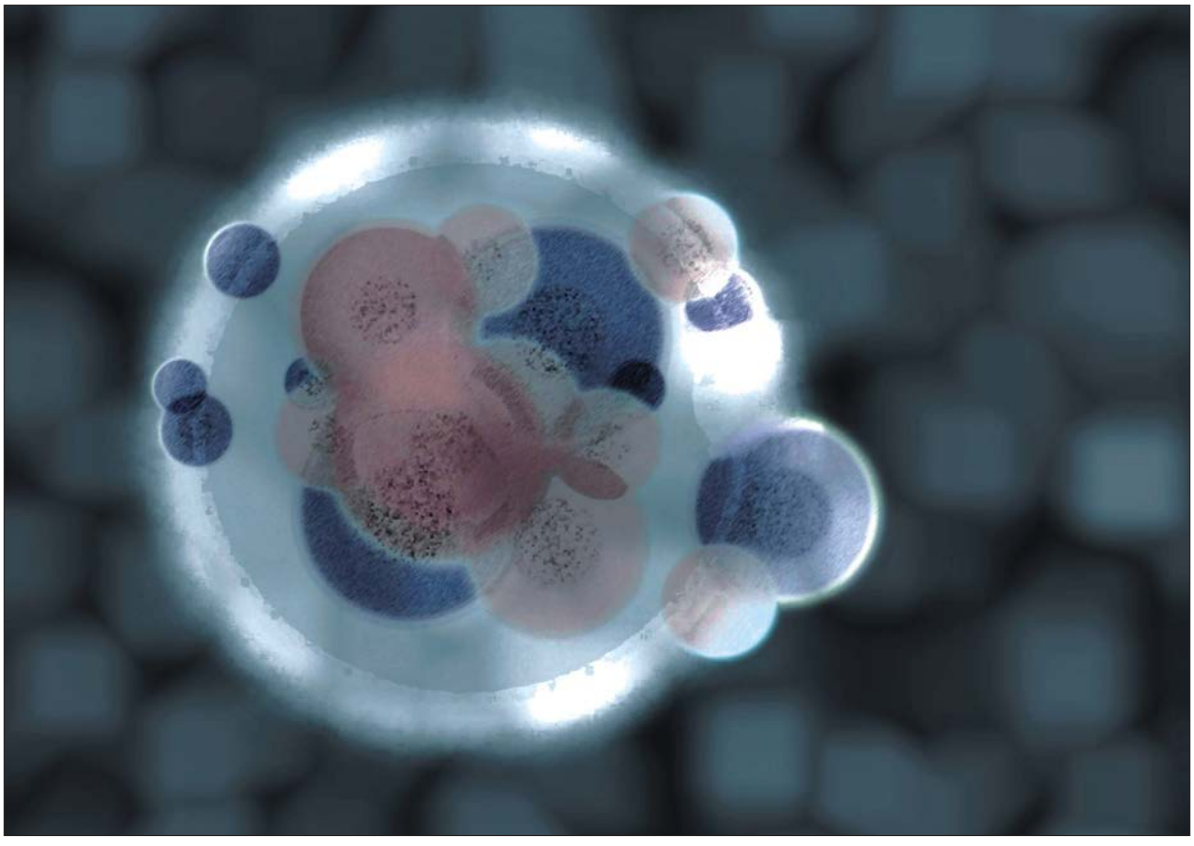

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 5 (3): 283-288, 2005
ISSN 1727-3048

Determination of *cryIAb* and *cryIAc* Copy Number in Transgenic Basmati 370 Rice (*Oryza sativa* L.) Plants Using Real-time PCR and its Comparison with Southern Blot

[1]Anwaar Ahmad, [1]Shahina Bano Maqbool, [2]Syed Anwar Hashsham and [1]Mariam B. Sticklen
[1]Department of Crop and Soil Sciences, 361 Plant and Soil Science Building,
[2]Department of Civil and Environmental Engineering, A16 Engineering Research Complex,
Michigan State University, East Lansing, MI 48824, USA

Abstract: We have developed a simple real-time PCR procedure for determination of transgene integration and copy numbers. Transgenic rice plants harboring *cry1Ab* and *cry1Ac* were produced via *Agrobacterium*-mediated transformation. Gene specific primers designed to amplify a relatively long amplicon, 542 bp for *cry1Ab* and 606 bp for *cry1Ac* were used to increase the specificity and sensitivity of the real-time PCR. The assay was carried out using the ABI Prism 7700 sequence detection system with Syber Green I as the fluorescence indicator. Using real-time PCR, we calculated 1-3 copies of transgenes (*cry1Ab*/*cry1Ac*) in our transgenic rice lines. The accuracy of real-time PCR results was determined with results obtained after Southern blot analyses. Comparison showed 75-100% accuracy of our Syber Green I real-time PCR with Southern blots.

