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Research Article

Molecular Characterization of chickpea genotypes and Identification of true hybrids by molecular markers

Bhavana Surduse[1], P. A. Mohanapure[1], V.C. Khelurkar[1], M. P. Moharil[1*], A.A. Sapkal[1], P. V. Jadhav[1], D.R. Rathod[1], S.B. Sakhare[1], A. W. Thorat[2] and R.B. Ghorade[2]

[1]*Department of Agricultural Biotechnology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India*

[2] *Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India*

Corresponding author e-mail: mpmoharil@gmail.com

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ABSTRACT

Chickpea (Cicer arietinum L.) is the third most important grain legume cultivated in the arid and semi-arid regions of the world. In the present study Six crossing combinations were executed in chickpea comprising Chanoli and PKV Kabuli 4 as female parents and Virat, BDNGK-798 and WR- 315 as resistant male parents. Total 54 markers including 13 SCoT, 31 SSR, 5 STMS, 3 RAPD, 1 SCAR, and 1 ISSR, used for parental polymorphism and polymorphic markers UBC-855, 66 % for TA-59 and 100 % for TA-110, TA-135 and GA-16 were further used to hybridity assessments of F1 plants. The PIC value for polymorphic markers ranged from 0.15 to 0.89 with an average value of 0.46. The highest PIC value was observed in UBC-855 marker (0.89), followed by TA-135 (0.62), TA-59 (0.50), and GA-16 (0.16) and lowest PIC value observed in TA-110 (0.15). From total crosses 31 F1 plants of six crosses were screened for true F1 hybridity assessment. STMS marker TA-59 was used for F1 hybrid purity assessment. This marker screened 31 F1 plants. TA-59 shows specific size amplicon in female and male parents. The results of this investigation proved that SSR markers are well polymorphic and more useful markers within species of chickpea genotypes to perform the molecular characterization and to test the genetic hybridity of F1 plants. Among the tested SSR markers TA-59, TA-110, TA-135, GA-16, UBC-855 shows high percentage of polymorphism and PIC value which will were more helpful for parental diversity analysis and hybridity assessment.

Keywords: Chickpea, genotypes and identification.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an ancient and third most important food legume crop after bean and pea, grown over 45 countries across five continents (Joshi et al. 2001). It is self-pollinating, annual, autogamous legume with chromosome number (2n=2x=16) (Tekeoglu et al.2000). The estimated genome size of chickpea is ~740 Mb (Arumuganathan and Earle,1991).

The ‘desi’ type chickpea is hardy in nature, gives better yield in Vidarbha region, Kabuli chickpea having soft seed coat and seems to have evolved from the desi types (Moreno and Cubero 1978). Chickpea plays a crucial role for supplying protein source; hence it is also called as the ‘poor man’s meat (Barman, 2012).

Chickpea protein rich in lysine and arginine but most deficient in sulphur containing amino acids viz. methionine and cystine. In general, kabuli type is rich in protein content than desi types. It is richer in calcium and phosphorus content than most other pulse crop (Singh et al. 2009).

Various abiotic and biotic stresses are the major bottleneck for increasing chickpea productivity. Biotic stress includes fungal, viral diseases, insects, nematodes, and parasitic weeds. Two fungal diseases, Ascochyta blight, caused by *Ascochyta rabiei* and *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *Ciceris* (Nene and Reddy, 1987), are major constraints for chickpea production. Fusarium wilt is wide- spread in the chickpea growing areas of the world and has been reported from at least 33 countries (Dubey et al. 2007). The annual yield losses due to wilt have been estimated at 10- 90 per cent (Jimenez-Diaz et al. 1989).

Eight distinct physiological races of *Fusarium oxysporum* have been identified (0,1A,1B/C, 2, 3, 4, 5 and 6), of which 1A, 2, 3 and 4 are prevalent in India (Haware and Nene, 1982) and the remaining races (0, 5 and 6) reported from Spain (Jimenez – Diaz et al. 1989). Race 0 and 1B/C causes yellowing syndrome, whereas 1A, 2, 3, 4, 5 and 6 lead to wilting syndrome. The disease is seed and soil borne. Symptoms of the disease can develop at any stage of plant growth, (Jiménez-Díaz et

al.1989). However, symptoms are usually more visible in the early stages of flowering, 6 to 8 weeks after sowing and can also appear up to pod stage ("late wilt").

