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DNA FINGERPRINTING OF NEW HIGH YIELDING GREENGRAM (*Vigna radiata* L. WILCZEK) VARIETY VBN 4 USING SSR MARKERS

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ABSTRACT

Precise identification of crop varieties is important for registration and certification of newly released varieties. Molecular markers are powerful tools, which helps in differentiating plant varieties at molecular level have been widely used for fingerprinting in a number of crop plants. The new greengram variety VBN 4 is derived from a cross combination PDM 139 x BB 2664 was released from National Pulses Research Centre, Vamban during 2019-20. The special features are high yield potential (1024kg/ha), moderate resistance to Mungbean Yellow Mosaic Virus (MYMV), Powdery mildew and resistant to Urdbean Leaf Crinkle Virus diseases with superior cooking quality traits. Morphological traits viz., size of trifoliate leaf, colour of the leaf and pod position are available to distinguish the new greengram variety VBN 4 from the ruling varieties VBN (Gg) 3 and CO 8. However, all these varieties cannot be distinguished with a single character alone. In the present study, a total of 27 SSR primers were used to differentiate VBN 4 from VBN (Gg) 3 and CO 8. Among them six primers viz., CEDG 198, CEDG 121, CEDG 271, CEDG 264, CEDG 141 and CP 08513 revealed polymorphism among varieties. Primers viz., CP 08513 and CEDG 264 showed polymorphism between all three varieties. Likewise, primers CEDG 121, CEDG 141 and CEDG 271 revealed polymorphism between VBN 4 and CO 8. One primer, CEDG198 was able to differentiate VBN 4 and VBN (Gg) 3. Hence these SSR primers can be used to differentiate these varieties at DNA level during seed certification.

Key words : Greengram VBN 4, SSR marker, DNA fingerprinting.

Introduction

Greengram [*Vigna radiata* (L.) Wilczek] is also known as goldengram, mungbean, mashbean and moong, is one of the most important pulse crops in the family Fabaceae (Leguminosae). It is a self-pollinated diploid species having a chromosome number of $2n = 22$ and an estimated genome size of 579 Mbp (Arumuganathan and Earle, 1991). Based on domesticated diversity data and archeological evidence, the origin of greengram is considered to be India and during the early domestication process, its cultivation migrated to other Asian countries and further into Africa, Australia, South America and other parts of the World (Fuller and Harvey, 2006; Kim *et al.*, 2015). It is regarded as quality pulse due to its excellent

digestibility and rich protein (25-28%), especially when combined with cereals. On account of its short duration, photosensitivity and dense crop canopy, it assumes special significance in crop intensification, diversification and hybridization (Mahalingam *et al.*, 2018). Since, greengram is a self-pollinated crop considerable variation exists among the cultivars (Bisht *et al.*, 2005).

Clear identification of varieties is important for registration and certification of crop varieties to curb supply of surplus seed materials and avoid marketing of same variety in different names by seed production agencies (Korir *et al.*, 2012). Generally, varietal identification involves DUS (Distinctness, Uniformity and Stability) testing system based on morphological and

traditional phenotypic evaluation following crop specific descriptors (IPGRI, 1991). As more and more number of cultivars are being developed from few elite parents, it becomes more and more difficult to distinguish varieties solely based on morphological characters. DUS test is greatly influenced by environmental conditions along with errors in data recording and storage. The majority of the breeding programs use a set of parents with similar pedigrees and features to combine desirable traits such as seed yield, cooking and oil quality, and resistance to biotic and abiotic stresses (Nie *et al.*, 2016).

Even the same genetic material is marketed with different trade names and it has become very difficult to protect the infringement of new crop varieties, which is a major problem for farming community at ground level. The current DUS testing requires detailed morphological characterization for at least two seasons to ensure the distinctive features between two varieties. This procedure is not only time consuming but also very expensive and laborious. This necessitated the identification of an authentic method for varietal identification viz., DNA fingerprinting to protect crop varieties by appropriate characterization and documentation (Amaravathi *et al.*, 2014). The last decade has showed drastic reduction in the cost of sequencing and genotyping due to advancement in technologies viz., genotyping-by-sequencing (GBS) (Dodia *et al.*, 2019; Kulkarni *et al.*, 2020), whole genome re-sequencing (WGRS) (Kumar *et al.*, 2020), ddRADseq, DArT, and DArT-sEquation (Vishwakarma *et al.*, 2016) and high density single nucleotide polymorphism (SNP) array (Gangurde *et al.*, 2020), which provided a much cheaper, time saving, and highly precise option for assessing the distinctiveness in plants including groundnut. Besides, it has the advantage of not being affected by gene expression during different crop developmental stages. Molecular markers are also not sensitive to environment and have high repeatability as well (Hayward *et al.*, 2015). Therefore, molecular markers are providing superior technical support in testing, approval and identification of new crop varieties (Jones *et al.*, 2016)

