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RESEARCH ARTICLE

Role of *AhMITEI* in the mutation and sub-specific differentiation of groundnut (*Arachis hypogaea* L.)

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SUMMARY

AhMITE1 is an active miniature inverted repeat transposable element (MITE) in peanut (*Arachis hypogaea* L). Twenty-one mutants belonging to different botanical types of groundnut was checked for (FST1-linked) site using *AhMITE1*-specifc PCR, which used a forward primer annealing to the 50-flanking sequence and a reverse primer binding to *AhMITE1*. The parent Dharwad early runner (DER) was found to be free of *AhMITE1* insertion at the FST1-linked site. Another EMS mutagenesis effort with VL 1, a Valencia mutant from DER, (28-2 and 110), showed insertion of *AhMITE1* into the FST1-linked site. Origin of NLM, a Virginia bunch type mutant from TMV 2, ainvolved excision of *AhMITE1* from the predetermined site. 28-2(s) and 110(s) obtained spontaneously from 28-2 and 110, respectively, were also associated with excision of the transposable element. Excision and insertion of *AhMITE1* at this particular site among the mutants their presence in all the Spanish types and absence in all Virginia and Valencia types; led to gross morphological changes resembling alternate subspecies.

Key Words : Groundnut, *AhMITEI*, Mutants, Subspecies

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Groundnut is believed to have originated in the Bolivian region of south America where the greatest diversity is found (Gregory and Gregory, 1976). There are about 70 species, most of them diploid (2n=2x=20). The cultivated groundnut is an allotetraploid having A and B genomes, which have been contributed by two diploid species. Groundnut [*Arachis hypogaea* (L.)] is classified into two subspecies, *viz.,* ssp. *hypogaea* (Krap. and Rig.) and ssp. *fastigiata* (Wald.) based on variation in morphology. Further, the ssp. *hypogaea* is bifurcated into var. *hypogaea* (Virginia bunch/runner) and var. *hirsuta* (Peruvian runner), and likewise ssp. *fastigiata* into var. *fastigiata* (Valencia), Peruviana, aequatoriana and var. *vulgaris* (Spanish bunch) (Stalker and Simpson, 1995). Only four botanical types namely, Virginia bunch (VB), Virginia runner (VR), Valencia (VL), and Spanish bunch (SB) are exclusively cultivated by the farmers owing to their agronomic attributes and market value. Kochert *et al*. (1996) suggested that *A. hypogaea* might have arisen as the result of single polyploidization event and the dramatic shifts in the morphology of plant organ arose as a result of changes in one or two major genes and a few modifier loci. The possible role of spontaneous and induced mutations in the evolution of abundant morphological variation in groundnut was also evident from various sub-specific changes brought about by mutations in a few breeding programmes (Mouli and Kale, 1982b; Prasad, 1989). Despite tremendous variation in morphology, physiology and agronomical traits, groundnut has shown limited variation at molecular level, isozyme and seed protein (Stalker *et al*., 1994); RFLP and RAPD (Paik – Ro *et al*., 1992). However, recent studies revealed polymorphism in the

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cultivated groundnut using amplified fragment length polymorphism (AFLP) (He and Prakash, 1997), RAPD (Bhagwat *et al*., 1997) and simple sequence repeat (SSRs) (Hopkins *et al.,* 1999) assay. But RAPD failed to differentiate different botanical types of groundnut. Miniature inverted–repeat transposable elements (MITEs) are widely distributed among animal and plant genome. MITEs are structurally characterized by relatively small (80- 500bp long) size, AT rich and non-coding interspersed elements with a terminal inverted repeat of 25bp. MITEs have the potential role in gene regulation, defining chromatin domains and genome organization. Miniature inverted repeat transposable elements (MITEs) are the predominant TEs among plant genomes (Shan *et al.,* 2005).Transposition preference for low copy genic regions emphasises the role of MITEs in modulating gene expression (Wessler, 1998) and aiding crop evolution (Shan *et al.,* 2005). Here we have made an effort to study the relevance of its transposition with the mutational and sub-specific differentiation. In the present investigation, *AhMITE*-specific primers were used to investigate its association in the 21 mutants using RAPD assay.

