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Characterization of *AhMITE1* transposition and its association with the mutational and evolutionary origin of botanical types in peanut (*Arachis* spp.)

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Abstract

AhMITE1 is an active miniature inverted repeat transposable element (MITE) in peanut (*Arachis hypogaea* L). Its transpositional activity from a particular (FST1-linked) site within the peanut genome was checked using *AhMITE1*-specific PCR, which employed a forward primer annealing to the 5'-flanking sequence and a reverse primer binding to *AhMITE1*. It was found that transposition activation was induced by stresses such as ethyl methane sulfonate (EMS), gamma irradiation, environmental conditions and tissue culture. Excision and insertion of *AhMITE1* at this particular site among the mutants led to gross morphological changes resembling alternate subspecies or botanical types. Analysis of South American landraces showed the presence of *AhMITE1* at the site among most of the subsp. *fastigiata* types, whereas subsp. *hypogaea* types predominantly missed the element, indicating its strong association. Four accessions of the primitive allotetraploid, *A. monticola* were devoid of *AhMITE1* at the site, indicating only recent activation of the element possibly due to the "genomic shock" brought with hybridization followed by allopolyploidization.

Keywords *AhMITE1* . Transposition activation . Peanut . Allopolyploidy . Subspecies . Botanical types

Introduction

Cultivated peanut (*Arachis hypogaea* L.) originated from a hybridization between diploid female species, *A. duranensis* with A genome and *A. ipaensis* with B genome (Kochert et al. 1996). A single allotetraploidization event involved either chromosome doubling in diploid F₁ (Favero et al. 2006) or unreduced gametes in F₁ (Harlan and deWet 1975). Occurrence of diploid parental and wild allotetraploid species (*A. monticola*) indicate that the eastern foothills of the Andes in the region of northern Argentina and southern Bolivia could be the area of origin of *A. hypogaea* (Krapovickas and Rigoni 1957; Kochert et al. 1996; Ravi et al. 2010). Allotetraploid *Arachis* species was then put under selection for domestication (Kochert et al. 1996), and the domesticated peanut dispersed to South and Central America where both natural and artificial selection produced many landraces of domesticated peanut.

On the basis of several morphological differences, two subspecies *fastigiata* and *hypogaea* have been recognized in the cultivated peanut. Subspecies *hypogaea* is characterized by alternate branching, absence of flowers on main axis, long duration (120-160 days) and presence of seed dormancy, while *fastigiata* is recognized by sequential branching, presence of flowers on main axis, short duration (85-130 days) and lack of seed dormancy. Based on the morphological differences, the two subspecies are subdivided into botanical varieties. Subspecies *hypogaea* is divided into var. *hypogaea* (Virginia bunch/runner) and var. *hirsuta* (Peruvian runner), whereas subsp. *fastigiata* is classified into var. *fastigiata* (Valencia), var. *vulgaris* (Spanish bunch), var. *aequatoriana* and var. *peruviana* (Krapovickas 1969, 1973). Subspecies *hypogaea* is thought to have originated by a mutation from within subsp. *fastigiata* (Singh 1988).

Cultivated peanuts exhibit a considerable variability for various morphological, physiological, and agronomic traits. However, limited variation is found at the DNA level

(Singh et al. 1998; Varshney et al. 2009; Khedikar et al. 2010) owing to the occurrence of a single polyploidization event followed by reproductive isolation from related diploid species (Young et al. 1996), self-pollination (Halward et al. 1991) and use of limited exotic germplasm in breeding programs (Knauff and Gorbet 1989). Hence it appears that most of the DNA level variation existing among the cultivated types stemmed from various mutations (Perry 1968; Mouli et al. 1979; Prasad et al. 1984; Prasad 1989) in comparison to recombination. In a previous study, we hypothesized the important role of mutations possibly involving the activation of cryptic transposable elements (TEs) in the origin and evolutionary differentiation of peanut (Gowda et al. 1996).

Miniature inverted repeat transposable elements (MITEs) are the predominant TEs among plant genomes (Wessler et al. 1995; Shan et al. 2005; Naito et al. 2006). Transposition preference for low copy genic regions underscores the role of MITEs in modulating gene expression (Wessler 1998; Zhang et al. 2000; Wessler 2001) and aiding crop evolution (Shan et al. 2005; Naito et al. 2006). Previously, we reported an active peanut MITE (*AhMITE1*), the transposition of which was associated with the high-frequency origin of late leaf spot (LLS) resistant mutants (Gowda et al. 2009). Here we have made an effort to study stress-induced activation of *AhMITE1*, and the relevance of its transposition with the mutational and evolutionary origin of botanical types.

