Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 1829-1835
Received: 14-01-2018
Accepted: 18-02-2018

Ajit Tiwari
Dept. of Plant Moleculat Biology and Genetic Engineering, Narendra Deva University of Agriculture and Technology, Faizabad, Utter Pradesh, India

KN Singh
Dept. of Plant Moleculat Biology and Genetic Engineering, Narendra Deva University of Agriculture and Technology, Faizabad, Utter Pradesh, India

Correspondence
Ajit Tiwari
Deptt. of Plant Moleculat Biology and Genetic Engineering, Narendra Deva University of Agriculture and Technology, Faizabad, Utter Pradesh, India

Transgene copy number

Ajit Tiwari and KN Singh

Abstract
It is 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.

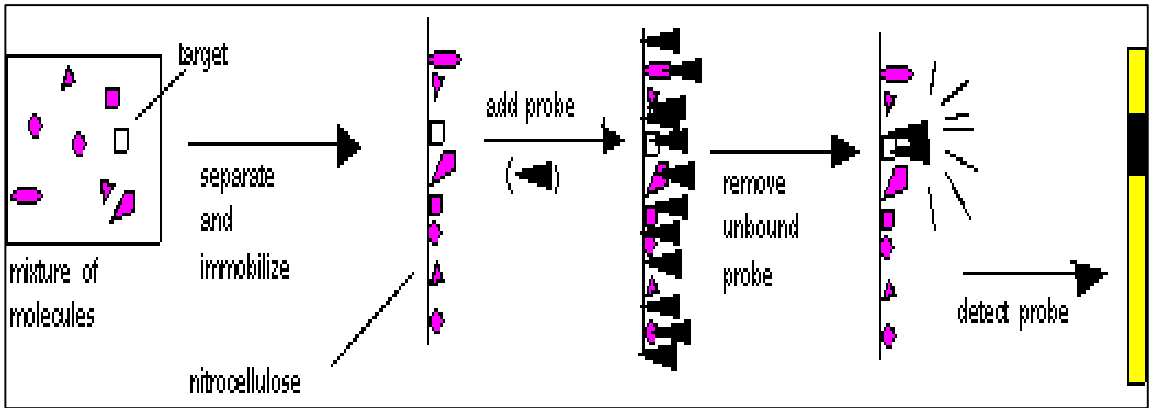
Keywords: Transgene, efficient methods, characterize transgenic, Determining transgene

Introduction
Genetic transformation is a widely employed tool in both basic research and commercial plant breeding programs. Transformation is the introduction of any DNA molecule into any living cell. Event indicates a unique transformation of plant by insertion of a particular transgenes. Application of transformation requires that transgenes be stably integrated and expressed in the plant genome. When trangenics plants are developed, it is essential to determine which plants contain the transgene and in how many copies. Transgenic plant must be characterized at the molecular level because the new DNA is randomly inserted into the plant genome. While multiple copies of the transgene are useful for over expression experiments, multiple copies of DNA integrated into one or more chromosomal locations affects the level and stability of gene expression sometimes resulting in transgene silencing. Most transgenic plants obtained via direct DNA delivery method, such as particle bombardment, electroporation, polyethylene glycol(PEG), etc., may consist of complex patterns of transgene integrations, i.e., multiple copies and or partial insertion of the integrated DNA. Less than 20% of the transgenic events generated using direct DNA delivery is play low copy integration (three copies or less). Never the less, estimating transgene copy no. is critical to the characterization and selection of candidate transgenic plants. No transformation method can completely control the no. of transgene integrations into the plant genome. Therefore, transgenic events should be screened as early as possible in the transformation process to identify multiple and low copy no. transformants. Such screening can be very difficult to handle, especially when the no. of independent transformed events is large.

Southern Analysis
Southern analysis utilizes a blot of digested genomic plant DNA hybridized with a labeled DNA probe corresponding to the transgene to produce an instructive band pattern. This method is a highly reliable procedure to estimate transgene copy no. However, Southern blot analysis is costly in terms of reagents, labor and time, and also requires a considerable amount of DNA fresh or frozen material and requires hazardous radioisotopes in some cases. Other methods that can be used for copy no. estimation include comparative genomic hybridization, fluorescence in situ hybridization, multiplex amplification probe hybridization, and microarray. Unfortunately, all of these methods share some of the limitations as Southern blot analysis.