MATERIALS AND METHODS

The experimental material comprised of 21 genotypes of groundnut representing Virginia bunch (3), Virginia runner (2), Valencia type (8), Spanish bunch (7), Dharwad early runner (1) was included (Table A) Dharwad early runner, a taxonomic freak was obtained from a cross between two *fastigiata* cultivars (Dh3–20 and CGC-1). DER on mutagenesis with ethyl methane sulphonate (EMS) yielded a very high frequency of mutants resembling all the four botanical varieties (Gowda *et al.,* 1996). On mutagenesis with EMS (0.5%) it yielded very high frequency of mutants representing different botanical types including VL1 mutant with resistance to rust (Gowda *et al.,* 1996), produced a DER revertant, VL 1 (R) in M_3 generation.The mutant and the revertant bred true in later generations. VL 1 yielded a high frequency of mutants with wide spectrum of variations for disease resistance reactions and morphological characters. The VL 1 derived mutants were evaluated for productivity and resistance parameters. A high level of resistance to late leafspot was evident in many mutants but these mutants, *viz.,* VL 1-28-2, VL 1-110 combined early maturity, moderate to high productivity and desirable pod and kernel features (Motagi *et al*., 1996). TMV 2 is a Spanish bunch variety which on mutagenesis with EMS (0.2%) resulted in a 'narrow leaf mutant' (NLM) with a shift to Virginia type (Prasad *et al*., 1984). TMV 2 and its mutant differ for several morphological characters. NLM on treatment with azacytidine resulted in 'broad leaf variants', *viz*., 'NLM broad' (BLM). Treatment of DER with EMS and 5-azacytidine has resulted in necrotic disease mimic mutants namely 'Spanish bunch necrotic' (SB necrotic) and 'Valencia necrotic' (VL necrotic),

Table A: List of mutants and their origin

Sr. No.	Mutants	Botanical types	Pedigree
1.	DER	-	Dh 3-20 x CGC -1
2.	SB 3	Spanish bunch	Gamma rays mutant of DER
3.	SB 6	Spanish bunch	Gamma rays mutant of DER
4.	VL 1	Valencia type	EMS mutant of DER
5.	VL 3	Valencia type	EMS mutant of DER
6.	VL 1 (dwarf)	Valencia type	Mutant of VL 1
7.	VL 1 (R)	Valencia type	Revertant of VL 1 resembling DER
8.	M 28-2	Spanish bunch	EMS mutant of VL 1
9.	M 28-2 (S)	Valencia type	Mutant of M 28-2 susceptible to LLS
10.	M 110	Spanish bunch	EMS mutant of VL 1
11.	M 110 (S)	Valencia type	Mutant of M 28-2 susceptible to LLS
12.	VB 1	Virginia bunch	Virginia bunch type mutant from DER
13.	VB 3	Virginia bunch	Virginia bunch type mutant from DER
14.	VR 8	Virginia runner	EMS mutant of VL 1
15.	SB necrotic	Spanish bunch	Disease mimic mutant from DER
16.	SB normal	Spanish bunch	Non- necrotic mutant from SB necrotic
17.	VL necrotic	Valencia type	Disease mimic mutant from DER
18.	VL normal	Valencia type	Non- necrotic mutant from VL necrotic
19.	TMV 2	Spanish bunch	Mass selection from Gudhiatham bunch
20.	NLM	Virginia bunch	EMS mutant of TMV2
21.	NLM broad	Virginia runner	Azacytidine induced mutant of NLM

respectively. In *Kharif* season, the experimental materials were sown on 26th September 2007. Regular cultivation practices were followed to raise a good crop and observations were recorded for the following characters according to 'groundnut descriptors'.Genotypes were grown in pots and genomic DNA was extracted from young leaves using cetyltrimethy lammonium bromide (CTAB) method (Saghai-Maroof *et al*., 1984). Genomic location of the *AhMITE1* was determined (Bhat *et al.,* 2008) by recovering its 50-flanking sequence tag from TMV 2, a Spanish bunch cultivar, using TAIL-PCR with a set of degenerate primers and nested primers designed based on the reported MITE sequence (Patel *et al*., 2004). Presence or absence of *AhMITE1* at this pre-determined site (FST1-linked site) in various genotypes/mutants was checked by developing an AhMITE1-specifc PCR. The forward primer (50 GGGAGAAGAAAAGGATGAGA 30) was designed on the basis of the AhMITE1 flanking sequence tag (FST1) recovered from the TMV 2 genotype of peanut (Bhat *et al*., 2008) whereas the reverse primer (50 TCTCATGAAGATGCTTTGGT 30) was specific to *AhMITE1* (Patel *et al.,* 2004). For *AhMITE1*-specifc PCR, A final volume of 20 ll containing 100 ng genomic DNA, 19 PCR buffer, 0.5 mM dNTPs, 10 pmol of each primer, and one unit of Taq DNA polymerase (Genei, Bangalore, India) was used for PCR. The amplification reaction was carried out in a Mastercycler (Eppendorf, Germany) by setting the conditions for one cycle of pre-denaturation (94^{0}C for 5 min), 35 cycles of denaturation (94^{0}C for 1 min), annealing (60^{0}C for 1 min), and extension (72^{0}C for 1 min). One cycle of final extension (72^{0}C for 1 min) was included before the PCR product was stored at 4^{0}C until further use. Presence of the PCR product was checked on 1.2 per cent agarose gel by electrophoresis. The PCR amplification would give a band of 242 bp when *AhMITE1* is inserted at FST1-linked site. Absence of 242 bp product with the same primers indicates the excision of *AhMITE1* from FST1-linked site.