In the recent years, molecular markers have offered an opportunity to identify the purity of F1 plants as they are found to be highly polymorphic, independent of environmental interaction and represent the genomic constitution of a plant. The use of markers is well known and is in routine now a-days in selection of desirable plants in several self- and cross-pollinated crop species.

Genetic purity of parental lines and hybrids is of crucial importance, as one percent reduction in purity of hybrid seed, results in a reduction of about 100 kg/ha in yield of commercial crop. Traditionally genetic purity is done by Grow-out Tests (GOT), based on morphological assay. This method requires space for growing the samples drawn, considerable time till flowering/maturity (one season) and labour for raising the crop. Low levels of polymorphism of sufficient numbers of molecular markers such as microsatellite or simple sequence repeats (SSRs) are the main constraints in chickpea breeding program. Hence, identification of polymorphic markers with respective to selected genotypes is the challenging and used of polymorphic markers in the hybridity assessment is important to hasten the breeding program of chickpea crop.

The objectives of the present study were to perform the parental polymorphism in the selected genotypes and identification of true crosses through polymorphic markers (SSR, ISSR, RAPD, SCAR and STMS) which will be further utilized for hybridity assessment and molecular breeding program to hasten the process of variety development.

MATERIALS AND METHODS

The present investigation was carried out during 2018-2020 at Biotechnology Centre, Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. The details of the experiments conducted, and materials used are presented below.

All molecular experiments conducted at Biotechnology Centre, Department of Agricultural Botany, Dr. PDKV, Akola. Field experiments related to breeding were carried out at Pulses Research Unit, Dr. PDKV, Akola.

Chickpea genotypes

Female line PKV Kabuli-4 and Chanoli (Dr. PDKV. Akola) and male Virat (MPKV Rahuri) BDNGK-798 (VNMKV, Parbhani) and WR-315 (ICRISAT) lines were kindly provided by Pulses Research Unit, Akola.

During present investigation six crosses combinations were executed in chickpea comprising PKV Kabuli 4 and Chanoli as susceptible female parents and BDNGK-798, Virat, and WR-315 as resistant male parent.

In Chanoli crosses were executed Chanoli X Virat, Chanoli X BDNGK-798 and Chanoli X WR-315. Similarly, for PKV Kabuli 4 were PKV Kabuli 4 X Virat, PKV Kabuli 4 X BDNGK-798 and PKV Kabuli 4 X WR-315.

DNA isolation

Good quality of DNA was isolated from the young leaves of parent and F_1 plants by following Cetyl Trimethyl Ammonium Bromide (CTAB) method described by Doyle and Doyle, 1987. Quantity and quality of DNA samples were assessed by Nano photometer by measuring O.D. at 260/280 nm. The quality of genomic DNA was confirmed on 0.8% agarose gel. Total 54 markers were used for parental polymorphism survey. The details of primers and their sequence along with melting temperature are mentioned in Table 1.

PCR amplification and Polyacrylamide Gel Electrophoresis

Parental polymorphism was carried out using 54 molecular markers. PCR amplification was performed in 20 µl reaction containing 10 X PCR buffer 17.5 mM $MgCl_2$, ~50 ng/µl sample DNA, 10 pmol primers, 10 mM of each dNTP, and 5U/µl of *Taq* DNA polymerase. The temperature profile varied as per primers, for SCoT/SSR/ STMS/ ISSR primers an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing was checked by using gradient PCR for every primer at 55 -62 °C for 45 sec, extension at 72 °C for 1 min and final extension at 72 °C for 10 min was followed by hold at 4°C. The separations of PCR products were resolved on 10 % PAGE.

Total 54 markers including 13 SCoT, 31 SSR, 5 STMS, 3 RAPD, 1 SCAR, and 1 ISSR, used for parental polymorphism and only polymorphic markers were further used to hybridity assessments of F_1 plants.

The observed banding pattern studied for parental survey is shown in Plate 1.