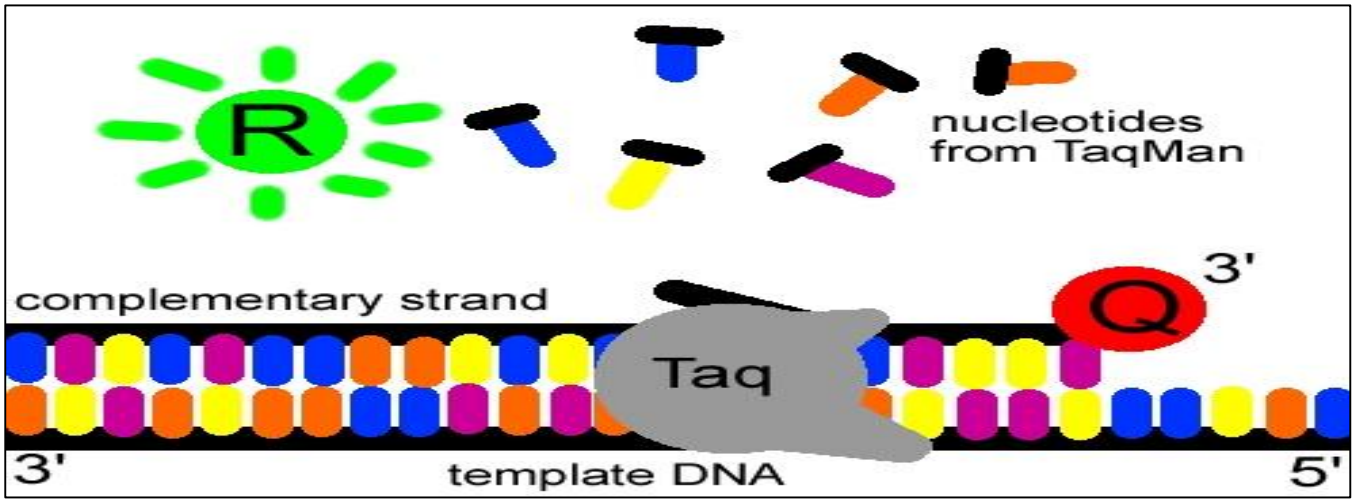
Steps
The mixture of molecules is separated.
The molecules are immobilized on a matrix.
The probe is added to the matrix to bind to the molecules.
Any unbound probes are then removed.

The place where the probe is connected corresponds to the location of the immobilized target molecule.



Polymerase chain reaction (PCR): PCR is one of the most sensitive techniques for detecting the integrated gene in the transgenic plant genome, and thus required for analysis. Quantitative real time PCR (qRT-PCR) technology relies on the ability to progressively monitor fluroscence emitted from nonspecific double stranded DNA binding dyes (SYBER Green I) or flurophore –labeled specific probes (TaqMan) that hybridize with target sequences during the exponential phase of the PCR reaction. Quantitative RT-PCR techniques have been reported for determining transgene copy no. in transformed plants.

The TaqMan® probe binds to the target DNA, and the primer also binds. Because the primer is bound, *Taq* polymerase can now create a complementary strand. Once the TaqMan® probe has bound to its specific piece of the template DNA after denaturation (high temperature) and the reaction cools, the primers anneal to the DNA. *Taq* polymerase then adds nucleotides and removes the Taqman® probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to give off its emit its energy. This is then quantified using a computer. The more times the denaturing and annealing takes place, the more opportunities there are for the Taqman® probe to bind and, in turn, the more emitted light is detected.



Transgene plants can be commercialized with improved agronomic characteristics or can be used as bioreactors or materials for research. When new transgenic plants are obtained, an early essential step is their molecular characterization. Many primary transformants must be analyzed because the new DNA is randomly inserted into the plant genome, often leading to a generation of plants with multiple transgene copies into integrated one or more chromosomal locations. The no. of copies of the exogenous gene inserted into the genome is one of the reasons for transgenic silence. Usually, plants with 1 or 2 integrations events yield high level expression of the exogenous gene, whereas lower and/or unstable transgene expression and transgene silencing has been associated with high no. of copies.

