sequence data, genomic and metabolomic platforms, etc. have been developed for many legume species. These efforts have led to development of large scale molecular markers, identification of various marker trait associations, construction of genetic and linkage maps, expressed sequence tags database, partial or whole genome sequences, physical and molecular maps, DNA chips and bacterial artificial chromosome (BAC) libraries. After the genome sequencing of three model species, *Medicago*, *Lotus* and *Glycine*, draft genome sequences have recently been made

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available in agronomically important food legumes, pigeonpea and chickpea while similar efforts are underway in groundnut and greengram. The new generation sequencing (NGS) and genotyping platforms such as 454/FLX sequencing and Illumina GoldenGate/Solexa have revolutionized plant genomic research as these generate millions of ESTs per run. With the increased amount of genomic resources, there are now tremendous opportunities to integrate these with the genetic resources for their widespread use in routine legume improvement programmes by integrating them with conventional breeding tools. As a result, the genomics assisted breeding (GAB) can now be successfully used in legume improvement and development of improved genotypes having improved agronomic and quality traits and resistance to biotic and abiotic stresses. This chapter discusses the developments made in development of legume genomics and their role in overall improvement of food legumes.

Keywords Genomic resources • Molecular markers • Genomic library • Whole genome sequencing • Comparative genomics • Genomics assisted breeding

Introduction

Legumes are important source of food, feed and fodder in many agricultural systems and are grown on a large scale in semi-arid tropics of the world. Grain legumes alone contribute 33 % of human protein nutrition (Vance et al. 2000) and have a unique ability to fix the atmospheric nitrogen in symbiotic association with *Rhizobium* bacteria, which not only enables them to meet their own nitrogen requirement but also benefit the succeeding crops. Improvement in agronomic and phenological traits of the legumes is crucial in order to improve their use as human food and sustainability of production system. Therefore, yielding ability, seed and quality characteristics, resistance to biotic and abiotic stresses, storability, etc. are receiving greater attention for the genetic improvement of legumes. There is also an increasing interest in improving nutritional characteristics of legumes with enhanced content of β-carotene, leutin, isoflavones and other nutraceuticals.

The way to development of better food and forage legumes requires a detailed knowledge of the different genes involved in biochemical pathways leading upto nutritional compounds, including the expression patterns and level of these genes and their interactions (Gepts et al. 2005). Genomic resources are important to understand the genetics of traits of interest and consequently, marker assisted backcross breeding (MABC), marker assisted recurrent selection (MARS) and advanced backcross (AB) breeding may be used effectively in legume improvement. A great success in this will be possible by combining genomic tools with rational selection of germplasm and precise phenotyping for traits of interest, termed as "genomics-assisted breeding" (Varshney et al. 2005). During the last two decades powerful genetic and genomic tools such as establishment of genetic and physical maps, expressed sequence tags (ESTs), bioinformatic tools, genome-wide sequence data,

genomic and metabolomic platforms have been developed for many legume species. This chapter gives a comprehensive view of development and utilization of genomic resources in major food legume crops.

Genomic Resources in Legumes

Over the past many years, there has been an increased focus on application of powerful genomic approaches to major legume species with an aim of generating genomic resources that will not only be of use in these species but also facilitate crop improvement in other species also. Apart from two model legumes, *Medicago truncatula* and *Lotus japonicus*, efforts have been made in developing genomic resources in common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), soybean (*Glycine max*), pigeonpea (*Cajanus cajan*), *alfa alfa* (*Medicago sativa*), chickpea (*Cicer arietinum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), pea (*Pisum sativum*) and peanut (*Arachis hypogaea* L.). However, these legumes differ greatly in their genome size, base chromosome number, ploidy level, and compatibility status (Table 11.1). The efforts have led to development of large scale molecular markers, identification of various marker trait associations, construction of genetic and linkage maps, expressed sequence tags (EST) database, partial or whole genome sequences, physical and molecular maps, DNA chips and bacterial artificial chromosome (BAC) libraries in all these crops (Table 11.2 and 11.3). Among the agronomically important food legumes, draft genome sequence has recently been made available in pigeonpea (Singh et al. 2012; Varshney et al. 2012) and chickpea (Varshney et al. 2013a) and similar efforts are underway in groundnut.

Table 11.1 Variation in basic chromosome number and genome size among legume species

Species	Basic chromosome number (X)	Genome size (Mb)
Peanut (*Arachis* spp.)	10–20	1,260–2,890
Lupin	5–13	468–1,177
Common bean (*Phaseolus vulgaris*)	11	637
Cowpea (*Vigna unguiculata*)	11	620
Pigeonpea (*Cajanus cajan*)	11	858
Soybean (*Glycine max*)	20	1,115
Lotus japonicus	6	472
Pea (*P. sativum*)	7	4,400
Lentil (*L. culinaris*)	7	4,063
Chickpea (*C. arietinum*)	8	740
Alfalfa (*M. sativa*)	8	800–900
Vicia faba	7	–
Medicago truncatula	8	500–550

Table 11.2 Current availability status of genomic resources in pulses

Genomic resources	Chickpea	Common bean	Pigeonpea	Cowpea	Lentil	Mung/Urdbean	Fieldpea
Mapping populations	+	+	+	+	+	+	+
BAC based resources							
BAC libraries	+	+	+	+	–	+	+
BAC-end sequences	+	+	+	+	–	–	–
Physical map	+	+	*	+	–	–	–
Second and third generation DNA markers							
Genomic SSRs	+	+	+	+	+	+	+
Genic or EST-SSRs	+	+	+	+	+	+	+
SNP	+	+	+	+	–	–	+
DArT	+	+	+	–	–	–	
SFP	–		+	+	–	–	–
Transcriptomic resources							
ESTs	+	+	+	+	+	+	+
Transcriptome assemblies	+	+	+	+	+	+	+
Published genetic maps							
Population specific (inter-specific/inter-subspecific and cultivated)	+	+	+	+	+	+	+
Consensus maps	+	+	+	+	–	–	+
Whole genome sequence	+	*	+	*	*	*	*

