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Molecular mapping and tagging of genes in crop plants

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Abstract

In India, molecular mapping and tagging of agronomically important genes using RFLP and RAPD markers have been carried out in three different crops: rice, mustard and chickpea. In rice, tagging of genes for resistance to gall midge and blast has been accomplished. Molecular mapping of cooking quality traits in rice is in progress. For fingerprinting rice cultivars, suitable probe enzyme combinations have been identified. In mustard, a partial RFLP linkage map has been constructed and one of the yellow seed-coat colour loci has been mapped. Significant associations of RFLP markers with quantitative traits have also been established. Potential use of RAPD markers to identify heterotic groups among mustard accessions has been demonstrated. In chickpea, the occurrence of considerable interspecific DNA polymorphism as revealed by RAPD analysis has facilitated construction of a partial linkage map.

Introduction

Ever since the method of constructing molecular linkage maps in humans using restriction fragment length polymorphism (RFLP) markers was outlined by Botstein *et al.* (1980), there have been continuous efforts in many laboratories of the world directed at construction and application of linkage maps in crop plants. This particular development has contributed tremendously to our understanding of plant genome organisation and has greatly facilitated tagging of many agronomically important genes in a variety of crop plants. The most exciting outcome of elaborate gene tagging efforts is the development of protocols to clone genes of unknown products. Availability of markers for complex quantitative traits has opened up a promising approach, marker assisted selection, whereby genomic regions containing favourable alleles at the quantitative trait loci (QTL) can be selected indirectly but efficiently even for low heritable traits.

In India, molecular mapping was initiated in the early 1990s. During the last five years there has been considerable progress in this area concerning three important crops: rice (*Oryza sativa*), mustard (*Brassica juncea*) and chickpea (*Cicer arietinum*). Given below is a cropwise review of the work done in different laboratories in India.

Rice

Rice is the most important cereal crop, contributing about 43% of the total food grain production in India. The crop yield is seriously affected by numerous insects and diseases. Of the several insect species which infest and feed on the crop, stem borers, plant hoppers, leaf hoppers and gall midge cause considerable damage. Among the diseases, blast, sheath blight and brown spot caused by fungi, bacterial leaf blight and viral diseases like tungro and grassy stunt are the most serious. For minimising losses due to insects and diseases, it is essential that multiple resistance to most major insects and diseases must be incorporated in new high yielding varieties and hybrids. Since sources of resistance against most of the serious insects and diseases are available, molecular-marker aided transfer of these genes to desired genetic backgrounds for con-

structing high yielding genotypes is emerging as the method of choice.

Molecular tagging of several qualitative traits in rice has been facilitated by three major factors: i) availability of molecular linkage maps (McCouch *et al.*, 1988); ii) easy access to a large number of DNA probes through the Rockefeller rice network; and iii) availability of suitable genetic stocks. With the availability of single/low copy sequence genomic DNA clones from Cornell University, Ithaca, through the Rice Network, molecular tagging work in rice was initiated in India. Subsequently, PCR based random amplified polymorphic DNA (RAPD) markers were incorporated for hastening gene tagging.

Tagging the gall midge resistance gene *Gm2*

Gall midge (*Orseolia oryzae* Wood-Mason) is a serious pest of rice in India, Southeast Asia and South China. The female gall midge lays eggs on the leaves near the base of the plant. The newly hatched larvae burrow into the shoot interior and feed on the shoot meristem. This leads to formation of tubular galls resembling onion leaves, which are called silver shoots. Such a modified shoot does not produce panicle, thus causing severe yield losses.

A total of five distinct gall midge biotypes have been recognised in India. Additional biotypes are known to exist in other rice growing areas of the world. Chaudhary *et al.* (1986) studied the inheritance of resistance to gall midge and identified two dominant genes, *Gm*1 and *Gm*2. Subsequently, Sahu *et al.* (1990) identified a recessive gene for resistance which was designated as *Gm*3.

