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Short communication

Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation

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Abstract

Particle bombardment and *Agrobacterium*-mediated transformation are two popular methods currently used for producing transgenic maize. *Agrobacterium*-mediated transformation is expected to produce transformants carrying fewer copies of the transgene and a more predictable pattern of integration. These putative advantages, however, tradeoff with transformation efficiency in maize when a standard binary vector transformation system is used. Using Southern, northern, real-time PCR, and real-time RT-PCR techniques, we compared transgene copy numbers and RNA expression levels in R_1 and R_2 generations of transgenic maize events generated using the above two gene delivery methods. Our results demonstrated that the *Agrobacterium*-derived maize transformants have lower transgene copies, and higher and more stable gene expression than their bombardment-derived counterparts. In addition, we showed that more than 70% of transgenic events produced from *Agrobacterium*-mediated transformation contained various lengths of the bacterial plasmid backbone DNA sequence, indicating that the *Agrobacterium*-mediated transformation was not as precise as previously perceived, using the current binary vector system.

Abbreviations: ADH – alcohol dehydrogenase; *bar* – phosphinothricin acetyltransferase gene; CaMV 35S – Cauliflower Mosaic Virus 35S RNA Promoter; GOI – gene of interest; GUS – β-glucuronidase; *nos* – nopaline synthase of *Agrobacterium tumefaciens*; *NPK* – *Nicotiana* protein kinase; PCR – Polymerase chain reaction; RT – Reverse transcription

Results

Generation of transgenic maize plants

We selected a subset of 24 *Agrobacterium*-derived events (designated A4-n) and 12 bombardment-derived events (designated P84-n) generated from eight independent experiments using the constructs illustrated in Figure 1. The gene of interest, *Nicotiana* protein kinase gene (*NPK1*), was regulated by a modified 35S promoter and a *nos* terminator (Kovtun et al. 2000). Expression of this gene is expected to enhance stress tolerance in maize (H-X. Shou, unpublished). Biolistic gun-mediated or *Agrobacterium*-mediated transformations using maize Hi II immature zygotic embryos were conducted as described (Frame et al. 2000; 2002). In both transformation methods, selection was carried out on 2 or 3 mg/L bialaphos. Average transformation efficiencies were 7.1% for the bombardment method and 3.3% for the

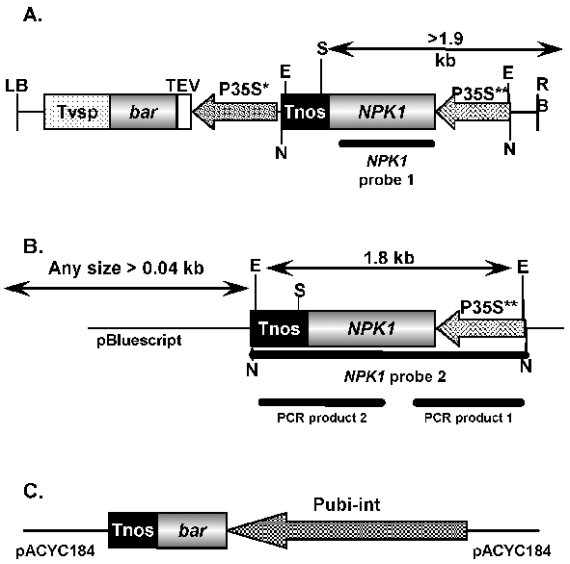

Figure 1. Constructs for maize transformation. (A) pSHX004, binary vector construct in *Agrobacterium strain* EHA101 for *Agrobacterium*-mediated transformation; (B) pSHX002 and (C) pBAR184, constructs used for co-bombardment transformation. LB, left border; RB, right border; *bar*, phospinothricin acetyl transferase gene; *NPK1*, *Nicotiana* protein kinase gene; P35S*, 2x CaMV 35S promoter; P35S**, a modified 35S promoter; Pubi-int; ubiquitin promoter with its intron (Christensen and Quail 1996); TEV, tobacco etch virus 5' untranslated region (Carrington and Freed 1990); Tnos, nopaline synthase terminator (Depicker et al. 1982); Tvsp, soybean vegetative storage protein terminator (Mason et al. 1993); T35S, CaMV 35S terminator; E, *Eco*R I; N, *Not* I; S, *Stu* I; NPK1 probe 1: 0.8 kb coding region of *NPK1* gene; NPK1 probe 2: 1.8 kb NPK1 gene with its promoter and terminator.

