Available online at www.ijpab.com

International Journal of Pure & Applied Bioscience

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **2 (4):** 100-105 (2014)

Research Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Real time PCR based detection of transgene copy number in transgenic chickpea lines expressing *Cry1Aa$_3$* and *Cry1Ac*

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ABSTRACT

Quantitative real-time PCR technique was used to determine the copy number of cry1Aa$_3$ and cry1Ac in transgenic chickpea lines. Gene specific primers designed to amplify relatively long amplicons (400 - 600 bp), were used to increase the specificity and sensitivity of real-time PCR using Syber green I as the fluorescence indicator. Significantly accurate estimate of single transgene copy in the transformants was achieved. Estimated copy number in transgenic lines using real-time quantitative PCR was found to be same as analyzed by southern hybridization. The study shows the effectiveness of real-time PCR in estimating transgene copy number in an efficient manner in transgenic chickpea plants.

Key words: *Real-time PCR, Chickpea transformation, Agrobacterium, copy number, Syber green.*

INTRODUCTION

Genetic transformation has been widely exploited to study biology and development of plants as well as to obtain commercial crops with improved agronomic characters. Plants with one or two transgenes are preferred for stable and high-level expression of transgene. Therefore an early screening of transformants with one or two copies of transgene and transgene homozygotes in subsequent generations would be highly desirable and beneficial, especially for crop plants that require a lot of space and long time to grow[18]. Efficient plant transformation procedures need sensitive assays for the analysis of putative transformants as well as to determine the transgene insertion events in terms of copy numbers[1]. Multiple transgene copies are more likely to cause transgene silencing[5,9,15].

Southern hybridization analysis is traditionally used to determine transgene copy number. Though used in routine and reliable, the method is time consuming, laborious, and requires large amount of DNA[11]. To overcome these constraints, quantitative real-time PCR assay was developed to determine transgene copy number in some plants[1,2,4,8,14,17]. Real-time PCR detects PCR products as they accumulate and utilizes speed, throughput and quantitative accuracy for amplification of a specific sequence of DNA or RNA[1,6,7]. Real-time PCR was developed to quantify the template DNA via continuous monitoring of fluorescent signal. It depends on the identification of the first cycle that generates a signal over the background level, which is calculated as the threshold cycle (C_t). SYBR green I is an intercalating fluorescence minor-groove DNA binding dye that exhibits enhanced fluorescence when bound to a dsDNA which provides a simple generic method for detection of amplification product[12,16]. Ahmad *et al.*[1] presented the SYBR green I copy number assay using gene specific primers for the amplification of relatively long amplified product to ensure enhanced sensitivity and accuracy in a short time period and determine the transgene copy number in transgenic rice plants. However, to date, the use of qRT-PCR for estimating transgene copy number in transgenic chickpea has not yet been investigated. In the present study the transgene copy number of the *cry1Aa$_3$* and *cry1Ac* gene in transgenic chickpea lines was determined by using gene specific primers designed to amplify a relative long amplicon, 492 bp for *cry1Aa$_3$* and 533 bp for *cry1Ac* by real-time PCR using Syber green I as the fluorescence indicator with optimized PCR conditions.

MATERIALS AND METHODS

Plant material

Four independent chickpea lines one carrying *cry1Aa*$_3$ [10] and three expressing *cry1Ac* (Table 2) were used for real time PCR based analysis and southern hybridization. Non-transgenic control chickpea plant was used as negative control.

DNA isolation

Total genomic DNA was isolated from young leaves of transformed chickpea plants by CTAB method[3]. The plasmid DNA was isolated following the alkaline-lysis mini-prep method[13]. Quantitative estimation of the standard amount of the template was done using ND-1000 Nano-drop spectrophotometer (Thermo Scientific).

Real-time PCR

A simple quantitative real-time PCR procedure was used to determine transgene integration and copy number in transgenic chickpea plants as described by Ahmad *et al.*[1]. Experiments were conducted using Applied Bio-systems 7500 Real-Time PCR System with 96 well microtiter plates. Reaction was set-up in total volume of 25µl containing, Maxima SYBR Green qPCR Master Mix (1X), 0.3µM each primers, 100ng template DNA and Thermal cycling conditions were: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at X °C for 30 sec and extension at 72°C for 30 sec. The data acquisition was set at extension step of each cycle.

The primer sequences used in the reactions for the amplification of 492bp and 533bp fragments of the *cry1Aa*$_3$ and *cry1Ac* with annealing temperatures are listed in Table 1. Standard curve for transgene copy number was generated using pBinAR at 0, 10, 10^2, 10^4, 10^6 and 10^8 copies. Analysis of data and threshold cycle (Ct) determination were accomplished using the ABI 7500 Detection Software. The fluorescence of the SYBER Green I was monitored at the end of the elongation step of each PCR cycle.