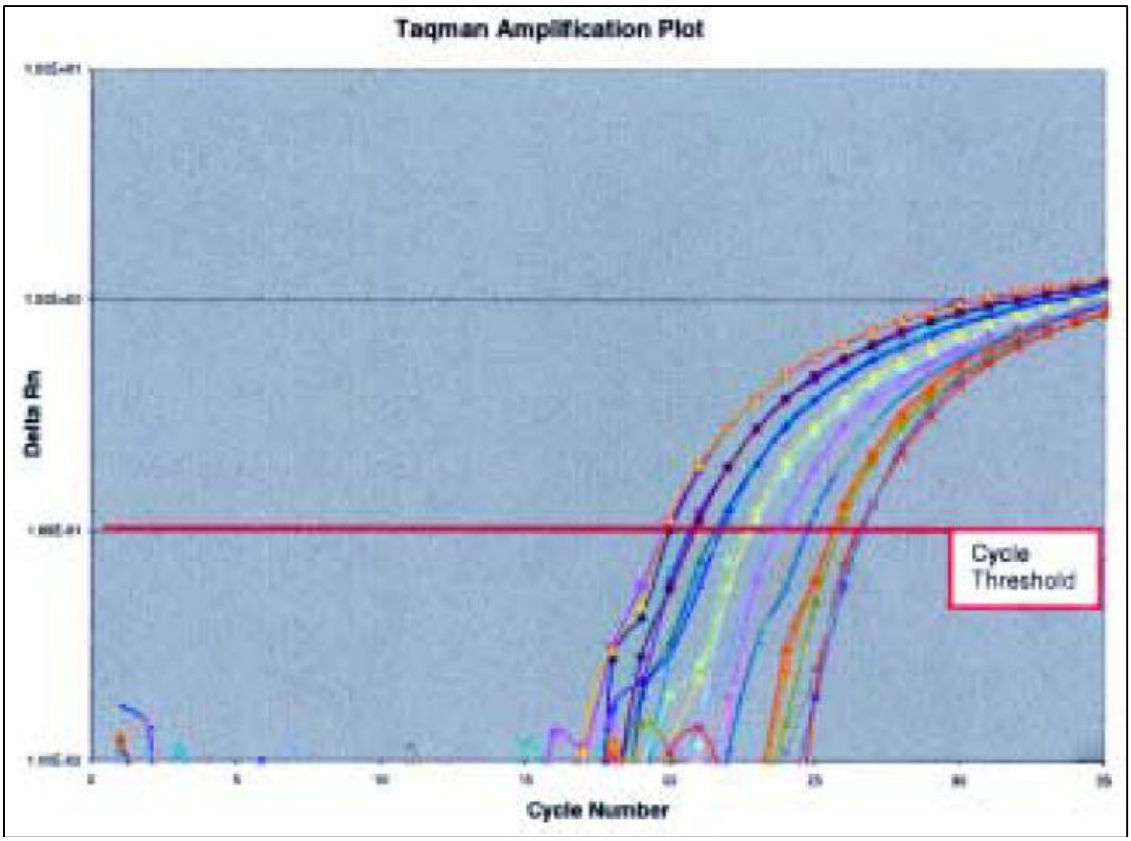
Researchers have developed a more sensitive and accurate real time flurorescent quantitative PCR technique for determining no. of transgenes copies in transformed plants, such as transgenic tomato and maize called TaqMan quantitative real–time PCR. This technique offers major advantages manipulations (greatly reducing the risk of carryover contamination), use of small amounts of starting material, and high throughput capacity.

Quantitative real-time PCR assay for determining transgene copy number in transformed plants

The development of transgenic events can be limited by many factors. These include expression levels, insert stability and inheritance, and the identification of simple insertion events. All of the factors can be related to the copy number of the transgene. Traditionally, copy number has been determined by laborious blotting techniques. Researchers have developed an alternative approach that utilizes the fluorogenic 5′nuclease (TaqMan) assay to quantitatively determine transgene copy level in plants. Using this assay, hundreds of samples can be analyzed per day in contrast to the low throughput