Progress has been made through conventional breeding at international and national research institutions in the last three decades towards the development of new varieties. Several morphological characters are available to differentiate the varieties. The environmental influences on morphological characters and time factor make it difficult to collect the morphological data, besides other limitations are unambiguous differentiation of genotypes (Dogney, 2002; 2005). To overcome this problem molecular markers can be used because they

offer a faster and reliable technique for precisely assessing the genotypes of a plant (Meena *et al.*, 2015; Dhurwe *et al.*, 2017). One among the proven technique is DNA fingerprinting. DNA fingerprinting technique was invented by Alec Jeffreys during 1985. While it is very difficult to differentiate the crop varieties by the morphological descriptors, DNA fingerprinting makes it possible for researchers to pinpoint specific fingerprint and accurately identify crop varieties (Ragul *et al.*, 2018). Several research organizations in India and across the globe have started offering DNA testing for plant varietal identification. DNA fingerprinting is used by plant breeders of both private and public sectors for identification of crop varieties. More importantly, Protection of Plant Variety and Farmers Right Authority (PPV and FRA, 2001), Govt. of India made a DNA fingerprint as an additional mandatory requirement for the notification of new crop varieties released both by the Central and State Variety Release Committees (CVRC & SVRC). With this background, an attempt was made in this study to identify a molecular marker to differentiate the newly released greengram variety VBN 4 from the other ruling varieties.

Materials and Methods

The experimental material comprises the newly released greengram variety VBN 4 from National Pulses Research Center, Vamban (Fig. 1) and check varieties viz., VBN (Gg) 3 and CO 8. The variety VBN 4 is a cross derivative between PDM 139 x BB 2664. The duration ranges from 65-70 days and suitable for cultivation in all the pulses growing season's viz., *Kharif*, *Rabi*, *Summer irrigated* and *Rice fallow* seasons of Tamil Nadu. DNA fingerprinting work has been carried out with a total of 27 Simple Sequence Repeat (SSR) markers. These were randomly selected from the SSR markers reported by Isemura *et al.* (2012) in mungbean (Table 1).



Fig. 1 : Field view of greengram VBN 4.

Table 1 : List of primers and its sequences.

Primer codes	Forward/Reverse	Primer sequence 5'-3'
CEDG 003	F	CCACTTCTCTTGACTTTGC
	R	GACCAAAGTGAAGCCAAGAG
CEDG 008	F	AGGCGAGGTTTCGTTTCAAG
	R	GCCCATATTTTACGCCAC
CEDG 020	F	TATCCATACCCAGCTCAAGG
	R	GCCATACCAAGAAAGAGG
CEDG 027	F	ACTTGGGGTTTGAGATGTGG
	R	TCATTTTGGCCACTCAGTGC
CEDG 060	F	TGCATCGTCTCATGCGACCAG
	R	ATTCTGGCAGCGGAGAAACC
CEDG 076	F	GGTGGTTTACTTACTGGCATT
	R	GGTCTATCCACCATCTATCAA
CEDG 092	F	TCTTTTGGTTGTAGCAGGATGAAC
	R	TACAAGTGATATGCAACGGTTAGG
CEDG 097	F	GTAAGCCGCATCCATAATTCCA
	R	TGCGAAAGAGCCGTTAGTAGAA
CEDG 107	F	GAAGTTGACCTTCAATGGAGAAAA
	R	TTGTAGCGTAAAATTAATCCACGC
CEDG 121	F	CTTCAAATAATGTTGAGGCATA
	R	CAATACATAAATAACCTTTTCTGC
CEDG 127	F	GGTAGCATCTGAGCTTCTTCGTC
	R	CTCCTCACTTGGTCTGAAACTC
CEDG 141	F	CCAGGCATCCATGATGACC
	R	GAAGTTGTTGGTAATGGTTGCCTC
CEDG 156	F	CGCGTATTGGTGACTAGGTATG
	R	CTTAGTGTTGGTTGGTCGTAAGG
CEDG 180	F	GGTATGGAGCAAAACAATC
	R	GTGCGTGAAGTTGTCTTATC
CEDG 191	F	CAATAAGCAATCTGTGGAGAG
	R	CTGCAGGAACTTGAATTGC
CEDG 198	F	CAAGGAAGATGGAGAGAATC
	R	CCTTCTAAGAACAGTGACATG
CEDG 245	F	GATAGAGCTTAAACCCCTC
	R	CTTTTGATGACAAATGCC
CEDG 254	F	CGATGTCTCTTGCTTCAAGG
	R	GTGAAGGACTAGCCAAGTTTG
CEDG 264	F	GATTCCCTTCCTAGCTATGG
	R	CTGCTGGACATGAAGATTACG

