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Genome-wide Analysis of *MuDR*-related Transposable Elements Insertion Population in Maize

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Abstract: Insertional mutagenesis has now been widely used to knockout genes for functional genomics. The maize *Mutator* transposons hold an advantage of high activity to construct large mutant libraries. In this study, a *MuDR* line was used to cross with an elite Chinese maize inbred line Z31. A total of 1 000 M_1 individuals were planted and self-pollinated to generate their M_2 families. Experiments were conducted to investigate the insertion specificity of *MuDR* related transposable elements. Six hundred and ninety-five *MuDR* inserted flanking sequences were isolated with a modified MuTAIL-PCR method and analyzed with bioinformatics. Three hundred and seventy-four non-redundant insertion sites were identified and 298 of them were mapped to a single locus on the integrated maize map. The results revealed some prominent features of the *MuDR*-related insertions of maize: random distribution across the 10 chromosomes, preferential insertion into genic sequence and favoring some classes of functional genes.
Keywords: *Zea mays*; *Mutator* (*Mu*) transposons; *MuDR* elements; Flanking sequence; Insertion sites; *Mu*TAIL-PCR

全基因组分析玉米 *MuDR* 转座因子插入突变体库

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摘 要：在功能基因组研究中，插入诱变被广泛用于基因敲除。玉米 *Mutator* 转座子因其具有较高的转座活性常被用于构建大型玉米插入突变体库。本研究利用具有活性 *MuDR* 因子的玉米材料与优良玉米自交系 Z31 杂交，获得 1 000 个 M_1 单株，自交构建 M_2 群体，研究 *MuDR* 因子在基因组中插入位点特性。利用优化的 *Mu*TAIL-PCR 方法分离出 695 条 *MuDR* 插入位点侧翼序列，经初步生物信息学分析得到 374 条非冗余的插入位点，其中的 298 条序列能够被定位在玉米基因组物理图谱单个位点上。实验结果揭示了 *MuDR* 因子插入的一些特性：在 10 条染色体上随机分布，偏向于插入到基因序列中，并在某些功能基因中有明显插入偏好。
关键词：玉米; *Mutator* 转座子; *MuDR* 因子; 侧翼序列; 插入位点; *Mu*TAIL-PCR

Gene knockout has become a powerful and indispensable tool in molecular genetics and functional genomics. A comprehensive collection of gene knockouts allows us to understand the relationship between the phenotypes and mutations of genes[1].

Diverse approaches have been used to develop comprehensive gene knockout resources, which are necessary for forward and reverse genetic analysis in plants. *Arabidopsis thaliana* has provided a good model using Flanking Sequence Tags (FSTs) approach via insertional mutagenesis populations[2-3]. Several methods were primarily applied to systematically amplify and sequence the genomic DNA flanking the T-DNA tags from each mutant. Then these FSTs were searched against the public DDBI/EMBL/GenBank GSS database to obtain the genome annotations. An improved FST approach was adopted in rice[4-6] and the latest release of OryGenesDB database contained 171 000 FSTs (http://orygenesdb.cirad.fr/index.html), which greatly accelerated rice functional genomic research[7].

Maize is comparable to rice as the model genetic system for genome studies[8-9]. Since the *Ac/Ds* transposable elements were discovered[10], the transposon mutagenesis approaches have been widely utilized in disrupting genes, isolating mutants and studying gene function in maize[11-12].

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Transposon insertional mutagenesis can be classified into low-copy and high-copy strategies[13]. The former contains *Ac* and *Spm*, which produce low genome-wide mutation rates and transpose preferentially to linked sites[14-16]. These populations cannot remove the interference of the background mutations due to the non-autonomous *Ds* elements in the genome. The high-copy system most commonly used in maize is *Mutator*[17].

Up to date, *Mutator* is the most active and mutagenic plant transposon discovered in maize[18-20]. Its properties of high copy numbers and high transposition frequency make it suitable for forward and reverse genetic analysis[21]. The *Mutator* transposon family is a two component system[22]. All the maize *Mutator* elements contain conserved −220 bp terminal inverted repeats (TIRs), but each class of elements contains specific and unrelated internal sequences[18,22]. Liu et al.[23] defined 21 novel *Mu* TIRs using a DLA-454 strategy, different from the TIRs reported previously. This system is regulated by autonomous *MuDR* elements, which control the transposition of themselves and the other classes of the non-autonomous *Mu* elements[24-25]. Besides, studies have shown that *Mu* insertions are heavily biased for transcribed regions of the genome[9,26-28].