Key words: *Agrobacterium*, gus, SYBR Green, TaqMan

INTRODUCTION

Production of transgenic plants via *Agrobacterium tumefaciens* is a routine procedure for most dicotyledonous and certain monocotyledonous plants. 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. Traditionally, scientists have been using Southern blotting[1] for identifying transgene insertion and copy numbers in transgenic plants. However, southern blotting is a labor intensive and relatively time-consuming technique.

Real-time PCR utilizes the speed, throughput and quantitative accuracy for amplification of a specific sequence of DNA or RNA[2-6]. Real-time PCR has become a popular technique since developed by Higuchi *et al.*[7]. Real-time PCR was developed to quantify the template DNA or to determine the transcribed RNA copies via continuous monitoring of fluorescent signal. This fluorescent signal generated by an introduced fluorescent reporter in the PCR reaction is proportional to the DNA concentration used for PCR. There are two basic chemistries that have been used in real-time PCR. One is the probe-based system, which requires a sequence-specific probe for quantitaion of the template of interest[5]. The second method utilizes intercalating fluorescence dyes that fluoresce only when bound to the double stranded amplified products[2]. Real-time PCR 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). The identification of the C_t value makes quantitation of DNA template in the PCR reaction precisely instead of measuring at the end of the reaction.

Sequence-specific probes e.g., TaqMan(R) system utilizes a fluorogenic probe (labeled with a fluorescent reporter dye on the 5' end and with a fluorescent quencher on the 3' end) that hybridizes within the target sequence flanked by regular PCR primers. Since, the quencher is located close to the reporter, it suppresses the reporter fluorescence. The 5' to 3' exonuclease activity of Taq DNA polymerase during PCR amplification degrades the hybridized probes, separates the two dyes and results an increase in fluorescence[4]. However, this technique requires a unique and specific probe for each template, which has to be designed and synthesized. Intercalating fluorescence dyes such as SYBR Green I provide a simple generic method for the detection of amplification product. SYBR Green I is a minor-groove DNA binding dye that exhibits enhanced fluorescence when bound to a dsDNA[8,9]. Although the SYBR Green I does not require probes, the quantification of template using dsDNA-binding dyes in this system is less sensitive[10]. To ensure the sensitive quantification of

Corresponding Author: Dr. Anwar Ahmad, Research Associate, University of Pittsburgh Cancer Institute Hillman Cancer Center, Research Pavilion 2.75 5117 Centre Avenue, Pittsburgh, PA 15213, USA
Tel: 412 623 7810 Fax: 412 623 7761 E-mail: ahmada2@upmc.edu

DNA, it is suggested to design highly specific primers and optimize the reaction conditions while using dsDNA-binding dyes.

Here, we present the use of SYBR Green I dye with real-time PCR to quantify the transgene copy number. This research was performed to show that SYBR Green I copy number assay, using gene specific primers for the amplification of relatively long amplified product, will allow the screening of transgenic plants with enhanced sensitivity and accuracy in a short time period. Present results suggest that real-time PCR could be used as an alternative to conventional Southern blotting for transgene copy number calculation saving considerable efforts and time.

MATERIALS AND METHODS

Plant materials: Five independent *Agrobacterium*-mediated *cry1Ab* and *cry1Ac* transformed Basmati 370 indica rice lines (Table 1)[11] were selected to develop the real-time PCR and Southern blotting. Untransformed wild type indica rice plant was used as negative control.

DNA isolation: Genomic DNA, from transgenic rice lines and untransformed wild type indica rice, was isolated using a phytopure plant DNA extraction kit (Amersham-Pharmacia Biotech, Piscataway, NJ). DNA was quantified spectrophotometrically and also flourometrically using Pico Green (R) double stranded DNA quantitation kit (Molecular Probes, Inc., Eugene, OR).

