Application of Next Generation Sequencing and Genotyping Technologies to Develop Large-Scale Genomic Resources in SAT Legume Crops

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ABSTRACT

Molecular markers and genetic maps are the pre-requisites for trait mapping and genomics-assisted crop improvement. However, very limited genomic resources were available until recently for the legume crops important in the semi-arid tropics (SAT). As a part of several initiatives, species-specific genomic resources are now being developed in most of these legume crops. For instance, using simple sequence repeat (SSR)-enriched libraries and bacterial artificial chromosome (BAC)-end sequence mining approaches, nearly 1,500- 3,000 novel SSR markers have been developed for chickpea, pigeonpea and groundnut. In addition, next generation sequencing technologies like Roche 454/FLX and Illumina/Solexa, in addition to Sanger sequencing, are being used to sequence the transcriptomes of reference or parental genotypes of mapping populations of chickpea and pigeonpea to access the gene space and develop functional markers. Based on Sanger and 454/FLX transcript reads, transcriptome assemblies have been developed for chickpea (103,215 tentative unique sequences, TUSs) and pigeonpea (127,754 TUSs) that are being characterized using genome sequence data of Medicago and soybean. In parallel, RNA of four chickpea and twelve pigeonpea genotypes, that represent parents of different mapping populations, have been sequenced by using Illumina/Solexa sequencing approach that has resulted ca. 120 million reads for chickpea and 20 million reads for pigeonpea. Alignment of these Illumina/Solexa reads of these genotypes with transcriptome assembly of the respective species has provided a large number (tens of thousands) of SNPs. Selected set of SNPs are being used to develop large-scale SNP genotyping platform in chickpea and pigeonpea. By using the existing resource of SSR, SNP and DArT markers, high-density genetic maps are being developed in these species for trait mapping and molecular breeding. It is anticipated that molecular breeding practice may be routine and part of breeding activities in the SAT legumes in coming future.

Key Words: Legume, chickpea, pigeonpea, sequencing, genotyping, genomic resource

INTRODUCTION

The Leguminosae is third largest family among the angiosperms and rank second after Poaceae with large number of domesticated species besides their importance in agriculture and economy. Grain and forage legumes are grown on over 190 million hectares, and their production is about 300 million metric tons across the world (Vance et al 2000). Grain legumes are also a rich source of essential

vitamins, minerals, and important amino acids (Duranti and Gius 1997; Grusak 2002). Traditionally legumes have been placed into three subfamilies (Mimosoideae, Caesalpinoideae, and Papilionoideae) with 730 genera and 19,400 species (Lewis et al 2005). With the notable exception of peanut, all important crop legumes namely soybean (*Glycine max*), peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), chickpea (*Cicer arietinum*), pigeonpea (*Cajanus cajan*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) fall into two Papilionoid clades, namely, Galegoid and Phaseoloid, which are often referred to as cool season and tropical season legumes, respectively (Lewis et al 2005).

Molecular markers and genetic maps are instrumental in several marker-assisted selection (MAS) and genomics-assisted breeding programmes for crop improvement (Varshney et al 2005b; Varshney et al 2006; Varshney et al 2009d; Varshney et al 2010b). However until recent past, in spite of availability large number of domesticated legume species, genomics research was in fact, extensively carried only in a few model legumes like *Medicago* (*Medicago truncatula* Gaertner), *Lotus* (*Lotus japonicus* [Regel] K. Larsen) and soybean (*Glycine max*; Vanden) (see Wilson et al 2004). Nevertheless, during the last five years concerted efforts are being made by ICRISAT and its collaborating partners from India and abroad towards developing genetic and genomic resources in semi-arid tropics' (SAT) legume species namely chickpea, pigeonpea and groundnut (see Varshney et al 2009b, 2010a). Some of these efforts have been summarized in this article.

