Role of Molecular Breeding in Genetic Improvement of Pigeonpea

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Abstract

Advances in molecular breeding tools and approaches in pigeonpea have allowed addressing many significantly scientific questions that are impossible to do so before. Recent progress in the development of genome-scale data sets for pigeonpea offers important new possibilities for crop improvement. This progress will enable biotechnologists to more rapidly and precisely target genes that underlie key agronomic traits. Among the most important agronomic targets are a series of abiotic and biotic stresses that limit crop productivity. Molecular analysis of germplasm collections with new-generation genomic tools will accelerate trait discovery through methods such as linkage and association mapping. Use of molecular markers in diverse mapping populations in pigeonpea will facilitate the construction of a genetic map, mapping, and map based cloning of disease resistance genes, quantitative trait loci (QTL) mapping, and the integration of phenotypic data across the different mapping populations. Moreover, organized genome resources, including physical maps and functional genomics tools, will facilitate the isolation of genes for resistance or tolerance to biotic and abiotic stresses. Molecular markers identified from these approaches that are associated with traits of importance to breeders should accelerate pigeonpea improvement *via* marker assisted selection (MAS) or transgenic approaches. 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 in an era of genomics-enabled molecular breeding in these legumes. Modern molecular breeding methods together with the power of genomics and genetic resources developed will revolutionize pigeonpea crop improvement, and consequently benefit farmers and consumers of this important pulse crop of India and the semi-arid regions of the world.

1. Introduction

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is the fifth most important pulse crop in the world and represents an important component of semi-arid and sub-tropical farming systems (Shanower et al., 1999). Globally, it is cultivated in 4.6 Mha with a production of 3.49 Mt. Nearly 70% of the pigeonpea production and 74% of the pigeonpea area is in India. Pigeonpea is a diploid species (2n=2x=22) and its genome comprises of 833.1 Mbp arranged into 11 pairs of chromosomes (Varshney et al., 2012b). Pigeonpea is a hardy and drought tolerant crop assuring sustainable returns from marginal lands with minimal inputs, hence it is considered as a very suitable crop for subsistence agriculture. Pigeonpea seeds contain about 20-24% protein and reasonable amounts of essential amino acids making it an important source of dietary protein, mainly in vegetarian-based diets (Bohra et al., 2012). As a member of the sub tribe *Cajaninae*, pigeonpea is contained in an early diverging lineage of tribe *Phaseoleae*, a monophyletic group of legumes that contains several of the world's most important food legumes including soybean, common bean, cowpea and mung bean (Greilhuber and Obermayer, 1998).

India is the world's largest producer of pigeonpea and the presumed center of origin (van der Maesen, 1990). Relative to most other crop legumes pigeonpea is highly drought tolerant, being able to retain productivity with less than 650 mm annual rainfall. Owing to its capacity for symbiotic nitrogen fixation, pigeonpea seeds have high levels of protein and they specifically enriched for amino acids that are often limiting in the human diet, including methionine, lysine, and tryptophan.

In resource poor areas of the world, pigeonpea serves as an important forage and cover crop, while the stems provide wood for tool making and fuel, and thatch for roofing. These factors, especially the ability to withstand elevated temperatures and limited water availability, add to pigeonpea's importance as a crop in semi-arid tropical (SAT) regions of the world, especially in the SAT of India where approximately 77% of global production occurs. Despite its importance in the SAT regions, little concerted research effort has been directed at either improvement or technology transfer in this crop. Thus, the pigeonpea production has remained static (Reddy and Faris, 1981) and a range of biotic and abiotic stresses continue reduce yields by 50% or greater (Marley and Hillocks, 1996). Among the most important limiting factors are Fusarium wilt, sterility mosaic disease, pod borer, soil salinity and water logging. Very recently, hybrid breeding technology based on the cytoplasmic-nuclear male-sterility (CMS) system has been implemented in the pigeonpea breeding programme at ICRISAT (Saxena et al., 2010), and this technology holds great potential to increase pigeonpea productivity.

2. Breeding and Production Constraints in Pigeonpea

In pigeonpea, plant growth as well as flowering is highly influenced by the environment. Hence, breeding for wider adaptation, a complex phenomenon is a major issue to be tackled. Although related wild species are a rich reservoir of not only resistance genes against various biotic and abiotic stresses but also of genes responsible for yield components such as pods plant^{-1}, length of fruiting branches, and number of primary branches plant^{-1}, use of inter-specifics in pigeonpea improvement have been limited. This is due to the poor crossability of cultivated *Cajanus cajan* tospecies other than the closest species, *Cajanus cajanifolia* and *C. scaraboides*. Biotechnology approaches, such as in vitro rescue and propagation of wide cross hybrids, in conjunction with the use of bridge crosses, may enable the transfer of novel genes from a wider range of germplasm within and outside the genus *Cajanus*. Ongoing efforts using molecular tools to examine taxonomic relationships within subtribe *Cajaninae* should clarify phylogenetic relationships within the subtribe, and may suggest parsimonious routes for trait introgression (Varshney et al., 2010).