Table 1. List of the molecular marker used in the present investigation

S.No.	Primers	Position	Nucleotide sequence	Annealing temperature ºC
1	TA-1	F	TGAAATATGGAATGATTACTGAGTGAC	58.9
		R	TATTGAAATAGGTCAGGCTTATAAAAA	55.9
2	TA-2	F	AAATGGAAGAAGAATAAAAACGAAAC	55.3
		R	TTCCATTCTTTATTATCCATATCACTACA	58.2
3	TA-3	F	AATCTCAAAATTCCCCAAAT	50.1
		R	ATCGAGGAGAGA AGA ACCAT	55.3
4	TA-14	F	TGACTTGCTATTTAGGGAACA	54.0
		R	TGGCTAAAGACAATTAAAGTT	50.1

5	TA-18	F	AAAATAATCTCCACTTCACAAATTTTC	55.9
		R	ATAAGTGCGTTATTAGTTTGGTCTTGT	58.9
6	TA-27	F	ACAATTCCACTTAATCTTTGC	52.0
		R	AATTTAGCCTACAGACACACACA	57.1
7	TA-28	F	TAATTGATCATACTCTCACTATCTGCC	60.4
		R	TGGGAATGAATATATTTTTGAAGTAAA	54.3
8	TA-37	F	ACTTACATGAATTATCTTTCTTGGTCC	58.9
		R	CGTATTCAAATAATCTTTCATCAGTCA	57.4
9	TA-59	F	ATCTAAAGAGAAATCAAAATTGTCGAA	55.9
		R	GCAAATGTGAAGCATGTATAGATAAAG	58.9
10	TA-64	F	ATATATCGTAACTCATTAATCATCCGC	58.9
		R	AAATTGTTGTCATCAAATGGA AAATA	53.8
11	TA-71	F	CGATTTAACACAAAACACAAA	50.1
		R	CCTATCCATTGTCATCTCGT	55.3
12	TA-96	F	TGTTTTGGAGAAGAGTGATTC	54.0
		R	TGTGCATGCAAATTCTTACT	51.2
13	TA-110	F	ACACTATAGGTATAGGCATTTAGGCAA	60.4
		R	TTCTTTATAAATATCAGACCGGAAAGA	57.4
14	TA-130	F	TCTTTCTTTGCTTCCAATGT	51.2
		R	GTAAATCCCACGAGAAATCAA	54.0
15	TA-135	F	TGGTTGGAAATTGATGTTTT	49.1
		R	GTGGTGTGAGCATAATTCAA	53.2
16	TA-180	F	CATCGTGA ATATTGA AGGGT	53.2
		R	CGGTAAATAAGTTTCCCTCC	55.3
17	TA-194	F	TTTTTGGCTTATTAGACTGACTT	53.5
		R	TTGCCATAAAATACAAAATCC	50.1
18	TA-200	F	TTTCTCCTCTACTATTATGATCACCAG	60.4
		R	TTGAGAGGGTTAGAACTCATTATGTTT	58.2
19	TAA-60	F	TCATGCTTGTTGGTTAGCTAGAAA	57.6
		R	CAAAGACATAATCGAGTTAAAGAAAA	55.3
20	TR-1	F	CGTATGATTTTGCCGTCTAT	53.2
		R	ACCTCAAGTTCTCCGAAGT	54.5
21	TR-19	F	TCAGTATCACGTGTAATTCGT	54.0
		R	CATGAACATCAAGTTCTCCA	53.2
22	TS-45	F	TGACACAAAATTGTCTCTTGT	52.0
		R	TGTTCTTAACGTAACTAACCTAA	53.5
23	TS-82	F	TCAAGATTGATATTGATTAGATAAAAGC	56.3
		R	CTTTATTTACAACTTGCACAACACTAA	57.4
24	STMS-2	F	ATTTTACTTTACTACTTTTTTCCTTTC	54.3
		R	AATAAATGGA GTGTAAATTTCATGTA	53.8
25	STMS-11	F	GTATCTACTTGTAATATTCTCTTCTCT	57.4
		R	ATATCATAAACCCCCCCAC	51.4
26	STMS-13	F	TATGTTAAAAGAGAAAGAAGAAGTGAT	55.9
		R	TTTTATTAGTTGTCGAAATGTATATCA	54.3
27	STMS-20	F	CTTNTCGTCATCATCGTTTTG	54.9
		R	CACCCTACTTTTTTCCACCAC	57.9
28	STMS-24	F	AAAGA CAGGTTTTAATCCAAAA	50.9
		R	CTAATCTTTCTTCTTCTTTTGTCAT	54.8
29	CS-27	F	AGCTGGTCGCGGGTCAGAGGAAGA	67.8
		R	AGTGGTCGCGATGGGGCCATGG TG	69.6
30	CS-27A	F	ACCTGGTCGCGGGTCAGAGGAAGA	67.8
		R	AGTGGTCGCGATGGGGCCATGGTG	69.6
31	GA-4	F	TTGCGTGTCAATCTCATTGG	55.3
		R	TCAACACCCCTAACTCGGA C	59.4
32	GA11	F	GTTGA GCAACAAAGCCACAA	55.3
		R	TTCTTGTCTGGTTGTGTGA GC	57.9

