Determining Transgene Copy Number Using Real-Time qPCR on the MJ Research® Opticon™ 2 Continuous Fluorescence Detection System

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Real-Time Detection

Application Note Vol.2, No.11

Abstract

Real-time quantitative PCR (qPCR) was used to investigate differences in transgene copy number from multiple transgenic lines of maize (Zea mays L.). Detection of amplified template was accomplished with SYBR Green I (SGI) chemistry using an MJ Research DNA Engine Opticon® 2 fluoresence detection system. Transgene levels varying from 2 to 21 copies per genome were detected among the lines, demonstrating the sensitivity and target-specificity of the Opticon 2 system for template quantification.

Introduction

The *MuDR/Mu (Mu)* transposable elements comprise a complex family of maize transposons that were originally identified in a *Mutator* line of maize with an exceptionally high mutation rate.[1] *Mutator* activity is controlled by the *MuDR* transposon, which consists of the *mudrA* transposase and *mudrB* helper genes, both of which are predicted to encode several distinct proteins as a result of alternative splicing.[2,3,4,5,6]

The *MuDR/Mu* transposons are the only eukaryotic elements that exhibit two types of transposition. In somatic tissues, *MuDR/Mu* elements exhibit "cut and paste" transposition, in which the element is excised from one site and inserted at a new site. This transposition results in an empty site at the original *Mu* element location. In contrast, just before meiosis and in haploid cells, *MuDR/Mu* elements exhibit "net replicative" transposition, in which the element is copied and inserted into a new site without being excised from the original site.[7,8]

Transgenic modification of maize is one approach being used to determine which *mudrA* and *mudrB* encoded proteins (termed *MURAs* and *MURBs*, respectively) contribute to specific transposition reactions. Maize lines expressing various combinations of these genes are generated by first crossing transgenic plants expressing individual *MURAs* to lines with or without *MURBs*, and then crossing resulting lines to lines expressing reporter alleles. The crossing schemes produce families in which as many as three transgenes must segregate appropriately. Consequently, each plant must be genotyped to verify that it has a *mudrA*, a *mudrB*, and a reporter allele.

In this study, a real-time qPCR protocol has been developed to genotype transgenic plants by determining *mudrA* transgene copy number. Total genomic DNA was used as the template for reactions utilizing DyNAzyme™ II DNA polymerase* in conjunction with the indicating fluorophore SYBR Green I. The accumulation of amplified nucleic acids was monitored by real-time fluorescence detection on the Opticon 2 system.

Materials and Methods

Quantitative PCR

A 20ng sample of DNA from each maize plant was used as the template for qPCR. DyNAzyme II DNA polymerase was obtained from Finnzymes (F-503L), and SGI was from Molecular Probes (S-7567). Reaction components were assembled in white Hard-Shell® microplates with white wells (MJ Research #HSP-9655) and sealed with ultra-clear strip caps (MJ Research TCS-0803). Volumes of individual components and final reaction concentrations are listed in Table 1.

Table 1. Quantitative PCR Reaction Components

Component	Stock concentration	Volume	Final Concentration
DNA sample	10mg/ml	2.0µl	1mg/ml
Forward Primer	(10µM)	0.4µl	0.2µM
Reverse Primer	(10µM)	0.4µl	0.2µM
Mg-free buffer	(10x)	2.0µl	1x
MgCl2	(50mM)	0.8µl	2mM
dNTPs	(6.25mM each)	0.4µl	125µM each
SYBR Green I	(10x)	1.0µl	.5x
DyNAzyme II	(2U/µl)	0.2µl	20U/ml
ddH_2O		12.8µl	
Total Volume		20 µl	

PCR primers were designed such that the forward primer hybridized within the S-epitope tag nucleotide sequence at the 5' end of the reading frame and the reverse primer hybridized within the *mudrA* coding sequence (see Figure 1). The primer set generated a 308bp amplicon: forward, 5'-GAG-ACC-GCC-GCG-GCC-AAG-T-3,' and reverse, 5'- TCA-TCC-AAT-ACA-TCT-GAT-ACT-AT-3'.