Agrobacterium method. R_1 seeds were obtained by out-crossing the transformants with untransformed Hi II or inbred line B73. Eighty-three percent (20 out of 24) and seventy-three percent (eight out of 11) of the *Agrobacterium*-derived and bombardment-derived events, respectively, segregated with a 1 to 1 ratio as expected for the transgene insertion at a single dominant locus (data not shown).

Copy number determination using Southern analysis and real time PCR

Southern blot assays were performed on a subset of transgenic events from each transformation method to estimate transgene copy number (Figure 2). For the bombardment-derived P84 events, genomic DNAs were digested with the restriction enzyme *Eco*R I, which excises a 1.8 kb fragment from the inserted pSHX002 DNA (Figure 1B). The digestion could also generate a fragment containing a small piece (0.04 kb) of the *nos* terminator, which could then be detected by the *NPK1* probe 2 (Figure 1B). The transgene copy number was estimated by counting the number of hybridization bands containing the *nos* terminator of the transgenic insertion (>0.04 kb, Figure 2A) and by comparing the density of the 1.8 kb drop-out hybridization bands with that of the positive control density (Figure 2A, CTR+). It was clear that most of P84 events contained numerous copies of the *NPK1* transgene (Table 1). All but two (P84-34 and P84-55) of the 11 transgenic events contained more than 10 copies of the transgene compared with the one copy positive control (CTR+). Events P84-44, P84-46, P84-50 and P84-54 might have more than 100 transgene copies.

For *Agrobacterium*-derived A4 events, genomic DNAs were digested with the restriction enzyme *Stu* I. Since the enzyme cuts only once in the T-DNA cassette, we could estimate the *NPK1* gene copy number by counting the number of hybridization bands. The copy number of the 11 A4 events ranged from one to nine (Table 1). Note that a common band around the 15 kb-position was observed (Figure 2B). This band was subsequently verified (Figure 2C) as a product of incomplete digestion of the genomic DNA used in Figure 2B.

Real time PCR using the Taqman assay (Boeckman et al., Bio-Rad Bulletin2620) was performed to quantify the copy number of the *bar* and *NPK1* transgenes. We used the dilution series of P84-44 genomic DNA to make the standard curves for the quantification of *NPK1* and *bar* DNA in all genomic DNA samples from the transgenic events. An intron sequence from *ADH1* (maize alcohol dehydrogenase gene 1, Osterman and Dennis, 1989), a known single copy gene sequence in maize was used as a baseline in our experiment. To account for variation in the amount of starting material between samples, the amount of *ADH1* DNA detected from the PCR reaction was used to normalize the amounts of *NPK1* and *bar* DNA, which are shown in Table 1. Since the A4-1 event had a single copy of the transgene in the Southern blot, we calculated the transgene copy number based on the amount of sample DNA relative to the A4-1 sample (Table 1). Copy numbers of the *NPK1* transgene detected from real time PCR analysis correlated with that from Southern analysis. The correlation coefficient between the *NPK1* transgene copies determined from the two methods (Southern and real time PCR) in the A4 events was 0.8455. Most A4 events