encountered with traditional methods. To develop the TaqMan copy number assay, they chose to utilize highly efficient Agrobacterium-mediated transformation system of maize. This transformation procedure generates predominantly low copy number insertion events, which simplified assay development. They have also successful applied this assay to other crops and transformation systems. The TaqMan assay is a real-time PCR detection technique in which the accumulation of PCR product is monitored directly during the progress of the reaction. Degradation of target-specific probe molecules by the 5′to 3′exonuclease activity of Taq DNA polymerase during each cycle of amplification produces an accumulation of fluorescence. Increased levels of fluorescence are directly related to the accumulation of PCR product and are detected during each cycle of amplification through the use of specialized instrumentation (ABI PRISM7700; Applied Biosystems, Foster City, CA, USA). Cycle thresholds (Ct) are assigned automatically to each sample according to the cycle at which the fluorescence exceeds a specific level above background (Figure 1). Samples with higher levels of template at the beginning of the reaction will amplify to detectable levels more quickly and yield a lower Ct. Because threshold values are determined during the logarithmic phase of amplification, PCR reagents are nonlimiting, and reactions will be most efficient. Therefore, the use of real-time (kinetic) detection techniques both simplifies and increases the accuracy of quantitative PCR assays.

In the assay described here, they use duplexed TaqMan reactions to accurately quantitate the level of a transgene relative to an endogenous calibrator gene. This relative quantitation approach provides a simplified, accurate alternative to using standard curves and absolute quantitation. Because the endogenous calibrator sequence remains constant relative to total genomic DNA, any variation in the relative level of the transgene to the endogenous gene is indicative of a difference in copy number. As shown in Figure 1, 2-fold differences in copy number are easily distinguished using TaqMan technology.



The TaqMan copy number assay allows the screening of hundreds of plants with greater accuracy within hours as comparedto weeks using conventional methods. This allows greater numbers of independent transformation events to be screened for desired traits in shorter time and at significant cost savings.

Method

Transgenic maize plants from the inbred line A188 were obtained via *Agrobacterium*-mediated transformation. The selectable marker phosphomannose isomerase (*pmi*) under the control of the *Zea mays* ubiquitin promoter was used.

DNA Preparation from Plant Samples

Genomic DNA for TaqMan copy number assays was isolated from maize leaves of transgenic and non transgenic plants. Genomic DNA was extracted using the Pure gene Genomic DNA Extraction kit (Gentra Systems, Minneapolis, MN USA). The dried DNA pellet was resuspended in 500 micro L TE.

Primers and Probes

For the endogenous control, primers and probes were designed specific to the *Zea mays* alcohol dehydrogenase (*adh*) gene. Probes were labeled at their 5'end with a reporter fluorophore [tetrachloro-6- carboxyfluorescein (TET) for *adh* andfluorescein (FAM) for *pmi*] and at the
3′end with the quencher fluorophore tetramethylrhodamine (TAMRA).

Table 1: Description of primers and probes

Target Gene	PCR Product Size (bp)	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Probe (5′→3′)
adh	71	GAACGTGTGT	TCCAGCAATCC	TGCAGCCTAACC
		TGGGTTTGCAT	TTGCACCTT	ATGCGCAGGGTA
pmi	60	CCGGGTGAA	GCCGTGGCCTT	TGCCGCCAACGA
		TCAGCGTTT	TGACAGT	ATCACCGG

Calculation of gene copy number

During the TaqMan reaction, the software accompanying the ABI PRISM 7700 instrument detects the accumulation of PCR product by the accumulation of fluorescence. Normalized fluorescence relative to established baseline levels (ΔRn) is plotted versus cycle number. A Ct value is obtained by drawing an arbitrary cutoff through the reactions so that the line passes through the log phase of each reaction. The sequence detection system software with the ABI PRISM 7700 instrument provides the cycle number at which the accumulation of fluorescence (PCR product) of a particular reaction crosses the threshold (Ct). The FAM Ct (*pmi*) value is compared to the TET Ct (*adh*) value to normalize the FAM Ct value of each reaction to the level of total nucleic acids present to yield ΔCt

[ΔCt = Ct(FAM) - Ct(TET)].

By comparing the ΔCt value of the unknown samples to the ΔCt of a known control, ΔΔCt is obtained

[ΔΔCt = ΔCt(Unknown)- ΔCt(Known)].

Copy number can then be calculated using the ΔΔCt value using the equation:
Copy Number = 2(**ΔΔ**Ct).

Southern Blot Hybridization

DNA (5–10 micro g) was digested with *Eco*RV subsequent hybridization with a *pmi* specific probe allows the identification of the border fragment between the insert and the plant DNA and provides an estimate of transgene copy number and number of insertion loci.