33	GA-16	F	CACCTCGTACCATGGTTTCTG	59.8
		R	TAAATTTCATCCTCTCCGGC	55.3
34	GAA-40	F	TTGACGCAGAGAACTCTCAA	55.3
		R	ATTGGTGTGATGGGTGGATT	55.3
35	GAA-42	F	CGCTTCAGTGTAGATATTATTCAAACA	58.9
		R	TCTCTCTTTCTCTTCAACACGC	58.4
36	GAA-44	F	AGCAAGCCCATGA TTTTCTC	55.3
		R	ATGA CATTCCAATCGGCTTC	55.3
37	GAA-45	F	TTGGGA TCCATTTCATCCAT	53.2
		R	GCCTGGA AGTCACACACTTG	59.4
38	GAA-46	F	TCTCCTGTGA ATGA ACCGA A	55.3
		R	CTGA GCAACAAAATCAGCCA	55.3
39	UBC-170	Seq.	ATCTCTCCTG	24.8
40	UBC-855	Seq.	ACACACACACACACACYT	56.3
41	OPC14-1	Seq.	TGCGTGCTTG	28.9
42	SCoT 11	Seq.	AAGCAATGGCTACCACCA	53.7
43	SCoT 12	Seq.	ACGACATGGCGACCAACG	58.2
44	SCoT 13	Seq.	ACGACATGGCGACCATCG	58.2
45	SCoT 14	Seq.	ACGACATGGCGACCACGC	60.5
46	SCoT 17	Seq.	ACCATGGCTACCACCGAG	58.2
47	SCoT 18	Seq.	ACCATGGCTACCACCGCC	60.5
48	SCoT 19	Seq.	ACCATGGCTACCACCGGC	60.5
49	SCoT 20	Seq.	ACCATGGCTACCACCGCG	60.5
50	SCoT 25	Seq.	ACCATGGCTACCACCGGG	60.5
51	SCoT 27	Seq.	ACCATGGCTACCACCGTG	58.2
52	SCoT 28	Seq.	CCATGGCTACCACCGCCA	60.5
53	SCoT 33	Seq.	CCATGGCTACCACCGCAG	60.5
54	SCoT 34	Seq.	ACCATGGCTACCACCGCA	58.2

Table 2. Details of the informative markers observed in present investigation

Primers	No. of amplicons	Monomorphic bands	Polymorphic band	Polymorphism %	PIC Value
TA - 59	3	1	2	66	0.50
TA - 110	2	-	2	100	0.15
TA - 135	4	-	4	100	0.62
GA – 16	4	-	4	100	0.16
UBC- 855	10	6	4	40	0.89
Total	23	6	16	406	2.32
Average	4.6	1.4	3.2	81.2	0.46

RESULTS AND DISCUSSION

Parental polymorphic survey

Variation between individual genotypes or between populations in a species, can be easily evaluated using a variety of molecular markers. Microsatellite marker is currently the most preferred molecular marker system owing to their highly desirable properties viz., abundance, hyper-variability, high reproducibility, and suitability for high throughput analysis also produces very high allelic variations even among very closely related varieties. Therefore, the 41 molecular markers were used in the parental polymorphic survey along with 13 SCoT markers.

Collard and Mackill (2009) describes that ATG start codons are incorporated into random primers to generate polymorphic fragments from the genome. Therefore, the 13 SCoT markers also used to study the start codon variation in the selected chickpea lines.

The polymorphic markers used in present study shows the discriminating banding pattern between parents. The details of the informative markers and their banding pattern is mentioned in the Table 2. and Plate 2.