Southern blot analysis: Two binary constructs KUB and KUC were used for genetic engineering of Basmati 370 indica rice plants (Fig. 1). In these constructs, *gus* gene was fused to either *cry1Ab* or *cry1Ac*. Southern blot analysis was carried out by digesting 10 μg of genomic DNA from R0 and R1 transgenic rice plants with *Bam*HI or *Hin*dIII restriction enzymes and fractionated on a 0.8% agarose gel. The digested DNA was transferred onto Hybond-N^+ membranes (Amersham-Pharmacia Biotech, Piscataway, NJ) and hybridized with ^{32}P labeled *gus* probe and the coding sequences of *cry1Ab* and *cry1Ac* (*Bam*HI and *Eco*RI excised 1.8 kb fragment from pKUB and pKUC) according to standard protocols[12].

Table 1: Determination of transgene (*cry1Ab/cry1Ac*) copy number in transgenic Basmati 370 indica rice lines and the correlation of Real-time PCR results with Southern blots

Lines[1]	DNA (ng)	Genome Copy No. (Y)	C_t[2]	Real-time PCR[3] Copy No. (X)	Transgene Copy No. (Z)	Southren[5] Copy No.	Accuracy[6] %
370-ub-2	100	$2x10^5$	21.62±0.40	$1.5x10^5$	0.75	1	75
370-ub-7	50	$1x10^5$	20.54±0.34	$3x10^5$	3.00	3	100
370-uc-1	50	$1x10^5$	22.10±0.42	$1x10^5$	1.00	1	100
370-uc-2	50	$1x10^5$	22.33±0.74	$0.9x10^5$	1.00	1	100
370-uc-3	100	$2x10^5$	20.55±0.13	$3.1x10^5$	1.60	2	80
Wild type	100	$2x10^5$	40.0±0.00	0	0.00	0	100

[1]Basmati 370 transgenic rice lines; [2]Average of three replicates; [3]DNA copies calculated using C_t values after Real-time PCR and standard curve. [4]Transgene (*cry1Ab/cry1Ac*) copy number calculated as Z=X/Y; [5]Transgene (*cry1Ab/cry1Ac*) copy number as determined by Southern blots; [6]Correlation of Real-time PCR results with Southern analyses

Real-time PCR: Experiments were conducted in the ABI Prism 7700 Sequence Detection System (Perkin Elmer/Applied Biosystem, Foster city, CA) using 96 well microtiter plates. The amplification reactions were prepared in a total volume of 25 μL using Advantage-GC Genomic PCR kit (Clonetech Laboratories, Inc., Palo Alto, CA) containing template DNA (0-100 ng), 100 nM of each primer and 40,000 times dilution of SYBR(R) Green I (Molecular Probes, Inc. Eugene, OR). The PCR was initiated with denaturation step at 94°C for 2 min and then followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 45 sec and extension at 72°C for 1 min.

The primer sequences used in the reactions for the amplification of 542 and 606 bp coding region of *cry1Ab* and *cry1Ac* are as follows:

cry1Ab/cry1Ac Forward primer: 5'ACAGAAGACCCTTCAATATC 3'
cry1Ab Reverse primer: 5'GTTACCCTGATTGATAGGC 3'
cry1Ac Reverse primer: 5'GTTACCGAGTGAAGATGTAA 3'

Standard curve for transgene copy number was generated using pKUC (Fig. 1) at 0, 10, 10^2, 10^4, 10^6 and 10^8 copies. Analysis of data and threshold cycle (C_t) determination were accomplished using the ABI Prism 7700 Sequence Detection Software. The fluorescence of the SYBR Green I was monitored at the end of the denaturation and the elongation step of each PCR cycle. Data represent mean of three replicates.

After amplification, a melting curve was acquired by heating at 95°C for 20 sec and then cooling to 52°C for 20 sec and then slowly heating (0.01°C/sec) to 95°C for 20 min with fluorescence data collection at 0.2°C intervals, using ABI Prism 7700's software.

Calculation of gene copy number: Calculation of copy number using the Southern blotting was performed by digesting rice genomic DNA with *Hin*dIII. Since, *Hin*dIII does not restrict within the cassette of *gus* gene in the construct used for transformation, therefore, the number of bands observed after hybridization with *gus* probe reflected the number of copies inserted into the rice genome.