DNA fragments were separated on a 0.8% agarose gel and blotted to positively charged nylon membrane following standard procedures (15). A *pmi*-specific restriction fragment was labeled with [32P]-dCTP by random priming using the ReadyPrimeII DNA Labeling Kit. Hybridization was carried out overnight at 65°C in hybridization buffer containing approximately 100 000 cpm of labeled probe. The first wash was carried out at 65°C with hybridization buffer for 15 min. The final wash was carried out at 65°C with hybridization buffer for 15 min. The membrane was exposed to Xray film at -80°C for two days.

The accuracy of the assay developed here can be assessed in two ways. First, by comparing results obtained from TaqMan assays to those from Southern blot analysis. A second approach to determining the level of accuracy of the TaqMan assay involves the comparison of results of replicate samples. The Ct difference between consecutive copy numbers diminishes as the copy number increases. Therefore, the most accurate differentiation is obtained between one and two copy events with discrimination capability diminishing as copy number increases.

Case Studies

Assessing copy number of MON 810 Integrations in commercial seed maize varieties by 5′ event-specific real-time PCR validated method coupled to 2_ΔΔCT analysis (Aguilera *et al*., 2008) [1].

The objective of the present study was to assess the applicability of the MON 810 5′ event-specific methods validated by the Community Reference Laboratory for Genetically Modified Food and Feed that is commonly used for quantitative purposes. This 5′ event-specific/hmgtaxon gene real-time polymerase chain reaction (PCR) protocol coupled to 2_ΔΔCT analysis was the chosen approach to determine the MON 810 insert copy number per haploid genome across 26 genetically modified commercial maize varieties. Variety DK 513 containing one copy integration per haploid genome was used as calibrator in each assay. Complementary data from end-point real time PCRs that targeted specifically the MON 810 insert were also analyzed. Global results assessed and guaranteed the genetic intactness of the transgenic integration per haploid genome for 24 out of the 26 commercial varieties studied, which showed no significant differences between 2_ΔΔCT values respect to the calibrator value. Conversely, two varieties showed no intact transgenic insert in their genomes. This validated analytical method was suitable for MON 810 detection and quantification purposes.

Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. (Huixia *et al*., 2004) [4].

Particle bombardment and *Agrobacterium*-mediated transformation are two popular methods currently used for producing transgenic maize. *Agrobacterium*-mediated transformation is expected to produce transformants carrying fewer copies of the transgene and a more predictable pattern of integration. These putative advantages, however, tradeoff with transformation efficiency in maize when a standard binary vector transformation system is used. Using Southern, northern, real-time PCR, and real-time RT-PCR techniques, we compared transgene copy numbers and RNA expression levels in R1 and R2 generations of transgenic maize events

generated using the above two gene delivery methods. Our results demonstrated that the *Agrobacterium*-derived maize transformants have lower transgene copies, and higher and more stable gene expression than their bombardment-derived counterparts. In addition, we showed that more than 70% of transgenic events produced from *Agrobacterium* mediated transformation contained various lengths of the bacterial plasmid backbone DNA sequence, indicating that the *Agrobacterium*-mediated transformation was not as precise as previously perceived, using the current binary vector system.