In polymorphic markers profiling, total alleles per locus were 4.6, whereas average number of monomorphic and polymorphic alleles were 1.4 and 3.2, respectively. The polymorphic percentage of markers were 40 % for UBC-855, 66 % for TA-59 and 100% for TA-110, TA-135 and GA-16, showed in Table 2. These finding also corelate with Solanki et al., 2010 who performed the parental polymorphic survey for five Lentil genotypes using sixty RAPD and 35 SSR markers and observed the twenty RAPD and 10 SSRs polymorphic markers which further used in the hybridity assessment of 24 F1 lentil individuals and confirmed the 5 true hybrids.

The PIC value for informative profiling ranged from 0.15 to 0.89 with an average value of 0.46. The highest PIC value was observed in UBC-855 marker (0.89), followed by TA-59 (0.50), TA-135 (0.62) and GA-16 (0.16) and lowest PIC value in TA-110 (0.15).

Identification of true F_1 hybrids

Molecular markers are efficient tool to identify and evaluate true F_1 hybrids. Therefore, the markers which are polymorphic were used for identification of true F_1 hybrids. The immense potentiality of molecular marker for the measuring the genetic purity as compare the morphological or biochemical rail has already been reported to several crop. PCR based marker has been employed successfully for parentage verification, hybrid identification and purity testing (Paran et al., 1995).

For analysing the genetic purity of hybrids, the banding patterns of polymorphic markers were compared and polymorphic markers between parents were identified. The size of a polymorphic marker was analysed on visual basis which was further used to test respective hybrids. The purity of the F_1s was confirmed when they showed the presence of male and female both parent alleles.

From total crosses 31 F_1 plants of six crosses were screened F_1 hybridity assessment. These plants were used to obtain F_2 plants for developing the wilt mapping population. Apart from all markers one informative marker were utilized for testing F_1 hybridity based on the parental polymorphism banding pattern.

F_1 hybridity testing by STMS TA-59 marker

STMS marker TA-59 was used for F_1 hybrid purity assessment. This marker screened 31 F_1 plants. TA-59 shows specific size amplicon in female and male parents. It shows amplicon of size 258bp in resistant male parent Virat, WR-315 and 257bp and 256 bp amplicon in female parent PKV Kabuli 4 and Chanoli, respectively.

STMS marker, (TA-59) screened on the 31 F_1 plants of crosses, Chanoli X Virat, Chanoli X BDNGK-798, Chanoli X WR-315, PKV Kabuli 4 X Virat, PKV Kabuli 4 X WR-315, PKV Kabuli 4 X BDNGK-798 the banding pattern were shown in Plate 3.

Four F_1 plants of cross Chanoli x BDNGK-798 shows presence of both amplicon of parents Chanoli (257bp) and BDNGK-798 (258bp). Hence all four F_1s were confirmed to be true hybrid. Similarly, one F_1 plants derived from Chanoli X WR-315 and two Chanoli X Virat confirmed to be true hybrid. Solanki et al.,2010 performed the hybridity assessments for 24 F1 plants and proves the importance of RAPD and ISSR markers in confirmation of true F1 individuals.

In crossed derived from PKV (Kabuli 4 x Virat) out of five F_1 plants two shows presence of both amplicon of parents PKV Kabuli 4 (256bp) and Virat (258bp). These two F_1s were confirmed to be true hybrids. Similarly, three from F_1 plants of crossed PKV Kabuli 4 x BDNGK-798 and one from cross of PKV Kabuli 4 X WR-315 were confirmed as true hybrid as shown in Plate 3.

The primer TA-59 was mapped on linkage group 2 on which genes for disease resistance were present (Winter et al., 2000). This marker gives the specific banding amplicon in male and female parent which is helpful to discriminate the true F1s hybrids.

Thus, determine hybrid purity important to develop a mapping population for reviewing the genetics or mapping of gene controlling desirable traits. The genetic improvement for wilt resistance can be made in lentil if genetics of this trait is known. This can only be studied if a correct segregating population is available. For obtaining a true segregating population, it is must that crossed seeds are to be true F_1s (Solanki et al.,2010.)

In chickpea, selfing is commonly occurred. Therefore, identification of true F_1 plants using molecular markers in present study will positively reduce chance of the further errors. Moreover, it will save time, efforts and money required to switch an incorrect segregating population.

CONCLUSION

The results of present work proved that SSR markers are well polymorphic and more useful to perform the molecular characterization and to test the genetic hybridity of F_1 plants. The polymorphic markers can be helpful for diversity analysis and speedup the breeding program.