Calculation of copy number using the real-time RCR took place by using the software accompanying the ABI Prism 7700 instrument that detects the accumulation of

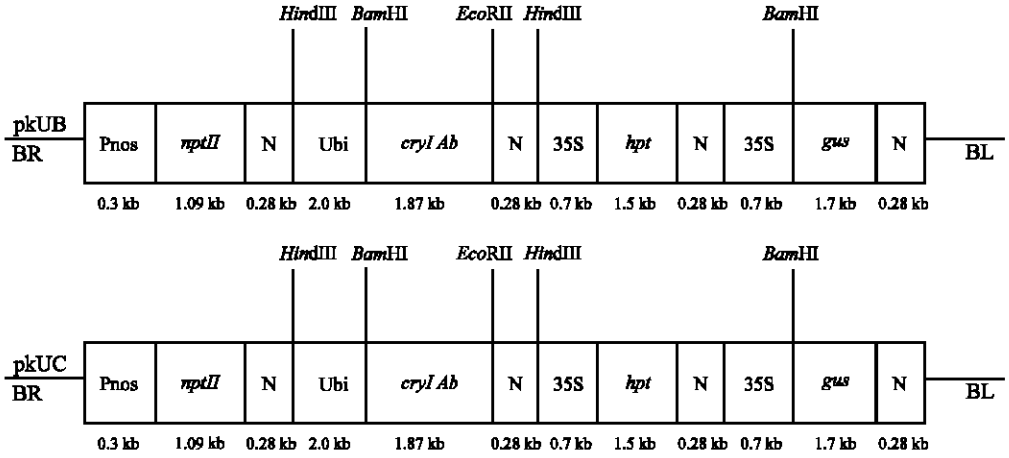

Fig. 1: Restriction map for the T-DNA of plasmid KUB and KUC. BR, T-DNA right border. BL, T-DNA left border. *cry1Ab* and *cry1Ac*, synthetic insecticidal genes from *B. thuringiensis*. *hpt,* Hygromycin phosphotransferase gene. *gus,* β-glucuronidase gene. *nptII*, Neomycin phosphotransferase gene. 35S, CaMV35S promoter. Ubi, Maize ubiquitin promoter. Pnos, Nopaline synthase promoter. N, 3' termination signal of nopaline synthase

PCR product by the increase in fluorescence. The accumulated fluorescence after normalization relative to established base line levels (ΔRn) was plotted against cycle number. The ΔRn values produce a real-time amplification plot for each well. The threshold was set at 10 times the standard deviation of the mean base line emission calculated between the 3rd and 10th cycles. The cycle at which the amplification plot crosses the threshold is defined as C_t, which reflects a positive result. The amount of DNA or transgene copy number in an unknown sample was calculated by interpolation from a standard curve C_t values generated using known amount of starting DNA concentrations. Since, one copy of rice genome contains 0.5 pg DNA[13], total rice genome copies (Y) in template DNA (50-75 ng) used in real-time PCR is 1-1.5 X 10^5 copies. Thus, the copies corresponding to each C_t 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

Molecular analysis of plants in R0 and R1 progeny: Southern blot hybridization has been used for determination of transgene(s) copy number in different plant species[14-16]. Southern hybridization experiments of transgenic plants using *Bam*HI showed different hybridization-banding pattern for each of the lines analyzed, which indicated the transgene integration in the rice genome and verified the independent transformation events. The results shown in Fig. 2a and b confirmed the integration of 1-3 copies of the transgene in R0 transgenic plants. Southern analyses of R1 progeny of independent transgenic lines 370-ub-3, 370-ub-4, 370-uc-1, 370-uc-3 and 370-uc-5 also confirmed the presence and inheritance of transgenes (Fig. 2a and b). Further southern analyses using *Hind*III confirmed the presence of full-length cassette for *cry1Ab* and *cry1Ac* genes with ubiquitin promoter and terminator (4.1 kb fragment) within the genomic DNA (Fig. 2c and d).

Development of standard curve for cry1Ab and cry1Ac using SYBR Green I: Quantification of target DNA using real-time PCR is done by generating a standard curve[2]. Therefore after optimization of primers and PCR conditions, we performed experiment with known amount of diluted DNA from 10-10^8 copies of *cry1Ab and cry1Ac* to generate a standard curve (Fig. 3a and b). The Ct values ranged from 12-40 cycles with a deviation of a maximum of 0.74 (Table 1). The C_t values were reproducible in replicate experiments. The correlation of coefficient (R^2) value was 0.9993 (Fig. 3b). There was a linear correlation between the amount of DNA used as template in the beginning of PCR reaction and the C_t values during amplification. Therefore, we used this standard curve to calculate the amount of DNA in our unknown transgenic samples.

