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*Short Communication*

# A narrow leaf groundnut mutant, TMV2-NLM has a G to A mutation in *AhFAD*2A gene for high oleate trait

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**Abstract**

**Based on the screening of groundnut mutant germplasm at Trombay, we identified a high oleate trait in a narrow leaf mutant (TMV2-NLM) that was previously isolated from ethyl methane sulfonate (EMS) mutagenized population of cv. TMV 2. The NLM had 78% improvement in oleic acid over its parent. PCR-RFLP and DNA sequencing analysis of mutated *AhFAD*2A gene in TMV2-NLM revealed a G to A mutation in the restriction site of *Hpy*99I of *AhFAD*2A but not any insertion of MITE or 441_442insA mutation in *AhFAD*2B gene.**

**Key words:** *Arachis hypogaea* L., *AhFAD*2A mutation, Gas chromatography, Narrow leaf mutant

The quality and flavour of groundnut oil is mostly conditioned by its fatty acid composition. The major fatty acids in groundnut oil are palmitic, stearic, oleic and linoleic acids, which make up about 90% of the total groundnut triacylglycerols [1]. Of these, palmitic and stearic acids are saturated, oleic acid is monounsaturated fatty acid (MUFA) and the linoleic is polyunsaturated fatty acid (PUFA). Higher amount of PUFA in groundnut oil increases the chances of oxidation, which leads to unpleasant odours and tastes [2]. While MUFA, in particular the oleic acid is less prone to oxidation and helps in extending the shelf life by delaying the development of rancidity [3]. Oleic acid has also been shown to increase insulin production, reverse the inhibitory insulin effect of TNF-$\alpha$ and ameliorate the diabetic symptoms of type II diabetes characterized by inflammation [4]. Thus development of high oleate mutant or genotype is of great importance in groundnut breeding programme. The mutated leaf trait 'narrow leaflet' was isolated in groundnut by several researchers in independent experiments [5]. Gujarat narrow leaf mutant is reported to be controlled by a single dominant gene while, Girnar 1 narrow leaf mutant was dwarf and conditioned by a recessive gene [6]. Induced mutagenesis of cv. TMV 2 by ethyl methane sulfonate (EMS) generated a narrow leaf mutant (NLM) in groundnut [7]. Present research reports an additional high oleate trait in this mutant and possible mutation in *AhFAD*2A gene.

Fifty five groundnut mutants at Trombay along with their four parents were grown in experimental field at Trombay, Mumbai. For fatty acid accumulation at different crop cutting stages, TMV2-NLM and TMV 2 were grown in three replications in a separate block and five plants were harvested at five-day interval starting from 80 days after sowing (DAS) to 120 DAS. Pods were harvested separately from each replication and dried well and stored at refrigerator (4°C) for further fatty acid analysis. Randomly selected ten seeds from each mutant of 2006 and 2007 rainy season (June to September) were ground to a fine paste using mortar and pestle in two replications. Approximately 0.2 g of ground seed meal from each mutant was extracted for oil and consequently the oil was converted to fatty acid methyl ester and quantified as described in Mondal *et al*. [8]. The percentage fatty acid data were Arcsine transformed and an analysis of variance was