GENETIC RESOURCES FOR MODERN BREEDING

In spite of an impressive number of germplasm accessions available at ICRISAT and in the genebanks, the pedigree analysis of the released cultivars, developed through traditional breeding approaches, indicated narrow genetic base in cultivated chickpea, pigeonpea and groundnut. With an objective to enhance the use of genetic resources in crop breeding, concepts of developing core collection (Brown 1989), mini core collection (Upadhyaya and Ortiz 2001) and core reference set (Glaszmann et al. 2010) were followed. In all these three legume species, all kind of above mentioned collections either have been developed or such efforts are in progress (Upadhyaya et al 2001a, b; Upadyaya and Oritz 2001; Upadhyaya et al 2002, 2003, 2006, 2008, Reddy et al 2005).

In addition to germplasm collections, mapping populations are other important class of genetic resources for use in trait mapping and marker-assisted breeding. Identification of appropriate genotypes that are genetically diverse in addition to contrast phenotype for a trait of interest is a quite challenging in the species like SAT legumes with low level of molecular diversity. Some efforts on identification of suitable genotypes, based on screening of candidate genotypes with some molecular markers, for making the mapping populations are underway at ICRISAT. For instance, a set of 32 contrasting pigeonpea genotypes for fusarium wilt (FW) and sterility mosaic disease (SMD) has been screened with 30 microsatellite or simple sequence repeat (SSR) markers and based on these results, diverse parental genotypes have been selected for developing the mapping populations for mapping resistance loci to FW and SMD (Saxena et al 2010b). However, it is important to note that though such mapping populations will be good enough for trait mapping, these are still not suitable to develop the good genetic maps for corresponding species. In these cases, use of one genotype from closely related species together with the other genotype from the cultivated species for developing inter-specific mapping populations also has been suggested. In case of chickpea and pigeonpea, inter-specific mapping populations are being used as reference mapping populations to develop the reference genetic maps (Table 1).

DEVELOPMENT OF GENOMIC RESOURCES

In the past molecular genetic diversity studies were conducted in chickpea, pigeonpea and groundnut by using anonymous molecular markers.

Table 1. Current status on the development of reference genetic maps for SAT legumes

Crop	Mapping population	No. of progenies/ type of mapping population	Markers mapped	Reference
Chickpea	ICC 4958 × PI 489777	131 RILs	1533	Nayak et al 2010; unpublished data
Pigeonpea	ICP 28 × ICPW 94	79 F2 lines	254	unpublished data
Groundnut	TAG 24 × ICGV 86031	318 RILs	191	Varshney et al 2009a; unpublished data

Examples are the use of random amplified polymorphic DNA (RAPD), or amplified fragment length polymorphism (AFLP) are (see Varshney et al 2007). These anonymous markers are either difficult to reproduce or to apply in the breeding programs. Microsatellites or simple sequence repeat (SSR) markers, however, on the other hand have been designated as markers of choice in plant breeding in several species (see Gupta and Varshney 2000, Varshney et al 2005a). Similarly, other high-throughput marker systems such as diversity array technology (DArT) and single nucleotide polymorphism (SNP) markers have become popular in many other crop species (Kilian et al 2005 Rafalski 2002). Therefore, there was a need for development of large number of user-friendly and informative markers, especially due to low level of genetic diversity in the intra-specific or cultivated germplasm, in these legume species.

Several national consortia or networks in India like Pigeonpea Genomics Initiative under Indo-US Agricultural Knowledge Initiative (Varshney et al 2010a), Chickpea Genomics Consortium and Groundnut Genomics Consortium under National Fund for Basic and Strategic Research of Indian Council of Agricultural Research (ICAR), Transcript Mapping project sponsored by Department of Biotechnology (DBT) as well as international projects or platforms such as Legume Genome Evolution sponsored by National Science Foundation (NSF), USA and Tropical Legume I of Generation Challenge Programme and Bill and Melinda Gates Foundation have facilitated development of genomic resources at large scale in these legume crops. Some of these resources have been summarized in following sections.