+ available; – not available; *in progress

Table 11.3 Important genomic resources in major food legumes developed in last 5 years

Genomic resources	Crop	References
BAC libraries and BAC end sequences	Chickpea	Thudi et al. (2011)
	Common bean	Córdoba et al. (2010)
	Pigeonpea	Bohra et al. (2011)
	Cowpea	Yu (2012); http://www.comparative-legumes.org/pages/resources
Large scale SSR/SNP markers	Chickpea	Thudi et al. (2011); Hiremath et al. (2012): Gaur et al. (2012)
	Common bean	Hyten et al. (2010)
	Pigeonpea	Raju et al. (2010); Bohra et al. (2011); Dubey et al. (2011); Kassa et al. (2012)
	Cowpea	Muchero et al. (2009); Lucas et al. (2011)
High throughput genotyping platforms		
DArT arrays	Chickpea	Varshney et al. (2010)
	Common bean	Briñez et al. (2011)
	Pigeonpea	Yang et al. (2011)
GoldenGate/KASPar assays	Chickpea	Hiremath et al. (2012); Gaur et al. (2012)
	Common bean	Cortés et al. (2011); Hyten et al. (2010)
	Pigeonpea	Kassa et al. (2012)
	Cowpea	Muchero et al. (2009); Lucas et al. (2011)
First genetic maps	Mungbean	Isemura et al. (2012)
	Pigeonpea	Yang et al. (2011); Bohra et al. (2011, 2012)
High density genetic maps	Chickpea	Thudi et al. (2011), Hiremath et al. (2012), Gaur et al. (2012)
	Common bean	Galeano et al. (2011)
	Cowpea	Muchero et al. (2009); Lucas et al. (2011)
Physical maps	Chickpea	Zhang et al. (2010)
	Common bean	http://cmap.comparative-legumes.org
	Cowpea	http://phymap.ucdavis.edu/cowpea/
Draft genome sequences	Pigeonpea	Singh et al. (2012); Varshney et al. (2012)

Genome Sequences

The three model species, *Medicago*, *Lotus* and *Glycine* are the first legume crops to have their genomes sequenced. Among these, *M. trancatula* and *L. japonicus* were chosen for genome sequencing largely because of their small diploid genomes (ca. 500 and 471 Mb in size), shorter life cycle and availability of supportive resources (Young et al. 2005). The information generated by genome sequencing of these two species has provided greater insight into their gene structure as well as their physical and genetic maps. Though, the sequencing of both these species was initiated at almost the same time, the approaches used for sequencing differed slightly in these. While for sequencing of *Lotus* genome, a modified BAC-by-BAC approach followed by draft sequencing of the selected regions of the genome was followed, in *Medicago* genome sequencing project,

a traditional BAC-by-BAC approach was followed, though it was focused on the euchromatic part of the genome. In *Medicago*, 0.6–0.7 of the estimated euchromatic genomic region has been sequenced, capturing about 0.60 of the genes (Kumar et al. 2011). The sequencing is expected to be completed soon; having an assembly of c. 300 Mb and capturing about 0.90 % of the genes. In case of *Lotus* genome also, considerable progress has been made with sequencing of about 0.67, covering 0.91 % of the gene space (Sato et al. 2008). In both cases, however, the traditional type of sequencing method was used.

For sequencing of soybean, a Phaseoloid legume, another genome sequencing method- whole genome shotgun (WGS)- was used. Soybean is an excellent representative of polyploid species and it was chosen as a model legume for sequencing (Gepts et al. 2005) due to its moderate genome size (ca. 1,115 Mb), available infrastructure (Jackson et al. 2006) and also due to its economic importance (Nunberg et al. 2006). Soybean WGS comprises 950 Mb of assembled and anchored sequences representing about 0.85 of the predicted genome size. It has been predicted that the soybean genome has 46,430 protein coded genes and about 0.75 of these genes are there in multiple copies (Schmutz et al. 2010). Though this approach is powerful and fast, but it is largely suitable to smaller and less complex genomes. Another Phaseoloid legume, common bean, a diploid species, has medium sized genome (588–637 Mb) (Bennett and Leitch 2012). Besides the small size, it was chosen for genome sequencing due to availability of good amount of genomic resources such as availability of 9X physical map, BAC libraries, 25 linkage, 83,530 ESTs and knowledge of the genic (0.29) and repetitive (0.49) portions of the genome (see Kumar et al. 2011). Its extensive macrosyntentic relationships with soybean has also favoured its candidature for best model species for soybean and other legume species in order to develop new SSR and SNP markers and also for identification of candidate genes.

Most recently, the draft genome sequence has been made available in chickpea, the second most important grain legume after soybean. In *kabuli* chickpea variety, CDC Frontier, ~738 Mb long draft WGS sequence has been reported which contains 28,299 genes (Varshney et al. 2013a). Re-sequencing of 90 more chickpea genotypes was also done which provided an access to millions of genetic markers and low diversity genome regions that may be useful in the development of superior varieties with enhanced drought tolerance and disease resistance. The genome map will also help tremendously in harnessing genetic diversity by broadening the genetic base of cultivated chickpea genepool. In pigeonpea, draft genome sequence has been made available by two independent groups almost at the same time (Singh et al. 2012; Varshney et al. 2012). For generating the genome sequence in this crop, the ICRISAT led team used Illumina next-generation sequencing platform to generate 237.2 Gb of sequence, which along with Sanger-based bacterial artificial chromosome end sequences and a genetic map, was assembled into scaffolds representing 72.7 % (605.78 Mb) of the 833.07 Mb pigeonpea genome. Genome analysis predicted 48,680 genes for pigeonpea and also showed the potential role that certain gene families have played throughout the domestication of pigeonpea and the evolution of its ancestors. In another independent approach by Singh et al. (2012),