After amplification, a melting curve was acquired by heating at 95°C for 15 sec, then cooling to 60°C for 15 sec and then slowly heating (0.1°C/sec) to 95°C for 20 min with data collection at 0.2°C intervals, using ABI 7500's software.

Southern Hybridization Analysis

Genomic DNA (20 µg) samples from transgenic chickpea plants were digested with *Hind* III separated on 0.8% agarose gel, and blotted on nylon membrane. The PCR amplified fragment of *cry1Aa*$_3$ and *cry1Ac* gene was eluted from the gel using QI Aquick Gel Extraction Kit (Qiagen Inc., USA) and was labelled by non-radioactive process using Biotin following the manufacturers manual (Biotin Decalabel™ DNA Labelling Kit, Fermentas). Biotin Chromogenic Detection Kit (Fermentas) was used to detect hybridized biotin labelled probe on the nylon membrane following the kit's manual.

Calculation of gene copy number

Calculation of copy number using the real-time PCR was done by using the software accompanying the ABI 7500 instrument that detects the accumulation of PCR product by increase in fluorescence. The accumulated fluorescence after normalization relative to established base line levels (ΔRn) was plotted against cycle number giving the amplification plots and Ct values. The transgene copy number in transgenic plant sample was calculated by interpolation from a standard curve Ct values generated using known amount of starting DNA concentrations. Since, one copy of chickpea genome contains 0.95 pg DNA (Bennett, 1995), total genome copies (Y) in template DNA (100 ng) used was 1.05×10^5.Thus, the copies corresponding to each Ct value of transgenic event (Z) was calculated as the ratio of amount calculated using standard curve (X) to the total amount of target DNA (Y) used in the real-time PCR (Z= X/Y)

RESULTS AND DISCUSSION

Southern hybridization analysis

Southern hybridization analysis of the genomic DNA of transgenic chickpea plants digested with *Hind* III, probed with *cry1Aa*$_3$ and *cry1Ac* gene specific biotin labeled probe confirmed the integration of single copy of the target gene into the transgenic chickpea plants genome (Fig. 2a and b).

The non-identical hybridization patterns of different transformation events of transgenic chickpea plants of cv. HC-1 (TH2-44 and TH2-166) and cv. C-235 (TC2-33) showed independent single copy integration of the *cry1Ac* gene into the transgenic chickpea plants (Fig. 2b).

Development of standard curve for using SYBR Green I

Quantification of the target genomic DNA using real-time PCR was done by generating a standard curve with known amount of diluted plasmid DNA from 10-10^8 copies of *cry1Aa*$_3$ and *cry1Ac* gene which was used to calculate the amount of DNA in unknown transgenic chickpea samples (Fig. 1a and b). The Ct values obtained by real-time PCR amplification ranged from 26-40 cycles and were reproducible. There was a linear correlation between the amounts of DNA used as template in the beginning of PCR reaction and the Ct values during amplification with a correlation coefficient (R^2) value of 0.984 (Fig. 1b).

Copy number of *cry1Aa*$_3$ and *cry1Ac* gene in transgenic chickpea plants

The Ct values and the calculated copy numbers of *cry1Aa*$_3$ and *cry1Ac* gene of transgenic chickpea plants using standard curve are given in the Table 2. Single copy of the transgenes *cry1Aa*$_3$ ($T_4$3-28, $T_4$6-19) (cv. C-235) and *cry1Ac* (TH2-44, TH2-166) (cv. HC-1); TC2-33 (cv. C-235) in transgenic chickpea plants was detected by real-time PCR (Table 2). No PCR amplification of genomic DNA was recorded in the non-transgenic chickpea control plant by real-time PCR. A simple quantitative real-time PCR procedure was used to determine transgene integration and copy number in transgenic chickpea plants using gene specific primers designed to amplify a relatively long amplicon to increase the specificity and sensitivity. Ahmad *et al.*[1] optimized the conditions for real-time PCR using SYBR Green I for specific targets by increasing the length of the amplicon three (from 200-600bp) to reduce the non-specific amplification, which might be a problem in case of using low (50-200bp) amplicons.

Accuracy of SYBR Green I copy number detection

Dissociation curve analysis was used for product differentiation after PCR amplification which enabled to assess the specificity and the purity of real-time PCR reaction (Fig. 1c). A single strong and consistent temperature of melting (Tm= 81.5°C) of the PCR products was observed after melting curve analysis in all of the samples except in no template control and non-transgenic chickpea DNA samples, where no amplification was observed.