SSR MARKERS

In case of these three SAT legume species, very few SSR markers were available, until recent past, in the public domain. For the last five years, ICRISAT in collaboration with its partners has been developing SSR markers by using three approaches: (a) sequencing the SSR +ve clones from SSR enriched libraries, (b) mining the BAC (bacterial artificial chromosome)- end sequences (BES), and (c) mining the transcript sequences generated by either Sanger sequencing or next generation sequencing approaches such as 454/FLX sequencing (Figure 1). By using one or combination of above-mentioned approaches about 1,700 novel SSR markers have been developed in chickpea (Nayak et al 2010, unpublished), 3100 in pigeonpea (Saxena et al 2010a, see Varshney et al 2010a, unpublished) and about 1,500 in groundnut (Mace et al 2007 Cuc et al 2008; Gautami et al 2009 unpublished).

DArT MARKERS

Although smaller set of DArT arrays were developed earlier for pigeonpea (Yang et al 2010), chickpea and groundnut at DArT Pty Ltd. (Australia), these were not found very effective for detecting the polymorphism in cultivated germplasm of these species. Therefore, diverse germplasm lines including the parental genotypes of different mapping populations were used for developing extended DArT arrays at DArT Pty Ltd in collaboration with ICRISAT. As a result, extended DArT arrays with about 15,360 features have become available for each of three species.