the whole genome of pigeonpea was assembled using long sequence reads of 454 GS-FLX sequencing with mean read lengths of >550 bp and >10X genome coverage, resulting in 510,809,477 bp of high quality sequence. Total 47,004 protein coding genes and 12,511 transposable elements related genes have been predicted in this study. Further, 1,213 disease resistance/defense response genes and 152 abiotic stress tolerance genes were also identified. This genome sequence was also used to identify large number of hypervariable pigenpea simple sequence repeat (HASSR) markers, 437 of which have been experimentally validated for PCR amplification and high rate of polymorphism among pigeonpea varieties. These markers will be immensely useful for fingerprinting and diversity analysis of pigeonpea germplasm and molecular breeding applications. Efforts are already underway to make the draft genome sequence available in peanut very soon. However, in most of the other food legumes, with the exception of pea (*P. sativum*), alfalfa (*M. sativa*), peanut (*Arachis hypogaea*) and cowpea where some progress has been made recently, lesser genomic information is available. In cowpea, genome filtering method has been used for sequencing and analyzing the gene-rich regions (hypomethylated portion of the cowpea genome). This has led to development of >250,000 gene-space sequence reads (GSRs) with an average length of 610 bp yielding ~160 Mb of sequence information (Timko et al. 2008). Among the GSR dataset, 29 % of the sequences annotated using the *Arabidopsis* gene ontology (GO) was involved to encode the majority of cellular enzymes and components of amino acid, carbohydrate and lipid metabolism. Besides, a total of 5,888 GSRs had homology to genes encoding transcription factors (TFs) and about 5 % of the total annotated sequences in the dataset have represented transcription associated factors (TAFs). This information can be utilized in mapping and tagging the genes for agronomically important traits in legumes.

BAC/BIBAC Resources

The bacterial artificial chromosome (BAC) and binary bacterial artificial chromosome (BIBAC) libraries are good genomic resources that allow genome sequencing, development of new molecular markers and physical map, and map based cloning of genes (Tao et al. 2001). In several legume species, these libraries have been developed with varying clone sizes from 100 to 150 kb. In chickpea, a BIBAC library of 23,780 clones, with an average insert size of 100 kb and a coverage of 3.8 genome equivalents, was prepared for facilitating the development of not only genomic SSRs but also gene specific SSRs (Rajesh et al. 2004).

In soybean, a genome-wide physical map has been constructed from more than 78,000 BAC clones, representing 9.6X genome. It consisted of approximately 2,905 contigs which were estimated to span 1,408 Mb in physical length (Wu et al. 2004). More than half of the length of the physical map was anchored to the genetic map using 388 DNA markers. Earlier, using molecular markers, physical map from BAC clones could also be related to the genetic map by locating existing genetic markers on the contigs (Lewers et al. 2002). These contigs work as a starting point for

positional cloning of specific genes which has accelerated the discovery of genes underlying phenotypes of agronomic interest (Liu et al. 2001; Xu et al. 2001). BAC libraries have also been used to generate SSR markers leading to identification of two genomic regions involved in resistance to the soybean cyst nematode (Cregan et al. 1996, 1999). Moreover, these also helped in fine mapping of genes leading to identification of tightly linked markers for marker-assisted selection. In cowpea, 60,000 BAC clones were assembled into a 10X physical map and efforts are already underway to anchor the cowpea physical map to the emerging SNP-based genetic linkage map. In common bean, sequencing of 89,000 BAC ends has yielded a 9X draft physical map which represents 62 Mb of genome sequence or 9.5 % of the common bean genome (Schlueter et al. 2008). In this map, 540 markers derived from RFLPs, genes, ESTs and other sequences have been anchored, of which 84 are genetically mapped and provide linkage between the physical and genetic maps (see at http://phaseolus.genomics.purdue.edu/).

In pea (*Pisum sativum* L.), two BAC libraries, which are useful resources for the isolation of genes underlying disease resistance and other economically important traits have been constructed. These libraries separately contained 55,680 and 65,280 clones, of which ~1 % clones were from chloroplast origin (Coyne et al. 2007). In peanut also, the BAC libraries from the AA genome (*Arachis duranensis*) with 84,096 clones and from the BB genome (*A. ipaënsis*) with 75,648 clones having average insert size of 110 and 100 kb, have been constructed. An estimate based on the library average insert size and *A. duranensis* haploid genome equivalent to 1,260 Mb showed that the coverage of the AA genome BAC library is equivalent to 7.4X genome. However, for *A. ipaënsis*, the DNA-content determination is controversial and hence the BB genome BAC library for *A. ipaënsis* could represent from 2.7 to 5.3 the haploid genome equivalents of the species considering the earlier discrepancies in estimation of haploid genome size (Varshney et al. 2009b). The BAC-based resources developed in different species will have greater utility for subsequent genome analyses, because they provide the basis for a physical interpretation of other genetic and genomic resources within each species, and they will facilitate more detailed analysis of high value regions of the genomes of legumes.

Molecular Markers

In most of the legume species, several DNA-based marker systems such as single nucleotide polymorphism (SNP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs) or microsatellites, amplified fragment length polymorphisms (AFLPs) and hybridization based marker systems such as restriction fragment length polymorphisms (RFLPs) and diversity arrays technology markers (DArT) are now available. However, PCR (Polymerase Chain Reaction) based SSR and SNP markers are preferred by breeders because of their high reproducibility, high level of polymorphism and user friendliness. SSR markers have the advantage of being multi-allelic and co-dominant (Gupta and Varshney 2000). Further, these can easily be employed in genotyping of large segregating populations in a

cost-effective manner and with minimum infrastructure facilities. While in many crops, these have been extensively utilized, in pulses their use is still limited to only a few crops like chickpea and pigeonpea (Varshney et al. 2009a; Saxena and Nadarajan 2010). Among the different marker systems used in pulses and other crop plants, SNP markers are high throughput and cost effective (Varshney et al. 2012). Similarly, diversity array technology (DArT) marker system is used for diversity studies, saturating linkage maps and identifying alien introgressions. The following section describes the most popular molecular marker systems in legumes.