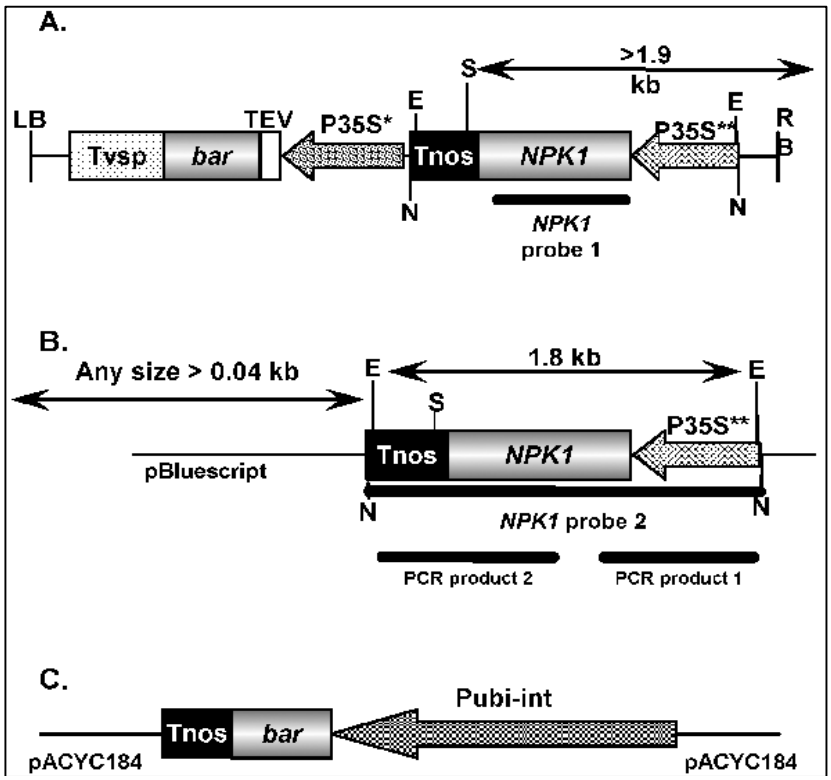
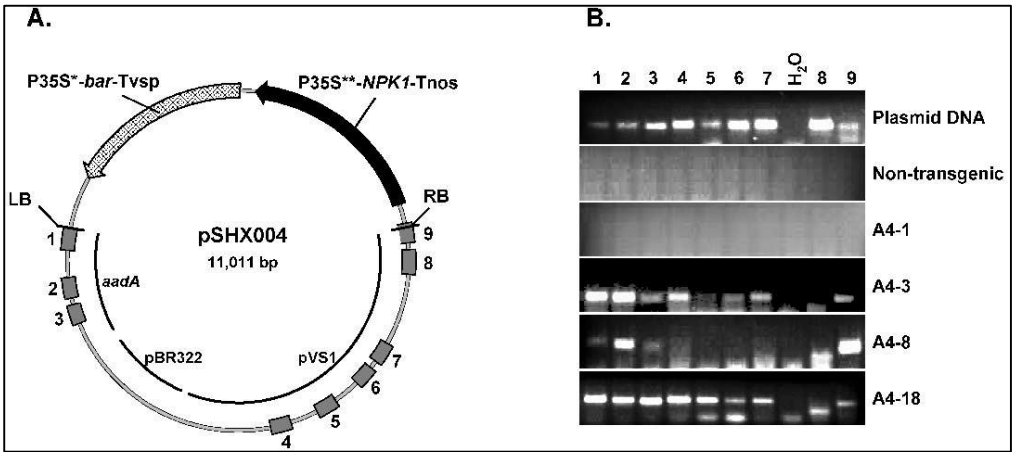


Fig 1: Constructs for maize transformation.

a. pSHX004, binary vector construct in *Agrobacterium strain* EHA101 for *Agrobacterium*- mediated transformation;
b. pSHX002 and
c. pBAR184, constructs used for co-bombardment transformation. LB, left border; RB, right border; *bar*, phospinothricin acetyl transferase gene; *NPK1*, *Nicotiana* protein kinase gene; P35S*, 2x CaMV 35S promoter; P35S**, a modified 35S promoter; Pubi-int; ubiquitin promoter with its intron TEV, tobacco etch virus 5' untranslated region, Tnos, nopaline synthase terminator Tvsp, soybean vegetative storage protein terminator, T35S, CaMV 35S terminator; E, *Eco*R I; N, *Not* I; S, *Stu* I; NPK1 probe 1: 0.8 kb coding region of *NPK1* gene; NPK1 probe 2: 1.8 kb NPK1 gene with its promoter and terminator.



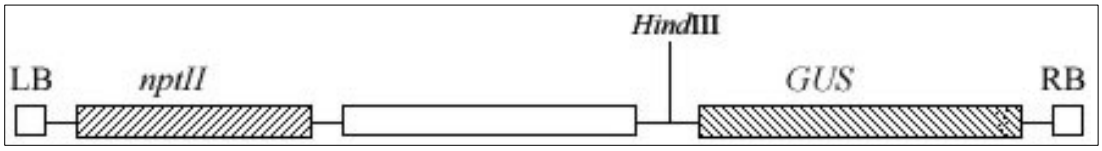
Integration of the *Agrobacterium* binary vector backbone DNA into transgenic maize events.