Materials and methods

Plant material

Genotypes tested for *AhMITE1* transposition included parents and mutants generated by various stresses (Table 1), and thirty three South American landraces representing different botanical types (Table 2) and four accessions of wild allotetraploid (*A. monticola*) with the AB

genome combination (Table 3). Parental wild types and their mutants developed through mutagenesis with EMS and gamma irradiation, and those recovered from spontaneous mutation were selected from our mutant collection. These stabilized mutants were phenotypically studied and characterized for morphological changes (Motagi et al. 1996). TAG 24 and its mutant [TAG 24 (M)] recovered through tissue culture as somaclonal variant were obtained from Bhabha Atomic Research Centre (BARC), Mumbai, India. South American landraces and accessions of *A. monticola* were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India.

Detection of *AhMITE1* insertion/excision

Genomic location of the *AhMITE1* was determined (Bhat et al. 2008) by recovering its 5' 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*-specific PCR (Gowda et al. 2009). The forward primer (5' GGGAGAAGAAAAGGATGAGA 3') was designed based on the *AhMITE1* flanking sequence tag (FST1) recovered from TMV 2 genotype of peanut (Bhat et al. 2008), whereas the reverse primer (5' TCTCATGAAGATGCTTTGGT 3') was specific to *AhMITE1* (Patel et al. 2004). Amplification of a 242 basepair (bp) product would indicate the presence of *AhMITE1* at FST1-linked site in the genome.

For *AhMITE1*-specific PCR, genomic DNA of various genotypes and mutants was isolated from young leaves collected at the 3-4 leaf stage of the seedling using GenElute™

Plant Genomic DNA miniprep kit (Sigma Aldrich). A final volume of 20 µl containing 100 ng genomic DNA, 1X 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. Amplification reaction was carried out in a Mastercycler[®] (Eppendorf, Germany) by setting the conditions for one cycle of pre-denaturation (94 °C for 5 min), 35 cycles of denaturation (94 °C for 1 min), annealing (60 °C for 1 min) and extension (72 °C for 1 min). One cycle of final extension (72 °C for 1 min) was included before the PCR product was stored at 4 °C until further use. Presence of the PCR product was checked on 1.2% agarose gel by electrophoresis.

Results

EMS and gamma mutagens and tissue culture could activate the transposition of *AhMITE1*. The parent Dharwad Early Runner (DER) did not amplify the 242 bp product (Fig. 1), hence was found to be free of *AhMITE1* insertion at FST1-linked site. Mutants generated by gamma irradiation (SB 2, SB 4, SB 5 and SB 6) and EMS (SB 10 and SB 11) treatment 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 for the erect growth habit, lack of seed dormancy and increased pod size compared to DER type (Table 1). Another EMS mutagenesis effort with VL 1, a Valencia mutant from DER, could generate three Spanish bunch mutants (28-2, 45 and 110). Compared to VL 1, these mutants had more number of primary and secondary branches, short main stem and primary branches, small leaves, high pod yield and test weight, and resistance to late leaf spot. They showed the 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 subsp. *hypogaea* from TMV 2, a Spanish bunch cultivar involved excision of *AhMITE1* from the pre-determined site, indicating a gross morphological change.

Two spontaneous mutants (VR 1 and VR 2) resembling Virginia runner were isolated from the Spanish bunch variety JL 24. These mutations represented the morphological changes from subsp. *fastigiata* to subsp. *hypogaea*, and accompanied the excision of *AhMITE1*. Likewise, 28-2(s), 45(s) and 110(s) obtained spontaneously from 28-2, 45 and 110, respectively, were also associated with excision of the transposable element. These mutants showed significant morphological changes such as susceptibility to late leaf spot and pod shape and size. Tissue culture induced somaclone, TAG 24 (M) from TAG 24 indicated excision of the element. The excision brought discernible changes like increase in number of branches, decrease in seed size and prostrate habit. Thus, origin of Spanish bunch mutants belonging to subsp. *fastigiata* were generally associated with insertion, while subsp. *hypogaea* mutants accompanied by excision of the TE from the FST1 site.

