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Molecular Characterization of Type II Transposable Elements in Cowpea [*Vigna unguiculata* (L.) Walp]

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Abstract

Previous genetic studies in cowpea [*Vigna unguiculata* (L.) Walp] have shown that an active bipartite transposable element (TE) is responsible for a range of mutant phenotypes of its leaf, stem and flower. Since type II TEs have not been characterized at the molecular level in cowpea, this study was initiated to survey the presence of type II TEs in the cowpea genome. Type II TEs: Enhancer/Suppressor-mutator (*En/Spm*) and Miniature Inverted-repeat Transposable Elements (*MITEs*) were isolated and characterized. The sequence identity between the *EnSpm* TE clones was 46% at the nucleotide level (NL) and 30% at the amino acid level (AL) while that of *MITEs* was 71% at NL and 63% at AL. These cowpea *En/Spm* TEs were 80% homologous with *En/Spm* elements of other crops at NL and 46% at AL. The *MITEs* were 96% similar at NL and 18% homologous at AL. DNA gel blot analysis confirmed the presence of the *En/Spm* TEs in cowpea. RT-PCR (reverse transcriptase polymerase chain reaction) analysis showed that the *VuEnSpm*-3 and the *MITE* clone, *VuPIF*-1 were actively transcribed in wild type and mutant cowpea tissues. Overall, our data show that multiple, divergent lineages of *En/Spm* and *MITEs* are present in the cowpea genome, some of which are actively transcribed. Our findings also offer new molecular resource to further investigate the genetic determinants underlying previously described mutant cowpea phenotypes.

Keywords

Cowpea, *En/Spm*, *MITE*, Transposable Element, *Vigna unguiculata*

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1. Introduction

Transposable elements (TEs), otherwise known as transposons or mobile genetic elements are widespread in pro- and eukaryotes, including plants and animals [1]-[3]. TEs were first characterized by Barbara McClintock using classical genetics [4]. They were later analyzed using molecular techniques [3] [5] [6].

In plants, TEs contribute significantly to the size, structure and plasticity of the genome [7]. For example, in maize and other plant species, up to 80% of the genome consists of transposable elements [8]. TEs also play an active role in genome evolution [3] [9] [10] by helping their hosts adapt to new conditions by conferring useful traits [11]. The identification and characterization of transposons in a given host can greatly assist genetic studies of that organism [12]. Transposable elements have been used for improvement in many crop plants such as sorghum, tomatoes, rice and maize [1] [4]; in maize, TEs were used to develop Striga tolerant lines [13], and they have been used as markers to assess genetic segregation in sorghum [14], for phylogenetic studies [15], gene tagging and reverse genetics in plants [16]-[18].

In eukaryotes, TEs are classified into two classes based on their mode of transposition: class I elements (retrotransposons), which move via an RNA intermediate, produced via reverse-transcription, before being inserted into the genome in a "copy and paste" manner. By contrast, class II elements move by a "cut and paste" mechanism without RNA intermediate. Type II TEs consist of an autonomous element—a transposase- and non-autonomous elements with terminal inverted repeats (TIR) of 10 - 200 bp [3]. They are usually characterized by target site duplications. Class II elements are classified into several super families [1], including the *Enhancer/Suppressor-mutator* (*En/Spm*) [19] [20]), the Activator/Dissociation (*Ac/Ds*), Mutator-Like Elements (*MULEs*), Mariner-Like Elements (*MLE*s) and Miniature Inverted-repeat Transposable Elements (*MITEs*) [21]. The *En/Spm* share a common sequence-5'-CACTA-3' at their TIR and the transposase is highly conserved among plants. They have been found and characterized in Gramineae [22], Leguminosae, Solanaceae, Chenopodiaceae, Alliaceae species [2] and Euphorbiaceae [23]. *MITEs* are characterized by their small size, usually less than 500 bp, lack coding capacity and have short TIRs. They can be further classified as Tc1/Mariner-like or PIF/Harbinger-like based on their association with established super families [24].