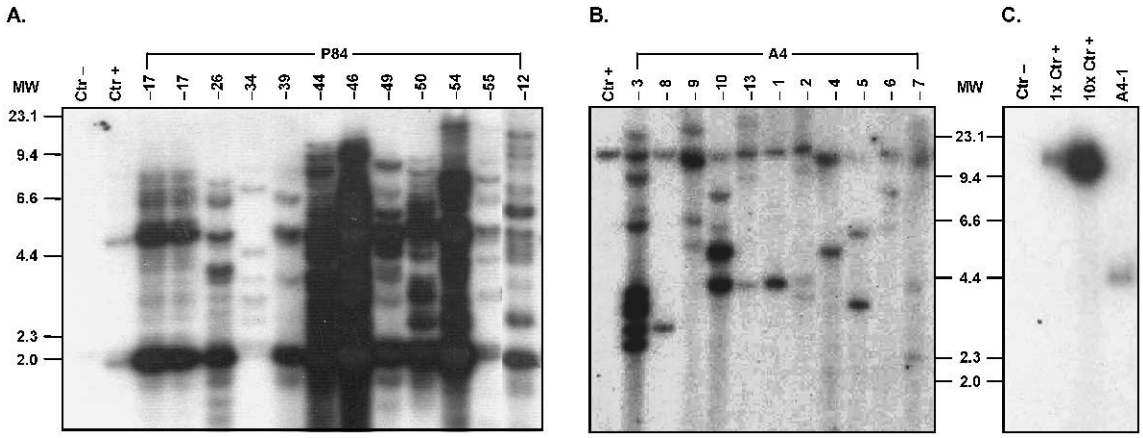

Figure 2. Southern blot analyses of P84 and A4 transgenc events. Ten micrograms of genomic DNA were digested with *Eco*R I (P84 events) or *Stu* I (A4 events) and probed with ^{32}P-labeled 1.8 kb NPK1 probe 2 or 0.8 kb NPK1 probe 1, respectively. Ctr-, negative control, genomic DNA from non-transformed maize inbred line B73; Ctr+, one copy of positive control, non-transgenic maize genomic DNA spiked with 27 pg of pSHX002 plasmid DNA digested with *Eco*R I (Blot A) or 62 pg of pSHX004 plasmid DNA digested with *Stu* I (Blot B). A. Bombardment-derived P84 events. B. *Agrobacterium*-derived A4 events. C. Southern analysis for verifying copy number of *Agrobacterium*-derived event A4-1. The 15 kb band seen in the A4-1 sample in Figure 2B was due to incomplete digestion of genomic DNA. 1x Ctr+ and 10x Ctr+ are one copy and ten copy positive control, non-transgenic maize genomic DNA spiked with 62 pg and 620 pg of pSHX004 DNA, respectively.

had similar copy numbers of the *NPK1* gene and *bar* gene, with a correlation coefficient of 0.9098.

Compared with the transgene copy number in A4 events, the majority of P84 events carried higher copy numbers of the *NPK1* gene. In only two of the 11 samples, P84-34 and P84-55, we could clearly count the hybridization bands from the Southern blot (Figure 2A). Real time PCR results indicated that the remaining events contained 8 to 277 copies of *NPK1* gene (Table 1). Interestingly, the *bar* gene which was co-bombarded with the *NPK1* construct had lower copy numbers in the same events, ranging from 1 to 21 copies (Table 1).

In summary, 92% (22 out of 24) of A4 events carried low copy number (<3 copies) and 8% carried medium (3 to 9 copies) to high (>10 copies) copy number of transgenes. Conversely, only 33% (4 out of 12) of P84 events contained low or medium copy numbers. Sixty-seven percent of the P84 events contained more than 10 copies of the transgene. Our data clearly shows that *Agrobacterium*-mediated transformation resulted in fewer copy numbers of the transgene in transformants.

Transcript level of transgene detected by northern analysis and real time RT-PCR

The amount of *NPK1* transcript varied significantly among the selected events as seen from northern analysis (Figure 3). These results were further confirmed by real time RT-PCR analysis. A4 events had a higher level of *NPK1* transcript than P84 events (Figure 4A and B). The average expression level of the A4 events was about four times higher than that of the P84 events. Forty-one percent of A4 events were high expressers (relative amount of NPK1 RNA $>$ 0.5), 55% were medium expresser (relative amount of NPK1 RNA between 0.1 to 0.5) and only 5% were low expressers (relative amount of NPK1 RNA $<$ 0.1). On the other hand, there were no high expressers among the P84 events based on these criteria. Forty-four percent of P84 events were medium expressers and 56% were low expressers. The amounts of RNA determined by real time RT-PCR were correlated with those from northern blot analysis in all the nine A4 events and 11 out of 12 P84 events. Event P84-49 had a higher transcript level in the real time RT-PCR analysis than that determined from northern Blot analysis. This may have been the result of a negative segregant plant being selected for northern analysis by PCR error.

