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Use of Quantitative Polymerase Chain Reaction for Determining Copy Numbers of Transgenes in *Lesquerella fendleri*

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Abstract: Problem statement: In transgenic plants, the number of transgene copies could greatly influence the level of expression and genetic stability of the target gene, thus it is important to develop an efficient method for accurate estimation of transgene copies. The quantitative Polymerase Chain Reaction (qPCR) technique is becoming more efficient nowadays to determine copy numbers of transgenes in transgenic plants, being used here, for the first time in quantifying copy numbers of transgenes in *Lesquerella fendleri*. **Approach:** The system utilized a known one copy gene, LfKCS4/5, from *L. fendleri* as an endogenous calibrator and the threshold Crossing point (Ct) measured by Applied Biosystem 7500 system to calculate the copy numbers of transgenes in primary transgenic lines (T0 generation). **Results:** The qPCR condition was optimized and each primer set had a PCR efficiency of 0.99 or 1.01. Our data demonstrated unambiguous 2-fold discrimination of the copy number of ß-glucuronidase gene (gusA) and hygromycine phosphotransferase II (hptII) genes in 12 T0 lines. Most of the lines contained one or two copies of each gene. Eight out of 12 samples (66.7%) showed more copies of gusA gene than that of hptII gene, suggesting rearrangements of the Transferred (T)-DNA. Possible modifications of the T-DNA cassette in *L. fendleri* are discussed based on main models of T-DNA integration in the plant genome. **Conclusion:** The qPCR described in this study is an efficient method and it is particularly useful in identification and selection of transgenic plants with desirable copy numbers at early stage.

Key words: *Lesquerella fendleri*, quantitative PCR, polymerase chain reaction, transgenes

INTRODUCTION

Lesquerella fendleri (A. Gray) S. Wats. (Brassicaceae), under development as a new industrial oilseed crop in the southwestern region of US, is valued for its unusual Hydroxy Fatty Acid (HFA). HFAs and derivatives are used as raw materials for numerous industrial products, such as lubricants, plasticizers and surfactants (Caupin, 1997).

Considerable efforts have been made to improve the productivity of *L. fendleri* through plant breeding (Dierig *et al*., 2006; 2004; Isbell *et al*., 2008). Because *L. fendleri* is amenable to *Agrobacterium*-mediated transformation (Skarjinskaia *et al*., 2003; Wang *et al*., 2008), alternative means to improve this crop exist through genetic engineering. When new transgenic plants are obtained, one essential step is to determine the copy number in the transgenic plants, because the copy number can greatly influence the expression level and genetic stability of the target gene. The number of transgene copies has traditionally been estimated by Southern analysis, in which a blot of digested genomic DNA is hybridized with a radioactive DNA probe corresponding to the transgene to produce an informative band pattern. The drawbacks of this method are laborious and time-consuming, especially when a large number of samples need to be estimated (Ingham *et al*., 2001). It requires a great amount of DNA and hazardous radio-isotopes (Mason *et al*., 2002). Moreover, Southern analysis often underestimates transgene copies when two copies of transgenes are inserted into one locus, because the digested DNA fragments containing each gene are very similar in size and thus indistinguishable by Southern analysis (Mason *et al*., 2002).

Recently, the development of quantitative Polymerase Chain Reaction (qPCR) methods for determining the transgene copy number has overcome the limitations of Southern analysis (Ingham *et al*., 2001; Mason *et al*., 2002). To date, qPCR technology

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has been applied to analyze copy number of transgenic plants, including soybean and peanut (Schmidt and Parrott, 2001), tomato (Mason *et al*., 2002), maize (Ingham *et al*., 2001; Shou *et al*., 2004; Song *et al*., 2002), rapeseed (Weng *et al*., 2004), wheat (Doshi *et al*., 2007; Li *et al*., 2004), rice (Jiang *et al*., 2009; Yang *et al*., 2005), tobacco (Subr *et al*., 2006), cotton (Yi *et al*., 2008), citrus (Omar *et al*., 2008), grape (Costa *et al*., 2009) and cassava (Beltran *et al*., 2009). To speed up the molecular analysis of transgenic plants, we describe here the development and application of a qPCR method that utilizes an endogenous calibrator and the threshold crossing point (Ct) calculated by the Applied Biosystem 7500 system for determination of transgene copy numbers in *L. fendleri*. The method presented is rapid, sensitive, robust and easy to optimize.