A. Map of construct used for *Agrobacterium*- mediated transformation.

B. PCR amplifications of backbone DNA fragments; lanes 1 to 9 correspond to the fragment 1 to 9 labeled on Figure 5A. P35S*-bar-Tvsp, bar gene with its double 35S promoter and *VSP* terminator; P35S**-NPK1-Tnos, NPK1 gene with its modified 35S promoter and *nos* terminator; LB, 25 bp direct repeat of left border; RB, 25 bp direct repeat of left border; 1 to 9: binary vector fragments outside the T-DNA region; coordinates of primers designed from these regions for PCR amplifications.

Estimating number of transgene copies in transgenic rapeseed by real time PCR assay with *HMG I/Y* as an endogenous reference gene (Haibo *et al.*, 2004) [5]

In transgenic plants, the number of transgene copies can greatly influence the level of expression and genetic stability of the target gene. Transgene copy numbers are estimated by Southern blot analysis, which is laborious and time-consuming, requires relatively large amounts of plant materials, and may involve hazardous radioisotopes. Here they report the development of a sensitive, convenient real-time PCR technique for estimating the number of transgene copies in transgenic rapeseed. This system uses TaqMan quantitative real-time PCR and comparison with a novel, confirmed single-copy endogenous reference gene, high-mobile-group protein I/Y (*HMG I/Y*), to determine the numbers of copies of exogenous β-glucuronidase (*GUS*) and neomycin phosphotransferase II (*nptII*) genes. The *GUS* and *nptII* copy numbers in primary transformants (T0) were calculated by comparing threshold cycle (*C*T) values of the *GUS* and *nptII* genes with those of the internal standard, *HMG I/Y*. This method is more convenient and accurate than Southern blotting because the number of copies of the exogenous gene could be directly deduced by comparing its *C*T value to that of the single-copy endogenous gene in each sample. Unlike other similar procedures of real-time PCR assay, this method does not require identical amplification efficiencies between the PCR systems for target gene and endogenous reference gene, which can avoid the bias that may result from slight variations in amplification efficiencies between PCR systems of the target and endogenous reference genes.



Graphic representation of the inserted tDNA region by *Agrobacterium*-mediated transformation.

TaqMan real-time PCR method for estimating the number of integrated copies of a transgene in transgenic rapeseed can be used as an alternative for Southern blotting, which is time-consuming and requires large amounts of fresh or frozen samples. Unlike other similar procedures, the flexibility of this method makes it appropriate in situations in which an accurate optimization of all reaction components is impossible or impractical (De Preter *et al*., 2002). A specific calibrator (sample containing 1 transgene copy) (Ingham *et al*., 2001; Song *et al*., 2002) is not needed because of the use of the single-copy endogenous gene. Moreover, identical amplification efficiencies between the PCR systems (Ingham *et al*., 2001) are not strictly required as long as the efficiencies are both adequate because the true efficiencies are used in the calculation expressed in equation Additionally, the quality of the information produced is higher than that generated by Southern blot analysis.

Using Real-Time PCR to Determine Transgene Copy Number in Wheat

In this study, quantitative real-time PCR was developed to determine transgene copy number in transgenic wheat. A conserved wheat housekeeping gene, *puroindoline-b*, was used as an internal control to calculate transgene copy number. Estimated copy number in transgenic lines using real-time quantitative PCR was correlated with actual copy number based on Southern blot analysis. *Puroindoline-b* is a monocot housekeeping gene that encodes the basic puroindoline b protein, which plays a direct role in affecting grain softness. Located on chromosome 5D, it exists as 2 copies in the hexaploid wheat genome.

Materials and Methods

Spring wheat cultivar "Centennials" was transformed by means of particle bombardment of immature embryos. Two different vectors were co-transformed into wheat-one carried the wheat streak mosaic virus coat protein gene (*WSMV-CP*), and the other carried the selectable marker *bar* gene. After bombardment, calli were selected for bialophos resistance. Herbicide-resistant calli were transferred to fresh media containing 3 mg/L bialophos and placed in growth chambers with a 16-h photoperiod until shoot development was observed. Shoots were transferred and grown on 1/2 Murashige and Skoog (MS) media containing 0.3% phytagel, 3% sucrose, and 3 mg/L bialophos. Shoots producing normal-appearing leaves and roots were considered to be putatively transformed.