The *Gm*2 gene for resistance to biotype 1 of gall midge was tagged recently at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi (Mohan *et al.*, 1994). To achieve this, a set of 40 F_{5-6} recombinant inbred lines (RIL) were employed. These lines were derived from a cross between the resistant variety Phalguna and the susceptible land-race ARC 6650 in the course of a breeding programme at the Directorate of Rice Research (DRR), Hyderabad, India. A total of 150 rice genomic DNA clones (McCouch *et al.*, 1988) were employed in combination with eight restriction enzymes. Only 43 probes differentiated the parents and, therefore, were used to genotype RI lines. Four markers closely segregated with *Gm*2 and two of these markers were tightly

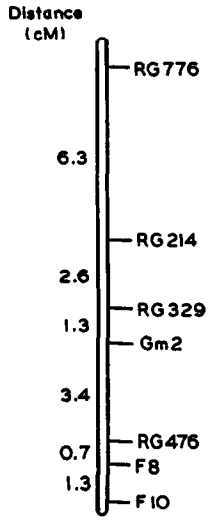

Fig. 1. Region of rice chromosome 4 in the vicinity of the *GM2* gene.

linked at 1.3 cM (RG 329) and 3.4 cM (RG 476) on either side of *Gm*2 on chromosome 4 (Figure 1).

To further confirm the map location of the *Gm*2 gene, 160 random primers were employed in conjunction with bulked segregant analysis. Of the 188 markers generated by these primers, two (F08 and F10) showed tight linkage with *Gm*2 at 4.1 cM and 5.4 cM respectively, on chromosome 4 beyond RG 476. RAPD analysis, therefore, did not generate any marker closer to the resistance gene than the RFLP markers. Nevertheless, the results from this study clearly indicated that: (i) the population already generated in the course of normal breeding programmes can be successfully used for molecular mapping; (ii) RAPD analysis is more useful in cases of narrow genetic diversity between the parents used to generate the mapping population, as for the *indica* × *indica* cross; and (iii) molecular tagging demands less time and effort, provided the above two factors are suitably combined.

Identification of markers for the *Gm*2 gene is of great significance from the application point of view. The flanking markers RG 329 and RG 476 will be useful in the transfer of this gene to desired genetic backgrounds. It has been estimated that use of both markers will reduce the error rate in selection to about 0.06% per generation, thereby providing higher efficiency for selection.

Considering that, for routine genotyping of a large number of segregants in gall midge resistance breeding programmes, a PCR-based marker is more useful

and economical than a RFLP marker, the above study was further extended (Nair *et al.*, 1994). The termini of the two specific RAPD fragments that showed linkage to *Gm2* obtained with the primers $F08_{1700}$ and $F10_{600}$ respectively, were cloned and sequenced. Based on the sequence information, new primers could be designed. It was interesting to note that these primers specifically amplified the marked genomic region. The F08 specific primers amplified selectively a 1.7 kb fragment in the susceptible plants, while F10 specific primers amplified a 0.6 kb fragment in the resistant plants. These primers will be of tremendous use in large scale screening of segregants.

Tagging of the blast resistance gene *Pi-10(t)*

Blast, caused by *Pyricularia grisea* Sacci, is the most devastating disease of rice. It occurs in all rice growing areas of the world and is most serious in upland rice, particularly under low temperature conditions during flowering.

Resistance to blast is classified as qualitative or complete and quantitative or partial, depending on the way the gene(s) affects pathogen reproduction. Inheritance of blast resistance has been extensively studied (Kiyosawa, 1981; Mackill & Bonman, 1992). Six genes (*Pi-2(t)*, *Pi-4(t)*, *IRAT 13*, *Pi-2h(t)*, *Pi-5(t)* and *Pi-7(t)*) for complete resistance have been mapped using RFLP markers (Yu *et al.*, 1991; Wang *et al.*, 1994). Ten genomic regions carrying genes for partial resistance (Wang *et al.*, 1994) have also been identified.

At M.S. University, Baroda, India, molecular tagging of a dominant blast resistance gene *Pi-10(t)* was accomplished (Naqvi, Kachroo & Chattoo, 1994). This was a new source of complete resistance identified in the cultivar Tongil. It offers protection against the race IB46 of the blast fungus. For tagging the *Pi-10(t)* gene, near-isogenic lines (NILs) in the BC_3F_2 generation were used. These lines were developed using the resistant cultivar Tongil and susceptible cultivar Co39. A total of 464 random primers were used in bulked segregant analysis of NILs. Two markers (PF6 and PH18) could be linked to *Pi-10(t)* on chromosome 5. High resolution mapping of this region of chromosome 5 was achieved using an F_2 population as well as a recombinant inbred population developed at IRRI, Philippines.