Cowpea [*Vigna unguiculata* (L.) Walp] is a drought-tolerant, fast-growing, and highly nutritious legume of particular importance in the semi-arid regions of tropical countries in Africa, Asia and southern America. Previous genetic studies in cowpea have shown that an active bipartite transposable element system is responsible for a range of mutations affecting leaf, stem and flower morphology and pigmentation [25]-[29]. As characteristic of most TE-induced mutations, the mutants were not stable; an example is that of the flower mutation, both mutant and wild type flowers were found on the same plants [30]. In this study, we present, for the first time, the isolation and molecular characterization of type II TEs, including members of the *En/Spm* and *MITE* superfamilies based on the analysis of these unstable mutants. Findings from this study will contribute to and possibly enhance the application of TEs in future breeding efforts of cowpea.

2. Materials and Methods

2.1. Plant Material and Nucleic Acid Extraction

Cowpea accessions and parental lines used in this study were mostly obtained from the Department of Crop Protection and Environmental Biology, University of Ibadan, Ibadan, Nigeria as listed on **Table 1**. Reduced Pet-

Table 1. The list of the mutants and wild types used for this study.

Name	Description	Source
Ife Brown	Non mutant cultivar	University of Ibadan, Ibadan, Nigeria
Ife BPC	Non mutant cultivar	IITA
Tvu 1	Wild sp	IITA
IBS2497_LM1	Unifoliate leaf form mutant	University of Ibadan, Ibadan, Nigeria
IBS2497_LM2	Non-petiolate and non-branching stem mutant	University of Ibadan, Ibadan, Nigeria
RFM	Rose-like flower mutant	University of Ibadan, Ibadan, Nigeria
RPM1	Reduced petal mutants	University of Ibadan, Ibadan, Nigeria
RPM2	Reduced petal mutants	University of Ibadan, Ibadan, Nigeria
Tvu 1509	Wild sp	IITA
Tvu 940151	Non mutant line	University of California Davis, Davis, CA, USA

al Mutants (RPM1 and RPM2); unifoliate Leaf form mutant (LM1); Rose-like Flower Mutant (RFM); non-petiolate and non-branching stem mutant (LM2); Ife brown and Ife BPC. The lines Tvu 1 and Tvu 1509 were obtained from the International Institute of Tropical Agriculture, Ibadan, Nigeria and Tvu 940151 was obtained from the University of California Davis, Davis, CA, USA. Total DNA was extracted from young leaves as described by [31]. RNA was extracted from leaves, stem and flowers as described by [32] and with the RNeasy kit following the manufacturer's instructions (Qiagen, Valencia, CA, USA).

2.2. Pedigree of Mutants

Mutants were obtained from crosses made by earlier workers on cowpea TEs [26]-[29], their pedigree is shown in **Figure 1**.

2.3. PCR Amplification of Fragments

Total DNA (250 ng) was amplified by polymerase chain reaction (PCR) in a PTC 100 Thermal Cycler (MJ Research Inc.) using the conditions and primers described by [2] [23] for the amplification of *En/Spm*-like transposases, and those used by [33] for the amplification of PIF/Harbinger-like *MITEs*. Others included primers for Mariner-like elements [24], *Ac/Ds* elements [34], Zaba elements [35] and Mutator-like elements [36]. Specific primers were used for the reverse transcriptase polymerase chain reaction (RT-PCR) were designed using Primer 3 program [37]. In all cases, PCR amplification was performed in a 50 µl reaction using 1 unit of Taq DNA polymerase (Bioline, USA).

2.4. Cloning Procedures and DNA Sequencing

PCR amplicons obtained from RFM were gel purified using the Qiaex gel extraction kit (Qiagen, USA). PCR amplicons were ligated into a pCR8-GWTOPO (Invitrogen, CA, USA) or pDrive (Invitrogen, CA, USA) and transformed into *Escherichia coli* DH5*α* competent cells according to standard procedures [31]. Following blue white selection protocol, recombinant clones were grown in liquid LB medium and plasmid DNA was isolated using ultra-pure plasmid kit (Baygene, USA). Inserts were sequenced using universal primers by the Iowa state University sequencing facility (Iowa, USA). All clones were sequenced in both orientations. The DNA sequences were manually edited and any sequence ambiguities were resolved by re-sequencing. For each PCR reaction, three to five independent plasmid clones were sequenced to enable detection of any size or sequence heterogeneity present in the clones.