MATERIALS AND METHODS

Plant material, bacterial strain: The *L. fendleri* seeds, WCL-LY2 (Dierig *et al*., 2001), were kindly provided by Dr. Dave Dierig (USDA-ARS, Arid-Land Agricultural Research Center, Maricopa, AZ). Plants were grown in a greenhouse at temperatures between 28°C (day) and 18°C (night), with supplemental metal halide lighting to provide a 15 h day length (1000-1250 μmol m^{-2} sec^{-1}). Mature female flowers were hand-pollinated and the seeds were harvested at about 49 days after pollination. An *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) carrying binary vector pCAMBIA 1301.1 (http://www.cambia.org), which contains gusA as a reporter gene and hygromycine phosphotransferase II (hptII) as a selection marker gene was used for plant transformation. An AGL1/pCAMBIA1301.1 culture was started with the inoculation of one clone in 1 mL Luria Broth (LB, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, 1 g L^{-1} glucose, pH 7) supplemented with 50 mg L^{-1} kanamycin and 100 mg L^{-1} carbenicillin shaken at 200 rpm overnight at 29°C, followed by sub-culturing of 0.1 mL of the overnight culture in 50 mL fresh LB for 24 h.

Tissue culture, transformation and regeneration: *Agrobacterium* cultures were centrifuged at 4,000 rpm for 10 min at room temperature (25°C) and the pellet was suspended to an OD_{600} of 0.5 in half strength Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962). *L. fendleri* seeds were surface-sterilized in a 0.25% (v/v) sodium hypochlorite solution for 15 min followed by five rinses in sterile deionized water. The seeds were germinated and grown aseptically on Germination Medium (GM) containing half strength of MS medium supplemented with 0.5% sucrose and 0.6% agar in a growth chamber at day/night cycle (16/8 h, 100 $\mu mol.m^{-2}.s^{-1}$) at 24°C (day) and 22°C (night). After 4-6 weeks, shoot tips together with 4-5 true leaves were sub-cultured and grown aseptically on GM for a continuous supply of leaf material.

To perform the transformation, leaves were harvested from plants after 4-6 weeks of sub-culturing and wounded by scratching slightly on the underside of leaf and then dipped in the half strength MS medium containing AGL1/pCAMBIA1301.1 for 5 min. Following the inoculation, leaves were blotted on sterilized filter paper and transferred to Callus and Shoot Induction (CSI) medium composed of Basal Medium (BM, half strength MS medium plus 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.7) supplemented with 1 mg L^{-1} 6 Benzylaminopurine (BA) and 0.1 mg L^{-1} Naphthaleneacetic Acid (NAA). After incubating the infected leaves in the growth chamber for 2 days, the leaves were cut into 5 mm segments and cultured on Callus/Shoot Induction (CSI) media plus 25 mg L^{-1} hygromycin (hyg) for transgenic selection and 100 mg L^{-1} timentin for inhibiting the *Agrobaterium* growth. In 6-8 weeks, yellow-greenish hygromycin resistant (hyg^R) calli started to appear on the leaf segments. The calli were than transferred to a timentin-free subculture medium CSI+ (CSI with hyg increased to 50 mg L^{-1}). Green shoots were developed from the calli on CSI+ medium in about 2-4 weeks after the transfer.

To eliminate chimeras, each shoot was cut into small pieces (about 1×1 mm) and placed on the CSI+ medium for shoot-regeneration. After 4 rounds of successive regenerations, shoots were sub-cultured on BM plus 1 mg L^{-1} BA, 1 mg L^{-1} Indole-3-Butyric Acid (IBA) and 50 mg L^{-1} hyg for multiplication. Shoots 10-15 mm in length were transferred to rooting medium (BM plus 1 mg L^{-1} IBA and 50 mg L^{-1} hyg). When a shoot developed 2-3 roots (usually in 3-5 weeks), it was than transferred to a Magenta box (Sigma, St. Louis, Mo) containing sterilized peat-vermiculite growth mixture (Sunshine mix #4, Planet Natural, Bozeman, MT) pre-soaked with 1 mg L^{-1} IBA water solution. After 8-10 weeks in the growth mixture, a well-developed primary plant (T0 generation) showing 8-12 normal leaves and 2-3 inch height was transferred to a 6-inch pot and placed under a transparent plastic cover for the first 2 weeks for acclimation in the greenhouse.