Tagging of genes for quality traits

In rice, the cooking quality traits include aroma, kernel elongation without significant increase in breadth after cooking, low starch gelatinization temperature (<70 °C), a low to intermediate amylose content (<25%) and medium gel consistency (41–60 mm). Rice cultivars which possess these traits, such as Basmati-370, are an important commercial commodity. However, Basmati varieties are tall, poor yielding and susceptible to a number of pests and diseases. Development of varieties by combining the high yielding potential of the semidwarfs with the grain quality characteristics of Basmati is an important breeding objective. In this context, identification of molecular markers for genes concerning cooking quality traits is a high priority area of research.

Intensive efforts at Cornell University, Ithaca, have led to molecular mapping of the gene *fgr* for aroma on chromosome 8 (Ahn, Bollich & Tanksley, 1992). Subsequently, a quantitative trait locus having a large effect on the expression of the trait kernel elongation (Ahn *et al.*, 1993) was also mapped on the same chromosome, interestingly revealing linkage between *fgr* and the QTL for kernel elongation.

The linkage between *fgr* and RFLP marker RG 28 has been confirmed in a different mapping population obtained from the cross Pusa 751 (aromatic) × IR 72 (nonaromatic) at the National Research Centre on Plant Biotechnology (NRCPB), Indian Agricultural Research Institute, New Delhi (Pandey, 1993; Mridula *et al.*, 1994). The map distance between the marker and the gene in this study, however, was larger (10 cM) compared to that observed (4.8 cM) by Ahn, Bollich & Tanksley (1992), probably due to a small mapping population. Keeping in view the advantages of RAPD analysis, this study has been extended (Mridula *et al.*, 1994). Based on bulked segregant analysis of the same F_2 population using 145 random primers, it was indicated that one of the primers amplified a specific fragment, giving a 1.2 kb band in the non-aromatic parent and the non-aromatic bulk but not in the aromatic parent and the aromatic bulk (unpublished). This putative linkage is being confirmed using a larger F_2 population generated from a different cross. Suitable mapping populations are also being developed to identify markers for other quality traits including kernel elongation, amylose content, gelatinization temperature and gel consistency.

Genetic diversity and DNA fingerprinting

RFLP marker technology has been employed to determine genetic diversity among *indica* cultivars at the National Chemical Laboratory, Pune, India (Ranjekar *et al.*, 1993). Using a dispersed repeat clone pOSMIC-2, genetic variability was assessed among 18 cultivars. The probe gave a monomorphic banding pattern with EcoRI and HindIII, while with DraI, EcoRV and ScaI digests, it hybridized to 5–7 restriction fragments and revealed polymorphism. Based on this, the cultivars could be grouped into two major clusters with three outgrouped ones. It was interesting to note that the grouping corresponded to the pedigree of the cultivars. Seven of the nine entries in one cluster had TN(1) as the common parent, while five of the six entries in the other cluster had IR8 as the common parent. These observations suggested potential use of this probe in studying genetic relationships among *indica* cultivars.

To generate cultivar-specific RFLP markers, this study was extended to include oligonucleotide probes specific for simple sequence repeats or microsatellites (Ramakrishna *et al.*, 1994). Six oligonucleotides, $(GATA)_4$, $(GACA)_4$, $(GCAT)_4$, $(GAA)_6$, $(CAC)_5$ and $(TG)_{10}$, were employed to genotype 13 accessions belonging to seven different species of *Oryza*. In spite of the fact that enzymes with six base pair specificities were used to digest DNA, it was difficult to obtain clear band resolution. For instance, the probe $(TG)_{10}$ gave a heavy background smear that did not allow analysis of fragment pattern. Better band resolution could be obtained with the probes $(GATA)_4$, $(GAA)_6$ and $(CAC)_5$. $(GATA)_4$, however, provided the highest resolution and distinguished all 13 genotypes. These probes also indicated intravarietal and somatic stability of the DNA fingerprints. Therefore, some of these oligonucleotide probes can be used for cultivar identification, germplasm characterisation and molecular mapping in rice.

