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Transcriptionally Active *Mutator*-like Transposable Elements in the Genome of Cassava (*Manihot esculenta* Crantz)

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Abstract: Randomly sequencing cDNA clones from a library made with mRNA expressed during the Post-harvest Physiological Deterioration (PPD) of cassava root led to the isolation of a clone with high similarity to an *Arabidopsis Mutator*-like transposable element (MULE) transposase. Southern hybridisation experiment using this cassava MULE transposase cDNA as probe revealed significant differences between the hybridisation pattern of the DNA digested by the isoschizomers, *Hpa* II and *Msp* I, suggesting partial methylation of cassava MULE sequences. We detected RNA transcripts of cassava *mudrA*-related elements under normal growing conditions by the northern hybridisation protocol. The transcripts expression increased in wounded cassava leaves and during PPD of cassava root. The implication and application of active *Mutator*-like transposable element in the genome of cassava is discussed.

Key words: Cassava, MULE, transposase, post-harvest-deterioration, transposons, *mudrA*

INTRODUCTION

Transposable element insertions causing phenotypic variation have been used for the identification, isolation and characterization of genes in plants (Walbot, 1992; Cardon *et al*., 1993; Aarts *et al*., 1995). In addition, transposable elements are proving useful in reverse genetics for the functional study of identified genes by acting as mutagens through insertion in the gene of interest (Sundaresan *et al*., 1995; Parinov *et al*., 1999; Miyao *et al*., 2003; Kuromori *et al*., 2004). Transposable elements are also being used as molecular tools in DNA fingerprinting, phylogenetic studies and molecular breeding. They have been used as DNA markers for the study of biodiversity in maize, pea and barley (Purugganan and Wessler, 1995; Pearce *et al*., 2000) and helped in generation of linkage maps in barley oat and pea (Kumar *et al*., 1997; Waugh *et al*., 1997; Ellis *et al*., 1998; Yu and Wise, 2000).

Mutator is a highly mutagenic transposable element system. Transposition preferentially occurs into low copy number DNA sequences, resulting in extremely high rate of mutations up to 50-fold above the spontaneous level and an unusually diverse nature of mutants (Robertson, 1978; Cresse *et al*., 1995; Raizada *et al*., 2001). These properties make *Mutator* useful for mutating and cloning many of the maize (*Zea mays*) genes (Walbot, 2000).

Robertson's *Mutator* transposons fall into a number of different categories, all of which share highly similar 220 bp terminal inverted repeats (TIRs), but each of which contains unique unrelated internal sequences (Chandler and Hardeman, 1992; Bennetzen, 1996). In addition, they typically generate a 9 bp target site duplication (TSD) of flanking DNA upon insertion. The autonomous (self replicating) and regulatory element, *MuDR* of the *Mutator* (*Mu*) system has been well studied in maize (Chomet *et al*., 1991; Hershberger *et al*., 1991; James *et al*., 1993). It consists of two convergently transcribing but non-overlapping genes, *murdA* and *murdB*. The larger, *murdA*, encodes the transposase while *murdB* codes for MURB. The latter is not essential for somatic excision in maize but appears to be required for germinal duplication (Lisch *et al*., 1999; Raizada and Walbot, 2000). There is no similarity between *mudrB* gene and sequences outside of maize in any of the public database and its precise function remains unknown (Lisch, 2002).

This *Mutator* system has been applied for the genetic analysis of this universally important food crop. *Mutator*-like transposable elements (MULEs) have also been found in many other plant species including *Lycopersicon esculentum* (Young *et al*., 1994), *Arabidopsis thaliana* (Le *et al*., 2000; Yu *et al*., 2000), *Oryza sativa* (Mao *et al*., 2000; Turcotte *et al*., 2001) and

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other cereals (Lisch *et al*., 2001). Transposition competence is the main requirement for most of the application of *Mutator* system in maize and other plants.