Copy number of *cry1Ab* and *cry1Ac*: We were able to quantify 100% of the transgenes in rice plants. The C_t values and the calculated copy numbers using standard

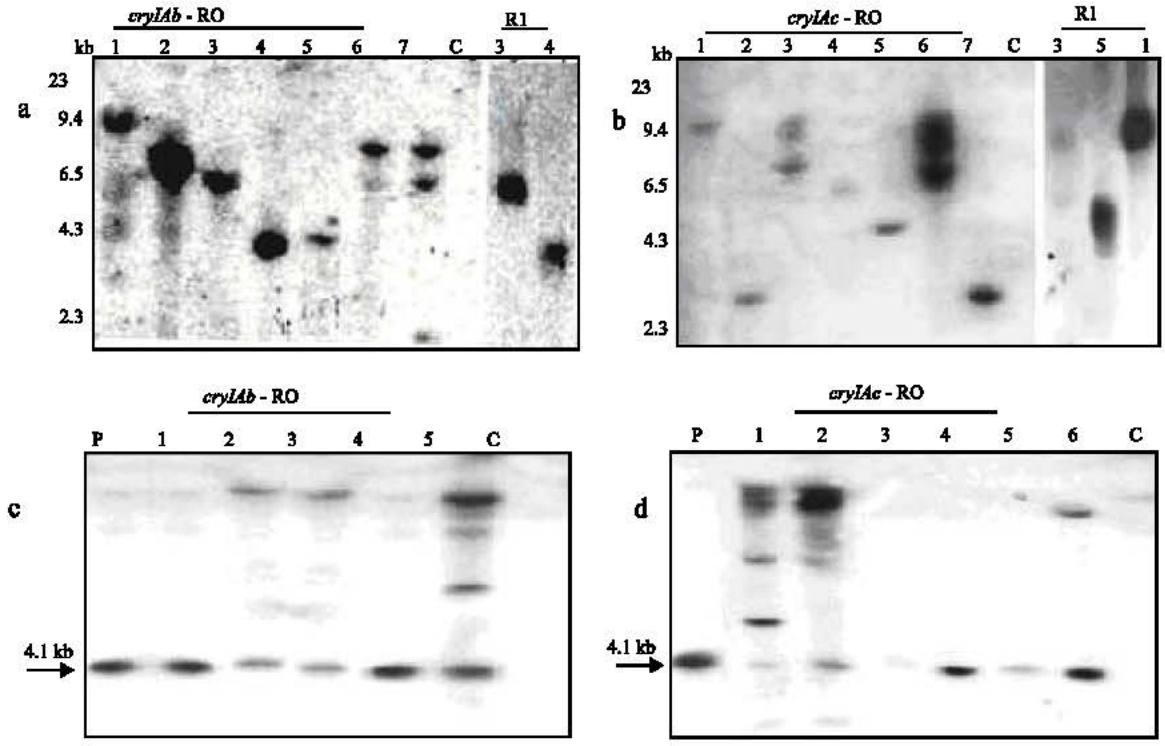

Fig. 2: Southern blot analyses of genetically engineered Basmati 370 indica rice plants obtained from *Agrobacterium*-mediated transformation. a and b) The DNA was restricted with *Bam*HI and probed with *gus* coding sequence in R0 and R1 progeny. c and d) The DNA was restricted with *Hin*dIII and probed with *cry1Ab* and *cry1Ac* coding sequences respectively. Lanes 1-7 in a: Basmati 370 transgenic lines 370-ub-1 to 370-ub-7 for *cry1Ab* and 1-7 in b: 370-uc-1 to 370-uc-7 for *cry1Ac*. P: plasmid DNA of *cry1Ab* and *cry1Ac*. C: DNA from non-transgenic plant

curve are given in Table 1. We calculated 1-3 copies of *cry1Ab* and *cry1Ac* in transgenic rice plants (Table 1). No amplification was recorded in the wild type rice plant DNA.

Accuracy of SYBR Green I copy number: Dissociation curve analysis has been used for product differentiation after PCR[17]. The dissociation curves analysis enabled us to assess the specificity and the purity of our real-time PCR reaction (Fig. 3c). We observed a single strong and consistent temperature of melting (Tm= 81.2°C) in all of our samples except in no template control (water) and wild type (non-transgenic) rice DNA samples, where no amplification was observed.