Mustard

Indian mustard (*Brassica juncea*) (AABB) is a natural amphidiploid of *B. campestris* (AA) and *B. nigra* (BB), having haploid chromosome number 18 and DNA content 1.15 pg. It is a major contributor to the improved edible oil economy in India. It ranks second in area and production among oilseed crops grown in the country, next to groundnut. Improvement in yield of oil per unit area remains the most important breeding objective in this crop. Stabilization of yield under adverse situations including biotic stresses caused by mustard aphid, alternaria blight, white rust and downy mildew is a major challenge. Genetic reduction in erucic acid content and glucosinolates of oil and meal, respectively, to non-toxic levels is also an important consideration in the quality improvement programme of mustard.

Following the publication of the first report on the occurrence of a high degree of inter- as well as intra-specific nuclear DNA polymorphism in the genus *Brassica* (Figdore *et al.*, 1988), there was a spurt in research activity directed at the construction of RFLP linkage maps in different cultivated *Brassica* species. Within a span of three years (1990–92), five maps were published in three of the *Brassica* species: two in *B. campestris* (Song *et al.*, 1991; Chyi, Hoenecke & Sernyk, 1992), two in *B. oleracea* (Slocum *et al.*, 1990; Landry *et al.*, 1992) and one in their amphidiploid *B. napus* (Landry *et al.*, 1991). RFLP markers for resistance to race 2 of *Plasmodiophora brassicae* that causes club root disease in cruciferous crops were identified (Landry *et al.*, 1992). Recently, molecular mapping of the *B. nigra* genome (BB) has been accomplished (Truco & Quiros, 1994).

Linkage map of mustard

Molecular mapping in Indian mustard (*B. juncea*) at NRCPB, Indian Agricultural Research Institute, New Delhi, was initiated following the construction of a PstI subgenomic library of mustard rich in low-copy sequence clones (Mohapatra, 1991; Mohapatra, Sharma & Chopra, 1992). These clones were characterised for their suitability to detect intervarietal nuclear DNA polymorphism in mustard and to identify the parents for construction of the mapping population. Based on morphological and molecular diversity, the cultivar Varuna and exotic collection BEC 144 were crossed and the F_2 population was generated. Feasibility of constructing a linkage map of mustard using the genomic DNA clones as probes was demonstrated (Sharma, Mohapatra & Sharma, 1994). Subsequently, a partial linkage map was constructed based on segregation of sixty-five RFLP markers (Mohapatra *et al.*, 1995). It consisted of 14 linkage groups and covered 407.9 cM of the *B. juncea* genome (Figure 2). The numbering of the linkage group was done in decreasing order of size. This is, however, likely to change as more markers are added to the map. Assigning these linkage groups to the constituent genomes is present-

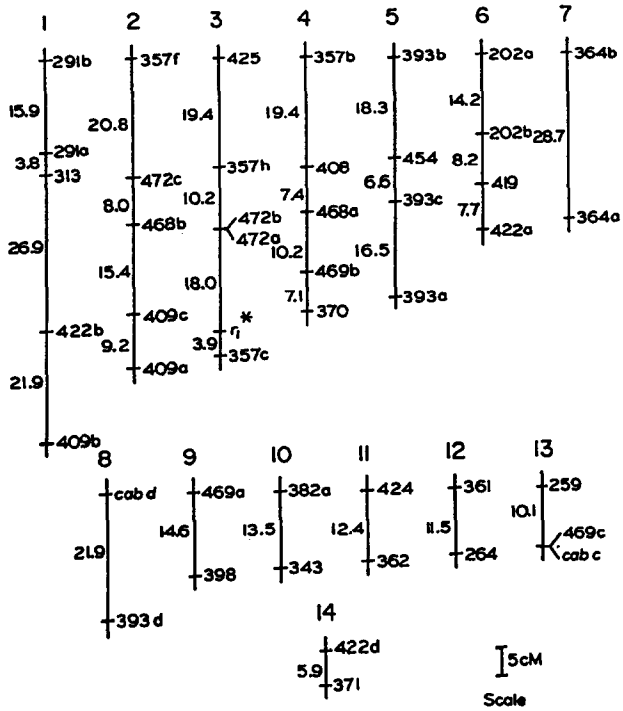

Fig. 2. RFLP linkage map of *Brassica juncea*. * – seed coat colour locus.

ly being carried out using genome-specific markers. Chromosomal localisation of the markers will be possible by employing chromosome addition lines which are being developed at NRCPB, IARI, New Delhi.