The successful use of transposon tagging lies in the identification and isolation of the genomic sequences flanking the insertion sites. Many PCR-based methods have been developed and some are optimized for *Mu* elements according to the conserved sequences in the inverted terminal repeats. Amplification of insertion mutagenized sites (AIMS) was a ligation-mediated method with the *Mu* primer biotinylated[29]. *Mu*TAIL was an adaptation of thermal asymmetrically interlaced (TAIL) PCR[30] to amplify flanking fragments in a complex pool of *Mu*-induced mutants[28]. Yi et al. combined elements of both *Mu*TAIL and AIMS into a procedure called *Mu*TA for co-segregation analysis[31]. Besides, an adaptor-mediated PCR-based method, Digestion-ligation-amplification (DLA), was developed to overcome difficulties of amplifying unknown sequences flanking known DNA sequences in large genomes[32]. Furthermore, an improved draft nucleotide sequence of the 2.3-gigabase maize genome has been released in 2009. All of these would greatly facilitate the *Mutator* tagging strategy in maize functional genomic research.

In our study, the M_1 population was generated by crossing the active *Mutator* transposon line as donor parent with the recipient parent Z31. The number of copies of the *MuDR* elements per mutagenic plant is one or two in the population. We observed the phenotypes of the M_1 and the M_2 generations and amplified the flanking sequences of the *MuDR* with a modified *Mu*TAIL-PCR method. The TAIL products were cloned and sequenced for further bioinformatics analysis. We attempt to construct our own *Mutator* insertional mutant populations of maize in China and create more novel mutants for the maize functional genomic studies.

1 Materials and Methods

1.1 Plant materials

MuDR-active line was used as the pollen donor in a cross with the maize inbred line Z31 (yellow kernel). The progenies of the cross were self-pollinated to produce the *Mutator* mutant stocks. The resulted kernels were planted and young leaves were harvested for genomic DNA extraction based on the protocol described by Settles [28].

1.2 Isolation of flanking sequences

Mu flanking sequences were amplified via a modified *Mu*TAIL-PCR[28]. Two nested specific primers were modified according to the *MuDR* TIR sequence reported as follows: TIR9-1: 5'-ATAGAAGCCAACGCCATGGC CTCCATTTCGTC-3'; TIR9-2: 5'-GGCCTCCATTTCGTC GAATCCCTT-3'.

The 12 arbitrary primers used in our experiment were designed.

Either 5% DMSO or glycerin was added to the PCR mixture. TAIL-PCR products were purified with QIAquick Gel Extraction Kit (QIAGEN, Germany) based on the manufacturer's instructions. The confirmed products were ligated to the pGEM-T Vectors (Promega, USA). Then the ligation products were transformed into the *E. coli* DH5α by electronic transformer (Electroporator 2510, Eppendorf), and plated onto the LA medium containing Ampicillin, X-gal and IPTG (40 mg μL^{-1}). Cloned inserts corresponding to the size of the fragment from the PCR were full-pass sequenced by an ABI3730XL sequencing facility (Applied Biosystems, CA) and the sequences were analyzed bioinformatically.

1.3 Sequence analysis

A homology search was first performed against the vector sequences of the plasmids using the NCBI VecScreen tool (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Then masked sequences were analyzed for the presence of the conserved *MuDR* TIR sequence using a BLAST program. Then a BLASTN was conducted against the B73 RefGen_v1 database to get the predicted location hits using the B73 Genomic Browse Tools with the expectation score cut-off $<10^{-12}$ (http://www.maizegdb.org/).

2 Results

2.1 *MuDR* elements insertion sites

Mu-specific fragments were identified and cloned by comparing its parental band patterns. Not all the 12 arbitrary primers were found to be suitable in our materials. Only four of them, BAD5, CTG1, SAD11, and SW41 yielded large products from *Mutator* lines (Fig. 1).

Totally, 695 specific *Mu*TAIL-PCR fragments were obtained and sequenced from about 5 000 M_2 individuals.

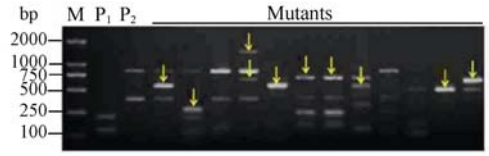

Fig. 1 *Mu*TAIL products from the DNA of parents and mutants using CTG1 arbitrary primer
M is DNA ladder marker DL2000. P_1 and P_2 are Z31 and *Mutator* active parent. The other 12 lanes show the secondary products of the TAIL-PCR. Arrows indicate the specific fragments.

Theoretically, a *Mu* flanking sequence should be amplified with 29 bp of *Mu*-TIR sequence downstream of the specific primer of the TAIL-PCR. Presence of the *Mu*-TIR sequence can identify the precise insertion site of the elements. The results showed that, except for 150 of them without partial *MuDR* TIR sequence, each of the remaining 545 sequences had a complete *MuDR* TIR sequence and indexed a known individual. Vector and quality-trimmed sequences were deposited to the NCBI gss database (dbGSS_ID: 28569169 to 28569485).