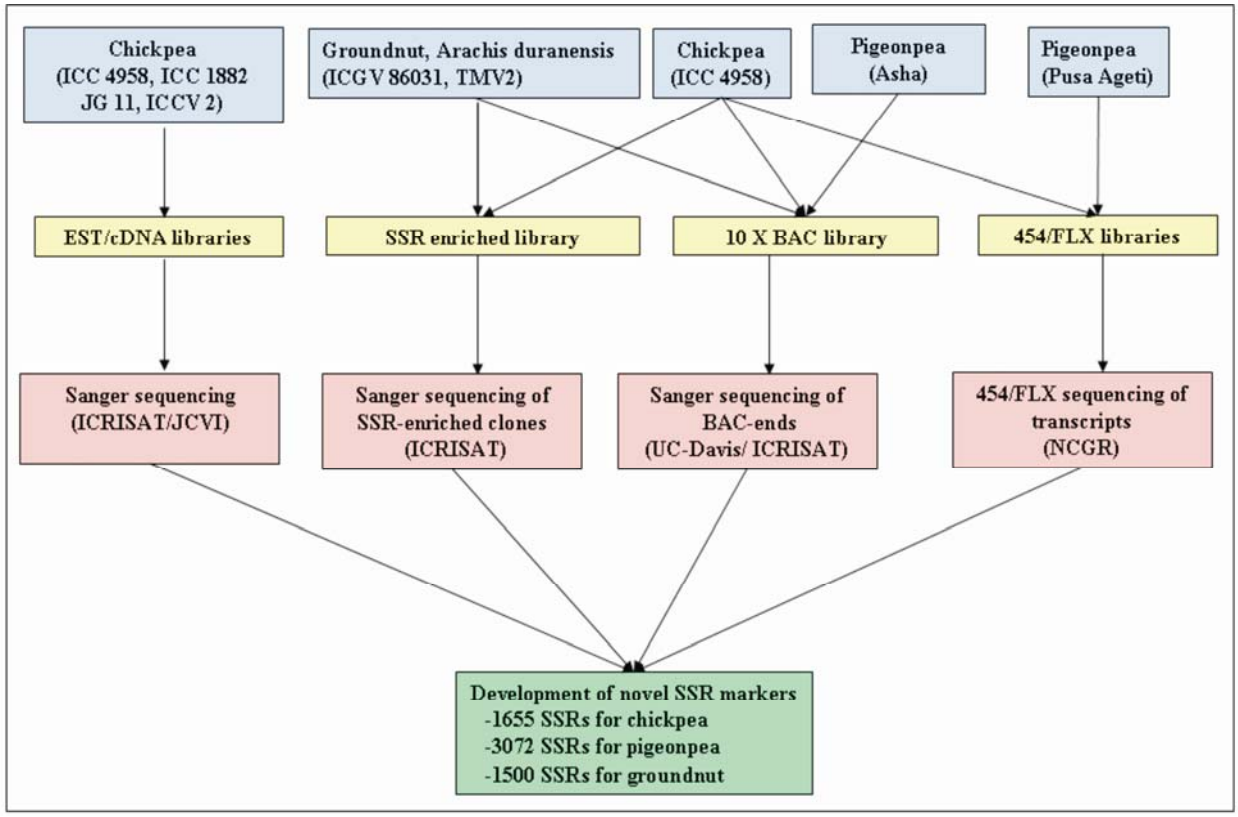

Figure 1. Strategies for developing microsatellite markers for SAT legumes. Microsatellite enriched libraries, bacterial artificial chromosome (BAC) end sequences and transcriptomic resources obtained through Sanger or 454/FLX sequencing are being employed for developing novel microsatellite markers.

TRANSCRIPTOMIC RESOURCE AND SNP MARKERS

An array of new generation sequencing technologies, popularly known as next-generation sequencing (NGS) technologies, have emerged in the recent past which drastically reduced the time and cost of sequencing compared to conventional sequencing (Varshney and Dubey 2009). Three major sequencing platforms that are currently being used in plant species include Genome sequencer FLX (Roche/454 Life Sciences, http://www.454.com/), Applied Biosystems SOLiD (http://www3.appliedbiosystems.com) and Illumina Genome Analyzer (http://www.illumina.com/) (Varshney et al 2009e). The NGS technologies like 454/FLX and Illumina/Solexa are being employed for sequencing the transcriptomes of parental genotypes of mapping population of chickpea and pigeonpea for developing the functional SNP markers. As Illumina reads are smaller in size, these needs to be aligned with a reference genome. In case of SAT legume species, however, reference genome sequences are not available. In such species, transcript assembly (TA) can be used for aligning Illumina/Solexa reads onto it (Figure 2). Therefore, Sanger as well as 454/FLX sequencing technologies have been used to develop TAs for chickpea and pigeonpea as mentioned below. By using Sanger sequencing, a comprehensive EST resource has been developed by sequencing drought- and salinity- challenge cDNA libraries for chickpea (Varshney et al 2009c) and FW- and SMD- challenged cDNA libraries for pigeonpea (Raju et al 2010). To enhance these transcriptomic resources, 454/FLX sequencing was conducted on normalized and pooled RNA samples collected from >20 tissues representing different parts of plant development.

