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GENETIC TRANSFORMATION AND HYBRIDIZATION

M. A. Schmidt · W. A. Parrott

Quantitative detection of transgenes in soybean [*Glycine max* (L.) Merrill] and peanut (*Arachis hypogaea* L.) by real-time polymerase chain reaction

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Abstract Quantitative real-time polymerase chain reaction (PCR) assays were designed that enabled the zygosity of transgenes in soybean [*Glycine max* (L.) Merrill] and peanut (*Arachis hypogaea* L.) to be determined. The two zygosity assays, based on TaqMan technology that uses a fluorogenic probe which hybridizes to a PCR target sequence flanked by primers, were both accurate and reproducible in the determination of the number of transgenes present in a cell line. In the first assay, in which TaqMan assays were performed on increasing amounts of a plasmid containing the transgene of interest, a linear relationship between the level of fluorescence and the template amount was produced. Using the resultant linear relationships as standard curves, we were able to determine the zygosity of both soybeans segregating for the *cry1Ac* transgene and that of a T_1 peanut segregating for the *hph* transgene. In the second assay, a relative determination of copy number (referred to as comparative Ct) was performed on transgenic soybeans by comparing the amplification efficiency of the transgene of interest to that of an endogenous gene in a multiplexed PCR reaction. Both methods proved to be sufficiently sensitive to differentiate between homozygotes and hemizygotes. These assays have numerous potential applications in plant genetic engineering and tissue culture, including the hastening of the identification of transgenic tissue, selecting transformation events with a low number of transgenes and the monitoring of the transmission of transgenes in subsequent crosses.

Keywords TaqMan · Transgenic progeny analysis · Zygosity

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M.A. Schmidt · W.A. Parrott (✉)
The University of Georgia,
Department of Crop and Soil Sciences,
3111 Plant Sciences, Athens, GA 30602-7272, USA
e-mail: wparrott@uga.edu
Fax: +1-706-5420560

Abbreviations *Bt*: *Bacillus thuringiensis* · Δ*Ct*: Normalized critical threshold · ΔΔ*Ct*: Comparative threshold value · Δ*Rn*: Normalized fluorescent value · *FAM*: 5-Carboxyfluoroscein · *TAMRA*: *N*,*N*,*N*′,*N*′-Tetramethyl-6-carboxyrhodamine

Introduction

The polymerase chain reaction (PCR) has proven to be tremendously useful in many scientific fields, including molecular biology, clinical diagnostics and forensics. Although PCR is able to detect the presence of a particular sequence of DNA, its main limitation is that the technique is qualitative rather than quantitative. Several approaches have been proposed to modify PCR to permit both reliable detection and quantification of the initial DNA target material (for review see Orlando et al. 1998). PCR-based assays for the quantification of DNA offer the distinct advantage over conventional copy number determination methods, namely Southern and dot blot hybridization, in that they use minimal amounts of starting material. Quantitative PCR methodologies have been employed to determine the load of infectious microbes and viruses (Desjardin et al. 1998; Higgins et al. 1998; Kimura et al. 1999; Nogva et al. 1999; Pusterla et al.1999), to monitor the copy number of genes involved in amplifications in cancerous cells (Bieche et al. 1998), to investigate the efficiency of gene transformation systems for human gene therapy (Becker et al. 1999) and to construct expression profiles of both endogenous (Gibson et al. 1996) and introduced genes (Studer et al. 1998; Fairman et al. 1999; Vaitilingom et al. 1999).