DNA extraction and qPCR: Genomic DNA from each T0 transgenic plant was isolated using a DNeasy Plant Mini Kit (Qiagen Sciences, Maryland, USA). Amplification reactions were carried out in a volume of 25 µL containing 20-1 ng of genomic DNA, 0.05 µM of each forward and reverse primers and 1x Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) using a 7500 Fast Real-Time PCR system (Applied Biosystems) and standard default thermal cycling conditions (initial step, 95°C for 10 min for polymerase activation, PCR (40 cycles), 95°C, 15 sec for melting, 60°C, 1 min for annealing and extending, dissociation step set by the system software).

Oligonucleotide primers were designed using Primer Express, version 3 software (Applied Biosystems). To ensure maximum specificity and efficiency during quantitative PCR, primer pairs were further tested for linearity of response by constructing relative standard curves on six serial 2-fold dilutions. The templates used for the standard curve analysis were mixed genomic DNAs from all samples. The 7500 system software analyzed the SYBR fluorescence and produced standard curves by plotting the log concentration of the starting quantity against the threshold Cycle (Ct). Data of a standard curve reported by the software includes slope, interception, correlation coefficients and Ct. For each primer set, standard curves were analyzed independently for at least three runs and standard curves repeatedly showing correlation coefficients of 0.99 or higher and PCR efficiencies (calculated by Efficiency = $[10^{(-1/\text{slope})}]$-1) between 98 and 102% were accepted. PCR product specificity was confirmed by melting-curve analysis and by electrophoresis on 4% agarose gel to ensure that PCR reactions were free of primer dimers and non-specific amplicons. Information on optimized primer pairs is listed in Table 1.

Transgene copy number calculation: A modified method (Weng *et al*., 2004) based on the equation of Livak and Schmittgen (2001) that does not require identical amplification efficiencies between PCR system for the target gene and internal control gene was used to calculate transgene copies. In brief, this method calculates copy numbers by obtaining the value of $X_0/R_0 = 10^{[(Ct,X - IX)/SX] - [(Ct, R - IR)/SR]}$ where I_X and I_R are intercepts of the standard curves of target and internal control genes, respectively, S_x and S_R are slopes of the standard curves of target and internal control genes, respectively and $Ct,_X$ and $Ct,_R$ are the detected threshold cycles of amplification of the target and internal control genes to a tested sample and the values are reported by the 7500 system software. If the copy number of the internal control gene (R_0) is well defined, the copy number of the target gene (X_0) can easily be deduced from the I_X, I_R, S_X, S_R, $Ct,_X$ and $Ct,_R$ in the tested sample. The values from representative standard curves are listed in Table 2, including slopes (S_X and S_R) and intercepts (I_X and I_R) for the target genes, ß-Glucuronidase (GUS) and hygromycin phosphotransferase II (hptII), (both genes are in Genbank accession number AF354045) and for the internal control gene LfKCS45 (a single copy endogenous gene encoding a root-specific 3-ketoacyl-CoA synthase, Genbank accession number AY695435 (Moon *et al*., 2004), correlation coefficients, PCR efficiencies and Ct range. For each plant DNA sample, triplicate sets of PCR reactions with primers of the hptII, gusA and LfKCS45 and duplicate negative controls (reaction samples without DNA templates), were prepared and run in a 96-well plate. The PCR experiments were repeated three times for each plate to ensure that similar results are obtained.

Table 1: Primers used in the qPCR assay

Abbreviated Gene Name	GenBank ID	Forward primer (5'-3') Reverse primer (5'-3')	Amplicon size (bp)	Position (bp)
LfKCS45	AY695435	CCAACTGCTTGTTCCGTCTTG GTCTTCGTTCTCCCGACTTGTT	66	1816-1881
hptII	AF354045	CGCGTCTGCTGCTCCATAC GGTCGCCAACATCTTCTTCTG	57	9234-9290
gusA	AF354045	ACAACGTCGTGACTGGGAAAA TGTGCTGCAAGGCGATTAAG	57	11108-11164

Table 2: Information of representative standard curves

Gene name	Ct range	Slope	Interception	Correlation coefficients	PCR efficiencies
LfKCS45	20-26	-3.3460	25.0723	0.9980	0.99
hptII	20-26	-3.3488	24.2839	0.9974	0.99
gusA	23-29	-3.2982	27.0508	0.9957	1.01

RESULTS

As described ((Weng *et al*., 2004) Material and methods)), the transgene copy number was determined relative to an endogenous reference gene or internal control gene using qPCR technology. In this study, the first essential step was to obtain an endogenous sequence with confirmed low copy number per genome. A single-copy endogenous gene is the best choice for this analysis to ensure sensitivity of detection. We searched the NCBI database and identified a known single-copy endogenous gene, LfKCS45 encoding a root-specific 3-ketoacyl-CoA synthase (Moon *et al*., 2004) as the internal control. To ensure maximum specificity and efficiency during qPCR, we designed gene specific primers (Table 1) and validated the primers by optimizing primer concentrations and performing standard curves analysis (Material and methods). The 7500 system software produced standard curves by plotting the log concentration of the starting quantity against the threshold Cycle (Ct) and also calculated the slope, interception and correlation coefficient for each standard curve.