A cDNA clone isolated from a library made from mRNA expressed during the PPD of cassava root proved to be a MULE transposase (Gbadegesin *et al*., 2003) suggesting that this family of transposons could be active in cassava. Cassava (*Manihot esculenta* Crantz), despite being the world's sixth most important crop in terms of production and the staple food of over 500 million in the tropical regions of the world (Beeching *et al*., 1998), is grossly understudied.

In this study, we have studied the methylation status of cassava MULE using Southern analysis of genomic DNA digested with restriction enzymes that are differentially sensitive to methylation. We also examined the MULE RNA expression in this important food crop. This will enable us to gain an insight into the activity of cassava MULE. Hershberger *et al*. (1995) have shown a correlation between the presence of the major *MuDR* transcripts and *Mutator* activity in maize.

MATERIALS AND METHODS

Plant material and DNA isolation: Young leaf samples for the isolation of DNA and total RNA were obtained from cassava cultivars grown in the green house at the University of Bath. The study was conducted between November 2003 and September 2004 in the department of Biology and Biochemistry, University of Bath, UK.

Sequence and phylogenetic analyses: DNA molecules were sequenced on an ABI 337 automated dye primer sequencer using universal primers for the cloning vector. Initial confirmation of sequence identity was by using BLASTN and TBLASTX searches against the GenBank non-redundant database at the default parameters (Altschul *et al*., 1997). Sequence data were aligned using CLUSTAL W (version 1.82) (Higgins *et al*., 1994). The PHYLIP program package version 3.63 (Felsenstein, 2004), available from the author at Department of Genetics, University of Washington, Seattle, Washington, was used for phylogenetic analysis. Consensus NEIGHBOR-joining trees (Saitou and Nei, 1987) were derived from equally parsimonious trees using the extended majority rule in the CONSENSE. Unless otherwise stated, distance matrices for phylogenetic analyses based on nucleotide sequences data were computed using DNADIST according to the Kimura 2-parameter model (Kimura, 1980). Nei and Li model (Nei and Li, 1979) of RESTDIST were used for those based on restriction fragments. Trees were drawn using TREEVIEW program version 1.6.6 available from the author, Roderic D.M. Page of the Taxonomy Unit, Department of Zoology, University of Glasgow.

DNA gel blot analysis: Cassava genomic DNA was isolated from young leaves by the method of Dellaporta *et al*. (1983). Restriction digestions of genomic DNA (5 μg each) were carried out using buffer and reaction conditions specified by the manufacturer (Promega). Blotting and hybridisation were performed using standard procedures (Sambrook *et al*., 1989).

Wounding and incubation procedures: Cassava leaves wounding stress was induced with a modified method of Takeda *et al*. (1998). In a set of experiments, leaf discs were prepared by cutting young cassava (cultivar MCOL22) leaves into discs of approximately 1 cm^2. In a second, the leaf segments prepared from young leaves were stabbed with the points of a forceps about twenty times. The leaf segments (stabbed or unstabbed) were then incubated on 0.05% 2-[N-morpholino] ethane sulphonic acid (MES)-KOH buffer (pH 5.7) at 25°C under room light (MES is generally used in tissue culture to prevent oxidative degradation of biomolecules). Leaf samples were taken at 2, 4, 6, 8, 12, 24 and 48 h from the incubation mix, quickly wrapped with aluminium foil and tucked into a vacuum flask of liquid nitrogen. These were immediately ground to fine powder using standard procedure and the powdered leaves stored at -70°C until required. Total RNA was isolated from the powdered samples of leaves as below. For the root the conditions for deterioration experiments were as described by Han *et al*. (2001).

RNA extraction and Northern blot procedures: Using 2 mL nuclease free microfuge tubes, RNA was extracted from the homogenized tissue using the Promega 'SV total RNA isolation system' with some modifications to the specifications of the manufacturer as follows: 350 μL of lysis buffer was added to 0.09 g of the ground tissue in the microfuge and mixed by inversion. Seven hundred microliter of RNA dilution buffer was then added and again mixing by inversion before centrifugation at 13,000 rpm for 10 min. The cleared lysate solution was then transferred to a fresh microfuge by pipetting without disturbing the pelleted debris otherwise the centrifugation step was repeated. Four hundred microliter of 95-100% ethanol was added to the clear lysate and mixed by pipetting 4 times. The mixture was then transferred to the 'spin column assembly' and spun at 13,000 rpm for 1 min. The rest of the protocol followed the procedure E (RNA purification by centrifugation) of the kit

manufacturer. Total RNA was extracted from cassava storage roots using the method of Chang *et al.* (1993).