2.5. Database Searches and Sequence Comparison

The sequences obtained from this study were compared with other sequences in the database by using BLASTN searches against the GenBank non-redundant database of the National Center for Biotechnology Information

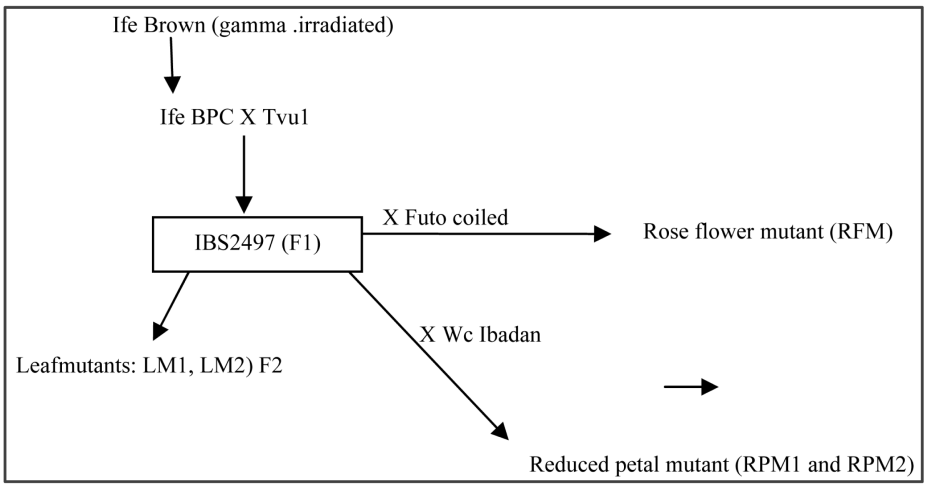

Figure 1. Pedigree of mutants.

(NCBI) using default parameters [38]. Cowpea TEs sequences were aligned using the programs QAlign [39], MEGA [40], and CLUSTAL W. Phylogenies of the clones were obtained using the relaxed dissimilarity algorithm of the Neighbour Joining program. Trees were constructed using Tree View [41].

2.6. Southern Blot Analysis

Twenty microgram cowpea DNA was digested overnight using EcoR1 and Hind III at 37˚C and separated on a 1% TAE gel following digestion. DNA was transferred to Hybond N+ nylon membrane (Amersham, USA) using standard procedures [31] as follows: the membrane was prehybridized at 42˚C in DIG easy HyB buffer (Roche, USA) in a hybridization oven. Probes were prepared using the DIG labeling kit (Roche, USA), and hybridization was carried out as recommended by the supplier (Roche, USA). After hybridization, the membrane was exposed to an X-ray film and developed using standard techniques.

2.7. RT-PCR Analysis

Primers were designed from the transposase gene of *VuEnSpm*-1, *VuEnSpm*-3 *and VuPIF*-1. The lists of the primers used are shown in **Table 2**. RT-PCR was carried out using RNA extracted from mutant and wild type plant tissues, including leaves, stems and flowers. The RNA was reverse transcribed into cDNA using the Superscript III reverse transcriptase of Invitrogen (CA, USA) according to the manufacturer's specifications. RT-PCR products were analyzed on a 2% agarose TAE gel.