RESULTS AND DISCUSSION

AhMITE and its flanking sequence-derived primers were used for amplification by polymerase chain reaction. The PCR products were separated on 1.5 per cent agarose gel electrophoresis. *Ah MITE* specific primer showed polymorphism with respect of 242 bp fragment. Out of 21 genotypes it was present in six genotypes and absent in others Table 1. The parent Dharwad early runner (DER) did not amplify the 242-bp product, hence was found to be free of *AhMITE1* insertion at the FST1-linked site. Mutants generated by gamma irradiation SB 6 amplified the 242-bp product with *AhMITE1*-specific PCR, indicating the insertion of *AhMITE1* into the FST1-linked site. These mutants resembled Spanish bunch type in their erect growth habit, lack of seed dormancy, and increased pod size compared with the DER type. Another EMS mutagenesis effort with VL 1, a Valencia mutant from DER, could generate two Spanish bunch mutants (28-2 and 110). They showed insertion of *AhMITE1* into the FST1-linked site. Contrastingly, EMS mutagenesis could also activate excision of *AhMITE1* from the FST1- linked site as observed with the narrow leaf mutant (NLM) from TMV 2. Origin of NLM, a Virginia bunch type mutant (with alternate branching and main stem flowering) belonging to spp. hypogaea from TMV 2, a Spanish bunch cultivar, involved excision of *AhMITE1* from the predetermined site, indicating a gross morphological change. Likewise, 28-2(s) and 110(s) obtained spontaneously from 28-2 and 110, respectively, were also associated with excision of the transposable element. It was present in all the Spanish types except SB 3 and absent in all Virginia and Valencia types; It was present in TMV 2 but absent in its mutant NLM and also in NLM broad; present in M 110 and M 28-2 but absent in VL 1 (Fig. 1).

Table 1 : Distribution of 242 bp band in mutants for AhMITE-specific primer

Sr. No.	Genotypes	Distribution of 242 bp band
1.	DER	_
2.	SB3	_
3.	SB6	+
4.	VL1	_
5	VL3	_
6.	VL1 dwarf	_
7.	VL1 (R)	_
8.	M28-2	+
9.	M28-2 (S)	_
10.	110	+
11.	110 (S)	_
12.	VB-1	_
13.	VB-3	_
14.	VR-8	_
15.	VL necrotic	_
16.	VL normal	_
17.	SB necrotic	+
18.	SB normal	+
19.	TMV-2	+
20.	NLM	_
21.	NLM broad	_

+ Presence of 242 bp band , - Absence of 242 bp band

Miniature inverted-repeat transposable elements (MITEs) belong to non-autonomous class II type (Osborne *et al.*, 2006). Their copy number is very high and varies from 3,000- 10,000 per genome. MITEs being a major component of interspersed repetitive sequences (genome-wide repeats), they serve as excellent tools for fine genomic analysis throughout the genome. In the present investigation, *AhMITE*-specific primers (Bhat *et al.,* 2008) were used to investigate genetic relations among the mutants and possible role of such elements in the inducting such mutations. *Ah MITE*-specific primer showed polymorphism with respect to 242 bp fragment. Out of

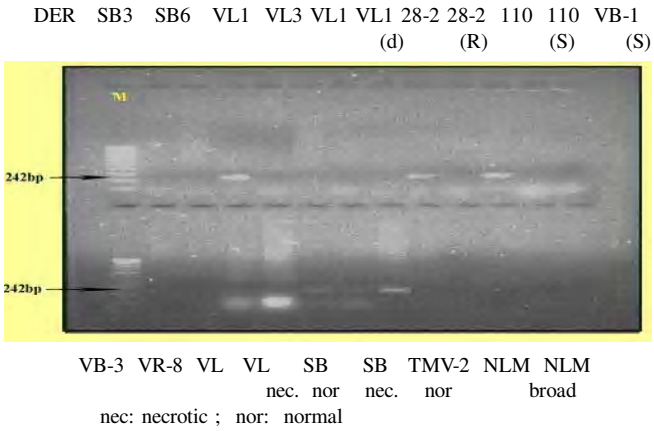

Fig. 1: Frequency of AhMITE – specific primer in 21 mutants. Amplification of the 242-bp product upon AhMITE1-specific PCR indicates the presence of the element at the FST1-linked site (M: 100 bp DNA ladder)