This association was further validated by checking South American landraces comprising native subspecies and botanical types for the presence of *AhMITE1*. Of the nine genotypes belonging to subsp. *hypogaea*, only one (ICG 10479) showed *AhMITE1* at the FST1-linked site. In the subsp. *fastigiata*, four of the eleven Valencia and seven of nine Spanish bunch, and one of two *peruviana* (ICG 11088) showed the *AhMITE1* insertion, while it was absent in two *aequatoriana* genotypes tested. Proportions of the genotypes within the two subspecies carrying *AhMITE1* at the FST1-linked site were compared statistically using z test (standard normal deviate test for proportion) (Nageswara Rao 2007). Since the calculated z value (2.06) was significantly higher than the critical value of 1.96 at the 5% level of

significance, it was concluded that the majority of the landraces belonging to subsp. *fastigiata* types had *AhMITE1* at FST1-linked site, but subsp. *hypogaea* types lacked it.

Four accessions of the allotetraploid *A. monticola* (AABB) along with a cultivar (TMV 2) were screened for *AhMITE1* at FST1-linked site to trace the possible time of transposition during the course of evolution and peanut domestication. It was found that all the accessions of *A. monticola* were devoid of the element at the site, indicating that insertion occurred late in the evolutionary differentiation of *A. hypogaea* into subspecies and botanical types.

Discussion

Among plants, allopolyploidy is common (Stebbins 1951; Masterson 1994; Leitch and Bennett 1997), and considered “dynamic” (Soltis and Soltis 1995) and a major force in evolution compared to diploidy (Liu and Wendel 2003). It is known to play a major role in genome diversification and adaptation (Cronn and Wendel 2004). However, the underlying genetic and molecular basis of allopolyploidy-mediated evolution remains obscure. Recent studies indicate that epigenetic mechanisms such as DNA methylation, chromatin remodeling and RNA-silencing regulate reprogramming of the gene expression and developmental patterns in allopolyploids (Chen 2007). Methylation of cytosine and histone modifications play a key role in regulating the activity of transposable elements (TE) belonging to both long terminal repeats (LTR) and MITEs (Wessler et al. 1995; Madlung et al. 2005) that are known to induce transposon-mediated variations fueling adaptation and evolution (McClintock 1984).

Numerous reports record activation of a rice MITE, *mPing*, by diverse stresses such as gamma rays (Nakazaki et al. 2003), tissue culture (Jiang et al. 2003; Kikuchi et al. 2003), environmental conditions (Jiang et al. 2003) and genomic shock due to interspecific

hybridization (Shan et al. 2005). *En-Spm*-like transposons of *Arabidopsis thaliana* showed enhanced transpositional activity following remodeling of CG methylation upon allopolyploidization (Madlung et al. 2005). Hence, it appears that the response of a plant to such stresses may involve epigenetic changes leading to transpositional activity (Chen 2007).

In peanut, of the several copies of MITE identified using Southern hybridization, a particular MITE was induced by diethyl sulfonate (DES) to cause high oleate mutation (Patel et al. 2004). We named it as *AhMITE1*, and determined its site of integration in TMV 2, a Spanish cultivar by recovering a 151 bp 5'-flanking sequence tag (FST1) (Bhat et al. 2008). Homology search indicated that a small region (23/24bp) of the FST1 corresponded to a genomic sequence of *A. batizocoi* (GenBank Acc. No.DX508954) of the methylation filtered library (LibID703) (Bhat et al. 2008). So the genomic location of *AhMITE1* as identified by the FST1 was referred to as FST1-linked site.

Analysis of several spontaneous and induced mutants originating from mutagenesis (EMS and gamma-irradiation) and tissue culture showed *AhMITE1* transposition. EMS induced NLM from TMV 2 involved excision of *AhMITE1* from the FST1-linked site. Most interestingly, this mutation involved morphological changes from subspecies *fastigiata* (TMV 2) type to those resembling *hypogaea* (NLM) like sequential branching to alternative branching and main stem flowering to main stem non-flowering. Likewise, many mutations as characterized previously (Motagi et al. 1996), were associated with drastic morphological changes resembling alternate subspecies or botanical types, and the presence of *AhMITE1* at the FST1-linked site was predominant in subsp. *fastigiata*. Like in the previous studies (Prasad et al. 1984; Gowda et al. 1996), origin of mutants belonging to subsp. *hypogaea* from subsp. *fastigiata* types was more common than the other way round. This could be due to higher chances of excisions of *AhMITE1* at FST1-linked site compared to fresh insertions at that site.