Table 3: Ct values of 3 genes for each transgenic line from the qPCR assay

Sample	Ct (KCS45)	Ct (hptII)	Ct (gusA)
149	24.30 ± 0.10	24.64 ± 0.11	26.34 ± 0.10
174	24.50 ± 0.08	24.65 ± 0.06	27.55 ± 0.14
178	24.44 ± 0.01	24.66 ± 0.07	26.48 ± 0.06
213	24.54 ± 0.05	24.70 ± 0.05	26.60 ± 0.04
312	24.71 ± 0.10	25.00 ± 0.05	26.70 ± 0.09
319	25.86 ± 0.03	24.61 ± 0.01	26.33 ± 0.04
322	25.95 ± 0.04	24.61 ± 0.09	26.34 ± 0.05
324	24.39 ± 0.08	24.61 ± 0.02	27.35 ± 0.08
344	23.98 ± 0.09	24.22 ± 0.03	26.79 ± 0.06
350	24.41 ± 0.04	23.69 ± 0.06	26.46 ± 0.06
376	24.08 ± 0.02	23.26 ± 0.02	25.40 ± 0.05
378	24.24 ± 0.01	23.40 ± 0.10	25.24 ± 0.01

Data are expressed as mean ± SD of triplicate

Table 4: Estimated number of hptII and gusA copies for each line and possible rearrangements determined from the qPCR results

	hptII		gusA		
Sample	2 times (X_0/R_0)	Number of copies	2 times (X_0/R_0)	Number of copies	Rearrangement
149	0.92	1	1.93	2	Yes
174	1.05	1	0.95	1	No
178	1.00	1	1.93	2	Yes
213	1.04	1	1.91	2	Yes
312	0.95	1	2.00	2	Yes
319	2.74	2-3	5.68	5-6	Yes
322	2.92	3	6.00	6	Yes
324	1.00	1	1.02	1	No
344	0.99	1	1.13	1	No
350	1.90	2	1.92	2	No
376	2.04	2	3.21	3	Yes
378	2.08	2	4.00	4	Yes

Among the various PCR conditions tested, adjusting primer concentrations was the most effective way to optimize the PCR; low concentration of primers at 50 nM produced the best standard curves for each gene. The correlation coefficients of the standard curves of target genes, hptII and gusA and internal control gene LfKCS45 were very good (0.996-0.998, Table 2) and the PCR efficiencies were almost 100% (0.99-1.01, Table 2), indicating that all three genes had very similar reaction efficiencies over a range (2-64 fold) of initial DNA concentrations. Other information such as slopes and intercepts of the representative standard curves of hptII, gusA and LfKCS45 were also listed in Table 2. To estimate the transgene copy number, triplicate sets of PCR reactions were run for each DNA sample from T0 transformants; the reported Ct values were averaged over the triplicates and listed in Table 3. The standard deviations for the mean Ct values varied from 0.01-0.14 (Table 3), demonstrating low variability within replicates. Because the transgenic lines in the T0 generation are heterozygotes for the transgenes (hptII and gusA) and homozygotes for the endogenous gene (LfKCS45) (Mason *et al*., 2002), the numbers of copies of the target genes in T0 transformants should be calculated after the value of X_0/R_0 in equation, $X_0/R_0 = 10^{[(Ct,X-IX)/SX]-[(Ct,\ R-IR)/SR]}$ is doubled (Weng *et al*., 2004). According to the equation, the number of transgene (hptII and gusA) copies of 12 T0 transformants were estimated (Table 4). For the hptII gene, 7 lines had 1 copy, 3 lines had 2 copies and the remaining 2 lines had about 3 copies. Comparison of the results of each sample (Table 4) indicated that the copy number of gusA gene was not entirely consistent with that of hptII, suggesting T-DNA rearrangements during the process of chromosomal integration. Of 12 tested samples, 8 (149, 178, 213, 312, 319, 322, 376 and 378) showed rearrangements (Table 4).