Genomic DNA extraction for real-time PCR

Approximately 100 mg of seedling leaf tissue from transgenic and nontransgenic plants was ground to a fine powder in liquid nitrogen. DNA was isolated by using a DNA easy Plant Mini kit according to the manufacturer's instructions.

Southern Blot Analysis

DNA was extracted from wheat leaves. DNA was digested with appropriate restriction endonucleases and separated overnight by means of 0.9% agarose gel electrophoresis with a constant 30 V. Separated DNA fragments were washed in alkaline buffer (0.4 M sodium hydroxide, 1 M sodium chloride) for 30 min to denature into single-stranded molecules and transferred onto a charged nylon membrane following standard alkaline transfer procedures. A 1.2-kb fragment containing the *WSMV-CP* sequence was used as a probe for Southern hybridization. The probe was labeled for chemiluminescent detection by using a Roche labeling kit according to the manufacturer's instructions. Southern hybridization was carried out by using a DIG High Prime DNA Labeling and Detection Kit II following the manufacturer's instructions, with some modifications:

Comparison of gene copy numbers as estimated by real-time PCR and Southern blot analyses

The copy number of 566B T1 *WSMV-CP* transgenic plants was first determined by means of Southern blots analysis, in which wheat genomic DNA was digested by *Bam*HI. *Bam*HI has only one recognition site in the transformation vector outside of the *WSMV-CP* gene. After hybridization with a probe containing *WSMV-CP*, *Bam*HI identified the border fragment between the insert and the plant DNA. An estimate of transgene insertion copy number can be determined by numbers of hybridization fragments. An assumed insertion

fragment of transgene cassette and the probe used for genomic DNA analyses are shown. Southern analysis indicated that all 566B T1 plants tested had multiple transgene copies, ranging from 3-6. All transgenic plants tested with Southern blot were used for real-time PCR analysis. To determine copy number of *WSMV-CP* in DNA samples, TaqMan real-time PCR for *puroindoline-b* (used as an internal control) and the target *WSMVCP* gene were done in the same tube. Calculation of transgene copy number was as described above. Estimation of transgene copy number by real-time PCR was compared with results from Southern blot analysis. Of 10 transgenic plants, 9 produced comparable results with both methods. In line 566B1, 16 copies were estimated by real-time PCR, but only 6 by Southern blot analysis. This discrepancy could be due to transgene insertions in this line that were tandemly inverted, yielding more than one hybridizing band of the same size with Southern blot analysis. Furthermore, depending on agarose gel electrophoresis resolution, bands of only slightly different sizes may not be detected. While speculative, these points may illustrate some of the disadvantages of Southern blot analysis in determining transgene copy number. In 566B5, 4 transgene copies were estimated by real-time analysis, but 3 or 4 were estimated by Southern blot analysis. This discrepancy may be due to poor agarose gel electrophoresis DNA band resolution.

Conclusion

The results obtained from qRT-PCR for quantifying transgene copy number were highly concordant with that using Southern blot analysis. In addition, the high sensitivity and efficiency of qRT-PCR allowed us to analyze more samples and quantify the transgene copy number more quickly and accurately.

References.

1. Aguilera M, Maddalena Q, Susana P, Gianni B, Anne M, Guy V. Assessing Copy Number of MON 810 Integrations in Commercial Seed Maize Varieties by 5 Event-Specific Real-Time PCR Validated Method Coupled to 2_ΔΔCT Analysis, 2008.
2. David J, Sandra B, Stephanie M, Geneviève H. Quantitative Real-Time PCR Assay for Determining Transgene Copy Number in Transformed Plants. Bio Techniques. 2001; 31:132-140.
3. Craig W, Gargano D, Scotti N, Nguyen TT, Lao NT, Kavanagh TA *et al*. 2005.
4. Huixia S, Bronwyn R, Steven A, Kan W. Assessment of transgenic maize events produced by particle bombardment or Agrobacterium-mediated transformation. Molecular Breeding. 2004; 13:201-208.
5. Haibo W, Aihu P, Litao Y, Chengei Z, Zhiliu, Dabing Z. Estimating number of transgene copies in transgenic rapeseed by Real-Time PCR Assay with HMG I/Y as an endogenous reference gene. Plant Molecular Biology Reporter. 2004; 22:289-300,