Table 1. Trangene copy number estimation.

Events	Real Time PCR				Southern Blot
	Relative amount of DNA[a]		Copy #[b]		Copy #[c]
	NPK1	*bar*	*NPK1*	*bar*	*NPK1*
Agrobacterium −derived events					
A4-1	1.4	8.0	1.0	1.0	1
A4-2	3.8	19.3	2.7	2.4	3
A4-3	14.8	138.0	10.5	17.3	9
A4-4	3.9	40.5	2.8	5.1	1
A4-5	3.2	14.2	2.3	1.8	2
A4-6	1.9	7.5	1.4	0.9	2
A4-7	2.5	18.3	1.8	2.3	3
A4-8	1.0	6.9	0.7	0.9	1
A4-9	4.1	20.9	2.9	2.6	3
A4-10	2.8	3.3	2.0	0.4	4
A4-13	2.3	9.6	1.6	1.2	2
A4-14	4.5	14.1	3.2	1.8	NA
A4-15	2.4	14.8	1.7	1.8	NA
A4-16	1.5	9.7	1.0	1.2	NA
A4-17	1.7	5.5	1.2	0.7	NA
A4-18	4.9	31.9	3.5	4.0	NA
A4-19	1.7	17.4	1.2	2.2	NA
A4-20	2.0	5.3	1.4	0.7	NA
A4-22	1.0	12.2	0.7	1.5	NA
A4-23	2.0	11.7	1.5	1.5	NA
A4-24	0.9	2.1	0.7	0.3	NA
A4-28	1.7	10.9	1.2	1.4	NA
A4-29	3.0	16.8	2.1	2.1	NA
A4-32	2.6	5.7	1.8	0.7	NA
Bombardment −derived events					
P84-12	138.1	164.9	98.6	20.6	23
P84-17	41.8	53.9	29.9	6.7	19
P84-26	62.9	49.7	44.9	6.2	16
P84-28	0.7	9.3	0.5	1.2	NA
P84-34	2.9	9.1	2.0	1.1	1
P84-39	11.0	30.0	7.9	3.8	14
P84-44	170.3	103.9	121.7	13.0	35
P84-46	388.2	145.5	277.3	18.2	44
P84-49	56.9	28.1	40.6	3.5	28
P84-50	36.9	16.8	26.4	2.1	29
P84-54	211.1	70.8	150.8	8.9	42
P84-55	7.4	4.1	5.3	0.5	5

[a]Relative amount of DNA was calculated using the amount of *NPK1*or *bar* DNA derived by the amount of adh1 DNA in each event; [b]Copy number of *NPK1* or *bar* was calculated using the relative amount of DNA od for each line devided by that of A4-1; [c]The copy numbers were estimated from the Southern blots (Figure 2)by counting the number of hybridization bands for A4 events or using a densitometer for P84 events, respectively; NA Not accessed.

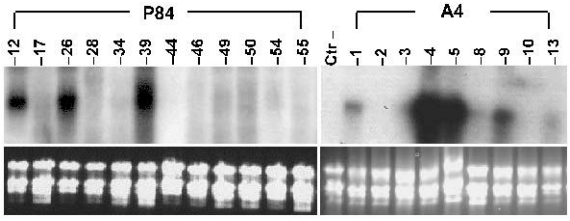

Figure 3. Northern blot analysis of P84 and A4 transgenic events. The ethidium bromide gel shown below the northern blot is the total RNA quantity loading control. The RNA gels were probed with a ^{32}P-labeled NPK1 probe 1. RNA from non-transformed maize inbred event B73 used as negative control (Ctr–).