Copy Number Comparison Studies: We compared our real-time PCR copy number results with results (Fig. 3 and Table 1) obtained after Southern blot analyses (Fig. 2 and Table 1). Both results matched perfectly. The difference was that the real-time PCR gave results in few hours and the southern blot took several days.

The amplicon size in real-time PCR is generally short[18]. However, our results show that we have been able to optimize conditions for real-time PCR using SYBR Green I for specific targets by increasing the length of the amplicon three folds (from 200-600 bp) to reduce the non-specific amplification, which may be a problem in case of using low (50-200 bp) ampliconsin real-time PCR. Further, it was possible to characterize the transgene specifically and accurately in a short interval of time and with little efforts. The real-time PCR used also did not require any post-PCR manipulations because the C_t values reflect the exact picture of PCR products. In conclusion, we recommend the use of real-time PCR to determine transgene copy number due to its simplicity, efficiency and costs savings. This report may prove valuable in analysis of transgenic products without generating specific probes to be used in real-time PCR, such as in TaqMan(R) assay[3].

Level of expression of *cry1Ab* and *cry1Ac* in transgenic rice plants used in this study and their effect on controlling of insects has been reported elsewhere[11].

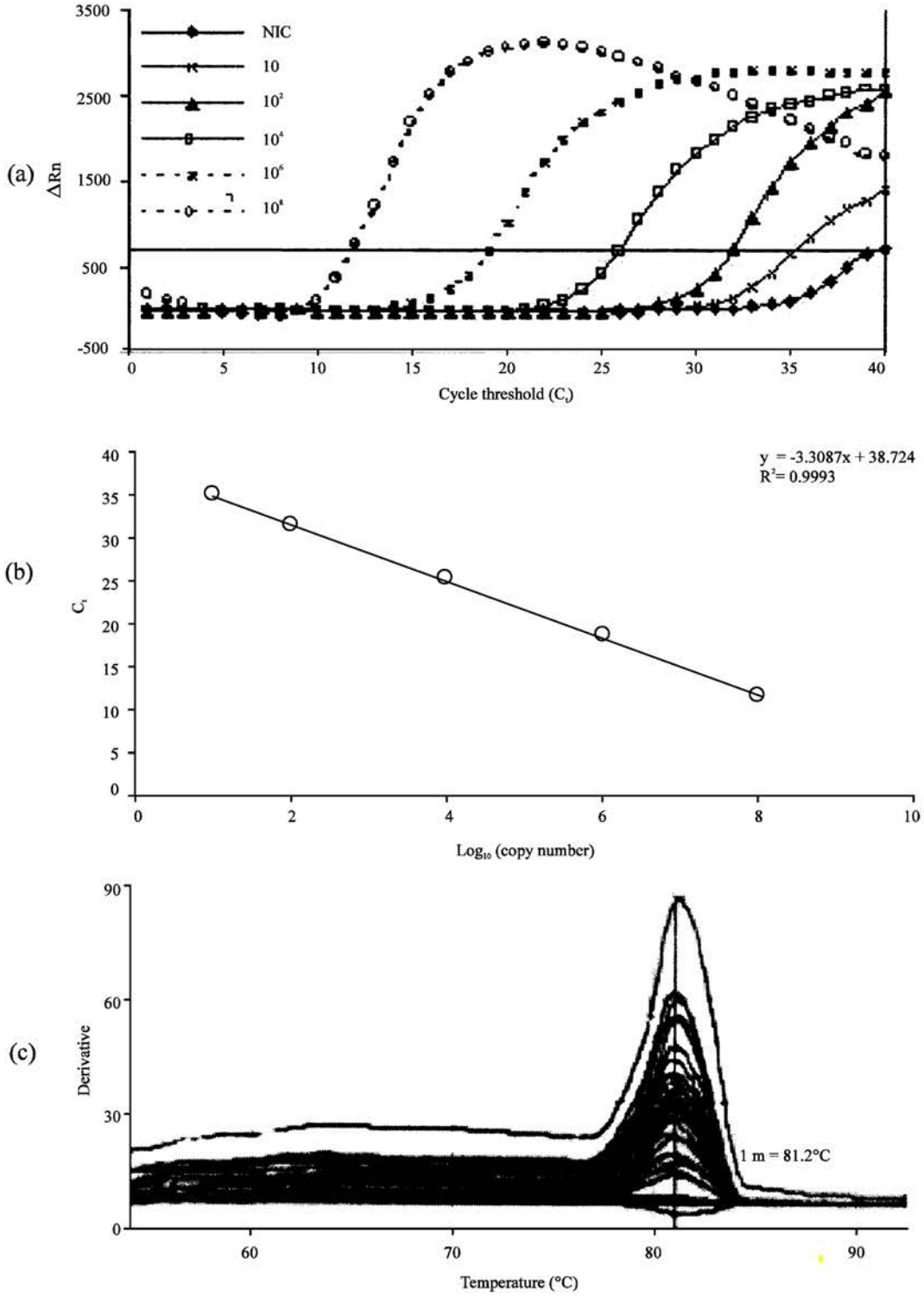

Fig. 3: *cry1Ab*/*cry1Ac* PCR product detection in Real-time. (a) Amplification plot generated using known amounts of pKUC. (b) Standard curve for the data presented in a. (c) an overlay of melting curve derivative profiles following Real-time-PCR showing peaks for known amounts of pKUC and *cry1Ab*/*cry1Ac* transformed plants

ACKNOWLEDGMENTS

We would like to thank Dr. Jeff Landgraf, Genomics technology support facility, Michigan state University, East Lansing, MI for technical advice on running the ABI 7700 Sequence detection system. We also thank Dr. T.A. Thorpe, (Ed.) IAPTC and B, for granting the permission to reuse the Fig. 2 from Ahmad *et al.*[11]. Financial support for this work from Intramural Research Grant Program of the Office of Vice President for Research at Michigan State University, Plant Breeding and Genetics program and Office of International Students and Scholars of Michigan State University is highly acknowledged.

REFERENCES

1. Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98: 503-517.