Despite the importance of pigeonpea in semi-arid regions of the world, little concerted research effort has been directed towards pigeonpea crop improvement. A number of factors are responsible for the poor productivity, including lack of improved cultivars, poor crop husbandry, pests, and diseases. Major diseases include Fusarium wilt (*Fusarium udum* Butler), sterility mosaic disease (Sterility mosaic virus) and phytophthora blight (*Phytophthora drechsleri*), and pests such as gram pod borer (*Helicoverpa armigera*), Maruca (*Maruca vitrata*), pod fly (*Melanagromyza obtusa*), plume moth (*Exelastis atomosa*) cause substantial reduction to pigeonpea production every year. Furthermore, sensitivity to abiotic stresses like water-logging, common in this rain fed crop during early stages, and stress from low water conditions in the later stages, and salinity also reduce pigeonpea production. Conventional breeding approaches for pigeonpea improvement have been in use for several decades but have had limited success in overcoming these biotic and abiotic challenges to stable crop production (Varshney et al., 2007; Saxena, 2008).

3. Current Status of Pigeonpea Breeding Research

Breeding in pigeonpea has been more challenging compared to other food legumes due to various crop specific traits. Pigeonpea is an often cross pollinated crop, with an insect-aided natural out crossing range from 20 to 70% (Saxena et al., 1990) that has limited the use of efficient selection and mating designs possible in self-pollinating species. Pure line breeding, population breeding, mutation breeding, and wide hybridization have been used for development of new varieties in pigeonpea and have led to incremental improvements in the yield potential of this crop. To overcome this bottleneck, two genetic male-sterility (GMS) systems were discovered in pigeonpea (Reddy et al., 1978; Saxena et al., 1983). Despite a 30% yield advantage over the non-hybrids, the GMS based hybrids could not be commercialized due to high cost of hybrid seed production.

The yield-jump observed in the GMS hybrids encouraged the development of the alternative and more efficient cytoplasmic-genetic male-sterility (CGMS) system (Tikka et al., 1997; Saxena and Kumar, 2003; Wanjari and Patel, 2003). As a result of intensive hybrid development programme at ICRISAT in collaboration with its partners, the first CMS-based hybrid GTH^{-1} was released in India in 2004. Another CMS-based pigeonpea hybrid, ICPH 2671 was developed using *C. cajanifolius* (A4 cytoplasm) at ICRISAT in 2005 (Saxena, 2008), that has been released as ''Pushkal'' by Pravardhan Seeds for cultivation in several states of India such as Andhra Pradesh, Karnataka, Madhya Pradesh, and Maharashtra. Continued hybrid-technology based improvements in pigeonpea yield potential, together with ongoing efforts to breed for resistance to biotic and abiotic stresses (Fusarium wilt, sterility mosaic, pod borer, etc.) are likely to lead to increased area under pigeonpea hybrids, contribute to increased crop returns for farmers and sustainable pigeonpea production (Varshney et al., 2010).

4. Genetic Resources and Their Utilization in Modern Breeding

Analysis of molecular diversity and pedigree information of the cultivars developed through classical breeding approaches

indicated a narrow genetic base in cultivated genepool of pigeonpea. However, a large number of accessions for pigeonpea are present in several genebanks of the world (Bohra et al., 2011a). The ICRISAT genebank stores the largest number of accessions of pigeonpea. In order to enhance the use of available genetic resources in crop breeding, the concept of core collection (Brown, 1989), mini core collection (Upadhyaya and Ortiz, 2001) and core reference set (Glaszmann et al., 2010) has been followed. These collections are now available in pigeonpea (Bohra et al., 2011a).

Development of mapping populations using identified genetic resources for trait mapping and marker-assisted breeding is the subsequent important task. Ideally, the suitable genotypes for developing the mapping population should be genetically diverse and with contrast phenotype for the trait of interest (Saxena et al., 2010a). However, due to narrow genetic diversity in the cultivated genepool of pigeonpea, identification of such genotypes is quite challenging. Nevertheless, by using conventional approaches as well as by using molecular diversity information, several mapping populations have been developed at ICRISAT and its partner institutes. Although these mapping populations are good for marker trait association, they may not be suitable for developing dense genetic maps. Therefore, interspecific mapping populations have also been developed and used for developing genetic maps with higher marker density in pigeonpea (Varshney et al., 2012a).

