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# Real Time quantitative PCR Analysis of Transgenic Maize Plants Produced by *Agrobacterium*-mediated Transformation and Particle Bombardment

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**Abstract:** Transgenic plants can be produced by particle bombardment and *Agrobacterium*-mediated transformation systems. As the new DNA is randomly inserted into the plant genome, often resulting in gene silencing in the transgenic plants with multiple transgene copies integrated into one or more chromosomal locations, estimation of transgene copy number is vital to genetically modified (GM) crop research. Transgene copy number is usually determined by means of Southern blot analysis, which is time consuming and laborious. In this study, quantitative real-time PCR technique was used to compare the RNA expression levels and transgene copy number of the *bar* gene in $R_0$ generation of transgenic maize lines generated by particle bombardment and *Agrobacterium*-mediated transformation methods. Our results demonstrated that transgenic events obtained from the *Agrobacterium*-mediated transformation method expressed the *bar* transgene at higher levels than those derived from bombardment transformation. Moreover, the copy number of the *bar* gene in the *Agrobacterium* derived transgenic events was lower than that in the bombardment derived events for the same inbred line.

**Key words:** Real time PCR, maize transformation, *Agrobacterium tumefaciens*, copy number, biolistic, transgene expression.

## INTRODUCTION

In transgenic plants, transgene copy number can greatly influence the expression level and genetic stability of the target gene. Thus, making estimation of transgene copy number and determination of the expression levels an important area of genetically modified crop research.

The analysis of transgenic plants can be as important as the transformation process itself. After producing the primary transformants, it is very important to decide which plants contain the transgene and in how many copies[7]. While multiple copies are useful for over-expression experiments, single or low copy transformation events are preferred for most applications because they are stable over several generation of subsequent breeding[24].

The analysis of transgene integration has been recently relied on PCR-based methods, which require only small amounts of plant material and are easily automated for high-throughput quantitation. Conventional PCR is commonly used for screening large numbers of putative transformants for transgenic sequences[23] and as an alternative to marker selection for segregation analysis[18]. However, conventional PCR analysis is plagued by false positives from contamination because a band with the size of the desired product is counted as signal, regardless of the intensity. Transgene copy number has traditionally been estimated by Southern analysis, although recently other methods, including comparative genomic hybridization, fluorescence in situ hybridization, multiplex amplifiable probe hybridization and microarray have been applied to copy number estimation. Unfortunately, all of these methods are laborious and time-consuming, require considerable amount of DNA from fresh or frozen samples and often involve the use of hazardous radioisotopes[34].

Real time PCR (ABI Prism 7700 Sequence Detection System) has been recently established as a rapid and sensitive technique for precise quantitation[13].

With real-time PCR as a quantification tool, it is possible to rapidly analyze large numbers of putative transformants from high-throughput transformation procedures[7]. Real-time PCR was initially used in plant research as a highly specific and sensitive detection

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tool. Specificity allows identification of plant pathogens by pathogen specific sequences and the sensitivity allows for the detection of small amounts of genetically modified plants in food. Since detection can also be performed with conventional PCR, the potential of real-time PCR is best realized when quantification is required[7].

In the present study, we used the real time PCR assay for fast and accurate estimation of the exogenous *bar* gene expression levels and copy numbers in transgenic maize plants produced by particle bombardment as compared to the *Agrobacterium*-mediated transformation method.

## MATERIALS AND METHODS

**Plant Material and Preparation of Explants:** Donor plants of the Egyptian inbred lines Giza 650, Sids 34, Sids 62 (Gz 650, Sd 62, Sd 34) and the American line A188 were grown in the field. The plants were self pollinated and immature embryos explants were excised 10 days after pollination to be used as explants.

**Plasmids and Bacterial Strains:** The standard binary vector pTF101.1 (9.2 kb) was used in transformation experiments. The plasmid contains the *bar* selectable marker gene driven by the CaMV 35S promoter (p35S). The bacterial strain EHA101 was used with the binary vector pTF101.1 for *Agrobacterium*-mediated transformation. The binary vector and the *Agrobacterium* strain were kindly provided from Dr. K. Wang, the director of the Plant Transformation Facility, Iowa State University.

**Particle Bombardment:** Immature embryos (1-2 mm in length) were aseptically excised and incubated for four to seven days at 25ºC on $N_6$ based (10) callus induction medium ($N_6$-Ag) containing 1.7mg/l silver nitrate and 2% sucrose[4].