3. Results

3.1. Identification of Type II Transposable Elements in Cowpea

PCR amplicons were obtained from the all the type II TEs investigated namely: *En/Spm*, *MITEs*, *Ac/Ds*, *MLEs* and mutator elements. However, subsequent BLAST searches showed that sequences with significant homology to previously characterized TE were observed only for *En/Spm* and *MITEs.* In case of *Ac/Ds*, MLE and the mutator elements, GenBank searches did not retrieve sequences with significant sequence homology to previously identified *Ac/Ds* and mutator elements. Four *En/Spm* clones of about 650 bp each were sequenced and analyzed. These sequences were submitted to the NCBI GenBank with the following accession numbers: FJ526201, FJ526202, FJ526203, and FJ526204. A total of five *MITEs* clones of approximately 500 bp each were sequenced with only two showing significant homology to previously identified *MITEs*, and in particular to the PIF/Harbinger subfamily. These two sequences were also submitted to GenBank with accession numbers GQ422756 and GQ422757. The *En/Spm*-like elements described here are the first class II TE reported in *Vigna unguiculata* using molecular techniques. *En/Spm*-like elements were present in all mutant and wild type cowpea genotypes used in this study as assessed by PCR analysis (**Figure 2(a)**). Likewise, MITE-like sequences were present in all cowpea genotypes based on PCR analysis (data not shown). The southern blot analysis confirmed the presence of *EnSpm* (**Figure 2(b)**). Five to six distinct bands were obtained in the cowpea lines under **Figure 2**.

Table 2. Characteristics of cowpea type II TE characterized in this study.

Type of TE	Targeted region and forward and reverse primers/reference	Cowpea TE (GenBankAcc No)	Top BLAST hit (E value)
En/Spm	Transposase F: 5'GGAAAACTAATATGATT CGACATAATATTGAYITIATGC R: 5'CATAGACAGATGTCCATATC TTTCAAASADRTACATCCA3'[2]	*VuEnSpm*1 (FJ526201)	*Glycine max* william 82 clone GMWBb173L02 (3e−120)
		*VuEnSpm*2 (FJ526202)	*Glycine max* william 82 clone GMWBb173L02 (2e−136)
		*VuEnSpm*3 (FJ526203)	*Glycinemax*clone 77G7b (6e−85)
MITEs	Transposase F: 5'ATGICKMIRRTTRAACAAYTC3' R: 5'GGIGCHHTIGATGGHACWCA3' [33]	*VuPIF*1 (GQ422756)	*Lotus japonicus clone* (4e−16)
		*VuPIF*2 (GQ422757)	*Dendrocalamus minor* clone *DM*-1 *PIF*01 *transposon PIF-like* (3e−11)

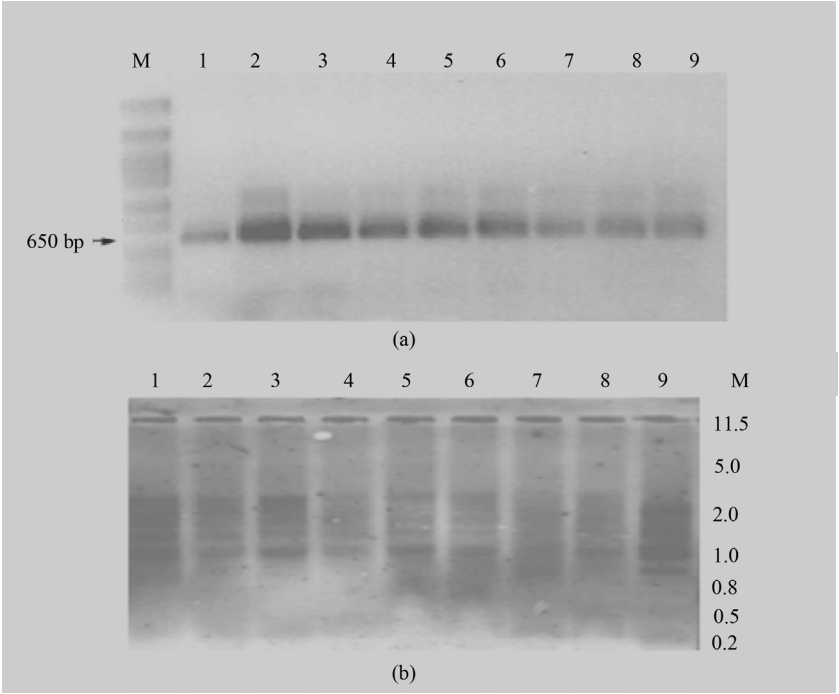

Figure 2. (a) PCR amplification of the *En/Spm* fragment in the cowpea lines used in the study; M = Pst1 size marker, 1 = Ife brown, 2 = Ife BPC, 3 = Tvu1, 4 = LM-1, 5 = RPM-1, 6 = RPM-2, 7 = RFM, 8 = LM-2, 9 = Tvu 94051; (b) Southern blot of different cowpea genotypes using *VuEnSpm* clone1 as probe: 1 = Ife brown, 2 = Ife BPC, 3 = Tvu1, 4 = LM-1, 5 = RPM-1, 6 = RPM-2, 7 = RFM, 8 = LM-2, 9 = Tvu 94051, M = Pst1 lambda digest (size marker). Arrows point to some distinct bands.