A fluorogenic real-time PCR technique, TaqMan, based on a 5′ nuclease assay first described by Holland et al (1991), is increasingly receiving attention as a way to quantitate gene copy number and expression. TaqMan technology uses a fluorescent dual-labeled DNA probe that hybridizes to a PCR target sequence flanked by PCR primers (Fig. 1). The probe is labeled at the 5′ end with a fluorescent molecule and at the 3′ end with a quencher

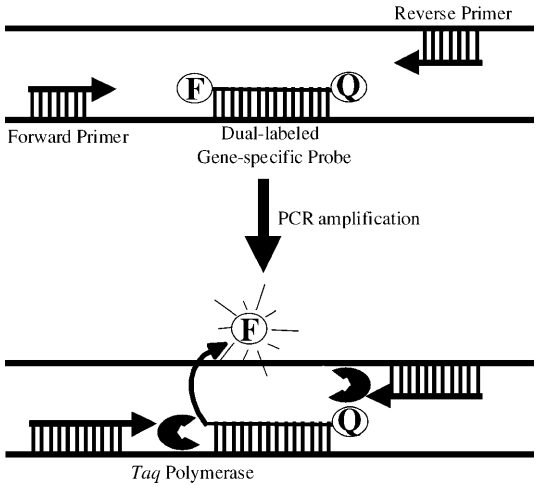

Fig. 1 Diagrammatic representation of how the 5′ exonuclease activity of *Taq* polymerase is utilized in real-time quantitative PCR. *F* denotes a fluorochrome, and *Q* denotes a quencher molecule. With each PCR cycle, a single fluorochrome is released which, separated from the quencher, begins to fluoresce. The fluorescent signal can be quantitated and, given the number of PCR amplifications that have occurred, used to calculate the amount of template DNA in the starting material

molecule. In an intact probe, the 5′ and 3′ labels are in close proximity, and the quencher silences the fluorescent signal that would otherwise be generated by the 5′ fluorescent label (Livak et al. 1995). During PCR amplification, the 5′ exonuclease activity of *Taq* polymerase degrades the internal probe, liberating the fluorescent dye molecule, which subsequently produces a fluorescent signal (Fig. 1). This fluorescent signal is proportional to the amount of PCR product generated which, in turn, is proportional to the quantity of initial DNA template in the sample.

Although TaqMan has been used successfully in numerous clinical applications it has been applied scantly to plant molecular biology. Some plant pathogens, such as *Ralstonia solanacea*, the causal agent of bacterial wilt (Weller et al. 2000), *Clavibacter michiganensis*, the potato ring rot bacterium (Schaad et al. 1999), the tobacco rattle *Tobravirus* (Mumford et al. 2000) and the potato mop top *Pomovirus* (Mumford et al. 2000) have been detected and quantitated by TaqMan. In addition, this technique has been used to determine the relative amount of transgenic plant products in certain foods (Vaitilingom et al. 1999).

Plant breeders introgressing transgenes need to be able to distinguish between homozygotes and hemizygotes in segregating progeny. Currently, progeny tests are the only accurate method available, thus adding months to the time needed to identify homozygous progeny. TaqMan offers a faster alternative to distinguish homozygotes from hemizygotes. In the study reported here, a quantitative real-time PCR assay was designed that would determine the zygosity of transgenes in both soybean [*Glycine max* (L.) Merrill] and peanut (*Arachis hypogaea* L.). Zygosity was determined by real-time PCR in two ways: (1) the absolute quantification of transgene copy number was determined by the generation of an external standard curve relating the level of fluorescence to template quantity, and (2) the relative determination of copy number was found by comparing the amplification of the transgene of interest to that of an endogenous gene in a multiplexed reaction. Both zygosity assays proved sufficiently sensitive to differentiate between homozygous and hemizygous transgenic lines.

Materials and methods

Transgenic plant material

The soybean lines used contain two transgenes: *Bacillus thuringiensis* (Bt) *cry1A(c)* and hygromycin B phosphotransferase (*hph*). The soybean plants were identified as being either non-transgenic, homozygous or hemizygous for the *cry1Ac* transgene by Southern blot analysis (Stewart et al. 1996) and by progeny segregation analysis. Likewise, the peanut transgenic line used segregated for *hph*. The number of integration sites in the peanut line had been determined previously by Southern blot analysis to be two copies (Magbanua et al. 2000).

DNA isolation and quantification

Genomic DNA was extracted from freeze-dried leaf tissue using the CTAB method (Doyle and Doyle 1990). DNA samples were measured on a TKO 100 mini-fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.) that has both fixed excitation and emission wavelengths of 365 nm and 450 nm, respectively. The DNA-binding dye bisbenzimide Hoechst 33258 (Calbiochem, Darmstadt, Germany) was used according to the manufacturer's instructions.