SSRs

Since the SSR markers are the markers of choice in legume improvement, their availability has great significance in legume species for practical purposes. Over the years, a large number of SSR makers have been developed for many legume species by using following approaches individually or in combination (Varshney et al. 2012): (a) construction and sequencing of SSR enriched genomic DNA libraries, (b) sequencing and mining the BAC (bacterial artificial chromosome)-end sequences (BES) for SSRs, and (c) mining the transcript sequences generated by either Sanger sequencing or next generation sequencing (NGS) approaches such as 454/FLX sequencing (for details see Kumar et al. 2011). Most recently, 487 novel markers including 125 EST-SSRs, 102 SNPs, 151 intron targeted primers, 109 EST polymorphisms have been developed in chickpea (Choudhary et al. 2012). Similarly, about 2,000 new SSRs have also been developed earlier using genomic DNA libraries (Nayak et al. 2010; Gaur et al. 2011), ESTs (Varshney et al. 2009b), BAC end sequences (Thudi et al. 2011) and 454/FLX transcript reads (Garg et al. 2011a, b). These markers are also in use in other legume species including cowpea (768 BAC end sequence-BES-SSRs), lentil (100 genomic SSRs) and common bean (ca. 500 SSRs) (see Kumar et al. 2011). In peanut, ca. 6,000 markers are now available for use (Pandey et al. 2012). Most recently, 3,072 BES-were developed in pigeonpea (Bohra et al. 2011). Besides 3,583 SSRs from ESTs (Raju et al. 2010) and 454/FLX sequences (Dubey et al. 2011 and Dutta et al. 2011) are also available for molecular marker assisted breeding programmes.

DArT

DArT marker system has tremendous use for diversity studies and identification of alien introgressions, especially from wild species into the cultivated ones. Recently, by using 1225 DArT markers in the cross between *C. platycarpus* and *C. cajan*, 2–5 % *C. platycarpus* genome-carrying genes for disease and insect resistance were observed (Mallikarjuna et al. 2011). Yang et al. (2011) developed first generation array comprising 6,144 clones in pigeonpea. Similarly, ICRISAT has developed

Table 11.4 EST database of food legumes (as on 28 Nov 2012 at NCBI)

Common name	Botanical name	EST submitted in NCBI
Soybean	*Glycine max*	1468424
Burclover	*Medicago truncatula*	286175
Cowpea	*Vigna unguiculata*	189593
Chickpea	*Cicer arietinum*	46064
Pigeonpea	*Cajanus cajan*	25577
Mungbean	*Vigna radiata*	1604
Blackgram	*Vigna mungo*	311
Field pea	*Pisum sativum*	21837
Common bean	*Phaseolus vulgaris*	149769

DArT assays comprising 15,360 clones in chickpea, pigeonpea and groundnut and diversity study using these showed a narrow genetic diversity in the elite gene pool in comparison to the landraces and wild species (Varshney et al. 2012). Recently, DArT arrays have also become available in common bean (Briñez et al. 2011).

EST Databases

Extensive efforts have been made in sequencing expressed genomic regions obtained from tissues in different conditions and developmental stages, leading to deposition of large number of EST sequences in the public database (Kumar et al. 2011; Table 11.4). The EST databases provide an effective tool for gene discovery and generate raw material for the production of cDNA arrays for transcriptome analysis (Coram and Pang 2005a). As a result, these easily accessible EST sequences have emerged as cost-effective valuable source for in silico generation of markers and broaden the field of comparative mapping in species where limited or no sequence information is available. EST database provides the first insight into the genes that may be associated with root development and abiotic stress tolerance, particularly in crops like chickpea (Jayashree et al. 2005). EST libraries have been generated and analysed in chickpea for isolation of candidate genes controlling defence mechanism in *Ascochyta* blight (Coram and Pang 2005b). The identified ESTs have putative relationships with proteins involved in drought tolerance and hence provided a useful resource for identification of candidate gene or mining the alleles responsible for drought avoidance and tolerance in cool season legumes (Buhariwalla et al. 2005).

Microarrays or DNA Chips and Transcriptome Analysis

Microarrays or DNA Chips are important tools of functional genomics for identifying the network of genes underlying the expression of agronomically important traits (Meyers et al. 2004). These can be developed from hundreds of thousands ESTs or cDNA libraries available in model and other legume species (Table 11.5).