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performed using IRRISTAT 2.0 software [9]. Total genomic DNA was extracted from young leaves of TMV 2 and NLM as described earlier [10]. Polymerase chain reaction (PCR) was performed for *AhFAD*2A gene with sense primer, 5'-GATTACTGATTATTGACTT-3', and antisense primer, 5'-CCAACCCAAACC TTTCAGAG-3' [11]. For *AhFAD*2B, two different primer combinations were used: primer bF19 forward (5'-CAGAACCATTAGCTTTG-3') and primer R1/FAD reverse (5'-CTCTGACTATGCATCAG-3') for MITE insertion in between 665 and 666$^{th}$ bases [12] and primer 1344 forward (5'-GGAGCTTTAACAACACAA-3') and primer 1345 reverse (5'-ATATGGGAGCATAAGGGT-3') for insertion of 'A' in between 441 and 442$^{nd}$ bases [13]. PCR was performed with 1 U of *Taq* DNA polymerase in a total reaction volume of 25 µL as described in Mondal *et al*. [8]. Subsequently, the amplified DNA fragments for both *AhFAD*2A and *AhFAD*2B were purified separately with GenElute$^{TM}$ PCR clean up kit (Sigma, St. Louis, MO, USA). Purified PCR (20 µL ~ 200 hg) products were digested with 2 U of *Hpy*99I and *Hpy*188I endonuclease (New England Biolabs, Ipswich, MA), respectively for *AhFAD*2A and *AhFAD*2B, at 37°C for 2 h 30 min. The digested products were separated on a 2% agarose gel stained with 0.1% ethidium bromide. After getting a confirmation that mutation was present only in *AhFAD*2A gene, the purified *AhFAD*2A-PCR products from TMV 2 and NLM were cloned, sequenced and analyzed as described previously [8]. Extracted DNA and protein sequences from both TMV 2 and NLM were aligned with ClustalW2 tool (available at http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Fatty acid analysis in 55 mutants and their four parents detected a significant variability for palmitic, oleic and linoleic acids. Among the mutants, TMV2-NLM contained the lowest amount of palmitic acid in its seed while TGM 62 contained the highest. TGM 53 and TGM 62 had more palmitate content compared to their parent, BAU 13 (Table 1). The mutant, TMV2-NLM had the highest amount of oleic acid and proportionately the lowest palmitic and linoleic acid in its seed oil (Table 1). The mutant showed 78% improvement in oleic acid and concomitant 58% decrement in linoleic acid content compared to TMV 2. Oleic acid accumulation showed a biphasic increment in this mutant. The oleic acid level increased at slower rate from 80 to 95 DAS and then it reached saturation. This saturation in oleic acid content continued upto 105 DAS followed by a steep rise in oleic acid and decrease in linoleic acid content in TMV2-NLM upto maturity (120 DAS) (Fig. 1a & b). The first phase of oleic acid increment in parent TMV 2 was similar to NLM while, the second phase of increment was absent (Fig. 1a & b). Although the basal oleic acid level is comparatively higher in NLM than TMV 2, the main determinant for high oleate mutation lies in the activity of oleic to linoleic conversion at 105 DAS onwards in the developing cotyledons. Cultivated groundnut is an amphidiploid and hence both *AhFAD*2A and *AhFAD*2B encoded the oleoyl PC desaturase activity. Mutations in any of these two or both can be primarily responsible for high oleate trait in groundnut. This impairment of oleoyl PC desaturase activity in TMV2-NLM was reflected in the alternation of *AhFAD*2A sequence as revealed by PCR-RFLP of partial *AhFAD*2A gene product (826 bp) through *Hpy*99I digestion (Fig. 2A). But no predicted mutations (MITE insertion or 441_442 'A' insertion) were detected in *AhFAD*2B in this high oleate mutant (Fig. 2B). DNA amplification with 'bF19' and 'R1/FAD' primer pair in both TMV 2 and TMV2-NLM generated similar PCR fragment (~1200 bp), suggesting absence of any MITE elements in the coding region of *AhFAD*2B gene in the mutant (Fig. 2B). Further, no alternation of *Hpy*188I restriction pattern of amplified PCR product by primers 1344 and 1345 in TMV2-NLM compared to TMV2 indicated absence of 'A' insertion in between 441 and 442$^{nd}$ bases of AhFAD2A (Fig. 2B). *In silico* translation of this mutated *AhFAD*2A DNA of TMV2-NLM revealed three effective amino acid changes, one from arginine (R) to glycine (G) at sixth position; one glycine (G) to aspartic acid (D) at 30 position and one aspartic acid (D) to asparagines (N) at 150 position of AhFAD2A protein (Fig. 3). All these amino acid changes corresponded to the respective nucleotide changes at 104, 177 and 536 position of mutated *AhFAD*2A DNA of NLM. Earlier, site-directed mutagenesis proved that high oleate phenotype in US peanut varieties was due to the alternation from aspartic acid-150 to asparagine (D150N) in AhFAD2A protein [14]. The mutant NLM was generated through the 0.2% EMS mutagenesis and had alternate branching habit [7]. The chemical mutagen EMS acts as an alkylating agent to change the nucleotide base during DNA replication and thus induces G:C to A:T transition. In spite of typical G:C to A:T transition, the occasional errors during the repair processes may lead to transversion during the treatment with EMS. Prasad *et al*. [7] reported that the TMV2-NLM had the capacity to produce higher dry matter and had resistance to *Cercospora* leaf spot. The low specific leaf area (SLA) in TMV2-NLM had imparted a greater drought tolerance index (based on