Thus, determining hybrid purity is important to develop true mapping population for studying the nature of genetics associated with trait of interest. Because use of segregating F2 population having mixture of selfed and true F1 plants hampers the results of genetic mapping and purity of population. In chickpea selfing and mixture are commonly occurred. Therefore, identification true F1 plants using markers in the present study will certainly reduced the efforts and time of breeding program. Moreover, it will helpful to speedup the breeding technique as compare to traditional one which helpful to farmers community.

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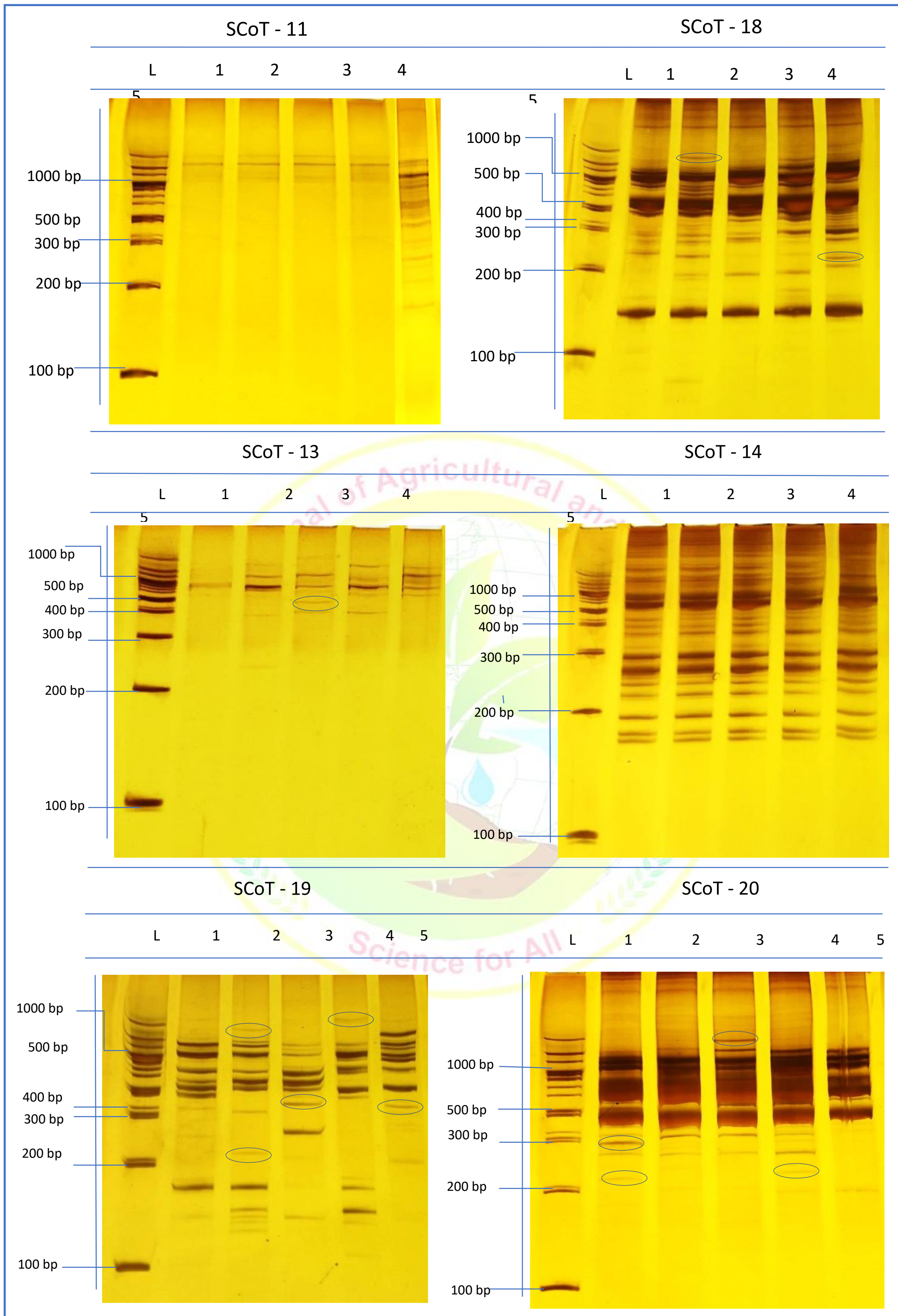