2.2 Homology analysis of the flanking sequence

Analysis of 545 sequences by the ClustalX (V2.0.12) software, 374 non-redundant insertion sites were obtained. Of the 374 sequences, the overall average length was 500 bp, including 25 sequences in the length ranged from 25 bp to 100 bp, 153 sequences in the range of 100–499 bp, 183 sequences in the range of 500–999 bp and 13 sequences longer than 1 000 bp. The homology search of the trimmed sequences using the BLASTN tool found that 298 sequences contained 29 bp TIR sequences as expected and the others having significant similarity (E-value<10^{-12}) with the maize genomic sequence mapped in the maize genome.

2.3 Distribution of *MuDR* insertions along chromosomes

Based on BLASTN searches of the released BAC and cDNA sequences, we used an in silico protocol to locate *Mu*TAIL sequences to make sequence information more visual in functional genomics. Predicted locations of 298 *Mu*TAIL are shown in Figure 2. Their distribution across the 10 chromosomes of maize was nearly random.

2.4 Distribution of *MuDR* insertion sites relative to transcribed maize sequences

The genomic sequence could be roughly divided into three classes: genic region, transposable element (TE)-related and intergenic sequences. The observed numbers in each of the three sequence classes are 497, 22, and 26, respectively. Only 22 (~4%, 22/545) sequences contain repeated sequences, having significant similarity with TE-related sequences. In addition, the remaining 523 sequences could be uniquely mapped to the maize genome. Taken together, *MuDR* insertions observed strongly disfavored the TE-related sequences and exhibited a preference for transcribed regions.

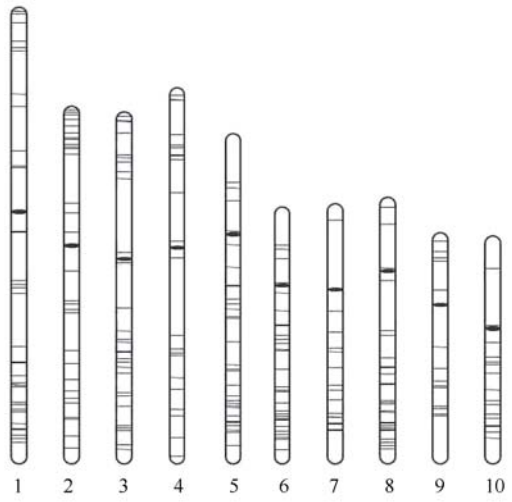

Fig. 2 Distribution of *MuDR* insertion sites among chromosomes
The predicted locations of 298 *Mu*TAIL sequences were determined by BLASTN analysis as described in the materials and methods. The black ovals show centromeres and the lines indicate the relative positions on the chromosomes.

BLASTP results showed that 265 of the 374 non-redundant insertion sites were found to be relevant to predicted genes with function annotations. These insertions were categorized into four disjointed regions: promoters, 5'-UTR, exons, introns, and 3'-UTR. The observed numbers of insertions that occurred in each of the four regions are shown in Figure 3.

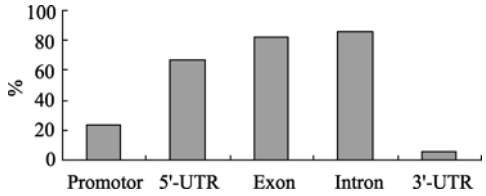

Fig. 3 Distribution of *MuDR* insertion sites within transcribed maize sequences
Abscissa: positions of the insertion sites; longitudinal coordinates: number of tested insertion sites.

2.5 Classification of *MuDR* target genes function using Gene Ontology (GO)

To gain a better perspective of the functional roles of the insertion sites in maize, we looked for targets enrichment in Gene Ontology (GO) molecular function and biological process categories[33]. The targets were annotated by using the GO annotations available from the B73 RefGen_v1. of the predicted targets, 71% had GO assignments whereas only 68% of the genes in the entire refined set were associated with GO terms. BiNGO (Biological Networks Gene Ontology)[34] was used to study targets enrichment and to construct a hierarchical ontology tree in Cytoscape[35], as shown in Figure 4. We found that *MuDR* elements preferentially target genes involved in a wide spectrum of regulatory functions and