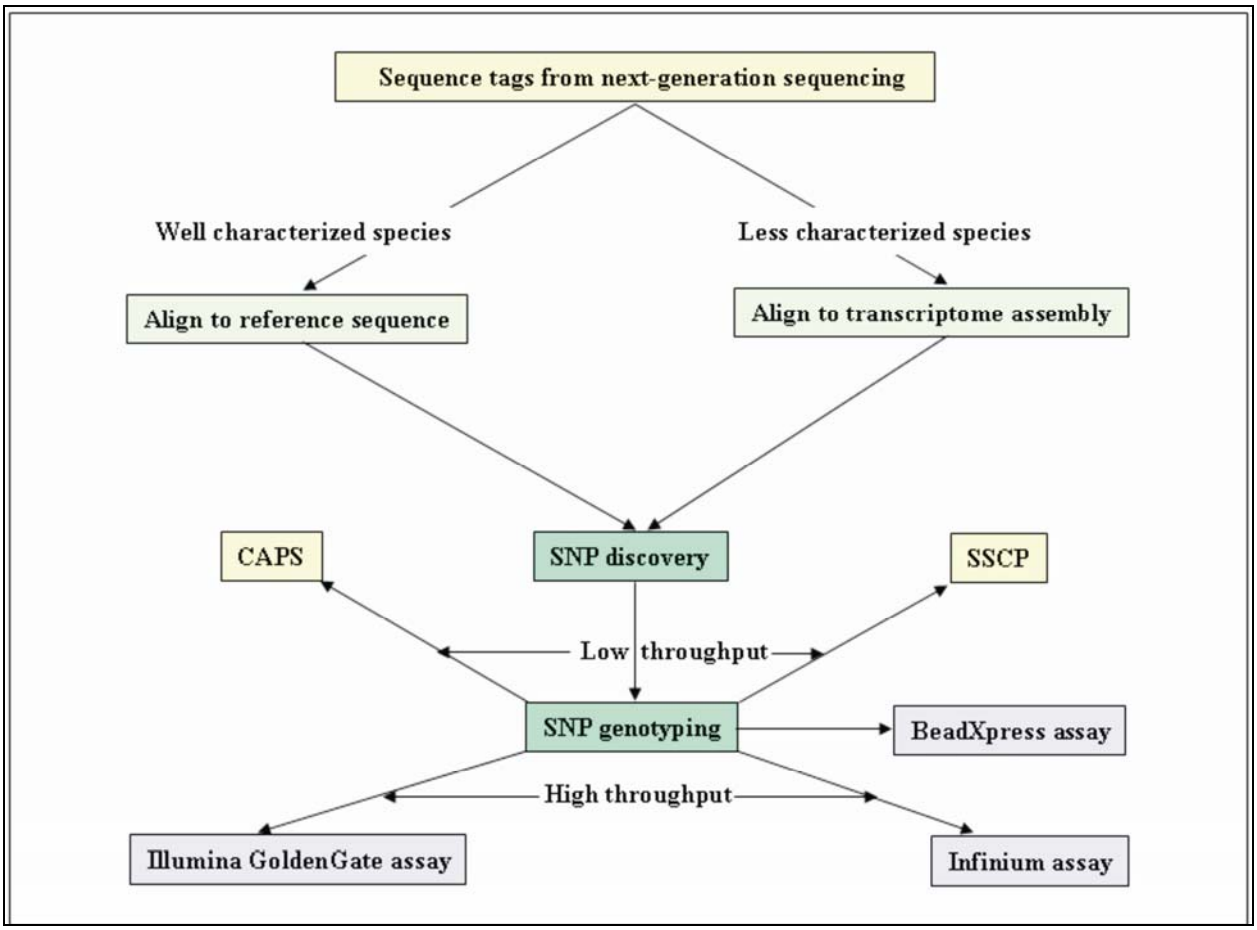

Figure 2. A scheme showing development of SNP markers and genotyping. The sequence reads generated through NGS technologies like 454/FLX, Illumina/Solexa sequencing need to be aligned with either reference genome sequences or transcriptome assemblies. Depending on the need, SNP resource can be used for developing low, moderate and high-throughput genotyping assays.

As a result, 435,018 transcript reads for chickpea and 494,353 transcript reads for pigeonpea have been generated. Cluster analysis of these transcript reads with Sanger ESTs generated at ICRISAT as well as those available in public domain provided transcript assembly of chickpea (Ca TA) with 103,215 tentative unique sequences (TUSs) and pigeonpea (Cc TA) with 127,754 TUSs. As mentioned above, Solexa/Illumnia sequencing has been carried out on parental genotypes of mapping populations of chickpea and pigeonpea. RNA sequencing of four chickpea and twelve pigeonpea genotypes has resulted ca. 120 million reads for chickpea and 20 million reads for pigeonpea. Alignment of these Illumina/Solexa reads onto TAs of respective species has provided a large number (tens of thousands) of SNPs in each of these species. In addition, SNP markers for chickpea and pigeonpea have been developed by University of California-Davis, USA (Doug Cook) in collaboration with ICRISAT and National Centre for Genome Resources, USA (NCGR, Greg May/ Andrew Farmer) based on conserved othologos sequence (COS) markers of legumes.