3.2. Comparisons within the Cowpea *En/Spm* and MITE Clones

For each type of TE, the different clones were aligned to assess the level of diversity present within their class. Sequence analysis of the three clones designated *VuEnSpm*1, *VuEnSpm*2 and *VuEnSpm*3, showed only 46% similarity at the nucleotide level and 30% at the amino acid level. Thus, the *VuEn/Spm* elements showed considerable sequence diversity. *VuEnSpm*1 and *VuEnSpm*2 are highly homologous with 72% nucleotide identity, while *VuEnSpm*3 showed only 41% and 52% nucleotide sequence identity to *VuEnSpm*1 and *VuEnSpm*2, respectively. All three clones contained an ORF of 192 amino acids encoding part of the *En/Spm* transposase as expected, which is an indication of possible active transposons. A similar alignment was made for the two clones *of VuPIF*, designated *VuPIF*1 and *VuPIF*2. These sequences were 71% similar at the nucleotide level and 63% at the amino acid level.

3.3. Comparison between TEs from Cowpea and Other Plants

A multiple sequence alignment of cowpea TEs and other plant species followed by phylogenetic analysis showed the genetic relationship between *VuEnSpm*1, *VuEnSpm*2, *VuEnSpm*3 and *En/Spm*-like transposons (**Figure 3**) as follows: *VuEnSpm*1 and *VuEnSpm*2 were more closely related to *En/Spm* of *Pisum sativum* while *VuEnSpm*3 was more closely related to *En/Spm* of *Cicer arietinum* and *Manihot esculenta*. However, *En/Spm-like* elements from *Beta* and *Allium spps* were grouped with other leguminous plants such as *Lens culinaris*, *Cajanus cajan* and *Lens esculentum*. Overall, top BLAST hits were obtained for leguminous plants (**Figure 3**) and they showed a higher level of relatedness to one another.

Similarly, when a BLAST search was performed using the *VuPIF* sequences, top hits included *Sorghum halenpense* and *Dendrocalamus minor* (**Table 1**). In addition, more hits with sequences from mostly monocot plants such as *Pennisetum glaucum*, *Zea mays* and *Saccharum* hybrid cultivar clones, with a nucleotide sequence identity ranging from 90% to 99% and amino acid identity of 67% to 72% were obtained from the BLAST

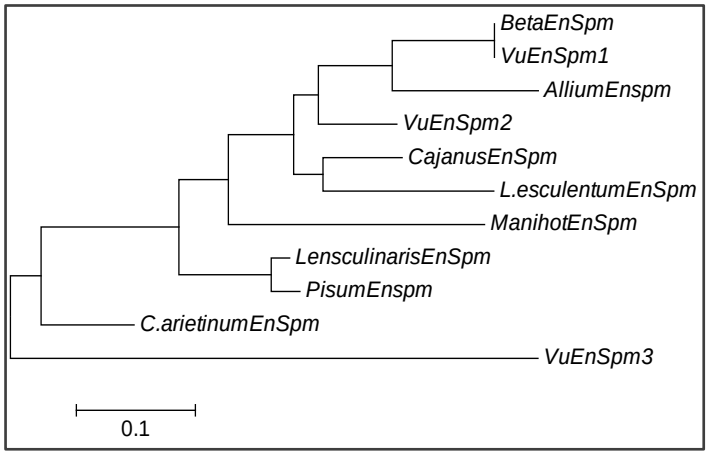

Figure 3. Dendrogram showing the relationship between *VuEn/Spm* clones and those from other plants from BLAST search.

search. When the conserved regions of the top hits were aligned with the *VuPIF* clones, the phylogenetic analysis showed two groups with the two *VuPIF* clones together and the second group consisted of 2 sub-groups of monocots and the only dicototyledonous crop (**Figure 4**). The overall mean distance between the amino acid of *PIF TEs* from other crops was 82%, hence a similarity of 18% from the analysis with MEGA version 4.