The gene gun (Bio-Rad Biolistic PDS-1000/He) was used for the transformation of maize immature embryos. Particle bombardment experiments and selection of the transgenic materials were carried out as described by El-Itriby *et al*.[12]. Embryogenic scutellar tissues were placed on osmotic medium (callus induction medium supplemented with 0.2M mannitol and 0.2M sorbitol) for four hours and the osmotic treatment was continued for 16 hours after bombardment. Maize scutellar tissues were bombarded once at 1100 psi with sterilized gold particles (1.0µm) coated with plasmid DNA according to the modified protocol described by Zhong *et al*.[36]. Transformed maize tissues were incubated in darkness at 25ºC for four days before selection started.

***Agrobacterium*- mediated Transformation:** *Agrobacterium*-mediated transformation experiments were carried out according to the protocol described by Assem *et al*.[3]. After infection, embryos were transferred to the surface of cocultivation medium. Plates were incubated in darkness at 20°C for 3 days, after which, embryos were transferred to 28°C on resting medium. The components of the infection, cocultivation and resting media were described by Frame *et al*.[15].

**Selection of Transgenic Plants:** After 7 days on resting medium (for the Agro-experiments) and 7 days on bialaphos free callus induction media (for the bombardment experiments), calli were transferred to selection medium containing callus induction medium supplemented by 1.5 mg/L bialaphos. In addition to the bialaphos, the medium for the Agro-experiments was also supplemented by 250 mg/l cefotaxime. Calli were incubated on this medium for 2 weeks, then, they were further transferred to selection medium containing 3 mg/L bialaphos and subcultured every 2 weeks. Putatively transgenic events were identified and selected within 2 months and transferred to regeneration medium I for 2 weeks, followed by regeneration medium II for rooting as described by Frame *et al*.[14].

**Molecular Analysis of the Transgenic Plants:** Genomic DNA was extracted by CTAB method according to Aitchitt *et al*.[2] from leaves of putatively transgenic maize plants produced by bombardment and *Agrobacterium*-mediated transformation and from control (untransformed) plants. PCR technique was used to screen $T_0$ generation of transgenic plants for the presence of the transgene. The presence of the *bar* gene in the putatively transgenic plants was determined by amplification of a 484 bp PCR fragment corresponding to the *bar* gene using the primer pair specific for the *bar* gene, verifying transgenic events. Southern blot analysis was employed to confirm the integration of the bar transgene in these events.

To carry out the real-time PCR analysis, we choose four *Agrobacterium*-derived events and four bombardment derived events generated from independent experiments representing the four maize inbred lines, i.e. Sd62, Sd34, Gz650 and A188.

**RNA Isolation:** RNA isolation from one gram of all samples has been done according to Wang *et al*.[32]. Primer Express® software (version 1.0) was used to design and select primer and probe sets using default parameters according to the manufacture's recommendation. The chosen primer pair (RT-*bar* `5-GTCTGCACCATCGTCAA CCA-3` and RT-*bar*

`5-GCGGCTCGGTACGGAAGT-3`) were used to amplify 51 bases amplicon. Ribosomal RNA (rRNA) was used as an internal control as it constitutes 85-90% of total cellular RNA. In addition, various rRNA transcripts are transcribed by a distinct polymerase[27]. The rRNAs have been shown to be more reliable than some other housekeeping genes[5].

**Real time PCR analysis:** Two-step real time reactions were performed. In the first step, cDNA was synthesized using SuperScript® II RNase H as described by the manufacture. The second step involved the quantitation assays using the kinetic PCR instrument (ABI PRISM® 7900 Sequence Detection System "SDS"). Using SYBR® Green RT-PCR kit the reaction contained 4 ng of cDNA, 1ul of 10 nM of the specific primers, 7.5 ul of SYBR® Green PCR Master Mix, and up to 15 ul of deionized water. Results were analyzed using ABI PRISM® SDS software (version 2.0), and calculated using the comparative threshold cycle $C_T$ method according to the manufacturers' instructions for data normalization. Two different sets of 18S rRNA were used as internal control[25].

## RESULTS AND DISCUSSIONS

This work was designed to investigate the ability to determine the level of transgene expression as well as the copy number in transformed plant samples by using real-time quantitative PCR analysis.

To minimize the influence of uncontrollable factors during development of transgenic plants, a single plasmid (pTF101.1) and immature embryos of the same ears were used for both *Agrobacterium* and particle bombardment- mediated transformation. All the tissue culture procedures were the same except that resting and selection medium for *Agrobacterium*-mediated transformation contained cefotaxime to repress the growth of *Agrobacterium*.

About 500 maize immature embryos were used as target explants in the transformation experiments using the biolistic gene gun and the *Agrobacterium*-mediated transformation systems. Transgenic events were recovered from each transformation method and regenerated after selection on bialaphos containing media. Leaf painting assay using the herbicide "basta®" revealed some herbicide resistant lines. The transformation efficiency among different experiments ranged between 1 to 2 % for the Egyptian lines while it was about 4 % for the A188 line. Molecular analysis using PCR specific forward and reverse primers showed the presence of the 484 bp fragment corresponding to the *bar* transgene in the herbicide resistant events, (Fig.1). Moreover, Southern blotting analysis confirmed the integration of the transgene in the genome of the transgenic plants, (data not shown).