HIGH-THROUGHPUT SNP GENOTYPING PLATFORMS

As a large numbers of SNPs have been identified in both chickpea as well as pigeonpea, in the first instance, pilot GoldenGate assays for 768 SNPs in each chickpea and pigeonpea have been developed

at UC-Davis in collaboration with ICRISAT and NCGR. In addition, ICRISAT is in progress of developing the 2nd set of GoldenGate assays for 1536 SNPs in each of these two species. These SNP assays are being employed to genotype the mapping populations as well as core reference sets.

GENOME MAPPING AND MOLECULAR BREEDING IN SAT LEGUMES

Genomic resources developed at ICRISAT and its partners are being utilised for construction of genetic maps and for mapping the traits of interest to breeders so that genomics-assisted breeding can be realized in these legume crops.

GENETIC AND TRANSCRIPT MAPS

Until recently in the SAT legumes either no genetic map was available or genetic maps with lower marker density were available for intra-specific mapping populations. For instance, the first SSR-based genetic linkage map was developed for groundnut based on TAG 24 × ICGV 86031 recombinant inbred line (RIL) mapping population (Varshney et al 2009a). Currently this map has about 200 SSR loci (Table 1).

In case of chickpea, although some genetic maps were available based on the reference mapping population (ICC 4958 × PI 489777), majority of times, these maps were developed by using anonymous markers like RAPD and AFLP or with smaller number of SSR markers. With the intent of extending this genetic map, and enhancing the number of easily scorable markers, a comprehensive chickpea genetic map with 521 loci has been developed by Nayak et al (2010). Furthermore, genotyping data have been obtained for large number of marker loci on this mapping population and development of a high-density genetic map is in progress. Owing to the dearth of molecular markers, there is no published genetic map available for pigeonpea. However recently, ICRISAT, UC-Davis and DArT Pty Ltd have made some efforts on genotyping the inter-specific mapping population (ICP 28 × ICPW 94) with SSR, SNP and DArT markers (Table 1).

As above mentioned maps are being used for integrating gene/transcript based SNP marker loci, the integrated maps are also being referred as 'transcript map'. In case of chickpea, for instance, SNP markers based on Solexa/Illumina sequencing or allele-specific sequencing of candidate genes, EST-derived SSR and SNP markers as well as conserved intron spanning primers (CISPs) are being developed and used for mapping at ICRISAT in collaboration with National Institute of Plant Genome Research (NIPGR), New Delhi.

TRAIT MAPPING

For realizing genomics-assisted breeding in the SAT legumes, it is important to identify the markers associated with tolerance to abiotic stress such as drought and resistance to biotic stress such as FW-, SMD- in pigeonpea, foliar diseases in groundnut. Like genome mapping, efforts have been very slow in the area of trait mapping in the SAT legumes. Nevertheless, some efforts have been initiated at ICRISAT to identify the markers associated with drought tolerance in chickpea and groundnut and for foliar diseases in groundnut. As drought is a complex trait, root traits such as root biomass, root length, root volumes etc. are being targeted as component traits in chickpea while transpiration efficiency (TE), specific leaf area (SLA) and soil plant analytical development (SPAD) chlorophyll meter reading (SCMR) are also targeted as additional component traits for drought tolerance in case of chickpea. By using two mapping populations (ICC 4958 × ICC 1882, ICC 283 × ICC 8261) in chickpea for SSR genotyping and extensive phenotyping for root traits, a genomic region harbouring QTLs for several drought related traits has been identified on LG 5. The QTLs present in this region contribute up to 30% phenotypic variation and therefore this region represents a candidate region for introgression in chickpea lines for enhancing drought tolerance. In case of groundnut, a detailed analysis was conducted with a number of programmes like QTL Cartographer, QTL Network and Genotype Matrix Mapping (GMM) on genotyping and phenotyping data on TAG 24 × ICGV 86031 population to identify main effect as