Following reaction assembly, plates were transferred to the DNA Engine Opticon 2 real-time detection system (MJ Research). Amplification was performed according to the program listed in Table 2. The primer annealing temperature was experimentally determined using the temperature gradient feature of the DNA Engine Opticon 2 system (data not shown). The temperature at which fluorescence was

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Table 2. Quantitative PCR Program for Transgene Template Amplification

1. 94°C, 3min
2. 94°C, 15sec
3. 63°C, 20sec
4. 72°C, 30sec
5. 85°C, 1sec
6. Plate read
7. Go to step 2, 42 more times
8. 72°C, 10min
9. Melting curve analysis: 65°C to 98°C, 0.2°C/read, 1sec hold
10. 72°C, 10min
11. 10°C, Forever

END

measured during each cycle was set below the T_m of the specific amplicon.[11]

A dilution series of a plasmid harboring the transgene was mixed with 20ng maize genomic DNA that lacked the transgene. This method controlled for PCR-inhibiting compounds that were residual in the maize genomic DNA preparations. Analysis of these samples was used to generate a standard curve of the log of template quantity versus C(t) cycle.

Data Collection and Analysis

The cycle threshold C(t) line was set using the signal/noise ratio option in the Opticon Monitor™ software (MJ Research). The setting used was 10 standard deviations above the mean fluorescence value of the first 3–7 cycles for each sample or standard. This threshold was automatically applied, which ensured consistent analysis of individual samples.

Opticon Monitor software automatically constructed the linear standard curve and determined the transgene copy numbers in the maize samples by interpolation. The maize genome size was estimated to be 2500Mb, and template equivalents of plasmid DNA standards were calculated relative to this genome complexity. All transgenic plants in this study arose from the same parental line and, as such, are expected to have equivalent genome complexities. All samples were analyzed in triplicate within the same plate.

Results

Quantification of Transgene Amounts Using Real-Time qPCR

Real-time qPCR with SGI was performed to investigate *mudrA* transgene copy number in 10 transgenic lines containing a *mudrA* construct. All of these lines were transformed with the same plasmid DNA preparation by the Plant Transformation Facility at Iowa State University (http://www.agron.iastate.edu/ptf/Web/mainframe.html). A schematic of the transgene construct is shown in Figure 1.

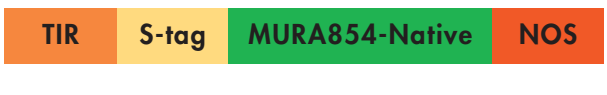

Figure 1. Schematic representation of the mudrA transgene (not to scale). The transgene is transcribed from the native promoter (TIR), and is engineered to incorporate a unique S-protein tag (S-tag) to serve in various downstream applications. The transcription terminator (NOS) is derived from the nopaline synthase gene.

Table 3. Mean and standard deviation of C(t) values for three replicate measurements of one sample from each of ten maize transgenic lines.

Sample	Mean C(t) ± Standard Deviation
1.	31.22 ± 0.12
2.	33.10 ± 0.30
3.	33.51 ± 0.26
4.	31.60 ± 0.35
5.	33.72 ± 0.41
6.	31.28 ± 0.20
7.	35.60 ± 0.36
8.	31.85 ± 0.26
9.	34.63 ± 0.43
10.	30.69 ± 0.19

The real-time assay was precise and reproducible, as indicated by the low variation in C(t) values across replicates of the same sample template. Table 3 lists the mean ± standard deviation of C(t) values for three replicates of one sample from each transgenic line. The standard deviation ranged from 0.12 to 0.43.

The standard curve generated from duplicate samples of a dilution series of the plasmid harboring the transgene is presented in Figure 2. The concentration of the plasmid ranged from 1,200 initial copies to 1.2 X 10^6 initial copies. For each reaction, plasmid DNA was mixed with 730 genomic equivalents of maize DNA.

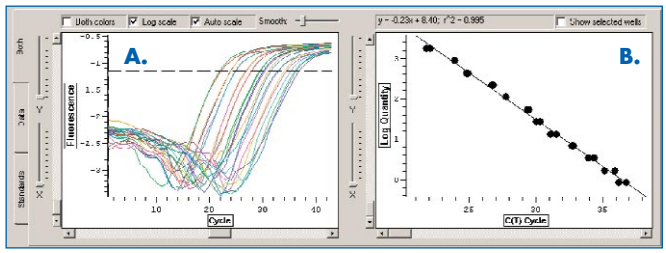

Figure 2. Standard curve for the transgene amplicon, generated from a plasmid serial dilution. A. Quantitation curves for the dilution series, which ranged from 1,200 copies to 1.2 X 10^6 copies of initial template per reaction. Duplicate reactions were performed at each starting concentration. B. Standard curve plot of log copy number vs. C(t) value. Square of correlation coefficient, r^2= 0.995.