Copy Number Comparison Studies

The real-time PCR based transgene copy number (Fig. 1) matched perfectly with the results obtained with Southern hybridization analysis (Table 2). The real-time PCR copy number results were compared with the results obtained after Southern hybridization analysis which matched perfectly. Further, it was possible to characterize the transgene specifically and accurately in a short interval of time and with little efforts. Compared with Southern blot analysis, real-time PCR is rapid and the DNA quantity requirement for real-time PCR is about 1/200th that of Southern hybridization analysis[11]. Thus, real-time PCR allows the determination of transgene copy number at an early stage of plant growth by reducing the quantity of plant tissue required. Therefore, low or single copy transformation events may be selected by real-time PCR early in the transformation process.

In conclusion, this study demonstrates the utility of SYBR green in the real-time PCR method for estimating the transgene copy number in transgenic chickpea plants by a simple, efficient and cost effective method. This method can be used as an alternative for Southern blotting and hybridization, which is time-consuming and requires large amounts of fresh or frozen samples.

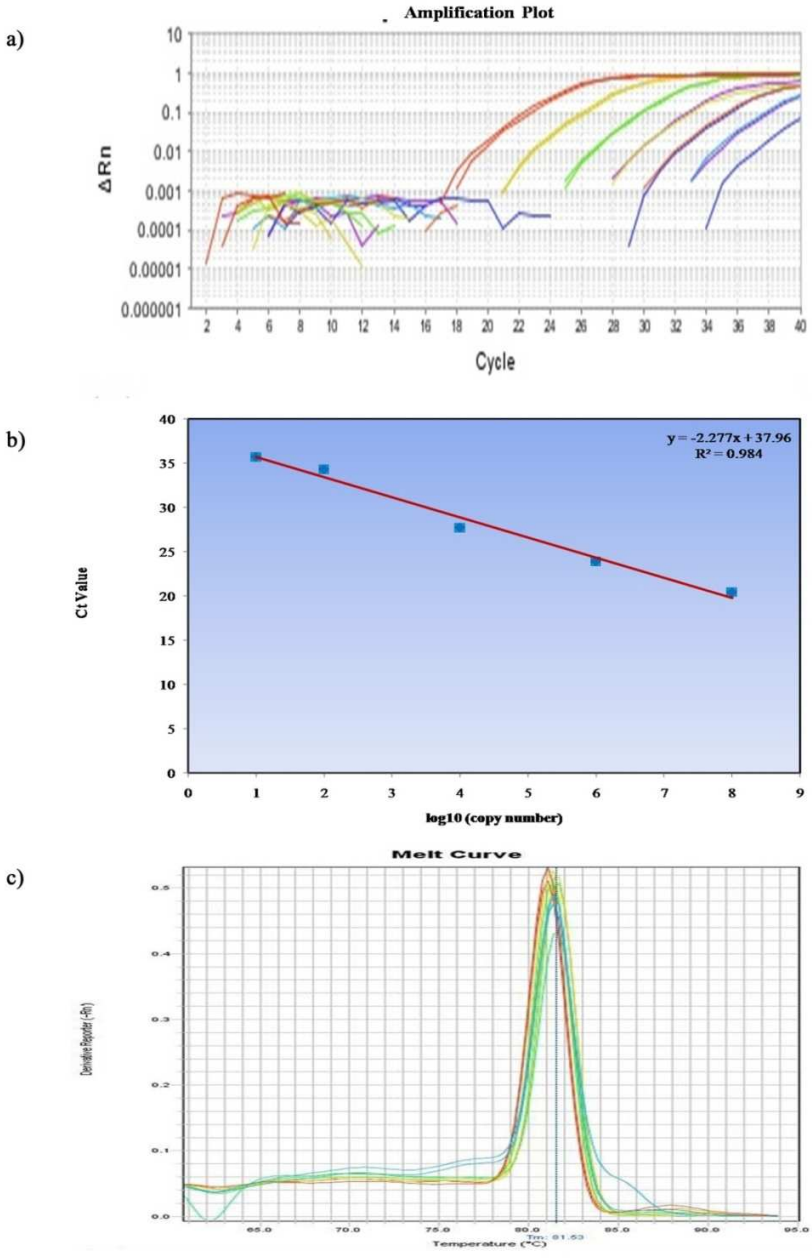
Table 1 List of primer pairs used for PCR analysis of transgenic plants.