3.4. Reverse Transcriptase PCR (RT-PCR) Analysis

To assess whether the TE isolated from cowpea are actively transcribed, RT-PCR reactions were conducted using cDNA from both the wild (non-mutant) and mutant types of different plant tissues. RT-PCR amplicons were obtained for *VuEnSpm*3 and *VuPIF*1 while *VuEnSpm*1 did not show any transcript in all the mutants and wild type tested. Tissue-specific amplifications showed that *VuPIF*-1 transcripts are present in both mutant and wild type tissues with expression of two copies (faint) of *MITEs* transcripts in the leaves of the unifoliate leaf mutant (LM1) compared to wild type leaf tissues (Ife brown) (**Figure 5**). In addition, only *VuPIF*-1 transcripts were present in the stem tissues of the branching habit mutant LM2 (**Figure 5**) while it was absent in the stem of wild type (Tvu 1) (not shown). A similar result was obtained in the flowers of the Rose-like Flower Mutant (RFM) where *VuEnSpm*-3 transcripts are more pronounced in the mutants than in the wild type (not shown). **Figure 5** shows typical amplification obtained from the different plant parts.

4. Discussion

This study aims to authenticate the presence of type II TEs in cowpea, *Vigna unguiculata* and to provide an insight into the type of TEs potentially implicated in mutations reported in previous studies through classical breeding. To our knowledge, this is the first report of molecular characterization of type II TEs in cowpea. The protein BLAST result of the TEs showed that there are partial open reading frames (ORFs) that encode products involved in transposition. The *VuEnSpm* encodes a transposase while the *VuPIF*, was found to be a non-functional transposase protein. These findings will constitute a useful source of information to the crop's genome annotation [42].

Also, it has shown the degree of genetic similarity of clones of these types of TEs in cowpea .Similar levels of divergence were obtained in the *En/Spm* of chickpea [2]. The multiple alignment and phylogenetic analysis with similar types of TEs in other crops shows the relationship between these organisms relative to cowpea TEs. This finding is comparable to the findings of [2], who reported that *En/Spm*-like transposon sequences from legume species cluster together. Overall, our data suggests that lineages of *En/Spm* and *MITEs* are present in cowpea. *En/Spm*-like transposable element was present in low copy in all the cowpea varieties investigated. The southern blot analysis revealed about 5 - 6 bands, which may be an indication that *En/Spm*-like transposons are present in the cowpea genome in low copies. This is similar to the report of [2] of medium and low copies of this type of TEs in the plants they studied. A low copy number has been reported for many other *En/Spm*-like elements

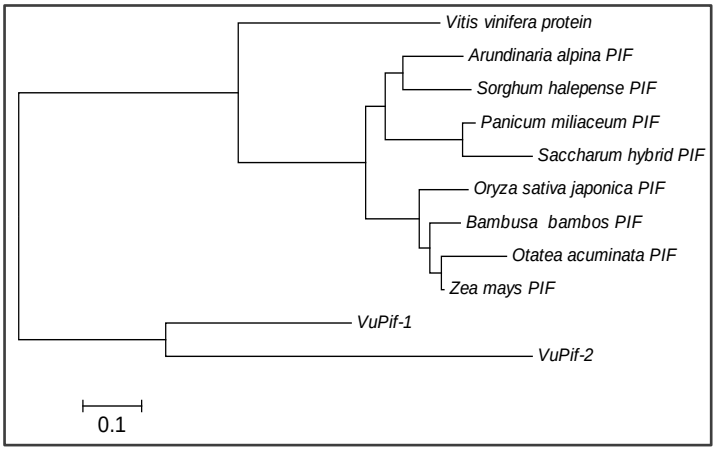

Figure 4. Dendrogram showing the relationship between *VuPIF* clones and those from other plants from BLAST search.