*Table 1 continued...***Table 1 continued...**

CEDG 271	F	GCACTAAAGTTAGACGTGGTTC
	R	CACTCCCACTGCCAAACAAGG
CEDG 279	F	GGTCTTTCTAAGCGGAGCAC
	R	CTGCCTCTCTACACAAGTGG
CEDG 282	F	CAGCAACAAGACATGGAGTG
	R	GGTGACCACTTAGACAGAC
cp 05325	F	GTCCAAGTGGAAGAAAGAAGGGGT
	R	GGAGCTACCTTTTCAGTGTGGAGC
cp 08513	F	GCAGGAAGAAAGTCGTTTACTGCAA
	R	GATATCAAGGACCTTCCTCCCCAC
GATS 11	F	CACATTGGTGCTAGTGTCCG
	R	GAACCTGCAAAGCAAAGAGC
VrD1	F	CAGCTTCTTGTCTTGTCTCC
	R	CGAATGTGCACAGGTGGTGT
Bm 170	F	AGCCAGGTGCAAGACCTTAG
	R	AGATAGGGAGCTGGTGGTAGC

Total genomic DNA of each parents were extracted from fresh young leaf tissues using the CTAB method (Lodhi *et al.*, 1994). The collected samples were grinded in pestle and mortar by using preheated (around 65°C) CTAB buffer. Around 500µl of CTAB buffer was added. Extracted samples were taken into eppendorf tubes and incubated in the water bath for 30 min at 65°C. After incubation, 300µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added into the tubes and inverted twice to mix and kept in centrifuge for 10 min at 10000 rpm. The supernatant was collected in the fresh tubes. Then the tubes were added with 500µl of chloroform: isoamyl alcohol (24:1) and inverted twice to mix. Then the tubes were kept in centrifuge for 5 minutes at 10000 rpm. Then the aqueous layer was transferred in to the new eppendorf tubes. An amount of 0.7 volume of isopropanol (stored at -20°C) was added to each sample and inverted once in mix and kept it overnight at 4°C. The samples were centrifuged at 8000 rpm for 10 min on the next day. The supernatant was discarded from each sample and the pellets settled in the bottom were air dried for 30 min. A quantity of 50 µl of TE buffer was added into each sample and stored it overnight at 4°C. RNase (3 µl) was added into each sample to exclude the RNA contamination on the following day and leave it at 65°C for 30min.

The concentration of DNA was determined by com-paring against lamda DNA and diluted to 1 ng/ pl for marker analysis. PCR was performed using a total volume of 10 µl containing 10 X Taq buffer + MgCl₂ (15mM) 1.0 µl, dNTPs (2 mM) 1.0 µl, Primers 5 µ M

each of forward and reverse primers, Magnesium chloride (25mM) 0.2 µl, Taq polymerase (3 IU / µl) 0.3 µl, Sterile double distilled water 4.5 µl and Template DNA (10ng/ µl) 2.0 µl. The DNA was amplified in a Eppendorf vapo.protect PCR System under the following conditions: 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, with a final extension step of 72°C for 20 min. The PCR products were separated on 6% polyacrylamide gels were used for better separation and visualization of PCR amplified microsatellite products.

Results and Discussion

Characterization of cultivars, establishment of varietal identity and genetic purity of the seed lots are crucial for varietal improvement, protection of varieties and seed production. A rapid and reliable technique to verify the identity and assess the purity of seed lots is important in seed quality assurance programmes to meet the minimum seed certification standards of seed quality prescribed for the certified class. Hence in order to establish and maintain a high market reputation, all major seed companies, both in private and public sector, take necessary care to ensure highest quality standards (Dogney, 2005).

