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Chapter 11

Determination of Transgene Copy Number by Real-Time Quantitative PCR

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Summary

Efficient methods to characterize transgenic plants are important to quickly understand the state of the transformant. Determining transgene copy number is an important step in transformant characterization and can differentiate between complex and simple transformation events. This knowledge can be extremely useful when determining what future experiments and uses the transgenic lines can be utilized for. The method described here uses real-time quantitative PCR to determine the transgene copy number present in the genome of the transformant. Specifically, this method measures the relative transgene copy number by comparing it with an endogenous gene with a known copy number. This method is a quick alternative to the Southern blot, a method that is commonly used to determine gene copy number, and is effective when screening large numbers of transformants.

Keywords: Transgenic plants, Quantitative PCR, Gene copy number

1. Introduction

Transgenic plants are created for a number of reasons that include both basic research and industrial commercialization applications. The number of times the transgene inserts into the genome (copy number) directly relates to the gene's expression level, insert stability, and inheritance. If commercialization of the end product is the objective, the transgenic plants need to be characterized correctly because complex events that contain multiple transgenes are more difficult to commercialize. Complex transformation events that contain truncated inserts and inverted repeats commonly occur *(1)*, as opposed to simple insertions containing one copy of the transgene. Determination of transgene copy number to distinguish between complex and simple events, zygosity,

and correct characterization of the transgene can save time and resources in future generations.

Southern blotting has traditionally been used to characterize genomic DNA and determine gene copy number. In this method, genomic DNA is digested with restriction enzymes and hybridized with a DNA probe. The resulting band pattern provides information about the number of transgene copies. However, this method is time consuming and is difficult to perform on a large scale, which makes it inefficient to use when analyzing large numbers of transgenic plants. In addition, complex events that contain multiple transgenes in concatamers or other complex arrangements may result in Southern blots that are difficult to interpret. An alternative method that is both faster and easier to perform on large numbers of plants is real-time quantitative PCR (RT-qPCR). Where a typical Southern blot protocol takes days to perform after the DNA has been extracted, RT-qPCR reactions take hours to run and yield data in digital form for analysis. However, there may be cases where RT-qPCR is not feasible, or information about the arrangement of the transgenes in the genome is required, and in these cases nonradioactive Southern blot protocols can be used to compliment RT-qPCR (*see* the chapter "Nonradioactive Genomic DNA Blots for Detection of Low Abundant Sequences in Transgenic Maize").

PCR amplifies DNA in an exponential fashion. The amount of product is eventually limited in each reaction by reagents such as primers and dNTPs, resulting in a plateau phase in which the amount of product produced is limited. Each reaction then produces different amounts of product, independent of starting template amounts. Thus, the amount of PCR product is related to the template copy number during the logarithmic phase of amplification. It is only during this phase of amplification that it is possible to extrapolate back to the amount of starting template.

In real-time PCR, detection of product is observed during thermal cycling by monitoring fluorescence in the PCR reaction. There are two types of detection chemistries available for real-time PCR, gene-specific fluorescent-labeled probes, and nonspecific dsDNA binding dyes. The gene-specific probes include TaqMan® probes or molecular beacons and are a more sensitive method to quantify gene copy number. The use of gene-specific labeled probes requires purchase of the fluorescent probe in addition to the primers, thereby increasing the cost of the experiment. Rather than utilizing a gene-specific probe, we employed a fluorescent nonspecific dsDNA binding dye (SYBR green), which is incorporated into newly amplified DNA. Both specific and nonspecific PCR products will bind SYBR green, but a simple dissociation curve analysis at the end of the PCR run will determine whether nonspecific products are present (*see* **Note 1**).

A threshold cycle (C_t) for each amplification is assigned by the RT-qPCR software according to the cycle in which the fluorescence signal is significantly greater than the background noise. Samples with more starting template (i.e., higher copy number) will generate enough amplicons to cross the threshold level first, and therefore have a lower C_t value. The C_t value is therefore related to the copy number of the template DNA.

The PCR efficiency in part determines the number of cycles required to amplify a given target. PCR efficiency corresponds to the proportion of template molecules that are doubled every cycle. Efficiency is determined using a standard curve consisting of a dilution series for each primer pair used. The standard curves for two templates to be compared should be parallel with an R^2 close to 1.

Although it is possible to derive the starting amount of template present in a PCR reaction knowing the amount of product produced by relating it to a standard curve (absolute quantitation), it is often easier to compare the copy number of a gene of interest to the copy number of a gene with a known copy number (relative quantitation) *(2)*. The 2^{-Ct} method can be used to calculate the relative differences between the control and experimental samples, and requires careful selection of an endogenous control gene of known copy number *(3)*. Bubner et al. *(4)* used a 2^{-Ct} approach *(3)*, which compared a calibrator line (a transgenic line shown to have one copy by Southern blot analysis) with the experimental lines, and found that distinguishing between 1- and 2-copy lines was the limit of RT-PCR. However, Ingham et al. *(5)* found the correlation between Taqman assay and Southern blots to be much better, presumably due to the higher accuracy gained by using a gene-specific probe.