Table 11.5 Microarray data tools developed in legume species

Species	No. of genes/cDNA clones on microassays	Plant tissue from which cDNA library developed	Remarks	References
M. truncatula	2,268 cDNA	Arbuscular mycorrhiza root	–	Liu et al. (2003)
	6,000 unigenes	Root symbiotic interaction	Mt6k-RIT	Yahyaoui et al. (2004)
	2,26,923 high quality ESTs	The TIGR Gene Index databases	–	Lee et al. (2005)
	Mt6k-RIT clones as well as 6,144 unigenes	Different stage of root nodule development	–	Lohar et al. (2006)
	Mt6k-RIT extended with 2 k-set of cDNA clones	Flowering and pod development stage	Mt8k-RIT	Firnhaber et al. (2005)
	16,086 oligo probes from tentative consensus (TC) sequences	Different developmental tissues	Mt16kOLI1	http://www.eugrainlegumes.org
	Mt16LI1 oligo probes along with 384 probes targeting TFs and other regulators	Different developmental tissues	Mt16kOLI1Plus (This microarray chips represent 35–40 % genes of *M truncatula*	http://www.genetik.uni-bielefeld.de/MolMyk/
Pea	70mer oligonucleotide microarray derived from ~5,200 EST	Cotyledon and seed coat ESTs	Ps6kOLI1	http://www.grainlegumes.com/aep/r_d_projects/grain_legumes_glip/progress_in_glip/integrated_activities/gene_expression_profili1ng
Mungbean	EST 6,272 genes (a unigene set of 2,013 comprising of 973 contigs and 1,040 singletons and 262 genotype specific SNPs)	Etiolated seedlings	–	Kuhar et al. (2012)
Chickpea	2,100 EST 65,000–68,000 singletons	Root and collar tissue from 25 days old seedlings	–	Ashraf et al. (2009)
Phaseolus	2,900 cDNA clones	–		Gepts et al. (2008)

In *M.truncatula*, microarrays developed using EST and other oligo sequences were used to study the expression of genes involved in nodule formation during symbiotic association, and in development of flower, pod and seed. A commercial affymetrix chip with a 51 k GeneChip including cDNA-microarrays and 70-mer oligonucleotide microarrays of different tissues developed in this species are useful genomic resource for comparative analysis of gene expression in related grain and forage legumes. The use of these DNA microarrays/chips has led to identification of thousands of genes that are induced or repressed during the development of nodules and symbiotic nitrogen fixation (Kuester et al. 2004; Baier et al. 2007; Benedito et al. 2008; Jones et al. 2008). Several other studies have also investigated the transcriptional basis of seed development, differentiation, desiccation, plant responses to aluminium toxicity, and changes in nitrogen nutrition using microarray chips (Buitink et al. 2006; Verdier et al. 2008; Narasimhamoorthy et al. 2007; Chandran et al. 2008). Long-oligo arrays of *M. truncatula* have also been used effectively to identify the transcripts upregulated in alfalfa trichomes secreting the molecules during insect defense (Aziz et al. 2005).

In Pea (*Pisum sativum*), a microarray (Ps6kOLI1) consisting of 70-mer oligo probes targeting ~5,200 EST clusters assembled predominantly from cotyledon under GLIP has been developed primarily for identifying genes relevant to seed formation. Similarly in soybean, high-density expression arrays containing 18,000 cDNAs arrayed on a filter have been developed (Shoemaker et al. 2003) and three microarrays comprising low redundancy unigene sets of 27,513 clones (each microarray with 9,728 unigenes) have been constructed from a variety of cDNA libraries made from a wide range of organs at different developmental stages, disease-challenged tissues, and various stress conditions. These microarrays have been used to examine tissue specific gene expression and global expression in mutant isolines which led to identification of set of candidate genes potentially encoded or modulated by the mutant phenotype (Vodkin et al. 2004). The microarray tools developed in soybean have been used successfully to identify genetic markers closely linked to soybean aphid resistance gene *Rag1* (Kaczorowski et al. 2008), and genes involved in the soybean iron deficiency chlorosis response under iron deficient conditions (O'Rourke et al. 2007).

In chickpea, 768-feature microarray was developed that comprised 559 chickpea cDNAs, 156 grass pea cDNAs, 41 lentil resistance gene analogs (RGAs) and 12 controls. Using this microarray, the transcriptional change in genes responsible for different abiotic stresses was observed leading to identification of 2, 15 and 30 genes differentially expressing between tolerant and susceptible genotypes for drought, cold and high-salinity, respectively. These genes code for various functional and regulatory proteins. Significant differences in stress responses were observed within and between tolerant and susceptible genotypes highlighting multiple gene control and complexity of abiotic stress response mechanism in chickpea (Mantri et al. 2007, 2010). In case of lentil also, a cDNA microarray approach has deciphered the *Ascochyta* blight resistance (Mustafa et al. 2009).

New Generation Tools for Legume Genomics

High Throughput Sequencing/Genotyping Platform

New generation sequencing (NGS) and genotyping platforms such as 454/FLX sequencing and Illumina GoldenGate/Solexa have revolutionized plant genomic research by generating millions of ESTs per run. The advantage with these sequencing methods is that these are not limited by prior knowledge of transcribed sequences or predicted genes. Approximately 75 million ESTs have been generated in *M. truncatula* using an Illumina/Solexa resulting in quantitative expression data complement and extend Affymetrix Gene Chip data (Benedito et al. 2008; Young and Udvardi 2009). Next-generation sequencing may also become an attractive option for transcriptomics of non model species where DNA arrays are unavailable, especially if sequence lengths can be increased to facilitate alignment and contig assembly. Using 454/FLX sequencing at ICRISAT in collaboration with JCVI and NCGR, 435,184 and 496,705 sequence reads providing 44,852 and 48,519 contigs were obtained from chickpea and pigeonpea, respectively. These sequence data provide access to a significant fraction of the total transcriptomes of these crops, and are expected to aid in the analysis of drought tolerance, including candidate gene discovery and the development of molecular markers for breeding applications (Varshney et al. 2005). In another study, 2,496 ESTs were generated and utilized in chickpea for the development of 487 novel EST-derived functional markers including 121 EST-SSRs, 151 intron targeted primers, 109 EST polymorphisms (ESTP) and 102 SNPs (Choudhary et al. 2012). While EST-SSRs, ITPs and ESTPs were developed by in silico analysis of the developed EST sequences, SNPs were identified by allele resequencing and their genotyping was done using Illumina GoldenGate Assay. In groundnut, Sanger sequencing, which is slightly more extensive, has been conducted which resulted in 54,000 ESTs for cultivated groundnut (*A. hypogaea*) and 6,000 in the diploid *A. stenosperma.*