4.1. Genomic resources for SAT legumes

In the past, for genetic diversity analysis, a range of molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) have been used. However, SSR and single nucleotide polymorphism (SNP) markers have become the markers of choice for genetic analysis and breeding applications in pigeonpea (Varshney et al., 2007). While SSR markers have the advantages of being multi-allelic and co-dominant (Gupta and Varshney, 2000), SNP markers offer high-throughput and cost-effective genotyping options. Another high-throughput marker system, is diversity array technology (DArT), which became popular in many other crop species since no sequence information is needed for developing these markers (Killian et al., 2005). The last 5 years have witnessed significant progress in the area of development of genomic resources in pigeonpea that have made it 'genomic resource rich' crop from so-called 'orphan crop' (Varshney et al., 2012a).

4.1.1. SSR markers

Until recently, very few SSR markers were available in pigeonpea. During the last few years, a large number of SSR markers have been developed by using following approaches individually or in combination: (a) constructing 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. Using these approaches independently or in combination, about 3,200 novel SSR markers have been developed in pigeonpea (Saxena et al., 2010b; Bohra et al., 2011b; Dutta et al., 2011).

4.1.2. DArT markers

The DArT marker system has been widely used for constructing genetic maps and diversity analysis in Triticeae species (Neumann et al., 2011; Roy et al., 2011; Varshney et al., 2012a). In the case of legumes, the first-generation array comprising 6,144 clones was developed in pigeonpea (Yang et al., 2011). With DArT Pty Ltd, ICRISAT has developed DArT arrays comprising 15,360 clones in pigeonpea. Use of these DArT arrays, as expected, showed a narrow genetic diversity in the elite genepool as compared to landraces and wild species. The parental genotypes of mapping populations including intra-specific mapping populations in pigeonpea, when screened with the available DArT arrays, showed 9% polymorphism. In summary, it seems that DArT markers are not cost-effective or attractive marker system for detecting polymorphism in cultivated germplasm of pigeonpea. However, DArT markers may prove useful for introgression of segments from alien species to the elite varieties of the legume crops. For instance, in pigeonpea by using 1,225 DArT markers in the cross between *C. platycarpus* and *C. cajan*, 2-5% *C. platycarpus* genome carrying genes for disease and insect resistance was observed (Mallikarjuna et al., 2011).

4.1.3. Transcript assembly and SNP markers

Next-generation sequencing (NGS) technologies offer the ability to produce huge sequence data sets at relatively low cost in less time. These technologies are based on a combination of template preparation, sequencing, alignment and assembly of the genome. Although a number of second-generation or third-generation sequencing technologies have become available (Thudi et al., 2012), most commonly used sequencing technologies are 454 (454 Life Sciences, http://www.my454.com/), SOLiD (Applied Biosystems, http://www.appliedbiosystems.com) and Illumina (Illumina Inc., http://www.my454.com/) (Varshney et al., 2009).

Two NGS technologies namely 454 and Illumina together with Sanger sequencing technology have been used to characterize the transcriptomes of pigeonpea. For instance, 9,888 ESTs were developed for pigeonpea using Sanger sequencing technology

on Fusarium wilt (FW) and sterility mosaic disease (SMD)-challenged cDNA libraries (Raju et al., 2010). By analysing these ESTs together with the then available ESTs in public domain, 5,085 unigenes for pigeonpea were identified. In order to improve these transcriptomic resources, 454/FLX sequencing was undertaken on normalized and pooled RNA samples collected from>20 tissues representing different developmental stages of the plant. As a result, 494,353 transcript reads for pigeonpea have been generated (Dubey et al., 2011).

Three approaches were used for identification of SNPs. In the first approach, Illumnia sequencing was carried out on parental genotypes of mapping populations of pigeonpea. RNA sequencing of 12 pigeonpea genotypes has resulted ca. 128.9 million reads for pigeonpea (Kudapa et al., 2012). Alignment of these short reads onto TAs has provided a large number (tens of thousands) of SNPs in pigeonpea. The second approach of allele-specific sequencing of parental genotypes of the reference mapping populations of pigeonpea using conserved orthologous sequence (COS) markers has provided 768 SNPs for pigeonpea. In the third approach, allele-specific sequencing of candidate genes may be undertaken to identify SNPs. In brief, a large number of SNPs have become available for pigeonpea and high as well as low throughout and cost-effective genotyping platforms have been developed (Varshney et al., 2012a).