Ten microgram of total RNA per lane was electrophoresed on a 1.5% agarose gel containing formaldehyde and blotted onto a nylon membrane (Hybond N+, Amersham) according to standard procedures (Sambrook *et al.*, 1989). Hybridisations were carried out at 65°C overnight after which membranes were washed to a final wash stringency of 0.2X SSC, 0.2% SDS for 20 min at 65°C. The membranes were then exposed to a photographic film, incubated at -70°C and the autoradiography developed.

RESULTS

A cassava MULE transposase cDNA (*MecTP*) grouped with the *Arabidopsis* non-TIR MULES: The whole genome-sequencing project of *Arabidopsis* had revealed the presence of many MULEs in the genome of this model plant. A group of *Arabidopsis thaliana* TIR-MULEs and non-TIR-MULEs, as defined by Yu *et al.* (2000) from *Arabidopsis* genome sequence analysis, were used in a comparative study with the cassava element. *Arabidopsis mudrA*-like transposase identified in the BLAST searches using the cassava sequence was specifically included.

The nucleotide sequences of *mudrA*-related open reading frames of the selected *Arabidopsis* MULEs and of maize *mudrA* gene (Table 1) were mined from the NCBI database at (www.ncbi.nlm.nih.gov/). These were aligned with *MecTP* nucleotide sequence. The cassava *MecTP* nucleotide sequence aligned very well with the *mudrA*-related sequences of *Arabidopsis* and a region of conservation >60% between all the sequences are prominent (not shown). This confirmed the cassava sequence as an authentic *mudrA*-related gene or transposase.

Table 1: The *Mutator*-like transposable elements (MULE) nucleotide sequences used in the comparative phylogenetic analysis with cassava (*Manihot esculenta* Crantz), *MecTP*. The table shows the names used for the sequences in the multiple alignments/phylogenetic analysis, the corresponding botanical name and the geninfo identifier (GI) number

Sequence name	Source organism	Database GI number
AtMULE1	*Arabidopsis thaliana*	3510344
AtMULE2	*Arabidopsis thaliana*	30683392
AtMULE3	*Arabidopsis thaliana*	2832639
AtMULE4	*Arabidopsis thaliana*	2443899
AtMULE5	*Arabidopsis thaliana*	5041971
AtMULE6	*Arabidopsis thaliana*	4585891
AtMULE7	*Arabidopsis thaliana*	6007863
AtMULE8	*Arabidopsis thaliana*	3063438
AtMULE9	*Arabidopsis thaliana*	4519197
AtMULE10	*Arabidopsis thaliana*	2760316
AtMULE11	*Arabidopsis thaliana*	3319339
AtMULE12	*Arabidopsis thaliana*	4388816
mudrA	*Zea mays*	22374
MecTP	*Manihot esculenta* (Crantz)	