21 genotypes it was present in six genotypes and absent in others. It was present in TMV 2 but absent in its mutant NLM; absent in VL 1, present in its mutants 110 and M 28-2 but absent in the secondary mutants 110 (S) and M 28-2 (S); present in all Spanish type mutants of DER but absent in DER; present in all Spanish types but absent in Valencia and Virginia types indicating its association with certain induced mutations. Observation that the presence or absence of *AhMITE* in a specific genomic region was significantly associated with morphological variation indicated the possible role of *AhMITE,* and its implication in differentiation of groundnut subspecies. A detailed analysis of breeding behaviour of these mutants earlier revealed several unusual features such as, homozygous mutations, mutation outbursts, segregation distortions, somatic mutations, multiple character mutations and multiple alternate forms indicative of non-Mendelian turnover mechanisms (Gowda *et al*., 1996) and activation of cryptic transposable element (TEs) was implicated as one of the possible causes of mutation. McClintock (1984) predicted that any challenge/ stress or "genomic shock" can result in a preprogrammed response resulting in extensive restructuring of the genome. Various stresses to the genome are known to activate the cryptic transposable element (TEs) (McClintock , 1978; Wessler, 1988) and activation of these elements leading to insertion and excision leading to creation of mutants. On mutagenic treatment of DER and other genotypes, the mutagens EMS, gamma rays and azacytidine might have caused "genomic shock" or genomic stress. Thus the TEs, which are widely known to cause insertion mutations and chromosomal rearrangement and are most often evoked as macro evolutionary agents in the organismic evolution (Charlesworth *et al*., 1994), can be considered as the motors of the evolutionary changes in *Arachis hypogaea.* Association of *AhMITE* with Spanish types but not with others is indicative of the role of the elements in intra-specific differentiation of *Arachis hypogaea* and deserves more detailed analysis by way of validation in wide array of material and detailed molecular analysis of the events leading to the mutations. Many MITEs are known to be preferentially placed in the low copy genes and their promoters (Casa *et al.*, 2000; Zhang *et al.*, 2000; Jiang and Wessler, 2001), indicating their potential role in gene regulation, defining chromatin domains and genome organization. Therefore, MITEs have been implicated in the evolution of gene structure and function (Wessler *et al.*, 1995). MITEs can be used as a marker and MITE-based primers can be effectively used in mapping and fingerprinting of genomes (Casa *et al.*, 2000; Zhang *et al.*, 2000 and Jiang and Wessler, 2001). Significant morphological difference between the mutants and their respective parents (TMV 2, DER and VL 1) indicate genetic control. The mutagens used to generate these mutants are known to induce normally the local lesions (mutations) in limited regions of the genome directly, though indirectly many downstream genome-wide changes can take place. TMV 2 and DER derived mutants showed significant differences with their parent both at morphological and molecular level. Whereas, VL 1 derived mutants showed significant difference to their parent only at morphological level but not at molecular level. This observation indicates two possible types of mutations; one involved in TMV 2 and DER, and the other in VL 1. TMV 2 and DER upon mutagenesis must have undergone genomic-stress leading to activation of various transposable elements (TE) that transposed to many regions in the genome thereby imparting genome-wide lesions as revealed by RAPD markers. However diverse, but related phenotypic changes among TMV 2 and DER-derived mutants could be due to the preferential insertion of a key TE in a gene encoding for a transcription factor (TF) involved in various regulaons governing these phenotypes. Significant morphological variation, but limited molecular variation observed among the mutants and VL 1 parent could be due to a slightly different nature of mutation. In this case, the genomic-stress must have activated a single TE (rather than many) which transposed to TF encoding gene affecting the expression of many downstream genes. Involvement of very few TE(s) in such mutations would certainly explain the limited molecular difference between VL 1 and its mutants. However, the significant morphological variation among these mutants could be explained by the type, nature and strength of TF tagged with TE. This hypothesis can be validated by comparing the transcriptome of these mutants with their parent (VL 1). In order to check the possibility of a key TE in generating such diverse phenotypes, a candidate TE, *AhMITE* was employed, wherein the presence or absence of *AhMITE* in a specific location was traced by PCR.

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