The NGS platforms are also important tools for discovery of SNPs, especially in legumes having a narrow genetic base. Development of large-scale SNP markers may help accelerate linkage mapping and whole genome association (WGA) studies. In this connection, efforts have been made by several institutions for developing the SNP markers in cowpea, pigeonpea, chickpea and groundnut (reviewed by Varshney et al. 2009a, b). Recently, 26,082 SNPs have been identified in chickpea based on alignment of approximately 37 million Illumina/Solexa tags generated from ICC4958 and ICC1882 genotypes (Hiremath et al. 2011). In pigeonpea, 12,141 SNPs have been identified in ten parental genotypes based upon the alignment of 160 million reads against a transcriptome assembly (CcTAversin 1.0) (Dubey et al. 2011). Further, comparison of transcript reads from 12 different pigeonpea genotypes has led to identification of 28,104 novel SNPs (Varshney et al. 2012). Kudapa et al. (2012) developed a comprehensive transcriptome assembly for pigeonpea by

analysing 128.9 million short Illumina GA IIx reads, 2.19 million single FLX/454 reads and 18,353 Sanger expressed sequenced tags from more than 16 genotypes. Based upon the knowledge of intron junctions, 10,009 primer pairs were designed from 5,033 TACs for amplifying intron spanning regions (ISRs). These ISR markers will be immensely beneficial to accelerate breeding and genetic research in pigeonpea. Similarly, KASPar assays from another next generation SNP genotyping technology, have also been developed for 2,005 SNPs in chickpea (Hiremath et al. 2012) and 1,616 in pigeonpea.

Serial Analysis of Gene Expression

Serial analysis of gene expression (SAGE) is an approach that allows rapid and detailed analysis of thousands of transcripts. In case of chickpea, 80,238 26-bp tags representing 17,493 unique transcripts (UniTags) from drought-stressed and non-stressed control roots have been generated using SuperSAGE technology for the analysis of gene expression in chickpea roots in response to drought (Molina et al. 2008). Sanger sequencing has been used to a limited extent to access the chickpea and pigeonpea transcriptomes (27,000 and 13,000 ESTs, respectively).

RNAi and TILLING

Forward genetics which aims at identifying the responsible genes for a trait, can be performed through map based cloning and T-DNA and transpose insertional or insertion mutagenesis. This has been used widely for identification and cloning of genes for a known phenotype (Kumar et al. 2011). For example, in *L. japonicus*, two new *Sym* genes (*LjSym1* and *LjSym2*) have been isolated through map-based cloning approach. *LjSym2* is required for symbiosis involving both arbuscular mycorrhizal (AM) fungi and rhizobia in root nodules (RNs) while, the *LjSym2* gene encodes a receptor-like kinase (Endre et al. 2002). Another important approach is reverse genetics approach for which mutant population can be a valuable resource. Such mutant populations can be generated through T-DNA and retrotransposon insertions where gene sequences or a protein with unknown function are associated with responsible phenotype. Following this approach, a population in *M. truncatula* mutagenized by a tobacco retrotransposon, *Tnt1* has become an important resource for reverse genetics (D'Erfurth et al. 2003). Screening this population by sequencing of tagged sites led to the isolation of *M. truncatula"Pim"*gene (Benlloch et al. 2006).

More recently, RNAi technology or virus induced gene silencing, have become important resources for knowing the function of genes (Allen et al. 2004). In legumes, virus-induced gene silencing has been used in pea (Constantin et al. 2004). In soybean, RNAi induced gene silencing has been successful using transformation methods, either through biolistics or *Agrobacterium tumefaciens* (Reddy et al. 2003;

Subramanian et al. 2005; Nunes et al. 2006), hairy root transformation (Jackson et al. 2006), transposon mutagenesis (Jackson et al. 2006) and virus-induced gene silencing. Targeting induced local lesions in genomes (TILLING) or deletion-TILLING (de-TILLING) is a reverse genetics approach which uses knowledge of gene sequence having unknown function to know their function or phenotype. A large number of TILLING resources have been developed in several legume species (Table 11.6). Using this approach, approximately 2,000 individual germlines have been generated in *Medicago truncatula* (Vanden Bosch and Stacey 2003). Similarly, in *Lotus japonicusa* population of >40,000 mutants was developed through induced mutation by using 1 % v/v EMS comprising mutants defective for morphological, metabolic and nodule formation characters (Perry et al. 2003) and also the mutants having variant alleles of SYMRK and sucrose synthesis genes using TILLING procedure (Stracke et al. 2002; Horst et al. 2007). The TILLING resources developed in different legumes have provided notable functional genomic resources to the legume researchers towards knowing the function of genes.

Use of Genomic Resources in Legume Improvement

With the development of large scale genomic resources in major food legumes, there are now tremendous opportunities to integrate them with genetic resources for their widespread use in routine breeding practices and their integration with conventional breeding tools. As a result, the genomics assisted breeding (GAB) can now be successfully used in legume improvement for development of improved genotypes having resistance to biotic and abiotic stresses and improved agronomic traits. The available genomic resources have successfully been used in legumes for hybridity confirmation, diversity analysis studies, marker assisted breeding, genome wide selection and advanced back cross QTL analysis.