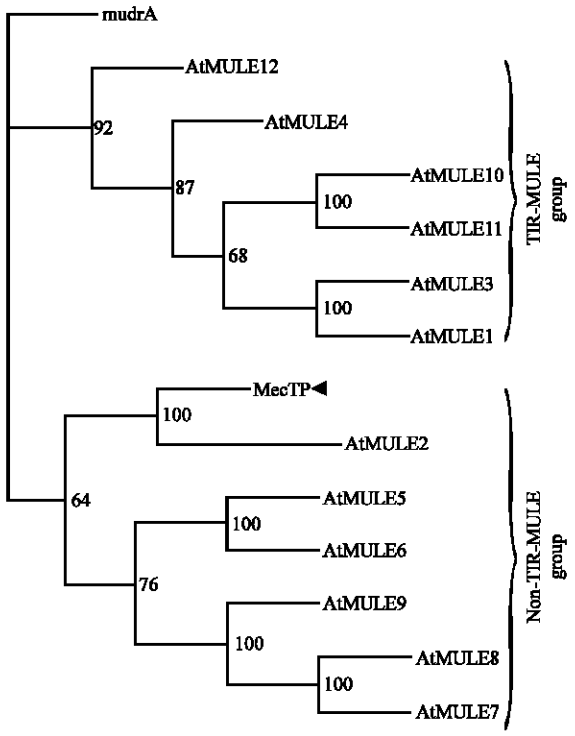

Fig. 1: Comparative phylogenetic analysis of cassava *MULE* transposase (*MecTP*) with Arabidopsis *mudrA*-like sequences. The tree is based on nucleotide sequences of *mudrA*-like fragments. It is a consensus NEIGHBOR-joining (PHYLIP) tree derived from distance matrix using Kimura 2-parameter methods. Outgroup sequence, *mudrA* of *Zea mays*, was used to root the tree. The two groups of MULEs defined by Yu *et al.* (2000) from analysis of *Arabidopsis* MULE sequences are shown. The cassava sequence is indicated with an arrowhead. Bootstrap values (100 replicates) are shown. The geninfo identifier (GI) numbers of the MULE sequences are shown on Table 1

The aligned nucleotide sequences data were used to compute a distance matrix and trees produced as described in the materials and methods. A consensus tree was derived from 100 equally parsimonious trees. The consensus tree rooted using maize *mudrA* sequence was drawn using TREEVIEW as shown in Fig. 1.

The two groups of MULEs from *Arabidopsis* genome sequence analysis (Yu *et al.*, 2000) stood out in the phylogenetic tree (Fig. 1). The analysis placed the cassava element, *MecTP*, in the clusters of *Arabidopsis* non-TIR-MULEs with 64% bootstrap value. Moreover, the sister sequence of *MecTP* on the phylogenetic tree is an *Arabidopsis* non-TIR MULE with the association of both supported by a bootstrap value of 100%.