Oligonucleotide primers and probes

All oligonucleotides for TaqMan were designed by Primer Express software (Perkin Elmer Applied Biosystems, Foster City, Calif.). The internal oligonucleotide probes specific for *cry1Ac* and *hph* were labeled at the 5′ end with FAM, whereas the probe specific for the endogenous gene, lectin (*Le1*) (Vodkin et al. 1983) was 5′ end-labeled with the fluorescent dye VIC (Perkin Elmer). The 3′ ends of all probes were labeled with the quencher dye TAMRA and phosphate-blocked to prevent extension during PCR cycling. The upstream *cry1Ac* PCR primer corresponds to the region from base 498 to base 516 (sequence 5′ AGGCTGCCAACCTGCACTT 3′); the downstream *cry1Ac* PCR primer corresponds to the region from base 564 to base 547 (sequence 5′ TCAAAGCCCCACCGTTGT 3′); and the *cry1Ac* probe lies between bases 518 and 538 (sequence 5′-FAM-TCGGTGCTCCGCGATGTCTCC-TAMRA-3′). The upstream *hph* primer corresponds to the region from base 669 to base 690 (sequence 5′ CGAGAGCCTGACCTATTGCAT 3′); the downstream *hph* PCR primer corresponds to base 730 to base 711 (sequence 5′ CAGGCAGGTCTTGCAACGT 3′); and the probe lies between bases 691 and 712 (sequence 5′-FAM TCCCGCCGTGCACAGGGTGT-TAMRA-3′). The *Le1* gene was used as a low-copy number endogenous control for the soybean samples in the comparative Ct method. The upstream *Le1* PCR primer corresponds to the region from base 1664 to base 1683 (sequence 5′ TCCCGAGTGGGTGAGGATAG 3′); the downstream *Le1* primer corresponds to base 1729 to base 1710 (sequence 5′ TCATGCGATTCCCCAGGTAT 3′); and the *Le1* probe lies between bases 1687 and 1708 (sequence 5′-VIC-TCTCTGCTGCCACGGGACTCGA-TAMRA-3′). The bases are numbered for each

gene in correspondence to those in GenBank accession numbers U63372, E00777 and K00821 for *cry1Ac*, *hph* and *Le1*, respectively.

PCR conditions and fluorescence detection

Each 25-µl PCR reaction contained 200 µ*M* each of dATP, dCTP and dGTP, 400 µ*M* dUTP, 3.5 m*M* $MgCl_2$, 1X TaqMan buffer, 200 n*M* probe, 0.125 U AmpliTaq Gold DNA polymerase (Perkin Elmer) and 0.01 U AmpErase uracil *N*-glycosylase (Perkin Elmer). For the generation of a standard curve, all primer concentrations were 900 n*M*. In the comparative Ct method, optimization of primer concentrations consisted of performing multiplexed real-time PCR amplifications using combinations of both endogenous and transgene primers at varying concentrations: 200 n*M*, 400 n*M*, 500 n*M*, 600 n*M*, 800 n*M*, 900 n*M* and 1,000 n*M* (data not shown). The combination of optimum primer concentrations for a multiplexed PCR reaction is achieved when the resultant amplifications show a reduction in ΔRn value but have little effect on ΔCt value (recommended by Applied Biosystems, Foster City, Calif.). For the multiplexed reactions of *cry1Ac* and *Le1*, the primer concentrations were 600 n*M cry1Ac* (transgene) and 600 n*M Le1* (endogenous) primers; for the multiplexed reactions of *hph* and *Le1*, the primer concentrations were 900 n*M hph* (transgene) and 600 n*M Le1* (endogenous) primers. Fifteen microliters of DNA (20 ng/µl) was used as a template for the TaqMan PCR amplifications. DNA extracted from a nontransgenic line was used as a no-template control for the specificity of the transgene primers and probes used in the amplifications. As a PCR no-amplification control, water was used as a template. In brief, the magnitude of the fluorescent signal produced for each reaction is indicated by a value ΔRn. This term is calculated by subtracting the ratio obtained by dividing the emission intensity of reporter by the emission intensity of quencher as measured prior to PCR cycling (R_n^-) from the same ratio at a given time during PCR amplification (R_n^+). A threshold was set approximately 10 standard deviations above the mean baseline emission, as calculated from cycles 3–15. The point at which an amplification curve crosses this threshold value is termed the critical threshold (ΔCt). For an absolute determination of DNA copy number, a standard curve was produced that related DNA starting copy number to the amount of fluorescence produced. To this end, serial dilutions of genome-equivalent amounts of a plasmid containing the transgene of interest were used as templates to generate a standard curve. For example, 0.17 pg of pSG/Bt, a 9.2-kb plasmid containing the *cry1Ac* gene, was calculated to be equivalent to 1 copy of 20 ng of the soybean genome (1.115 Mbp/1C, Arumuganathan and Earle 1991). Likewise, 82.5 pg of the plasmid pSG/Bt was used to represent approximately 500 copies of the soybean genome when 20 ng genomic DNA was used as the initial template material. PCR cycling parameters were 50°C for 3 min, 95°C for 10 min and then 45 cycles of 95°C for 15 s and 60°C for 1 min. Real-time detection of fluorescence emissions was performed on the ABI PRISM 7700 (Perkin Elmer). Data acquisition and analysis were performed using Sequence Detection software (Perkin Elmer). All samples were tested in triplicate.