Hybridity Confirmation

In most of the legumes species, making crosses is difficult as compared to cereals owing to small size of the flower and a weak peduncle supporting the bud. Legumes being self pollinated crops, have increased chances of selfing. Furthermore, differentiating between the selfed and F_1 plants is also difficult due to low phenological diversity between the selfed and crossed plants. Marker assisted identification of true F_1 hybrids is a robust and full-proof approach for identification of true hybrids and therefore increasing the efficiency of selection of desired recombinants. This approach is now being routinely used in identification of true F_1 plants in chickpea in the crosses between Pusa 256 × Vijay and Pusa 256 × WR315 at IIPR, Kanpur; C104 × WR315 and C 214 × ILC 3279 at ICRISAT; JG 74 × WR 315 at JNKVV, Jabalpur; Phule G12 × WR 315 at MPKV, Rahuri and Annigeri-1 × WR 315 at ARS

Table 11.6 TILLING resources in legumes

Species	Type of population	Population size (nos)	Mutagen used	Remarks	References
Medicago	Induced	5,000 M1 and 5,000 M_2	EMS (0.2 %)	9–12 alleles/1 kb target sequence	http://www.gl-http.com/products_services/technical_services/genomic_resources_from_glip/functional_genomics
		8,000 M_2		9.8 alleles	Triques et al. (2007)
Pea	Induced	48,000 M_2 and 5,000 M_2	EMS (0.2–0.3 %)	10–40 alleles/1 kb target sequence	-do-
	Natural	400 germplasm lines	–	Identification of hidden allele and association mapping	-do-
	Induced	4,817 lines	93 symbiotic mutants 26 genes involved in nitrogen fixation		Hofer et al. (2009)
Mungbean	Natural	25 lines	–	Single nucleotide polymorphisms (SNPs) and small insertions/deletions (INDELS) in a collection of *Vigna radiata* accessions	Barkley et al. (2008)
L. japonicus	Induced	45,600 M_2	(1 % v/v)	Population comprises to morphological, symbiotic and metabolic mutants of *L. japonicus*. Population was used to identify mutants for sucrose synthesis genes (LjSUS1 to LjSUS4)	Perry et al. (2003); Horst et al. (2007); Stracke et al. (2002)
	Induced	4,904 M_2	EMS		Perry et al. (2009)

Gulberga in a molecular breeding network project funded by Department of Biotechnology, Government of India. In lentil also this approach has been successfully applied with 21% F_1 plants identified at true F_1s and the others as selfed or admixtures (Solanki et al. 2010).

Diversity Analysis Studies

Moleculer markers greatly help in studying the availability and level of genetic diversity among the different gene-pools (Zong et al. 2009; Taunk et al. 2012). Diversity analysis studies in food legumes which have a comparatively narrow genetic base may also help in identifying contrast parents for development of ideal mapping population for a variety of uses. Comprehensive assessment of genetic diversity help identify and rescue the genetic resources at the verge of extinction (Polegri and Negri 2010). The genetic diversity estimates using molecular markers in different crops including pea demonstrated that no gain or reduction of genetic diversity has occurred in last five decades (van de Wouw et al. 2010).

Marker Assisted Breeding

Marker assisted recurrent selection (MARS) and marker-assisted backcrossing (MABC) are the two approaches of marker assisted breeding in legumes as well as other crops. MABC involves introgression of specific trait(s) from a donor parent into the genetic background of a recurrent parent using molecular markers (Hospital 2005). This approach can also be used to generate near-isogenic lines (NILs) or chromosome segment substitution lines (CSSLs) for genomics research, which are populations that are often used for genetic analysis of genes/QTLs and alien gene introgressions (Varshney et al. 2013b).Use of MAS is especially advantageous for traits with low heritability where traditional selection in difficult, expansive, or lacks accuracy or precision (Varshney et al. 2010).

MARS is used to estimate the marker effects from genotyping F_2 or F_3 population and phenotyping F_2 derived F_4 or F_5 progenies, followed by two or three recombinant cycles based on presence of marker alleles for small effect QTLs (Eathington et al. 2007). For MARS, identification of QTL in the population (generally good × good cross) is followed by crossing the lines carrying superior alleles for maximum QTLs to pyramid superior alleles in a single genetic background. The resultant recombinant lines are screened finally in the field to identify the best lines for their multi-locational evaluation and their possible release as a cultivar. The genetic gain achieved in MARS is higher because it captures several genomic regions at a time, and more number of major and minor QTLs (Bernardo and Charcosset 2006).

Knowledge of marker-trait association provides greater insight to the breeders in executing MAS in a better way for development of improved cultivars. The manipulation of the genomic regions having positive additive effects on traits of

interest can lead to maximum potential genetic gain through MAS, particularly for traits having low heritabilities and difficulties in scoring (Kumar et al. 2011). Soybean is the best example where use of markers in breeding programmes has been most successfully demonstrated (Pratap et al. 2012). In past several years, many improved varieties/lines for resistance to different SCN races (Arelli and Young 2009), phytophthora root rot and brown stem rot, insect resistance (Warrington et al. 2008); low linolenic acid content, yield (Concibido et al. 2003), mosaic virus resistance (Shi et al. 2009) have been developed. MAS has also been used successfully in common-bean to develop several lines which are resistant to rust (Stavely 2000; Faleiro et al. 2001), anthracnose (Alzate-Marin et al. 1999) and bean golden yellow mosaic virus (Miklas 2002). In peanut, markers linked with root knot nematode resistance were introgressed into cultivated background via amphidiploids pathway (Simpson et al. 2001). DNA fragment carrying nematode resistance gene was also introgressed selecting a recessive AhFAD2B allele using the linked markers for foreground selection (Chu et al. 2011). This led to development and release of the improved variety "Tiftguard High O/L". Currently, MABC is also being practiced for introgression and pyramiding *Fusarium* wilt and *Ascochyta* blight resistance gene into chickpea in India (Chamarthi et al. 2011; Varshney et al. 2012) by ICRISAT, IIPR and other collaborators in state agricultural universities. In one such project funded by Department of Biotechnology, Government of India, resistance to two races (*foc2* and *foc 4*) independently and pyramiding of resistance to two races (*foc1* and *foc3*) of fusarium wilt and two QTLs for resistance to Acsochyta blight is being undertaken using MABC and MARS and currently, various generations (BC_1F_2 to $BC_3F_{3:4}$) are available for the different crosses. Similarly, for drought tolerance, nine different chickpea varieties have been targeted (see Varshney et al. 2012). Efforts have also been initiated to use MARS in chickpea at ICRISAT, IARI and IIPR.