**Transcript Level of Transgene Detected by Real Time PCR:** In our study, The chosen transgenic maize lines, having different genetic background produced by particle bombardment and *Agrobacterium*-mediated systems (4 plants for each transformation method) (Table 1), were subjected to real time quantitative PCR analysis to detect the level of expression and the copy number of the *bar* transgene.

The threshold cycle ($C_t$) is the PCR cycle at which fluorescence exceeds background and a significant increase in fluorescence is observed[16]. $C_t$ value corresponds to PCR product accumulation, thus it is correlated with the starting template amount. A lower $C_t$ value implies a higher starting quantity of the nucleic acid target. Threshold is achieved during the exponential phase of PCR, where reaction components are not limiting, so $C_t$ values are reproducible. This leads to improved precision in DNA quantitation[16,20].

We calculated the transgene copy number according to the $2^{-\Delta\Delta C_t}$ method,[22,33,28,24,17]. Final copy number was calculated according to the following equation:

*Copy number* $= 2^{-\Delta\Delta C_t}$ *Where,* $\Delta\Delta C_t = C_t$ *(unknown sample)* $- C_t$ *(reference sample)*

As long as the target gene (*bar*) and "normalizer" (*18S*) have similar dynamic ranges, quantitation calculations are done by using the difference( $C_t$) in $C_t$ values between the target and the normalizer: $C_t = C_t$ (target, *bar* gene) – $C_t$ (normalizer, *18S* gene). This value is calculated for each sample to be quantitated, because the *18S* gene is presumably constant relative to total genomic DNA.

The present results revealed that the amount of *bar* transcript varied significantly among the selected events. The real-time assay was accurate and reproducible, as indicated by the low variation in $C_t$ values across replicates of the same sample template. Table (1) lists the mean ± standard deviation of $C_t$ values for three replicates of one sample from each transgenic line. The standard deviation ranged from 0.11 to 0.42, (Table 1).

The highest level of *bar* transcript (47.217) was obtained by the A188 line (American line) transformed by *Agrobacterium*, while the lowest level (21.207) was obtained by the Egyptian inbred line Gz 650 transformed by particle bombardment. The average expression level of the A188 event transformed by *Agrobacterium* was more than two times of the Gz 650 transformed by particle bombardment. Among the events transformed by *Agrobacterium* or bombardment, the A188 line revealed the highest level of expression of the *bar* transcript in comparison to the other

**Table 1:** The level of expression of transgenic maize $T_0$ plants produced by particle bombardment and *Agrobacterium* transformation methods.

| Plant # | Genotype | Method of transformation | PCR *bar* | Leaf painting | Gene expression level Mean $C_t$ ± standard deviation |
|---|---|---|---|---|---|
| | Sd62 | *Agrobacterium* | + | ++ | 28.124 ± 0.13 |
| | Sd62 | Bombardment | + | + | 23.237 ± 0.27 |
| | Sd 34 | *Agrobacterium* | + | +++ | 39.782 ± 0.22 |
| | Sd 34 | Bombardment | + | ++ | 26.361 ± 0.12 |
| | A188 | *Agrobacterium* | + | +++ | 47.217 ± 0.42 |
| | A188 | Bombardment | + | ++ | 29.875 ± 0.11 |
| | Gz 650 | *Agrobacterium* | + | +++ | 42.178 ± 0.23 |
| | Gz 650 | Bombardment | + | + | 21.207 ± 0.37 |

+++: Strong tolerance to the herbicide; ++: Moderate tolerance; +: Weak tolerance to the herbicide

**Table 2:** Average number of transgene insertions in each maize transgenic line.

| Plant # | Genotype | Method of transformation | Transgene copies/genome (mean ± standard deviation) |
|---|---|---|---|
| 1 | Sd62 | *Agrobacterium* | 6 ± 1 |
| 2 | Sd62 | Bombardment | 8 ± 1 |
| 3 | Sd 34 | *Agrobacterium* | 7 ± 2 |
| 4 | Sd 34 | Bombardment | 11 ± 1 |
| 5 | A188 | *Agrobacterium* | 3 ± 2 |
| 6 | A188 | Bombardment | 14 ± 2 |
| 7 | Gz 650 | *Agrobacterium* | 5 ± 1 |
| 8 | Gz 650 | Bombardment | 9 ± 1 |

Egyptian lines transformed by the two methods. Among the Egyptian lines, the highest average of transcript level of *bar* gene was revealed by the line Gz 650 transformed by *Agrobacterium*. The high expression of the transgene reflects the stability of this gene in the transgenic plants (Fig.1).