Results

Standard curve method

A standard curve of ΔCt values was generated from known starting DNA concentrations and used to determine the amount of DNA in an unknown sample by interpolation. To generate the curve, we performed TaqMan amplifications on serial dilutions of a plasmid containing the transgene of interest; a linear relationship between the level of fluorescence and the input amount of DNA was produced. In the case of Bt-soybean, plasmid DNA was diluted from a starting concentration equivalent to 500 copies of the soybean genome down to the equivalent of one copy of the soybean genome. Real-time quantitative PCR was performed on the plasmid standards (Fig. 2a). As the starting DNA quantity decreased, the cycle number needed to reach the critical threshold (ΔCt) of fluorescence increased. That is, the more cycles necessary to reach a certain level of fluorescence, the fewer the number of target sequences present in the initial template sample. Aberrant fluorescent readings were detected in some of the negative control samples. This is probably not due to polymerization that would occur in an actual PCR reaction, but rather can likely be attributed to the thermal cycling causing degradation of the probe and liberating some fluorescent molecules. The ΔCt values were then plotted against the log of the initial copy number of template DNA (Fig. 2b). The resulting standard curve had a linear regression coefficient of R^2=1.000. This standard curve was linear over 2.5 logs and thus, theoretically, could be used to accurately quantify up to 500 copies of a gene. By using the regression equation [ΔCt=–3.256 log (copy number) +22.409] that characterizes the standard curve and by measuring the ΔCt value of a given sample, we were able to determine the zygosity of Bt transgenic lines. The Bt lines tested were null, homozygote or hemizygote for the Bt transgene. Amplification profiles of a Bt-homozygote and -hemizygote are shown in Fig. 2c. For all of the lines tested, the transgene copy number determined using TaqMan corresponded with the zygosity data of these samples previously obtained by conventional methods (Stewart et al. 1996).

In a similar manner to that of the Bt-soybean lines, an absolute determination of transgene copy number was made on peanut T_1 plants containing the hygromycin resistance gene. A linear relationship (R^2=0.982) between the level of fluorescence and the log of the initial DNA template amount, equivalent to 1–1,000 copies of the peanut genome, was produced and used to determine the zygosity of the transgenics (Fig. 3a, b). A nontransgenic peanut line and water were used as specificity and PCR-negative controls, respectively. No amplification in any of the negative controls occurred, hence these reactions cannot be seen in Fig. 3a. It was determined that the peanut line tested contained two copies of the *hph* transgene. This result could be interpreted as the line being either a *hph* homozygote or a *hph* two-insertion hemizygote. The finding that this peanut line is a *hph* two-insertion hemizygote is consistent with its line history and with Southern blot hybridization data previously obtained on this sample (Magbanua et al. 2000).