DISCUSSION

We have developed a simple, rapid and effective qPCR method to estimate transgene copy number in transgenic *L. fendleri*. In this approach, the transgene copy is determined relative to an endogenous single copy gene, LfKCS4/5. Because the LfKCS4/5 sequence serves as an endogenous calibrator, it remains constant relative to the total genomic DNA of *L. fendleri.* Therefore, any variation in the relative level of the transgene to the endogenous gene is indicative of a difference in copy number. Also, the method incorporates the true amplification efficiencies of PCR systems in gene copy calculation, eliminating the need of having identical efficiencies between the PCR

systems which is a prerequisite when using the original equation (Livak and Schmittgen, 2001) but sometimes is impractical or impossible to obtain (Weng *et al*., 2004). Among 12 transgenic plant samples, we found 8 samples having a low copy number (1 or 2). This is consistent with the knowledge that *Agrobacterium*-mediated transformation gives rise to lower copy numbers compared to direct transformation methods such as particle bombardment (Kohli *et al*., 2003). By using qPCR, we revealed T-DNA rearrangement events occurring in 8 out of 12 samples (66.7%), i.e., because the hptII copy number did not agree with the gusA copy number. This 66.7% occurrence of rearrangement indicates that T-DNA rearrangements may be more common than generally thought. This discrepancy is likely associated with technique used, since the traditional Southern blotting technique examines only the transgene of interest, not the antibiotic selection gene and thus may not detect a large proportion of rearrangements. This qPCR method is more effective than Southern blot detection, as it allows simultaneous detection of the copy numbers of different genes and is more accurate in estimating transgene copy number. There is increasing evidence of T-DNA rearrangements in studies where qPCRs are utilized. For example, in tomato, of 15 transgenic lines investigated, at least 5 (i.e., 33.3%) showed rearrangements (Mason *et al*., 2002). In rice, the estimated number of lines with rearrangements was high at 45.8%, corresponding 11 of 24 evaluated lines (Yang *et al*., 2005). In rape seed (Weng *et al*., 2004) and cassava (Beltran *et al*., 2009), the number was 10% (2 of 20) and 13.3% (2 out of 15), respectively. The frequency of rearrangement may correlate to the host plant to be transformed and/or the transformation system, such as *Agrobacterium* strain, T-DNA vector and growth state of the *Agrobacterium* (Gelvin, 2003). In *L. fendleri*, we noted that the rearrangements showed more copies of the gusA gene than the *hptII* gene, indicating the T-DNA cassette must have undergone some kind of modification during the integration process in the plant genome. A T-DNA cassette consists of Right Border (RB), Left Border (LB) and genes of interests in between. The RB and LB delimit the T-DNA and are the only required cis-elements that determine the T-DNA cassette integrating into plant genome. According to the main model of T-DNA integration (Tzfira *et al*., 2004), T-DNA integration can be accompanied by some deletions in both ends; when they occur, deletions were more severe at the LB end (tail) compared with its RB end (head), because the RB is attached (or protected) by VirD2 protein which helps the integration. When two T-DNAs co-integrate to plant genome, T-DNAs can arrange to head-to-head or head-to-tail repeats prior to T-DNA integration. Tail-to-tail orientation occurs with less frequency. Moreover, large deletions (e.g., >1000 bp) occur particularly in one of the tails of the repeats at 100% frequency in head-to-head condition and at 21.6% in head-to-tail (Zhu *et al*., 2006). The T-DNA in pCAMBIA 1301.1 used in this study has the hptII gene located close to the LB (or tail) and the gusA close to the RB (head). A large deletion in the tail would truncate the T-DNA, resulting in loss of the hptII sequences and thus would be undetected by the qPCR method. Truncation of T-DNA may be a common phenomenon in rearranged T-DNAs in transgenic L. fendleri. Similar results were reported in rice (Afolabi *et al*., 2004), where truncation occured in 70% of the transgenes analyzed and ~20% of them contained only a part of DNA, e.g., from the middle to RB. To gain an improved understanding of the mechanism of T-DNA rearrangement and integration, *L. fendleri* may serve as a suitable model species.

CONCLUSION

As described above, we have developed a qPCR method that can be implemented as a tool for rapid and accurate determination of transgene copy number in *L. fendleri*. Rearrangements are common during integration of two or more T-DNAs in the genome. When it occurs, the gene constructed next to the left border of T-DNA is likely to be truncated. Because the required sample size for the qPCR method is very small, the transgene copy number can be determined while T0 transgenics are still in the tissue culture stage. This allows the selection of desirable T0 plants and saves greenhouse space, which increases the capacity of the transgenic event production pipeline.

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