The method described here in detail is the relative quantitation method that determines gene copy number by comparing the transgene amplification to that of an endogenous control gene that has a known gene copy number. This method meets the needs of transgenic plant producers as it is easy to perform and can be used to analyze a large number of transformants.

2. Materials

1. MX3000P real-time PCR system (Stratagene, La Jolla, CA).
2. Real-time PCR reagents Brilliant® SYBR® Green master mix (Stratagene, La Jolla, CA).
3. Optically clear PCR tubes 200 μL (Stratagene, La Jolla, CA).

4. DNA primers for transgene and endogenous control gene (0.5 µM final concentration).

5. Genomic DNA in tenfold dilution series for standard curve determination.

3. Methods

1. Set up two sets PCR reactions, each containing a dilution series (*see* **Note 2**) of the genomic DNA as the template. Prepare one set of reactions to amplify the gene of interest and the other to amplify the known copy number control sequence (*see* **Note 3**). Each reaction should be run in triplicate. PCR reactions that do not contain genomic DNA (no template control) or DNA polymerase should be included as negative controls.

2. The quantitative real-time PCR analyses were performed using the MX3000P real-time PCR system (Stratagene, La Jolla, CA).

3. PCR reactions contained 12 µL of Brilliant® SYBR® Green master mix (Stratagene, La Jolla, CA), 12 µL of ddH$_2$O, 1 µL of each primer (0.5 µM final concentration), and 1 µL of DNA (DNA amount as determined by the dilution series).

4. The cycling parameters were as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s, 55°C for 60 s, and 72°C for 30 s.

5. Verify that the PCR product of each reaction is of the expected size by ethidium bromide agarose gel electrophoresis and/or has a single peak in the dissociation curve analysis. Primer-dimers and spurious amplification products will interfere with the procedure (*see* **Note 1**).

6. Calculate the PCR efficiency for the gene of interest and the copy number control gene as follows: Calculate the slope of the line fit to a plot of Ct vs. amount of template. The PCR efficiency is calculated with the following equation:

Efficiency = $10^{(-1/\text{slope})} - 1$.

7. PCR efficiencies should be greater than 90% (*see* **Note 4**).

8. To calculate the ratio of the copy number of the gene of interest to the reference gene, select a template concentration in which both the gene of interest and the copy number control gene amplified well and apply the following equation:

$$\text{Ratio} = \frac{1 + \text{Efficiency}_{\text{gene of interest}}^{(\text{Ct gene of interest})}}{1 + \text{Efficiency}_{\text{control}}^{(\text{Ct control})}}.$$

4. Notes

1. Real-time PCR products can be measured by utilizing sequence-specific fluorescent probes or by nonspecific dsDNA binding dyes. In this method, SYBR green is used as the non-specific dye, which has an excitation wavelength of 497 nm and an emission wavelength of 520 nm. Because the SYBR green is nonspecific, it will also bind to primer-dimers, so it is necessary to optimize the PCR reaction to reduce or eliminate the primer-dimers that will interfere with the reaction. A dissociation curve analysis (or melting curve analysis) can be performed to determine whether primer-dimers exist in your reaction. Multiple peaks in the melting curve indicate the presence of nonspecific products and/or primer-dimers, and reaction conditions should be optimized to minimize this.

2. Prepare a twofold serial dilution series with at least five points. We suggest a range between 1 and 100 ng of genomic DNA.

3. The endogenous control gene should be a gene that is known to be present in a single copy. We have used globulin-1 of maize as our endogenous control gene *[(6)*, GenBank accession M24845). The *glb-1* control gene primer sequences are forward 5′-CACTGTGGAACACGACAAAGTCTG-3′ and reverse 5′-CTCACCATGCTGTAGTGTCACTGTGAT-3′. Other examples of single copy genes are *Adh*1 (accession number X04050), *hmg*a (accession number AJ131373), and *i*V*r*1 (accession number U16123) *(7)*.

4. If the PCR efficiency of the copy number control gene is less than 90%, then optimize the PCR reaction by changing reaction conditions such as annealing temperature, magnesium concentration, or the elongation and acquisition times. Designing new primers may be necessary if acceptable efficiency cannot be obtained by changing reaction conditions. Lastly, a different copy number control gene may be selected if a PCR efficiency of >90% is not achieved.

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