Comparative Ct method

The zygosity of Bt-insect resistance and hygromycin antibiotic resistance transgenes in soybean was also deter-

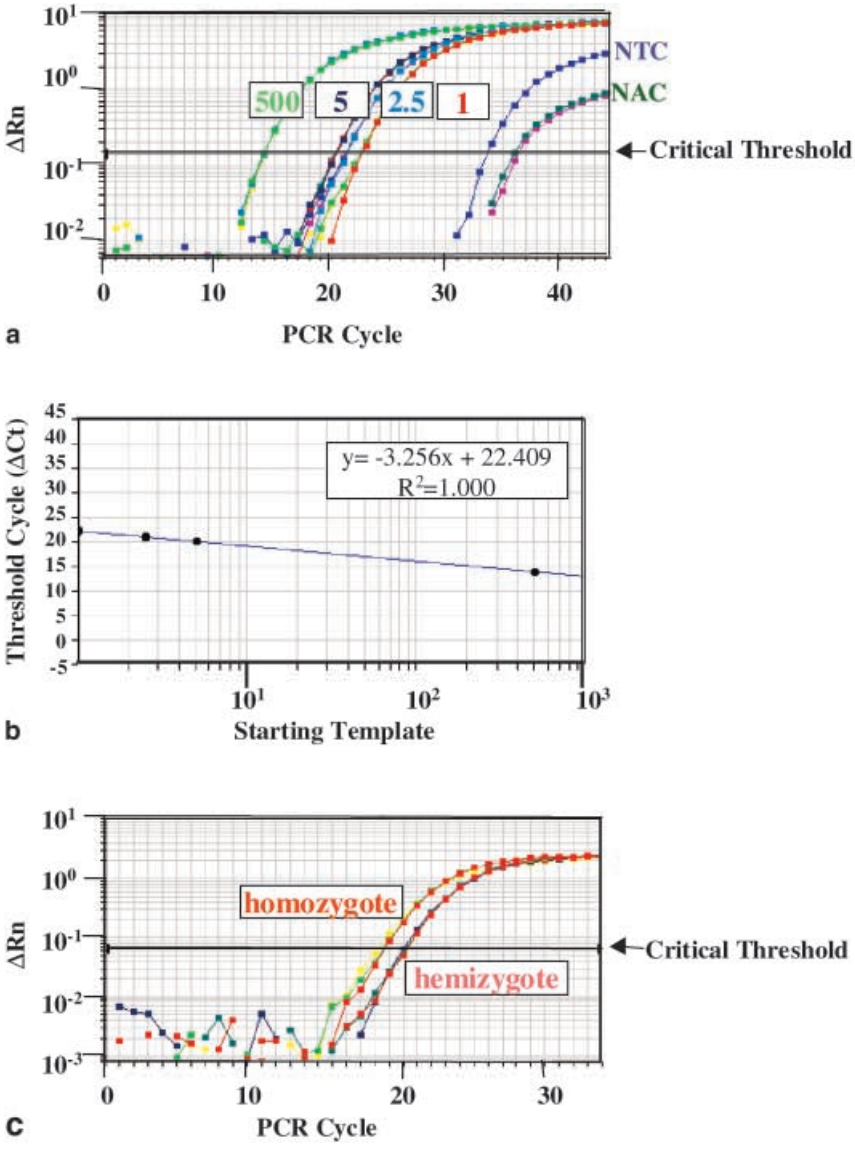

Fig. 2a–c Screen-captures from the software to quantify the *cry1Ac* transgene in soybean by the generation of a standard curve. **a** Amplification plots from the 7700 Sequence Detection System showing cycle number versus normalized fluorescent values (Δ*Rn*). *NTC* No template control, *NAC* no amplification control. **b** Standard curve for the *cry1Ac* transgene shown as the log of transgene-containing plasmid of known quantities (in genome equivalents) versus threshold cycle (Δ*Ct*), using data from **a**. Regression equation: ΔCt=–3.256 log(copy number) + 22.409. **c** Amplification profiles of *cry1Ac* homozygote and hemizygote segregants