**Table 1.** Variability in fatty acid contents among selected mutant and their parents

| Mutants | Pedigree | Plant type | C 16:0 | C 18:0 | C 18:1 | C 18:2 | C 22:0 |
|---|---|---|---|---|---|---|---|
| TGM 11 | Mutant of SP | SB | 9.56 | 0.67 | 57.93** | 20.01** | 3.29 |
| TGM 12 | Mutant of SP | SB | 9.13** | 1.04 | 63.84** | 17.77** | 2.33 |
| TGM 13 | Mutant of SP | SB | 9.31** | 0.90 | 56.31** | 25.03** | 2.47 |
| TGM 14 | Mutant of SP | SB | 10.75 | 1.87 | 52.08** | 26.64** | 2.75 |
| Spanish Improved (SP) | Parent | SB | 11.19 | 0.89 | 44.58 | 36.24 | 2.57 |
| TMV2-NLM | Mutant of TMV 2 | VB | 7.19** | 1.14 | 68.66** | 14.75** | 2.66 |
| TMV 2 | Parent | SB | 11.41 | 0.79 | 38.52 | 35.03 | 2.93 |
| TGM 24 | Variegated leaf | VB | 9.18 | 1.32 | 56.98 | 21.65 | 2.67 |
| TGM 36 | Mutant of TG 9 | SB | 9.44 | 1.78 | 53.66** | 24.06 | 2.56 |
| TG 9 | Parent | SB | 7.98 | 1.63 | 60.54 | 20.06 | 2.56 |
| TGM 39 | Extra large pod | SB | 8.39 | 1.74 | 59.55 | 20.60 | 2.13 |
| TGM 41 | Virescent mutant from USA | VB | 7.86 | 2.72 | 65.43** | 16.82 | 2.71 |
| TGM 53 | Mutant of BAU 13 | VB | 10.32** | 0.73 | 53.96** | 25.75** | 2.82 |
| TGM 55 | Mutant of BAU 13 | VB | 9.37** | 0.80 | 59.05** | 21.80** | 2.40 |
| TGM 62 | Mutant of BAU 13 | VB | 10.49** | 1.38 | 51.71** | 26.45** | 2.57 |
| TGM 63 | Mutant of BAU 13 | VB | 8.16 | 1.31 | 66.97 | 13.97 | 3.09 |
| TGM 64 | Mutant of BAU 13 | VB | 7.88 | 0.85 | 67.38 | 13.40 | 2.99 |
| BAU 13 | Parent | VB | 7.03 | 0.79 | 67.15 | 13.28 | 2.57 |
| LSD (1%) | - | - | 1.69 | 3.50 | 4.80 | 4.08 | 1.80 |

**Indicates the values differed from the respective parent at 1% level of significance.
SB = Spanish bunch, VB = Virginia bunch

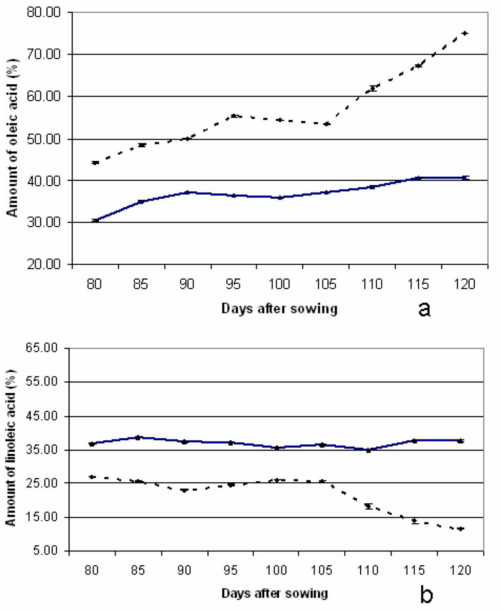


**Fig. 1. Oleic acid (a) and linoleic acid (b) accumulation in parent (TMV 2) and mutant (TMV 2-NLM) seeds at different crop growth stages. Note: Dotted lines represent TMV2-NLM and normal lines represent TMV 2**