well as epistatic QTLs. A large number of main effect QTLs as well as epistatic QTLs were identified by using the comprehensive analysis. These results indicate that drought tolerance in groundnut is probably contributed by several main-effect as well as epistatic QTL each with small phenotypic variation. Therefore, traditional molecular breeding approach like marker-assisted back crossing (MABC) may not be useful for enhancing drought tolerance in groundnut. In collaboration with University of Agricultural Sciences- Dhawad (UAS-D) and Directorate of Groundnut Research, Junagadh, some efforts have been made at ICRISAT to identify the makers associated with two main foliar diseases of groundnut i.e. late leaf spot (LLS) and rust. In this context, one RIL population comprising of 268 lines (TAG 24 × GPBD 4) was phenotyped extensively at UAS-D and genotyped at ICRISAT. Composite interval mapping (CIM) undertaken on genotyping and phenotype data yielded 14 QTLs for LLS (explaining 0.20 to 6.50% variation) and 16 QTLs for rust (explaining 0.10 to 55.20% variation). Interestingly a major QTL associated with rust ($QTL_{rust}01$), contributing 6.90 to 55.20% variation, was identified by both CIM and single marker analysis (SMA). A candidate SSR marker (IPAHM 103) linked with this QTL was validated using a wide range of resistant / susceptible breeding lines as well as progeny lines of another mapping population (TG 26 × GPBD 4). Therefore, this marker may be useful for introgressing the major QTL for rust in desired lines/varieties of groundnut through MABC approach. Similar efforts are underway in pigeonpea to map FW- and SMD- resistance.

MOLECULAR BREEDING

Once QTLs/genes are identified, the next challenge is to deploying these QTLs/genes in crop improvement programmes. Very recently, some efforts have been initiated at ICRISAT to introgress the major root trait QTL in three elite chickpea lines (JG 11, Chefe and KAK 2) in collaboration with Egerton University (Kenya), Ethiopian Institute of Agricultural Research (EIAR, Ethiopia), Lake Zone Agricultural Research Development Institute (LZARDI, Tanzania) and Indian Institute of Pulse Research (IIPR, India). After completing three cycles of MABC and two cycles of selfing, BC_3F_3 seeds have been generated. The generated lines will be used for agronomic performance under both irrigated and rainfed conditions in different environments. Similarly a multidisciplinary team from leading institutes ICRISAT, Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV, Jabalpur), IIPR, Kanpur, Mahatma Phule Krishi Vidyapeeth (MPKV, Rahuri) and Agricultural Research Station (ARS-Gulbarga) has initiated a molecular breeding network project, sponsored by DBT, on introgressing the resistance to FW- and *Aschochyta* blight in elite cultivars from different agroclimatic zones through MABC approach.

ICGGC AND IIPG FOR KNOWLEDGE SHARING AND COMMUNITY EFFORT

With an objective of knowledge sharing and enhancing collaborative efforts within chickpea and pigeonpea communities, two international platforms "International Chickpea Genetics and Genomic Consortium (ICGGC; http://www.icrisat.org/gt-bt/ICGGC/homepage.htm) and International Initiative on Pigeonpea Genomics (IIPG; http://www.icrisat.org/gt-bt/IIPG/home.html) have been established. These platforms should be useful for facilitating chickpea and pigeonpea research in the areas of genetics and genomics to understand genome architecture and to assist crop improvement.

TOWARDS A PROSPEROUS FUTURE OF SAT LEGUME GENOMICS APPLIED TO BREEDING

As it is evident from the details discussed, significant genomic resources have been developed in so called 'orphan legume crops' of SAT regions. It is believed that these crops have turned into "genome resource rich" crops from "orphan legume crops". It is possible now to undertake the molecular breeding in these legumes which was a dream even just five years ago. As significant advances are being made and costs is

going down in sequencing and genotyping technologies, development and use of genomic resources in breeding will be accelerated in coming future.

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