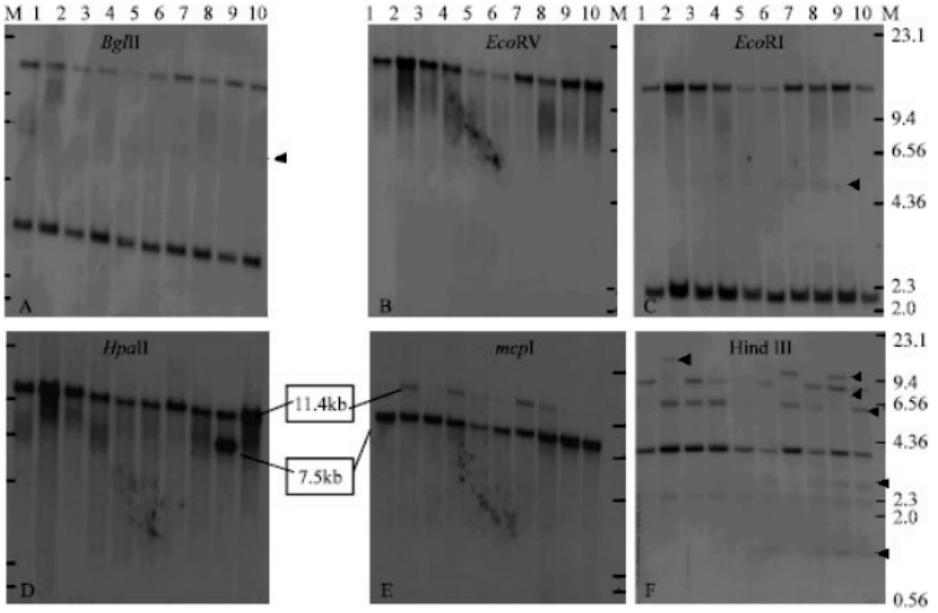

Fig. 2: Southern analysis of MULE transposase of cassava cultivars (1-10). Ten microgram of genomic DNA from each of 10 cassava cultivars; lanes 1 (MGA1), 2 (MNGA2), 3 (MDOM5), 4 (MNGA19), 5 (MCOL22), 6 (CMC40), 7 (MVEN77), 8 (CG402), 9 (SM627) and 10 (CM2177) were digested with *Bgl* II (Panel A), *Eco* RV (Panel B), *Eco* RI (Panel C), *Hpa* II (Panel D), *Msp* I (Panel E) or *Hin*d III (Panel F). The digested DNA were separated on 0.8% agarose gel, transferred to nylon membrane and hybridised with *MecTP* probe. *Hin*d III-digested lambda DNA was used as DNA size marker (M). The polymorphic bands from the *Eco* RI, *Bgl* II or *Hin*d III digests were

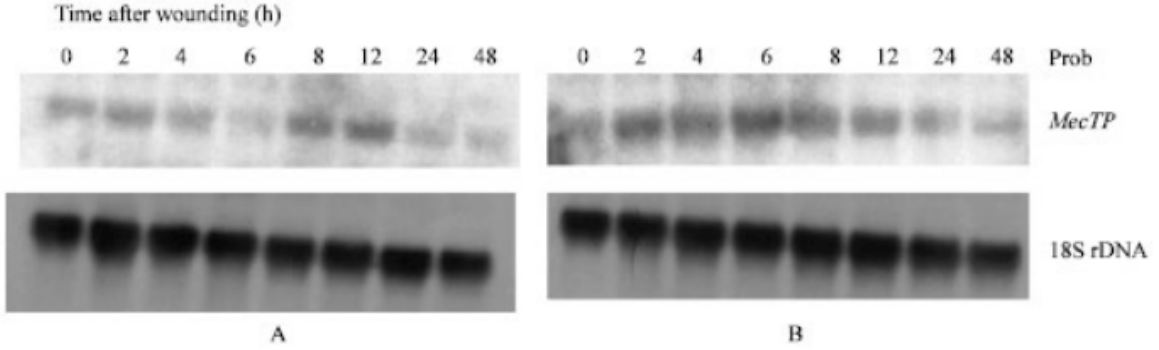

Fig. 3: Accumulation of cassava *mudrA*-like transcripts in the leaves of cassava after cutting (A) and cutting and wounding (B) over a time course (0-48 h). Cassava leaf segments stabbed (B) or unstabbed (A) (as described in the text) were incubated on 0.05% 2-[N-morpholino] ethane sulphonic acid (MES-KOH buffer (pH 5.7) at 25°C under room light. Leaf samples taken at 2, 4, 6, 8, 12, 24 and 48 h of incubation were used for isolation of total RNA. 0 point of time course represents untreated control. Ten microgram of the total RNA in each case were analysed by northern blot hybridisation with the indicated probes. 18S rDNA probe was used to assess quality and quantity of the loaded total RNA

Study on diversity and genomic organisation of MULEs in Cassava: Southern analysis: *MecTP*, the cassava transposase cDNA, was radiolabelled and used to probe each of the Southern blots of the DNA digests from 10 different cassava cultivars. Following high stringency washes (0.2 X SSC, 0.1% SDS, 60°C); strong signals were observed for all the digests (Fig. 2), The blot hybridisation revealed few hybridising bands in all the cultivars tested with all of the six enzyme digests except for *Hin*d III. This suggests that binding to heterologous and distant related sequences did not occur. Distant related sequences will be expected to form probably weaker but bands distinct from cassava MULE. The probe contained two *Hin*d III recognition sites which could explain the