In general, the real time assay revealed that the events transformed by the *Agrobacterium* showed high expression level of the *bar* transcript in comparison to the events from the same lines transformed by bombardment method. The American line A188 revealed higher level of expression of the *bar* transcript in comparison to the other Egyptian inbred lines. These results are in agreement with the leaf painting assay, were the *Agrobacterium* derived lines showed higher tolerance to the herbicide than the bombardment derived lines (Table 1).

A188 is considered one of the best maize inbred lines widely used in the transformation and regeneration of maize, it has high regeneration as well as transformation frequencies. Moreover, the A188 line is one of the parental lines used to generate the HiII line (A188 x B73)[1], which is considered the most ideal line for regeneration and transformation of maize and its transformation frequency is about 7.0 %[29] for the bombardment method and 5.0% for the *Agrobacterium* method[15]. On the other hand, the Egyptian inbred lines are considered as semi-tropical lines and show difficulty in tissue culture and their regeneration and transformation frequencies are relatively low[4,12].

The average transgene copy number per genomic equivalent for each maize line is presented in Table (2). The transgene was present at various levels in the different samples, with a range of approximately 10-fold, from 3 to 14 copies/genome. The Agrobacterium derived lines exhibited lower copy numbers for *bar* transgene per genome (from 3 to 7 copies) while the bombardment derived lines revealed higher copy numbers (from 8 to 14 copies).

Our data show that transgenic events obtained from the *Agrobacterium*-mediated transformation method express the *bar* transgene at higher levels than those derived from bombardment transformation. This result is in agreement with Shou *et al*.[29] where their results demonstrated that the *Agrobacterium*-derived maize transformants have lower transgene copies and higher and more stable gene expression than their bombardment-derived counterparts. Moreover, our results are in agreement with the current limited public information regarding transgenic cereal plants in which these two transformation methods are compared[11,35].

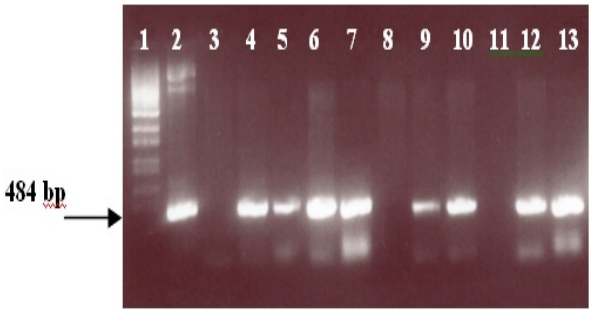


**Fig. 1:** PCR analysis of maize events transformed by *Agrobacterium* (9,10,12,13) and bombardment (4,5,6,7). Lane 1: 1 kb-DNA molecular weight marker, lane 2: Positive control (plasmid pTF101. 1), lane 3: negative control (untransformed DNA), lanes 8, 11: negative samples.

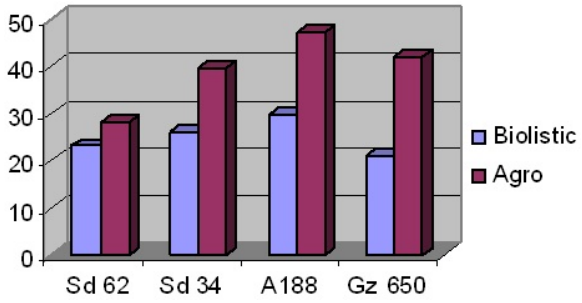


**Fig. 2:** Level of *bar* transgene expression in *Agrobacterium* and bombardment transgenic events as estimated by real time PCR.

On the other hand, real time PCR assay has also been used by[26] to determine the zygosity of transgenes in soybean and peanut. Yang *et al*.[34] used the real time PCR to confirm the results obtained by Southern blot analysis and determine the copy number and the level of expression in transgenic rice plants. With optimized PCR conditions they achieved significantly accurate estimates of one, two, three and four transgene copies in the $T_0$ transformants.

In conclusion, this study demonstrates the utility of the real-time PCR method for estimating the level of expression of integrated transgene and the transgene copy number in transgenic GM maize. Plant transformation methods such as *Agrobacterium*- and particle bombardment–mediated transformation result in multiple transgene copies at the same or different integration sites[19,31]. Southern blot analysis is usually used to determine transgene copy number. While routine and reliable, the method is time consuming, laborious, and requires large amounts of high quality DNA. To avoid these constraints, the quantitative real-time PCR assay was developed to determine transgene copy number in plants[8,9,17,30]. In addition, this method is sensitive enough to determine either a heterozygous gene deletion or duplication[6].

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