Among the cultivated species of peanut, subsp. *fastigiata* (both Spanish bunch and Valencia) were found to carry a *AhMITE1* insertion at the FST1-linked site, whereas genotypes classified under subsp. *hypogaea* rarely contained *AhMITE1*, indicating a strong association between *AhMITE1* transposition and intraspecific differentiation. Such a preferential activity of MITEs has been reported in *japonica* versus *indica* types of rice (Jiang et al. 2003). The exceptions like VL 1, a Valencia type mutant from DER, and some genotypes belonging to different botanical types in subsp. *fastigiata*, which were devoid of the element, indicated a probable role of additional mechanisms. Genotypes belonging to *A. hypogaea* subsp. *fastigiata* var *aequatoriana* missed *AhMITE1* at the site like many subsp. *hypogaea* types. Earlier efforts with AFLP markers also indicated its resemblance closer to subsp. *hypogaea* than to subsp. *fastigiata* (He and Prakash 2001).

Lack of *AhMITE1* at the FST1-linked site in four accessions of *A. monticola* but its presence in some forms of cultivated species indicates de-repression of MITE possibly due to “genomic shock” triggered by hybridization followed by allopolyploidization (Shan et al. 2005; Naito et al. 2006). To our knowledge, this is the first report indicating the role of a MITE in peanut genome differentiation that took place after allopolyploidization. This unique association supports the view that the subsp. *hypogaea* is much closer to wild allotetraploid (Paik-Ro et al. 1992; Singh et al. 1993; He and Prakash 2001), and that *AhMITE1* transposition at an FST1-linked site could have played a key role in the origin of subsp. *fastigiata*.

This study reveals a possible reason for extensive morphological diversity, in spite of low DNA polymorphism in the cultivated species. MITEs by virtue of their inherent property, preferentially get inserted into low copy genes and their regulators (Singh et al. 1998), and the transpositions are known to modulate gene expression due to methylation, gene disruption and frame shift mutations (Nakazaki et al. 2003; Shan et al. 2005). Such events could also be

important precursors generating morphologically distinct subspecies and botanical types. Genomic characterization of FST1-linked site for candidate gene and future elucidation of other MITEs/LTRs using transposon display technique (Van den Broeck et al. 1998) in mutant collection, landraces and wild types will pave the way for better understanding of peanut genome differentiation and domestication.

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Titles and legends to figures

Fig. 1. Mutagen induced transpositional activity of *AhMITE1* in some of the mutants and their parents. 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)

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Table 1. Activation of *AhMITE1* in peanut mutants

Parent	Mutant	Mutagen	<i>AhMITE1</i> *	Botanical type	Phenology
DER			0	--	Like ssp. <i>fastigiata</i> , but is prostrate in habit with short main axis, small seeds and fresh seed dormancy and resembles some primitive forms of <i>A. hypogaea</i> and some forms of wild tetraploid progenitor of groundnut, <i>A. monticola</i> in phenology
	SB 2, SB 4, SB 5, SB 6	Gamma	1	Spanish bunch	Increased pod size
	SB 10, SB 11	EMS	1	Spanish bunch	Increased pod size
VL 1			0	Valencia	Valencia mutant derived from DER
	28-2, 45, 110	EMS	1	Spanish bunch	Resistant to LLS, change in pod size and shape
28-2	28-2(s)	Spontaneous	0	Spanish bunch	Susceptible to LLS, change in pod

					size and shape
45	45(s)	Spontaneous	0	Spanish bunch	Susceptible to LLS, change in pod size and shape
110	110(s)	Spontaneous	0	Spanish bunch	Susceptible to LLS, change in pod size and shape
TMV 2			1	Spanish bunch	Cultivar
	NLM	EMS	0	Virginia bunch	Narrow leaf, drought tolerant
JL 24			1	Spanish bunch	Cultivar
	VR 1, VR 2	Spontaneous	0	Virginia runner	Subspecies shift from Spanish bunch to Virginia runner
TAG 24			1	Spanish bunch	Cultivar
	TAG 24 (M)	Tissue culture	0	Spanish bunch	With some features of subsp. <i>hypogaea</i>