2. Higuchi, R., G. Dollinger, P.S. Walsh and R. Griffith, 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology, 10: 413-417.
3. Heid, C.A., K.J. Stevens, K.J. Livak and P.M. Williams, 1996. Real-time quantitative PCR. Genome Res., 6: 986-994.
4. Holland, P.M., R.D. Abramson, R. Watson and D.H. Gelfand, 1991. Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA., 88: 7276-7280.
5. Lee, L.G., C.R. Connell and W. Bloch, 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucl Acid Res., 21: 3761-3766.
6. Livak, K.J., S.J. Flood, J. Marmaro, W. Giusti and K. Deetz, 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Applied, 4: 357-362.
7. Gachon, C., A. Mingam and B. Charrier, 2004. Real-time PCR: what relevance to plant studies? J. Exp. Bot., 55: 1445-54.
8. Witter, C.T., M.G. Herrmann, A.A. Moss and R.P. Rasmussen, 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques, 22: 130-138.
9. Ponchel, F. *et al.,* 2003. Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. BMC Biotechnology, 3: 18.
10. Morrison, T.B., J.W.J.T.W.C., 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. BioTechniques, 24: 954-962.
11. Ahmad, A., S.B. Maqbool and M.B. Sticklen, 2002. Expression of synthetic cryIAb and cryIAc genes in Basmati rice (*Oryza sativa* L.) Variety 370 via Agrobacterium-mediated transformation for the control of European corn borer (*Ostrinia nubilalis*). *In vitro* Cell Dev. Biol. Plant, pp: 213-220.
12. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Ed. New York: Cold Spring Harbor Laboratory Press.
13. Bennett, M.D.I.J.L., 1995. Nuclear DNA amounts in angiosperms. Ann. Bot., 76: 113-176.
14. Hiei, Y., S. Ohta, T. Komari and T. Kumashiro, 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J., 6: 271-282.
15. Zhong, H., B. Sun, D. Warkentin, S. Zhang, R. Wu, T. Wu and M.B. Sticklen, 1996. The competence of maize shoot meristem for integrative transformation and inherited expression of transgenes. Plant Physiol., 110: 1097-1107.
16. Cheng, X.Y., R. Sardana, H. Kaplan and I. Altosaar, 1998. Agrobacterium-transformed rice plants expressing synthetic *cry1Ab* and *cry1Ac* genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA, 95: 2767-2772.
17. Riri, K.M., R.P. Rasmussen and C.T. Witwer, 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Ann. Biochem., 245: 154-160.
18. Bustin, S.A., 2000. Absolute quantification of mRNA using Real-time reverse transcription polymerisation chain reaction assays. J. Mol. Endocrinol., 24: 169-193.