Primer Name	Sequence (5'- 3')	Amplicon size (bp)	Annealing temperature
cry1Aa$_3$ F	CCTCTGGCCAAACACGGAGACG	492	58°C
cry1Aa$_3$ R	ACCCTGAGGTCGAGGTCCTTGGT		
cry1Ac F	TTCTGCCCAAGGTATCGAAG	533	53°C
cry1Ac R	CAGAACGGTGAATCCAAGAG		

Table 2 Correlation between transgene copy number detection by real time PCR and Southern hybridization in transgenic chickpea lines

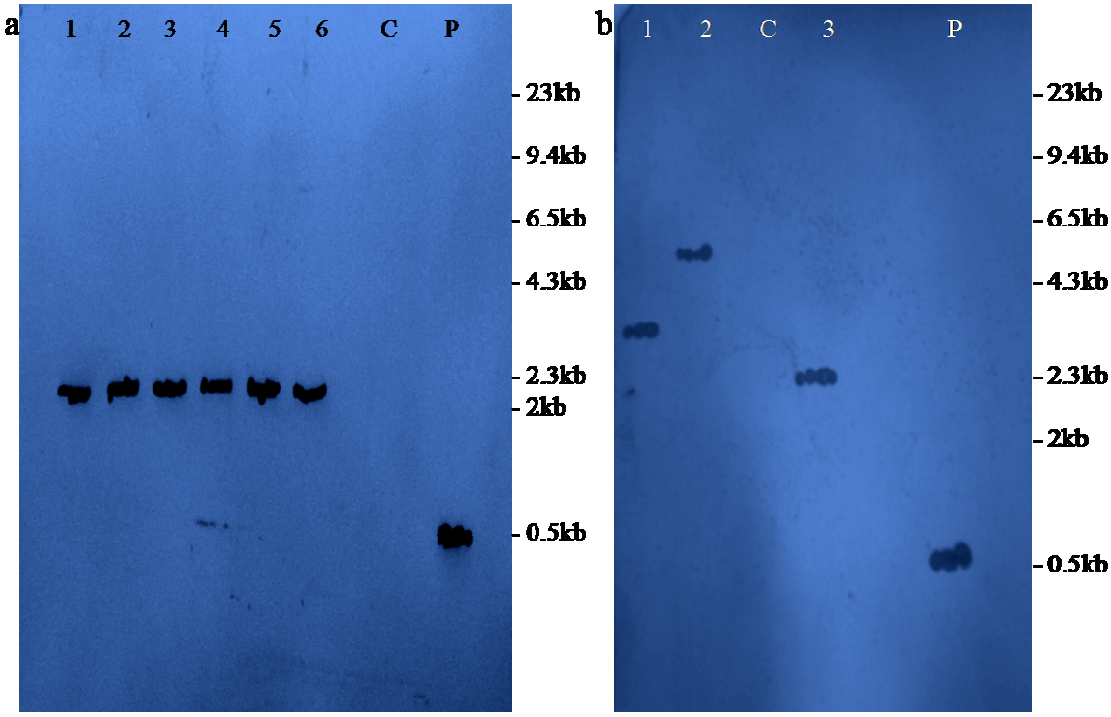
Chickpea lines	C_t value (mean±SE)	Genomic copy no. (Y)	Real-time PCR based copy no. (X)	Transgene copy no. (Z= X/Y)	Copy no. of transgene based on Southern hybridization
Control	40.0±0.00	$1.05x10^5$	0.00	0.0	0.0
$T_4$3-28	26.51±0.34	1.05×10^5	1.02×10^5	1.0	1.0
$T_4$6-19	26.72±0.52	1.05×10^5	0.85×10^5	0.8	1.0
TH2-44	26.43±0.36	1.05×10^5	1.12×10^5	1.0	1.0
TH2-166	26.69±0.27	$1.05 x10^5$	0.87×10^5	0.8	1.0
TC2-33	26.63±0.44	$1.05 x10^5$	$0.92x10^5$	1.0	1.0

Fig 1 The *cry1Ac/cry1Aa*$_3$gene PCR product detection in real-time PCR.



a) Amplification plots generated using known amounts of the pBinAR plasmid. b) Standard curve for data present in a. c) an overlay of melting curve derivative profile following real time PCR showing peaks for known amount of the pBinAR plasmid and *cry1Ac/cry1Aa*$_3$ transformed chickpea plants.

Fig.2: Southern analysis of the transgenic chickpea plants carrying *cry1Aa*$_3$ and *cry1Ac* gene



The genomic DNA samples were digested with *Hind*III restriction enzyme and PCR amplified product of target gene was used as a biotin labelled probe. a) Lanes 1- $T_4$3-28, 2- $T_4$3-43, 3- $T_4$6-19, 4- $T_4$6-41, 5- $T_4$7-10 and 6- $T_4$11-2 Transgenic chickpea plants carrying *cry1Aa*$_3$ gene. b) Lanes 1- TH2-44, 2- TH2-166 and 3- TC2-33; transgenic chickpea plants carrying *cry1Ac* gene; C- non-transgenic chickpea plant control; P- positive control.

Acknowledgments

Thanks are due to Dr. P Ananda Kumar, NRCPB, India for providing the synthetic *cry1Aa*$_3$ and *cry1Ac* gene constructs. We thank Dr. Minakshi for help and providing the facility for real-time PCR. We acknowledge UGC, New Delhi for Fellowship to KS.

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