The major task in the varietal release program is the identification of distinguishable morphological characteristics for the newly released varieties. Distinguishing morphological traits for VBN 4 and check varieties were presented in Table 2. Among the greengram varieties, VBN 4 is clearly distinct from ruling VBN (Gg) 3 for its size of trifoliate leaf and pod position.

The variety VBN 4 has large size of trifoliate leaf and pod position of above canopy. VBN (Gg) 3 has medium sized ovate leaves and intermediate pod position. Distinguishing the closely related varieties is a difficult task. In that case morphological descriptors will lose its utility as it has limited classifications. Hence, DNA level varietal differentiation has gained its importance for the

Table 2 : Distinguishing morphological traits for greengram varieties.

S. no.	Morphological traits	VBN 4	VBN (Gg) 3	CO 8
1	Size of trifoliate leaf	Large	Medium	Medium
2	Colour of the leaf	Dark Green	Light green	Light green
3	Pod position	Above canopy	Intermediate pod position	Above canopy

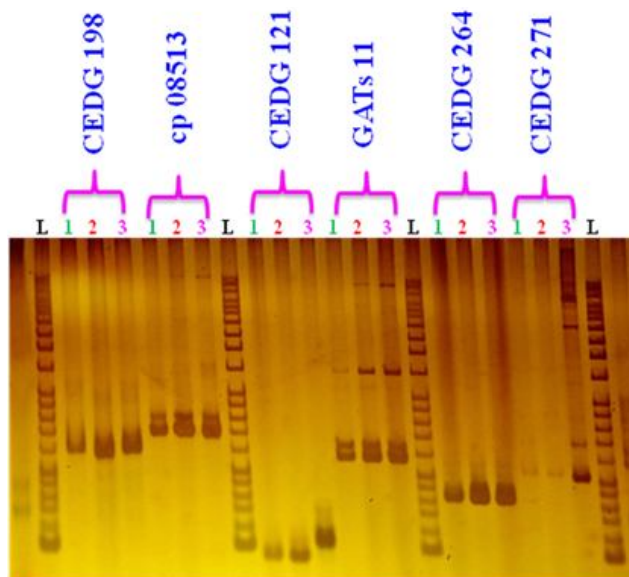
Table 3 : Polymorphic primers for the varieties VBN 4, VBN (Gg) 3 and CO 8 with base pair differences.

Primers/varieties	Base pair differences (bp)		
	VBN 4	VBN (Gg) 3	CO 8
CEDG 198	220	200	220
CEDG 121	100	100	130
CEDG 264	160	145	145
CEDG 271	220	220	200
CEDG 141	230	230	210
cp 08513	280	265	265

varietal identification. DNA fingerprinting will be a better choice of obtaining clear differences to pinpoint the particular variety.

The unambiguous identification of plant varieties is important in many areas of agriculture and plant biology research. New techniques based on DNA profiling provide novel approaches to varietal identification which offer advantages over traditional morphological comparisons. These advantages include great potential resolving power, data that can be analysed objectively, testing at all stages of development, and cost effectiveness (Morell *et al.*, 1995; Chakravarthi and Naravaneni, 2006).

A total of 27 SSR primers were used to differentiate VBN 4 from VBN (Gg) 3 and CO 8. These primers were selected from all the 11 linkage groups of mungbean reported by Isemura *et al.* (2012). Among them six primers viz., CEDG 198, CEDG 121, CEDG 271, CEDG



1-VBN 4; 2-VBN (Gg 3); 3-CO 8

Fig. 2 : DNA fingerprinting for greengram varieties VBN 4, VBN(Gg) 3 and CO 8.

264, CEDG 141 and CP 08513 had polymorphism among the varieties (Table 3 and Fig. 2). Primers viz., CEDG 198, cp 08513 and CEDG 264 revealed polymorphism between VBN 4 and VBN (Gg)3 varieties. Likewise, five SSR primers viz., CEDG 121, CEDG 264, CEDG 271, CEDG 141 and cp 08513 showed polymorphism between VBN 4 and CO 8. Hence these primers may be useful to differentiate these varieties at DNA level. This can be a potent tool in seed certification to find out the genetic purity at seed lot inspection.

Conclusion

To conclude, morphological descriptors are available to distinguish the variety VBN 4 from VBN (Gg) 3 and CO 8. However, all these varieties cannot be distinguished with a single character alone. The DNA marker will be an added tool to distinguish varieties. Out of 27 primers surveyed, six primers were useful to aid in DNA fingerprinting to differentiate these varieties.

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