The above studies revealed that a high degree of sequence duplication exists in *B. juncea*, which is associated more with presence–absence polymorphism. It was also indicated that the genomic regions that could be assessed using 64 random PstI genomic DNA fragments as probes contained more sites for HindIII than for EcoRI or EcoRV, as indicated by the size and the number of hybridizing fragments.

Tagging of the seed coat colour locus r_1

In the oilseed *Brassica*, yellow seed colour is considered to be desirable because of its reported association with low fibre content and high oil and protein percentage (Stringam, McGregor & Powlowoski, 1974). Therefore, attempts are being made to transfer this trait from *B. juncea* to *B. napus* (Rashid, Rakow & Downey, 1994). However, the trait is recessive to brown colour and is known only when the plant matures. Its transfer to desired genetic backgrounds, therefore, requires additional growing time and labour to identify the yellow seeded segregants.

Genetic analysis by several workers (Vera, Woods & Downey, 1979; Singh & Aruna, 1994) suggested a digenic mode of inheritance of the trait with duplicate gene action. Recessive alleles (r_1 and r_2) at both loci in the homozygous condition give rise to yellow seed coat colour, while dominant alleles at either one or both loci lead to brown coat colour. One of these loci was recently mapped by us using RFLP markers (Mohapatra *et al.*, 1995; Upadhyay *et al.*, 1995). As indicated in Figure 2, the markers BJG357c and BJG372a/b were located on either side of r_1 at 3.9 cM and 18.0 cM respectively on linkage group 3. Tight linkage between r_1 and BJG357c can be of use in early identification of yellow seeded segregants in breeding programmes.

Association of RFLP markers with quantitative traits

Several complex quantitative traits have been characterised using molecular markers in different crop species including maize, tomato and rice. In *B. juncea*, RFLP analysis of quantitative traits was carried out in our laboratory taking advantage of two factors. First, the parents used to generate the mapping population differed for a number of quantitative traits including days to 50% flowering, plant height, number of primary branches, siliqua density and oil content. Second, the range of variation in the mapping population was wide and it transgressed the parental limits either in one or in both directions for different traits. Many of the 48 individual F_2 plants which were genotyped possessed extreme phenotypes for each of the six traits studied (Table 1).

Employing single factor analysis of variance as outlined by Edwards, Stuber & Wendel (1987), twenty significant marker trait associations were identified (Sharma, Mohapatra & Sharma, 1994; Mohapatra *et al.*, 1995; Upadhyay *et al.*, 1995). The results, summarised in Table 1, indicated that: (i) the majority of the significant associations individually explained more than 10% of phenotypic variation, suggesting large effects of the quantitative trait loci (QTL) in the marked genomic regions on trait expression; and (ii) the determined gene action for the majority of the regions was partial dominance to dominance.

Table 1. RFLP analysis of quantitative traits in mustard indicating number of F_2 plants with extreme phenotypes, F values, Probability (P), proportion of phenotypic variation ($R^2 \times 100$) explained by individual marker-trait association and the gene action.

Trait	No. of plants with extreme phenotype	Marker	F value	P	$R^2 \times 100$	Determined gene action
DF	17	BJG 357h	4.11	0.023	13.6	PD to D
		BJG 364a	5.06	0.011	9.9	PD to D
		BJG 364b	5.41	0.008	12.9	PD to D
		BJG 398	3.44	0.041	13.5	A
		BJG 433	3.65	0.030	NA	PD to D
		BJG 472a/b	3.89	0.028	12.9	PD to D
PH	18	BJG 357f	3.92	0.028	15.3	A
		BJG 291b	3.69	0.033	12.1	PD to D
PB	5	BJG 59a	4.31	0.020	NA	OD
		BJG 364a	4.42	0.018	14.3	PD to D
SBP	20	BJG 202a	3.91	0.028	13.5	PD to D
		BJG 264	6.26	0.004	22.7	A
		BJG 357b	4.90	0.012	3.0	OD
		BJG 382b	5.90	0.006	11.9	PD to D
		BJG 426b	7.92	0.001	NA	OD
SSB	23	BJG 382b	6.74	0.003	14.8	PD to D
SS	16	BJG 357a	8.13	0.001	15.3	PD to D
		BJG 370	7.04	0.003	15.8	OD
		BJG 393a	4.45	0.019	3.8	OD
		BJG 408	9.54	0.001	33.2	A

*PD: Partial dominance, D: dominance, OD: overdominance, A: additive, NA: data not available.