4.1.4. Genetic and transcript maps

Efforts have been made only recently to develop genetic maps in pigeonpea. Based on an inter-specific mapping population (ICP 28×ICPW 94), DArT and bacterial artificial chromosome (BAC)-end derived sequence (BES) SSR markers were used for developing genetic maps. A total of 122 DArT markers were used to generate maternal genetic linkage map (270.0 cM) and 172 DArT markers were used to generate a paternal genetic linkage map. The average marker distance is 2.2 cM and 2.6 cM, marker^{-1} in maternal and paternal linkage maps, respectively (Yang et al., 2011). Another BES-SSR-based genetic map was developed by Bohra et al. (2011b) using 239 BES-SSR markers covering 930.90 cM with an average of 21 markers linkage group^{-1} and an average marker distance of 3.8 cM.

4.1.5. Trait breeding

Yield in pigeonpea is mainly affected by various abiotic stresses like drought and biotic stresses such as FW and SMD. To overcome the constraints related to production in pigeonpea using genomics-assisted breeding, identification or discovery of marker trait association between the trait of interest and a genetic marker is an important and starting point to work for crop improvement. Because of paucity of markers and non-availability of genetic maps, QTL mapping in pigeonpa have been very slow. However, during the last 5 years, ICRISAT in collaboration with its partners have made some progress towards QTL mapping for several production constraints (Varshney et al., 2012a).

In pigeonpea, QTL analysis provided six QTLs for SMD using ICP 8863×ICPL 20097 and TTB 7×ICP 7035 mapping populations. Of these one QTL (qSMD4) explaining 24.72% of phenotypic variance was identified on LG 7 (Gnanesh et al., 2011). Furthermore, four major QTLs explaining up to 24% phenotypic variation have been identified for fertility restoration (Rf) in pigeonpea (Bohra et al., 2012).

5. Molecular Breeding

Gepts (1999) discussed use of molecular markers for improving the efficiency of plant breeding programs because at the molecular level recognizing the presence or absence of a particular gene is independent of plant part or plant age. Also, in contrast to morphological traits, molecular markers are not influenced by various pleiotropic and epistatic interactions. The first step in molecular breeding, therefore, is to establish linkage between a gene and its marker locus. Subsequently specific DNA diagnostic tests can be applied to assist plant breeders in selection. The identification of useful breeding lines with the help of linked molecular markers is popularly known as marker-assisted selection (MAS). MAS is particularly useful for traits having low heritability where phenotypic selection would be poorly effective.

The RAPD markers were used with bulk segregation analysis to report an association of two loci for *Fusarium* wilt resistance (Kotresh et al., 2006). This preliminary report of an association between a molecular marker and a phenotype is the first of its type for pigeon pea and remains to be confirmed. The high level of intra-accession heterogeneity and the narrow genetic variation among cultivars has hindered the construction of molecular maps and marker trait association analysis in pigeon pea.

After identification of QTLs or genes responsible for trait of interest, the next step is to use this information in crop improvement. However, for successful introgression of QTLs in elite breeding materials or varieties, the targeted QTLs should be major QTLs that contribute>20% phenotypic variation. ICRISAT together with its partners, during the last few years, have developed significant amount of genomic resources that have already started to make an impact on trait mapping and molecular breeding in pigeonpea (Varshney et al., 2012a).

Recently, four new intra-specific genetic maps have been constructed based on BAC end sequence (BES) derived SSR markers. All these genetic maps together with the two intra-specific genetic maps reported in earlier study (Gnanesh et al., 2011), allowed development of a consensus genetic map comprising

339 loci with an average marker density of 3.1 cM. This is the first instance of integrating multiple component genetic maps in pigeonpea. Furthermore, grouping of markers into bins and associating them with polymorphism information content (PIC) values on the integrated genetic map will facilitate the selection of evenly distributed markers for various genetics and breeding studies including genetic mapping (for new populations), association or linkage disequilibrium (LD) studies, diversity analysis, or for practicing background selection in molecular breeding studies aimed at crop improvement in pigeonpea. The identification of major RF-QTLs would open new avenues for genomics-assisted hybrid breeding in pigeonpea (Bohra et al., 2012).

6. Prospects of Genomics-Assisted or Molecular Breeding in Pigeonpea

It is evident from the above discussions that as a result of concerted and coordinated efforts of several partners together with advances in genomics like NGS and high throughput genomic technologies, the pigeonpea so-called 'orphan legume crop' has become 'genomic resources rich crop' now (Varshney et al., 2009). Even the genome sequence has become available now for pigeonpea (Varshney et al., 2012c). Molecular markers have already become available for several important traits like FW, SMD and Rf in pigeonpea. Efforts have already been initiated to use the linked markers for molecular breeding applications for enhancing drought tolerance and disease resistance. It is anticipated that availability of large-scale genomic resources and cost-effective genotyping platforms as well as possibilities of outsourcing of genotyping work will accelerate trait mapping for other important traits in pigeonpea. This will eventually enhance adoption of molecular breeding for increasing crop productivity in pigeonpea.

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