mined by the comparative Ct TaqMan assay. In this method, multiplexed PCR reactions were performed whereby the amplification of the transgene of interest was compared to that of an endogenous gene. By amplifying both genes in the same reaction tube simultaneously, we achieved identical conditions. Zygosity of the transgene of interest was determined by comparing the ΔCt value of the transgene amplification to that of the endogenous gene amplification. It follows that the validity of this technique relies on the transgene and reference gene being amplified at approximately equal efficiency. In the comparative Ct assays described in this paper, the lectin gene (*Le1*) was used as an endogenous internal control. Molecular, genetic and biochemical data have shown that *Le1* is a single structural gene locus that controls the production of lectin in soybean seeds (Vodkin et al. 1983). However, through hybridization and sequence analysis, a divergent gene, having 85% homology at the DNA level to *Le1*, has been detected in the soybean genome (Vodkin et al. 1983). Even if the primers and probe used in the TaqMan assays were able to anneal to this *Le1* homolog, it would still mean that *Le1* is a low-copy gene. In practice, the number of divergent sequences in a genome a primer/probe may bind to is of little consequence. For the purpose of choosing a gene to use as an endogenous control in a comparative Ct multiplexed PCR reaction, any gene, regardless of how many homologous sequences may exist in the genome, may successfully be used. The success of the assay depends on the assurance that both the endogenous and transgene amplifications occur at approximately equal efficiencies. Equal efficiencies of the amplifications are achieved by testing various combinations of primer concentrations of the two PCR reactions and finding the conditions whereby one reaction is not out-competing the other and thereby exhausting the reaction components. Since the objective of this study was to quantitate transgene copy number in lines that contained one (hemizygote) or two (ho-