Average transgene copy number per genomic equivalent for each maize line are presented in Table 4. The transgene was present at various levels in the different samples, with a range of approximately 10-fold, from 2 to 21 copies/genome.

Table 4. Average number of transgene insertions in each maize transgenic line.

Sample	Transgene copies/genome (mean±STD)
1.	16 ± 1
2.	6 ± 1
3.	5 ± 1
4.	13 ± 2
5.	5 ± 1
6.	15 ± 1
7.	2 ± 1
8.	11 ± 2
9.	3 ± 1
10.	21 ± 2

Melting Curve Analysis

To assess reaction specificity and to verify product identity, melting curve analysis was performed following amplification. Figure 3 shows the melting curves for triplicate samples of the transgene from one representative maize line. The negative first derivative calculation shows a single peak at approximately 91.5°C, which represents the melting point (T_m) of the amplicon. The presence of a single peak at the same T_m seen in the standards (standard melting curve not shown) supports specific amplification of the transgene.

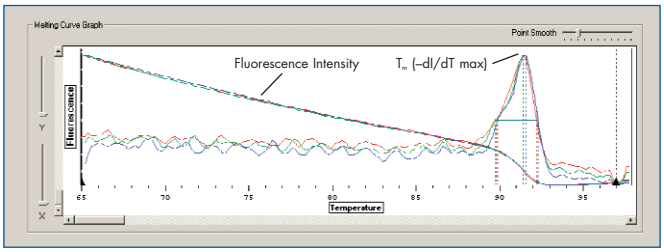

Figure 3. Melting curves depicting the fluorescence intensity and negative first derivative plots for the amplified transgene construct from triplicate measurements of a single sample from a maize plant line.

Discussion

In this study, we developed a real-time qPCR assay to determine the copy number of the *mudrA* transgene in several transgenic maize lines. Results showed that the transgene level varied from 2 to 21 copies per genome among the 10 independent lines. This level of variation is expected, because the use of particle bombardment for transformation of cultured maize embryos is likely to produce multi-copy arrays. In fact, complex transgene arrays containing multiple copies of the transgene plasmid have been previously reported after maize particle bombardment.[5]

Advantages of real-time qPCR for Transgene Quantification

Real-time qPCR can be used to detect and measure the absolute copy number of a transgene in the original lines derived for each transgene construct. Currently, a major limitation in maize experiments is finding individual carriers after completing complex crossing schemes. Such schemes can involve hundreds of plants and varying copy numbers of multiple types of transgene constructs. In some experiments, two discrete loci containing one or more transgenes might be produced. In such instances, the protocol we have developed would permit quantification of the copy number in lines inheriting one or both loci.

The specificity and high signal-to-noise ratio characteristic of real-time qPCR provides several advantages for quantification of transgene copies compared to a technique such as the Southern blot.[9, 10] Compared to real-time qPCR, the DNA blot hybridization technique is costly in terms of reagents, labor, and time: each lane requires 2µg of DNA for radioactive detection or 10µg for fluorescence detection. Consequently, a large tissue sample is required, and so experiments must be postponed until plants have regenerated enough tissue for surveying transgene copy number. Additional complications in determining copy number may exist if rearranged copies are present.

The unique forward primer based on the S-epitope tag sequence and the reverse primer complementary to the *mudrA* gene used in our experiments were designed to hybridize specifically within the transgene coding sequence. This approach should provide superior detection sensitivity by helping to avoid the potential complication caused by the two primers hybridizing to homologous *mudrA* targets present in the maize genome.[12]

The first step in characterizing transformed plants is verifying that the transgene has stably integrated into the chromosome. The second task is to estimate how many copies of the transgene are present and are contributing to the level of trangene expression. Given the relatively long life cycle of maize and the complexity of the genetic analysis, the high degree of accuracy and robustness of the real-time qPCR technique combined with the advantages of high-throughput and reduced assay costs, make this the preferred method over traditional blotting techniques.

Research supported in part by a grant to Virginia Wolbot from the National Institutes of Health.

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