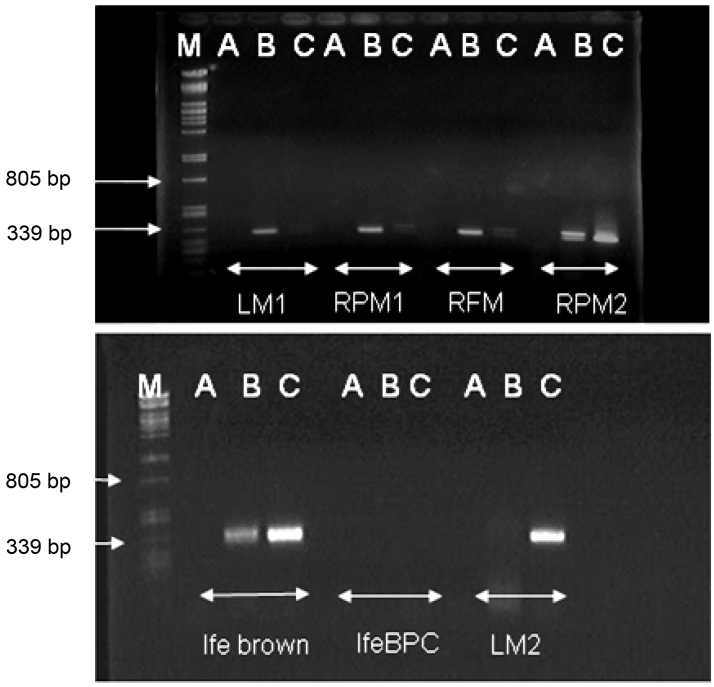

Figure 5. RT-PCR analysis for the *VuEn/Spm* and *VuPIF* clones on leaf, flower and stem samples (M = Lambda Pst1 digest, A = *VuEn/Spm*-1, B = *VuEn/Spm*-3 and C = *VuPIF*-1) of some mutants and wild types, LM1 = unifoliate leaf mutant, RPM1 = reduced flower mutant 1, RFM = "Rosa" flower mutant, RPM2 = reduced flower mutant 2, and LM2 = non-petiolate leaf mutant and non-branching stem mutant (LM1 analysis shown was on the unifoliate leaves); RFM, RPM1 and RPM2 were on the flowers, LM2 was on the stem while Ife brown and Ife BPC (parental lines/wild types) were on the leaf tissues.

in plants, including other legumes [2], Poaceae [22] as well as others plant species [43]-[46].

A substantial proportion of plant genomes are made up of transposable elements and they contribute both to their structure and evolution [24]. Therefore, the amplification obtained for the *En/Spm* in all the lines analyzed is an indication that the high mutability observed in cowpea mutants are likely due to the activities of transposable elements; with the *En/Spm* and *MITEs* also possibly responsible for these mutations. Therefore, the results

obtained in this study corroborates the presence of these types of class II TEs in cowpea and therefore supports the fact that the mutations observed through classical breeding might have been as a result of the activities of transposable elements. [25]-[28] described the previous work done in obtaining these mutants, which revealed that there was somatic instability and possible insertion and activity of TEs in the tissues in which the mutation took place.

The RT-PCR analysis provides additional evidence that these TEs are transcribed differently in the different plant parts/tissues investigated both in the mutants and in the parental lines/wild type. Firstly, the fact that RT-PCR amplicons were obtained for *VuEnSpm*3 and *VuPIF*1 while *VuEnSpm*1 did not show any transcript in all the mutants and wild type tested, suggests that *VuEnSpm*-3 is actively transcribed in the tissues analyzed as opposed to *VuEnSpm*-1. This corroborates the activity of TEs as a result of somatic instability by earlier workers.

5. Conclusion

In conclusion, this study presents evidence to confirm the presence of *EnSpm* TEs and *MITEs* in the cowpea genome. Our results will contribute significantly to the improvement of this crop by providing important genomic resource that was previously unavailable and open up avenues for cowpea TEs to be used in molecular marker development [14] or gene tagging [18]. Availability of complete cowpea genome sequence will further expand the discovery and application of TEs in cowpea genomic studies.

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