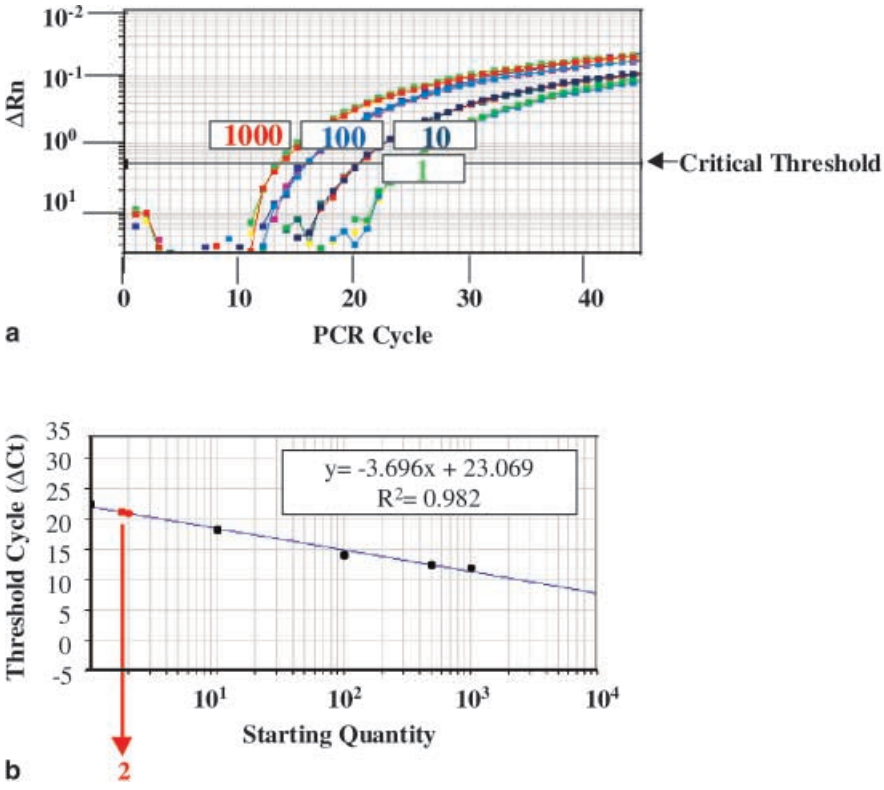

Fig. 3a,b Screen-captures from the software to quantify the *hph* transgene in peanut by the generation of a standard curve. **a** Amplification plots from the 7700 Sequence Detection System showing cycle number versus normalized fluorescent values (Δ*Rn*). **b** Standard curve for the *hph* transgene shown as the log transgene-containing plasmid of known quantities (in genome equivalents) versus threshold cycle (Δ*Ct*), using data from **a**. Regression equation: ΔCt=–3.696 log(copy number) + 23.069. Example of one tested peanut T_1 plant is shown in *red*. The finding of this line being a two-insert hemizygote, as opposed to the line being a homozygote, is consistent with line history and Southern hybridization data

Table 1 Comparative Ct values (*ΔΔCt*) for the zygosity determination of *crylAc* soybean progeny[a] (*SE* standard error)

Zygosity[b]	Replicate 1			Replicate 2			Replicate 3			Mean ΔΔCt ± SE
	FAM-Bt	VIC-lectin	ΔΔCt	FAM-Bt	VIC-lectin	ΔΔCt	FAM-Bt	VIC-lectin	ΔΔCt	
Homozygote	20.08	20.29	–0.21	20.16	20.34	–0.18	20.06	20.52	–0.46	–0.28±0.11
Homozygote	19.07	20.03	–0.96	18.10	20.26	–2.16	18.23	20.06	–1.83	–1.65±0.44
Homozygote	19.01	22.57	–3.56	20.05	22.45	–2.40	20.16	23.00	–184	–2.93±0.42
Homozygote	21.07	20.93	0.14	20.71	22.01	–1.30	21.14	21.59	–0.18	–1.34±0.54
Homozygote	19.07	21.47	–1.50	9.93	21.27	–1.34	20.03	21.39	–1.36	–1.40±0.06
Hemizygote	20.22	20.08	0.14	20.33	19.80	0.53	20.04	20.04	0.00	0.22±0.19
Hemizygote	22.89	21.84	1.05	19.75	20.48	–0.73	21.91	20.66	1.25	0.93±0.86
Null	0[c]	19.08	–25.92	0	19.16	–25.84	0	19.26	–25.74	–25.83±0.06
Null	0	20.98	–24.02	0	23.24	–21.76	0	21.75	–23.25	–23.01±0.81
Null	0	19.63	–25.37	0	19.56	–25.44	0	19.69	–25.31	–25.37±0.05

[a] Individual replicates are shown in this table to help explain how the final ΔΔCt values were obtained

[b] Homozygote average ΔΔCt=–1.52±0.47, hemizygote average ΔΔCt=0.58±0.50, null average ΔΔCt=–24.74±1.07

[c] Samples in which no fluorescence, and hence no amplification, was detected within the PCR cycling parameters were given a zero value

mozygote) insertions, then it follows that it would be sagacious, but not necessary, to use a low-copy endogenous gene as a control. The combination of 600 n*M cry1Ac* and 600 n*M Le1* showed a reduction in ΔRn with a corresponding little, to no, change in ΔCt value; therefore, this combination was deemed optimum to use in the determination of zygosity of Bt lines by the comparative Ct method (Table 1). In a similar manner, primer concentrations were optimized for multiplexed real-time PCR reactions involving the *hph* transgene and the *Le1* endogenous gene. Optimum primer concentrations were found to be 900 n*M* for *hph* and 600 n*M* for *Le1* and were then, in turn, used in reactions to determine the zygosity of *hph* lines (Table 2). The mean ΔCt values with their corresponding standard errors were calculated and used to determine the copy number for each sample. Comparative Ct values (ΔΔCt) were calculated by subtracting the ΔCt values from VIC-labeled endogenous gene reactions from the ΔCt values of the FAM-labeled transgene reactions. Delta delta Ct values were then used as an indication of the quantity of transgene copy number relative to that of the endogenous gene. In instances where no fluorescence was detected within the duration of the PCR reaction, it was presumed that no amplification occurred