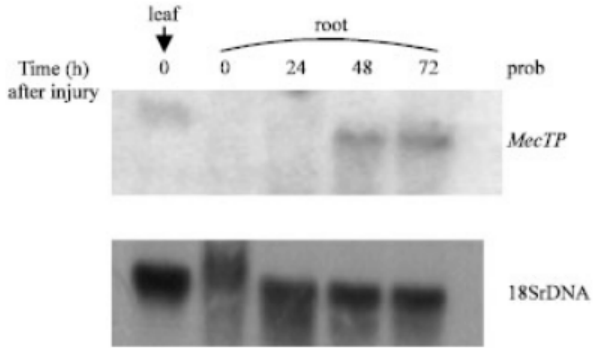

Fig. 4: Expression of cassava *mudrA*-like RNA transcripts during post-harvest storage of the root tuber. Total RNA was isolated from the whole root tissue after 0, 24, 48 and 72 h of initiation of PPD as explained in the text. Ten microgram of the total RNA in each case were analysed by northern blot hybridisation with the indicated probes. 18S rDNA probe was used to assess the quality and quantity of the loaded total RNA

presence of more than one band in the DNA digestions by this enzyme (Fig. 2 Panel F). The presence of more than one hybridising bands in the digestions with *Bgl* II, *Eco* RI and *Msp* I suggested that there might be more than one copy of MULE in the genome of cassava.

Effect of wounding on accumulation of *murdA* transcripts in cassava leaves: Wounding stress has been shown to induce the expression of the tobacco retrotransposon, *Tto1* (Takeda *et al*., 1998). Ten microgram of total RNA per lane was electrophoresed on a 1.5% agarose denaturing gel and blotted onto a nylon membrane as described in the material and method section. Hybridisation of the RNA on the membrane with *MecTP* probe, the cassava transposase cDNA, showed a ~2.8 kb transcript. This is the expected size of *mudrA* transcript (Hershberger *et al*., 1995; Joanin *et al*., 1997). The signal, representing expression of *mudrA*-like gene, is observed for all samples treated with cutting or cutting and stab wounding and untreated (Fig. 3). This implies that the gene is expressed in normal growing conditions of the cassava plants. There was no difference between uncut leaf sample (0) and leaf segments up to 6 h of incubation time. The transcript accumulation increased slightly at 8-12 h of incubation of the cut leaf segments but fell back to values comparable to the untreated control (0) at 24 and 48 h of incubation (Fig. 3A). Wounding by stabbing of the leaf segments enhanced the transcript accumulation faster than cutting without stab wounding. There was an increase from 2 h and peak value was seen at 6 h of incubation. At 24 h the transcript level is comparable to the untreated control (Fig. 3B).

Accumulation of *murdA* transcripts during post-harvest storage of cassava root tubers: The cassava transposase cDNA, *MecTP*, probe of filter membrane containing the total RNA extracted from fresh cassava storage roots and those undergoing PPD showed a ~2.8 kb transcript (Fig. 4). Expression of *mudrA* RNA transcripts in cassava root at 0 and 24 h after initiation of PPD is lower than what was observed for untreated leaf (Fig. 4). There was increased transcript expression at 48 and 72 h of root PPD indicating that cassava *mudrA*-like sequences are expressed later during PPD.

DISCUSSION

The availability of the whole genome sequence of *Arabidopsis* has enabled a detailed analysis of the pattern and extent of diversity of MULEs and these has revealed the presence of the two groups of MULEs. Unlike TIR-MULEs and the *Mu* elements in maize, the non-TIR-MULE elements are characterised by the absence of long TIR structures and low sequence similarity between termini of individual elements. However, there is high level of homogeneity between members of individual sub-groups (Yu *et al*., 2000). They are also characterised by their abundance in the genome and a relatively high frequency of members of non-TIR-MULE group, which encode a putative MURA-related protein (Yu *et al*., 2000). The analyses in this study grouped the cassava MULE transposase cDNA with the *Arabidopsis* non-TIR-MULEs.