*Amplification of 242 bp product upon *AhMITE1*-specific PCR (1: Yes, 0: No)

Table 2. Activity of *AhMITE1* at FST1-linked site among South American peanut landraces

SN	Landraces	Origin	Subspecies	Botanical type	<i>AhMITE1</i> *
1	ICG 1711	Bolivia	<i>fastigiata</i>	<i>vulgaris</i>	1
2	ICG 3746	Argentina	<i>fastigiata</i>	<i>vulgaris</i>	1
3	ICG 3775	Brazil	<i>fastigiata</i>	<i>vulgaris</i>	0
4	ICG 4750	Paraguay	<i>fastigiata</i>	<i>vulgaris</i>	1
5	ICG 5236	Chile	<i>fastigiata</i>	<i>vulgaris</i>	1
6	ICG 7190	Brazil	<i>fastigiata</i>	<i>vulgaris</i>	0
7	ICG 8567	Uruguay	<i>fastigiata</i>	<i>vulgaris</i>	1
8	ICG 15287	Brazil	<i>fastigiata</i>	<i>vulgaris</i>	1
9	ICG 9930	Zimbabwe	<i>fastigiata</i>	<i>vulgaris</i>	1
10	ICG 332	Brazil	<i>fastigiata</i>	<i>fastigiata</i>	1
11	ICG 5221	Argentina	<i>fastigiata</i>	<i>fastigiata</i>	0
12	ICG 6888	Brazil	<i>fastigiata</i>	<i>fastigiata</i>	1
13	ICG 8517	Bolivia	<i>fastigiata</i>	<i>fastigiata</i>	0
14	ICG 11144	Argentina	<i>fastigiata</i>	<i>fastigiata</i>	0
15	ICG 10890	Peru	<i>fastigiata</i>	<i>fastigiata</i>	1
16	ICG 15309	Brazil	<i>fastigiata</i>	<i>fastigiata</i>	1
17	ICG 10554	Argentina	<i>fastigiata</i>	<i>fastigiata</i>	0
18	ICG 8106	Peru	<i>fastigiata</i>	<i>fastigiata</i>	0
19	ICG 14630	Brazil	<i>fastigiata</i>	<i>fastigiata</i>	0
20	ICG 10044	Peru	<i>fastigiata</i>	<i>fastigiata</i>	0
21	ICG 10036	Brazil	<i>fastigiata</i>	<i>peruviana</i>	0
22	ICG 11088	Peru	<i>fastigiata</i>	<i>peruviana</i>	1
23	ICG 12625	Peru	<i>fastigiata</i>	<i>aequatoriana</i>	0
24	ICG 12719	Ecuador	<i>fastigiata</i>	<i>aequatoriana</i>	0
25	ICG 2857	Argentina	<i>hypogaea</i>	<i>hypogaea</i>	0
26	ICG 6993	Brazil	<i>hypogaea</i>	<i>hypogaea</i>	0
27	ICG 2381	Brazil	<i>hypogaea</i>	<i>hypogaea</i>	0

28	ICG 10479	Uruguay	<i>hypogaea</i>	<i>hypogaea</i>	1
29	ICG 6703	Paraguay	<i>hypogaea</i>	<i>hypogaea</i>	0
30	ICG 12276	Bolivia	<i>hypogaea</i>	<i>hypogaea</i>	0
31	ICG 12672	Bolivia	<i>hypogaea</i>	<i>hypogaea</i>	0
32	ICG 15207	Mexico	<i>hypogaea</i>	<i>hypogaea</i>	0
33	ICG 15206	Mexico	<i>hypogaea</i>	<i>hypogaea</i>	0

*Amplification of 242 bp product upon *AhMITE1*-specific PCR (1: Yes, 0: No)

Table 3. Activity of *AhMITE1* at FST1-linked site among the primitive allotetraploids of peanut

SN	Accession number	Species	Genome	<i>AhMITE1</i>*
1	ICG 13177	<i>A. monticola</i>	AB	0
2	ICG 8135	<i>A. monticola</i>	AB	0
3	ICG 8197	<i>A. monticola</i>	AB	0
4	ICG 8198	<i>A. monticola</i>	AB	0

*Amplification of 242 bp product upon *AhMITE1*-specific PCR (1: Yes, 0: No)

Fig. 1