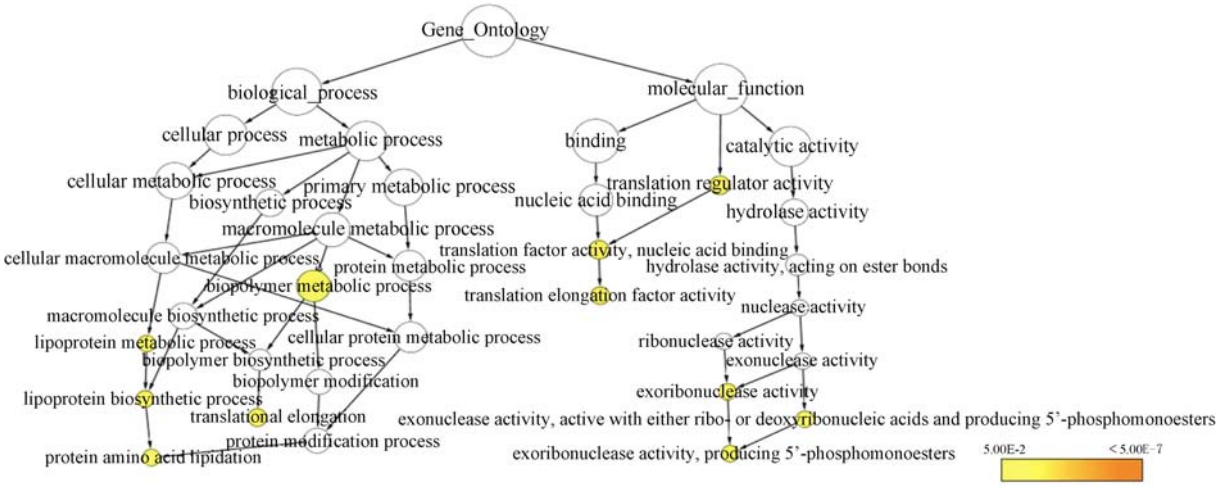

Fig. 4　Maize *MuDR* targets enrichment network based on GO molecular functions and biological processes
Significantly overrepresented GO terms were visualized in Cytoscape. The size of a node is proportional to the number of targets in the GO category. The color represents enrichment significance: the deeper the color on a color scale, the higher the enrichment significance. White color nodes are not enriched but show the hierarchical relationship among the enriched ontology branches.

selected biological processes including gene expression, transcription, metabolism, catalysis, and transport.

3　Discussion

3.1　Efficiency of the modified *Mu*TAIL-PCR

It has previously reported that *Mu*TAIL-PCR is highly effective to identify the causal *Mu* insertions rapidly[28]. We applied this method and made some improvement of the specific primers in order to identify genes disrupted by *MuDR* elements within a genome. We found that the TAIL products were more specific with less but bright bands in the agarose-gel (data not shown). In addition, about 80% of the isolated sequences contained the authentic 29 bp TIR sequence of *MuDR* elements. The data demonstrated that the modified *Mu*TAIL-PCR method is effective to get the *MuDR* flanking sequences.

3.2　Transposition features of the *MuDR* elements

As more and more genomic insertion sites of *Mu* transposons were amplified, the characterization of the *Mutator* system was made clear. For example, the *vp* loci of maize primarily acquires *Mu1*-related elements. Alleles of five loci (*vp1*, *vp8*, *vp10/vp13*, *vp14*, *vp15*) have been confirmed[36-39]. However, previous experiments showed that *sh1* locus seemed to primarily acquire mutations due to elements of the *MuDR* subfamily. Though lacking of any evidence of hotspot genes in our experiments, we found that some *MuDR* insertion sites were located in the same predicted gene within dozens of basepairs intervals. We conclude that *MuDR* elements have a strong preference for insertion into low-copy sequences, especially into the transcribed sequences which was consistent with the *Mutator* system.

3.3　Utilization of Mutator population

Thanks to the discovery of the maize *Mutator* system, many large-scale genomic projects have been initiated. A large number of *Mu* insertional populations were constructed to understand the phenotypic consequence of the loss of any gene within the genome[27-28,40-41]. Z31, an elite maize inbred line with high combining ability, was used to construct our own *Mu* insertion mutant population and get more novel mutants. Our strategy, based on molecular analysis of mutant lines using *Mu*TAIL sequencing, serves to anchor sequence-indexed insertional mutations caused by *Mu* insertions. It dramatically simplifies a reverse genetics project by allowing us to identify genes of interests and corresponding knockouts in silico.

4　Conclusion

Six hundred and ninety-five *MuDR* elements flanking sequences were isolated with a modified *Mu*TAIL-PCR method. Three hundred and seventy-four non-redundant insertion sites were identified and 298 of them were mapped to a single locus on the maize integrated map. The results revealed some prominent features of the *MuDR*-related insertions of maize. The construction of the population and the analysis of *MuDR* insertion sites were just part of our research. Two specific mutants will be meticulously analyzed and further research is in progress.

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