Plate 1. Start codon targeted (SCoT) marker profiling in Chickpea genotypes
Lane L- 100 bp, 1- Chanoli, 2- Kabuli-4, 3- Virat, 4- BDNGK 798, 5- WR 315

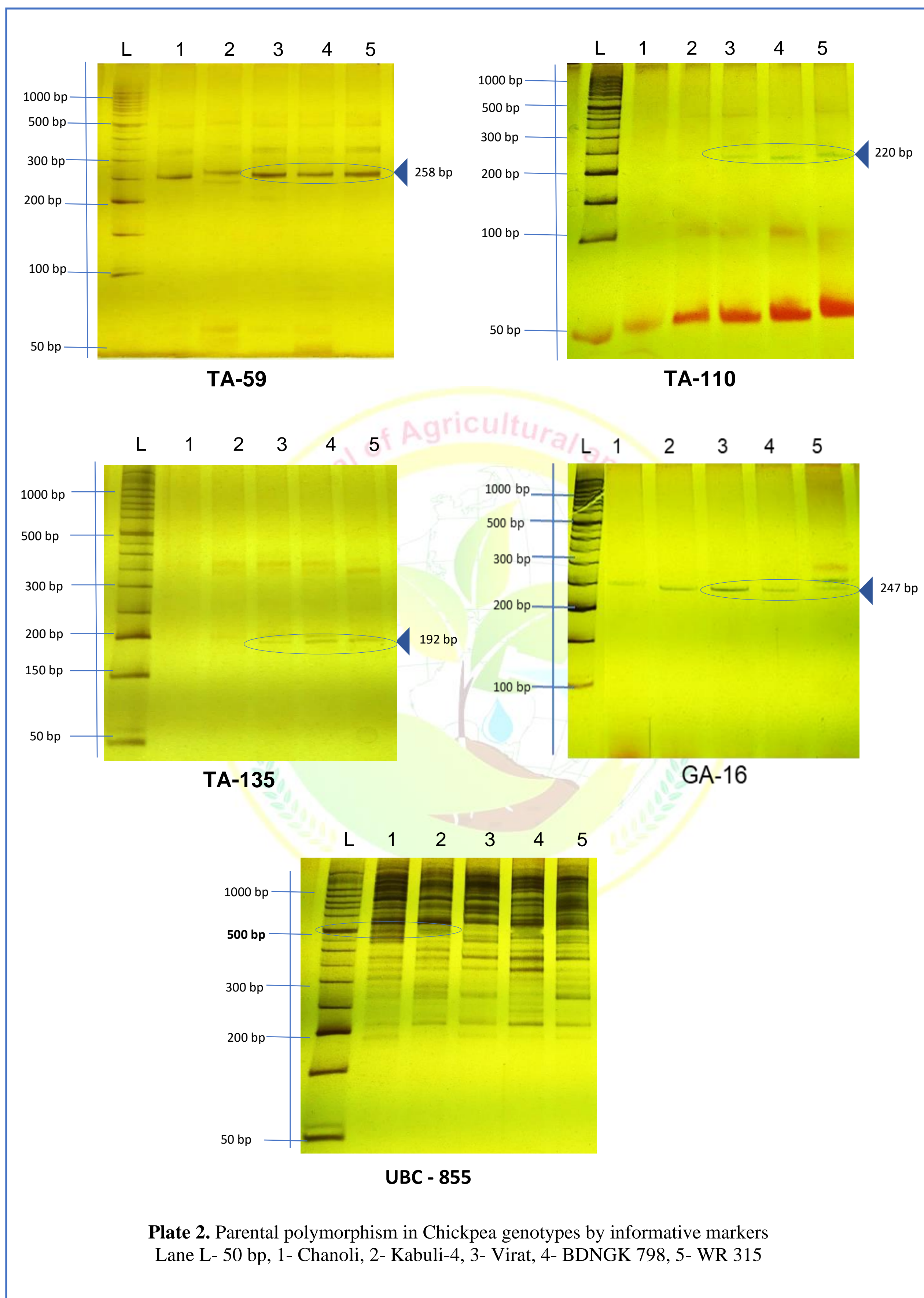

Plate 2. Parental polymorphism in Chickpea genotypes by informative markers
Lane L- 50 bp, 1- Chanoli, 2- Kabuli-4, 3- Virat, 4- BDNGK 798, 5- WR 315