Genomic library screening of cassava and preliminary analysis showed that multiple copies of cassava MULEs are present in its genome (data not shown). Reports have shown that *Mu* elements are widely distributed in both eukaryotes and prokaryotes making up a super-family. The *Mutator* system in maize is a diverse family of transposable elements composing of at least five different classes of elements, all of which share homologous TIRs but each contains completely heterogeneous internal sequences (Walbot, 1991; Chandler and Hardeman, 1992; Bennetzen, 1996). Recent genome-sequencing projects have revealed that *Mu* elements are widespread among plants (Young *et al*., 1994; Le *et al*., 2000; Yu *et al*., 2000; Mao *et al*., 2000; Turcotte *et al*., 2001; Lisch *et al*., 2001). In some cases multiple, almost identical copies of these elements have been identified especially in species with completely sequenced genomes (Yu *et al*., 2000; Mao *et al*., 2000). Detail sequence analysis of MULEs in the genome of cassava has not been carried out and repeated efforts to sub-clone fragments of λ clones that hybridised to the cassava transposase in this study proved unsuccessful. One cause of this may be in the nature of the sequence and the activity of the

cassava MULEs. For instance, the instability of *MuDR* elements in *E. coli*, particularly the portion encoding the 2.8 kb transposase transcript has been reported (Benito and Walbot, 1994). A similar problem with the full-length isolation and sequence characterisation of *Tdc A1*, the *En/spm*-like transposon of carrot, has been documented (Itoh *et al*., 2003). Research effort is on to circumvent this hurdle in cassava.

Southern hybridisation supports the diversity of cassava MULEs in the genome of all cassava cultivars tested. It also revealed hypomethylated sequences as shown by the contrasting differences in the hybridisation pattern of the DNA digested by the isoschizomers, *Hpa* II and *Msp* I. These two enzymes share the recognition sequence CCGG, but, while *Hpa* II is blocked by methylation at either C, *Msp* I is blocked by methylation at the external C only. The findings here suggest the partial methylation and not hypermethylation, of the cytosine residues in the sequence CCGG of cassava *Mutator*-like transposable elements for all of the cultivars tested. Under this methylation condition unmethylated external C in the sequence CCGG may occur more frequently and *Msp* I was able to restrict cassava MULE sequences at the recognition sequence CCGG more extensively than *Hpa* II. Hypermethylation of *Mutator*-like transposable element sequences defines epigenetic silencing, a widely discussed means of regulation of activity of these elements. In the absence of functional *mudrA*, cytosine residues in the termini of *Mu* elements are methylated consistent with a lack of activity (Chomet *et al*., 1991).

Southern analysis with the restriction enzymes digests of total genomic DNA using cassava transposase, *MecTP*, as probe revealed the presence of highly polymorphic bands among 10 cassava cultivars examined, particularly with some enzymes such as *Hin*d III. Similar polymorphism has been reported among the grass species in DNA blot that were probed with the conserved portion of *mudr*A from maize (Lisch *et al*., 2001). Scoring this polymorphism in the cassava cultivars, they were grouped into two clades but we consider the data to be so few that little confidence can be placed in this separation. Further studies of these elements with larger number of cultivars would greatly facilitate knowledge of the continuing co-evolution of transposable elements with their hosts, cassava in this case.

Northern hybridisation results showed that low levels of the transcript of transposase gene of cassava were produced under normal growing condition and are slightly upregulated by wounding. In addition, the transposase transcript expression is upregulated during the late stages of PPD. This finding is consistent with the fact that the cassava MULE transposase cDNA clone was isolated from a library made from mRNA expressed during the late phase of PPD of a cassava root (Gómez-Vásquez and Beeching, unpublished). In maize, blots performed on RNA from a variety of tissues and developmental stages indicated that the major transcript of *MuDR* might be ubiquitously expressed in active *Mutator* stocks (Chomet *et al*., 1991; Hershberger *et al*., 1995; Rudenko and Walbot, 2001). Moreover, MULE sequences have been found in the expressed-sequence–tag databases of some plants (Lisch *et al*., 2001).

Understanding the mechanism of action of this active cassava transposons system including their integration and excision from the genome will open up potentials for their application for cloning and mutation of genes in cassava as have been obtained in maize. This will contribute to the much-awaited biotechnological development of the world-class food crop, cassava.

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