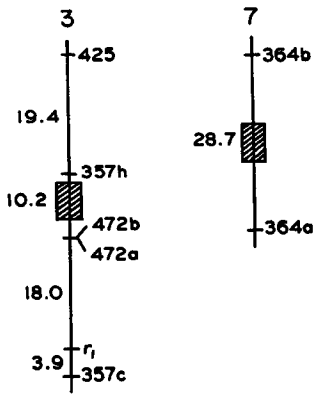

Fig. 3. Linkage groups 3 and 7 of *B. juncea* RFLP map. Shaded region indicate position of QTL for days to flowering.

With respect to days to flowering, results obtained by using F_3 data have confirmed the marker trait associations identified with F_2 data. Based on this, two genomic regions have been identified on linkage groups 3 and 7 respectively (Fig. 3). The late flowering parent BEC 144 alleles at the QTL on linkage group 3 enhanced the trait expression, while the alleles of the early flowering parent Varuna enhanced the trait expression on linkage group 7. However, the number of genomic regions so far identified for each of the quantitative traits is an underestimate. The use of more markers in conjunction with genotyping of phenotypic extremes and interval mapping will allow total genome coverage and help in the identification of additional genomic regions.

Genetic diversity versus heterosis

RAPD analysis was recently employed at Tata Energy Research Institute, New Delhi, to study genetic diversity among 23 (12 Indian + 11 exotic) *B. juncea* accessions and its relationship with yield heterosis (Jain *et al.*, 1994). The level of polymorphism detected among Indian accessions, employing 34 single random decamer primers and 6 double primer combinations, was found to be lower than that observed among exotic ones. Using Jaccard's similarity co-efficient and the unweighted pair group method with arithmetic averages (UPGMA), a dendrogram was constructed. Based on this analysis the accessions could be clustered into two groups. One group consisted of seven exotics, while the other group included twelve Indian accessions along with four exotics. These observations agreed well with the expected grouping based on ancestry and geographic origin, and further supported a polyphyletic origin of *B. juncea*.

It was evident that the correlation between genetic distance and yield heterosis was not consistent. However, the heterosis in the crosses between heterotic groups, i.e. in Indian × exotic hybrids, over the national check variety, was highest, followed by the crosses within heterotic groups, i.e. exotic × exotic and Indian × Indian hybrids. It was, therefore, suggested to employ RAPD analysis, at least for allocating genotypes of unknown origin to known heterotic groups. Such allocation of genotypes is expected to reduce costs by way of eliminating the generation and evaluation of within-group hybrids.

Chickpea

Chickpea is the third most important grain legume crop in the world. In India, it ranks first in both area and production among pulses. It is a diploid with $2n = 2x = 16$ and is self pollinated. Major factors which limit production in this crop include low yield potential, susceptibility to the highly damaging pod borer insect (*Helicoverpa armigera*), and diseases like *Aschochyta* blight and fusarium wilt.

Molecular mapping of the chickpea genome is being carried out at NRCPB, Indian Agricultural Research Institute, New Delhi. Since the availability of a large number of probes is a prerequisite for map construction, a subgenomic library was constructed in chickpea using 0.4 to 2.0 kb genomic DNA fragments obtained by PstI digestion. This library was rich in single/low copy sequence clones and contained 83.18% of such clones (Udupa *et al.*, 1993).

Of the 17 of these clones and five heterologous probes used in 65 probe enzyme combinations, only two clones revealed nuclear DNA polymorphism among five *desi* and five *kabuli* type chickpea cultivars. This study suggested the occurrence of narrow intervarietal nuclear DNA polymorphism, which limited the use of low/single copy sequence genomic DNA probes in molecular mapping in this crop. However, when this study was extended to include the wild species *Cicer reticulatum*, RAPD polymorphism could be detected even with four random primers (Udupa *et al.*, 1993).

Based on these observations, two mapping populations have been generated from two sexual interspecific crosses, *C. arietinum* × *C. reticulatum* and *C. arietinum* × *C. echinospermum*, in collaboration with International Crop Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad, India. RAPD analysis of F_2 plants from the cross of *C. arietinum* and *C. reticulatum* was carried out in our laboratory. A partial linkage map of chickpea has been already constructed (unpublished). Since *C. reticulatum* possesses some important traits such as resistance to *Aschochyta* blight, it will be possible to identify markers for these traits.

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