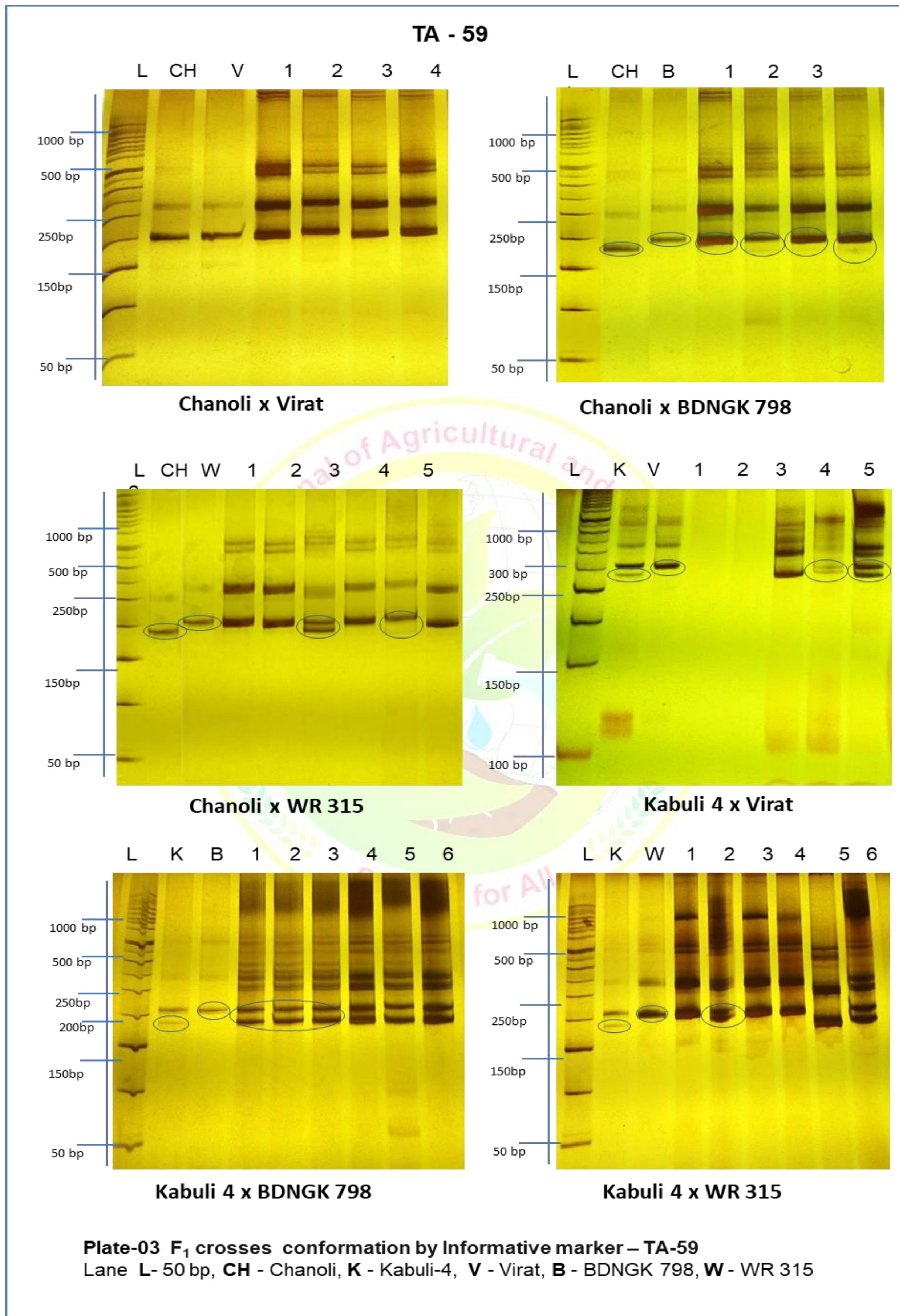

Plate-03 F_1 crosses conformation by Informative marker – TA-59
Lane **L**- 50 bp, **CH** - Chanoli, **K** - Kabuli-4, **V** - Virat, **B** - BDNGK 798, **W** - WR 315

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