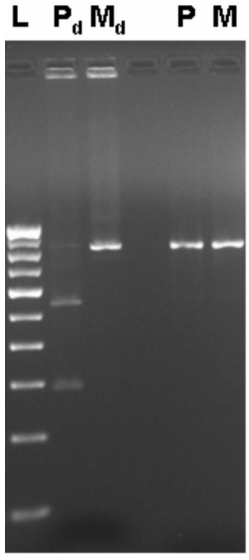


**Fig. 2A. Detection of *AhFAD*2A mutation in TMV 2-NLM as compared to the parent, TMV 2. Note: L = 100 bp DNA ladder, $P_d$ = *Hpy*99I digested PCR product from TMV2, $M_d$ = *Hpy*99I digested PCR product from NLM, P = undigested PCR product from parent (TMV 2), M = Undigested PCR product from NLM**

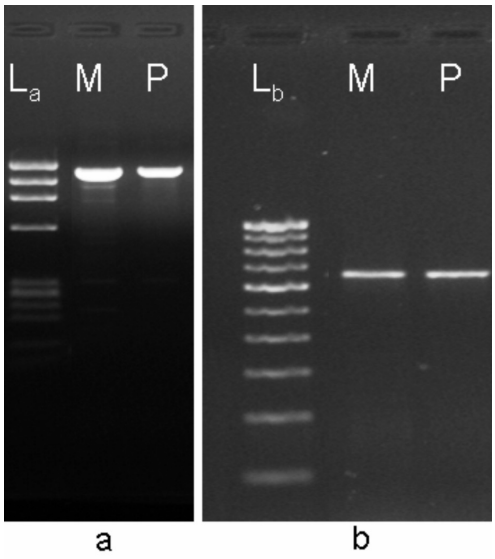


**Fig. 2B. Agarose gel analysis of *AhFAD*2B mutation for 665_666insMITE (a) and 441_442 'A' insertion (b). Note: $L_a$ = fX174 DNA *Hae*III digest, M = PCR product from mutant, P = PCR product from parent for MITE insertion (a) and M = *Hpy*188I digested PCR product of mutant, P = *Hpy*188I digested PCR product of parent for 441_442 'A' insertion analysis, $L_b$ = 100 bp DNA ladder (b)**

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TMV          MGAGGRVTKIEAQKKPLSRVPHSNPPFSVGQLKKAIPPHCFERSLFISFSYVVYDLLVAY 60
TMV2-NLM     MGAGGGVTKIEAQKKPLSRVPHSNPPFSVDQLKKAIPPHCFERSLFISFSYVVYDLLVAY 60
             ***** ***********************.******************************

TMV          LLFYIATTYFHKLPYPFSFLAWPIYWAIQGCILTGVWVIAHECGHHAFSKYQLVDDMVGL 120
TMV2-NLM     LLFYIATTYFHKLPYPFSFLAWPIYWAIQGCILTGVWVIAHECGHHAFSKYQLVDDMVGL 120
             ************************************************************

TMV          TLHSCLLVPYFSWKISHRRHHSNTGSLDRDEVFVPKPKSKVSWYNKYMNNPPGRAISLFI 180
TMV2-NLM     TLHSCLLVPYFSWKISHRRHHSNTGSLDRNEVFVPKPKSKVSWYNKYMNNPPGRAISLFI 180
             ****************************:*****************************

TMV          TLTLGWPLYLAFNVSGRPYDRFASHYDPYAPIYSNRERLLIYVSDSSVFAVTYLLYHIAT 240
TMV2-NLM     TLTLGWPLYLAFNVSGRPYDRFASHYDPYAPIYSNRERLLIYVSDSSVFAVTYLLYHIAT 240
             ************************************************************

TMV          LKGLGW 246
TMV2-NLM     LKGLGW 246
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**Fig. 3: Alignment of *insilico*-translated protein sequence of TMV 2 and NLM**

total dry matter) due to its higher relative water content and carbon exchange ratio in leaf tissue under moisture stress [15]. Further, this high oleate mutant TMV2-NLM was identified as a good general combiner for SPAD chlorophyll meter reading, SLA and carbon isotope discrimination character [16]. With this view, the mutant could be of an important parental source for breeding for drought tolerance with higher oil quality.

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