Table 2 Comparative Ct values (*ΔΔCt*) for the zygosity determination of *hph* soybean progeny (*SE* standard error)

Zygosity[a]	Mean ΔΔCt ± SE
Homozygote	–1.41±0.07
Homozygote	–1.35±0.76
Hemizygote	0.66±0.28
Hemizygote	0.08±0.06
Hemizygote	1.25±0.63
Hemizygote	0.45±0.34
Hemizygote	0.76±0.15
Null	–21.54±0.19
Null	–23.97±0.29
Null	–23.12±0.50

[a] Homozygote average ΔΔCt=–1.38±0.04, hemizygote average ΔΔCt=0.64±0.21, null average ΔΔCt=–22.88±0.87

due to a lack of the target sequence within the template DNA. Such samples were given a zero ΔCt value for the purpose of calculating a numerical ΔΔCt value. TaqMan zygosity assays consistently produced small, negative ΔΔCt values for homozygotes, small, positive ΔΔCt values for hemizygotes and large, negative ΔΔCt values for nontransgenics (Table 1, 2). The finding that each zygosity class (homozygote, hemizygote or nontransformant) had a discrete numerical category facilitated the efficient determination of transgene zygosity in the samples.

Discussion

This work demonstrates the ability to determine transgene zygosity in plant samples by using real-time quantitative PCR analysis. We evaluated two assays that allowed the zygosity of target genes to be determined in soybean and peanut samples. Both assays were shown to be accurate, reproducible and sensitive enough to distinguish between homozygous and hemizygous individuals. The standard curve assay was shown to have the potential to determine the absolute quantification of a range of copy numbers. This methodology should be capable of determining the zygosity of any gene in any DNA sample, given primers and a fluorogenic probe that are specific to the gene of interest. Still, it has the inherent shortcoming of being highly dependent on the quantification of the input target DNA. Any minor errors in the initial quantification of the sample or plasmid DNAs result in an inaccurate quantification of gene copy number. Nonetheless, this assay should be useful in numerous areas of plant genetic engineering and tissue culture. For example, the use of this technique should hasten the characterization of primary transformants. A distinct benefit of early analysis of transgenic tissue is that "escapes", as well as multiple-copy transformants which may exhibit co-suppression (Fagard and Vaucheret 2000), can be discarded prior to investing time and resources into their propagation and regeneration.

The need to precisely quantify both DNA template samples and standard plasmids was eliminated by measuring the zygosity of the transgenes relative to that of an endogenous gene present in the samples. Hence, the comparative Ct method has the added advantage of allowing a higher sample throughput, as it is less labor-intensive than the standard curve method. The comparative Ct method generates a relative quantification of transgenes and thus has a limited range of applications. It is ideally suited for large-scale screening of segregating F_2 populations to identify homozygous transgenic individuals, thereby negating the need for progeny tests. The prompt identification of homozygous lines would thus expedite plant transformation and breeding projects, especially those of species that have long generation times.

In addition to its many applications, real-time quantitative PCR offers many notable technical advantages over other quantitative PCR methods, such as competitive PCR and the use of intercalating dyes. First, TaqMan-based assays offer a higher degree of specificity than other PCR methods, since the only way that a fluorescent signal can be produced is by both primers and the probe hybridizing in the correct orientation to the target sequence. Second, zygosity in real-time PCR is measured during the log phase of amplification as opposed to an end-point determination. This offers a more accurate and reliable estimation of gene copy number as well as a larger range (five to nine orders of magnitude) over which copy number can be determined (Heid et al. 1996; Lie and Petropoulos 1998). Third, through the use of genomic DNA as a template, gene zygosity can be determined; by using cDNA as a template, the level of gene transcription can be studied. Fourth, there is no post-PCR analysis of samples, thereby minimizing handling time and lessening the likelihood of contamination that may lead to false positive results. Fifth, real-time assays have the potential of high sample throughput and automation: up to 96 samples can be prepared and analyzed in approximately 3 h.

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