Gene pyramiding is also a useful approach to achieve multiple and durable resistance (Shi et al. 2009). It has been successfully demonstrated in soybean where genes controlling resistance to CSN have been pyramided (Concibido et al. 2004). Similarly, QTLs/genes controlling tolerance to Phytophthora root rot and resistance to soybean mosaic virus have also been stacked in this crop (Shi et al. 2009; Li et al. 2010).

MAS for two QTLs available on separate linkage groups has been shown to be effective in imparting white mould resistance in common bean (Ender et al. 2008). Similarly, MAS for a major QTL associated with root-rot resistance was found to be effective and it imparted realized gain in plant biomass and vigour traits associated with root-rot complex in snap bean (Navarro et al. 2009). Utilization of MAS has also resulted in development of several improved cultivars in common bean and soybean, mostly in USA (Chamarthi et al. 2011; Pratap et al. 2012). In common bean three genotypes, USPT-ANT-1, ABCP-8 and ABC-Weihing have been released between 2004 and 2006 (Miklas et al. 2003; Mutlu et al. 2008). Similarly, a number of varieties (JTN5503, JTN5303, JTN5109, DS880) have been released in soybean also for resistance to diseases and soybean cyst nematode (Arelli et al. 2006, 2007; Arelli and Young 2009; Smith 2010).

Genome-Wide Selection

Genome-wide selection (GWS) or "genomic selection (GS)" is useful for complex traits that are controlled by many genes/ QTL, each with small effect (Chamarthi et al. 2011). This method predicts genomic estimated breeding values (GEBVs) of progenies, which are calculated for progenies, based on both phenotyping and genotyping data. These GEBVs are then used to select the superior progeny lines for advancement in the breeding cycle (Heffner et al. 2009; Jannink et al. 2010). Doubled haploid (DH) populations are very useful in GWS compared to F_2 populations, when many QTL control a trait (Mayor and Bernardo 2009). GWS can help breeders in reducing the frequency of extensive phenotyping as well as bypass the need of QTL mapping besides reducing the selection cycle, thereby having considerable savings of time. However, there is not much information available on use of GWS in legumes, although recent developments in plant genomics make it feasible to generate genome-wide marker data (using SNPs) to start GWS in breeding programmes. In the coming few years, GWS is expected to be used at least in soybean among the legumes.

Advanced Backcross QTL Analysis and Harnessing Variability from Secondary Gene Pool

Many a times the genes for traits of interest may not be available in cultivated/ primary gene pool of a species and it is necessary to explore the wild species/relative for them. However, owing to linkage drag, their use in conventional breeding programmes still remains restricted. It is now possible to recover the favourable alleles in elite germplasm avoiding associated linkage drag using molecular maps and integrative analysis. In the advanced backcross QTL (AB-QTL) approach, parallel discovery and transfer of desired QTL from an unadapted germplasm into selected breeding lines takes place (Tanksley and Nelson 1996). In AB-QTL, repeated backcrossing is done with the elite parent in wild × cultivated species cross and selection is imposed in advanced backcrossed (BC_2F_2 or BC_2F_3) populations. This approach reduces linkage drag as well generates phenotyping and genotyping data. The advanced backcross populations are simultaneously used to identify desirable genes/QTL through QTL analysis. Once favourable QTL alleles are identified, marker assisted selection in a few generations (3–4) can lead to development of near isogenic lines (NILs) which can be used for development of a variety. This approach has been successfully used in soybean and commonbean (Blair et al. 2003; Chaky et al. 2003). Fonceka et al. (2009) reported a successful effort for genome wide segment introgressions from a synthetic amphidiploids (*A. duranensis*×*A. ipaensis*) to a cultivated variety (Fluer 11) using molecular markers. The backcross (BC_1F_1 and BC_2F_1) lines carrying the wild genome segments with maximum recurrent parent genomic regions provided optimal distribution of the synthetic genome introgressions.

In another approach, introgression libraries are constructed which are made up of several introgression lines (ILs). The ILs are developed by repeated backcrossing of F_1s between wild × cultivated lines. This leads to distribution of donor (wild species) genome into the entire genome of ILs and consequently their expression in the phenotype. Such libraries have been reported to be developed in soybean using wild soybean species (*G. soja*) (Concibido et al. 2003) and groundnut from synthetic tetraploids (Fonceka et al. 2009).

Conclusions and Perspectives

In the past decade, proactive and coordinated efforts of the international legume community have ensured a significant progress in the development of genomic resources of food legumes which have led to a better understanding of their genome structure. These have also offered new possibilities for genetic improvement of not only grain legumes but also several other species, especially those where their development is costly. While the cost effective, polymorphic and reproducible markers such as SSRs, SNPs, etc. can be used by breeders in development of improved cultivars through marker assisted breeding employing MAS, MARS and MABC, high throughput sequencing can accelerate the development of new molecular markers. The marker-trait association will enable biotechnologists to more rapidly and precisely manipulate target genes underlying key agronomic traits, especially a series of abiotic and biotic stresses limiting crop productivity. This will be especially useful in developing such genotypes which suit the marginal environments of food legume growing areas of the world. Increased focus is required on development of organized genome resources including physical maps and functional genomic tools, TILLING populations, and microarray chips, which will facilitate the isolation of genes for resistance/tolerance to biotic and abiotic stresses. Ultimately, the availability of high-throughput and cost-effective genotyping platforms, combined with automation in phenotyping methodologies, will increase the uptake of genomic tools into breeding programs, and thus usher an era of genomics-enabled molecular breeding in legumes.

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