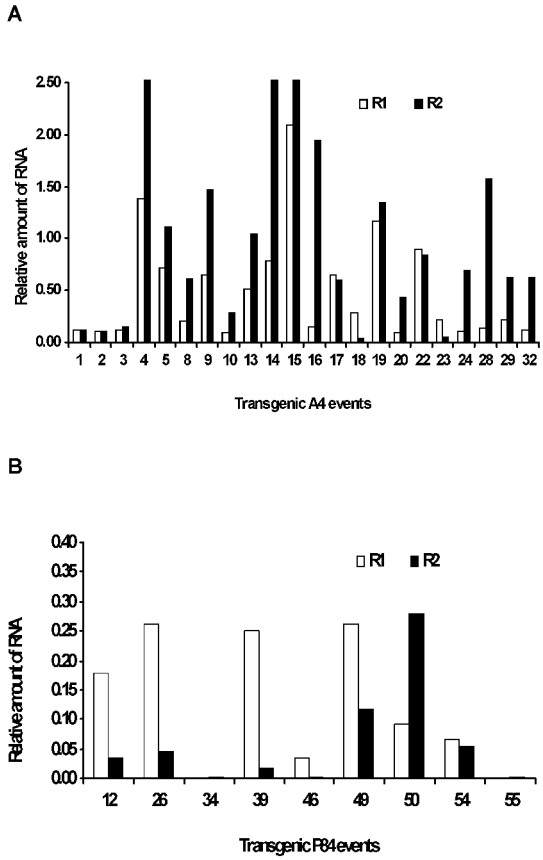

Figure 4. NPK1 transcript levels in the R_1 and R_2 transgenic plants. A. A4 events. B. P84 events. R_1, progenies of primary transformants generated through out-crossing the transformants with untransformed Hi II or inbred line B73. R_2 plants obtained from self-pollination of R_1 plants. Relative amount of RNA was calculated using the amount of *NPK1* transcript divided by the amount of 18S RNA in each sample.

Our data shows that transgenic events obtained from the *Agrobacterium*-mediated transformation method express the *NPK1* transgene at higher levels than those derived from particle bombardment transformation.

Table 2. Integration of vector backbone sequences into *Agrobacterium* − derived transgenic maize events.

Events	PCR Fragments ID*								
	1	2	3	4	5	6	7	8	9
A4-1	–	–	–	–	–	–	–	–	–
A4-2	+	+	+	+	+	–	–	+	–
A4-3	+	+	+	+	–	+	+	–	+
A4-4	+	+	+	+	+	–	–	+	+
A4-5	–	–	–	–	–	–	–	–	–
A4-6	+	+	+	+	+	+	+	–	–
A4-7	+	+	+	+	+	+	+	+	+
A4-8	+	+	+	–	–	–	–	–	+
A4-9	+	+	+	–	+	+	+	+	+
A4-10	–	–	–	–	–	–	–	–	–
A4-13	+	+	+	+	+	+	+	+	+
A4-14	+	+	+	–	–	–	–	–	–
A4-15	+	–	+	+	+	–	+	+	–
A4-16	–	–	–	–	–	–	–	–	–
A4-17	–	–	–	–	–	–	–	–	–
A4-18	+	+	+	+	+	+	+	+	+
A4-19	+	+	+	+	+	+	+	+	+
A4-20	+	+	+	–	–	–	–	+	–
A4-22	+	+	+	+	–	–	–	+	+
A4-23	+	+	+	+	–	–	+	+	+
A4-24	+	+	+	+	–	–	–	+	+
A4-28	+	+	+	+	–	–	–	–	–
A4-29	–	–	–	–	–	–	–	–	–
A4-32	+	+	+	+	–	–	–	+	+

*1-9: Fragments outside of T-DNA region. See Figure 5A for the coverages of these fragments.

NPK1 gene expression changes over generations

We tracked transgene expression from the R_1 to the R_2 generation in 22 A4 and nine P84 events. RNA expression levels in 77% (17 out of 22) R_2 A4 events and 11% (one out of nine) R_2 P84 events increased compared with their R_1 counterparts (Figure 4A and B). Average RNA levels doubled from the R_1 to R_2 generation for A4 events, while it was halved for P84 events. Our data indicates that transgene expression in *Agrobacterium*-derived transgenic events is more stable over generations than in the particle bombardment-derived events.

Integration of T-DNA binary vector backbone sequences

Nine pairs of primers (Table 2) covering regions outside the T-DNA borders were used in a PCR reaction to determine whether vector backbone sequences were also transferred to plant cells by *Agrobacterium* during transformation (Figure 5A). Results showed that 18 of the 24 (75%) A4 events (R_1 plants) carried some portion of an *Agrobacterium* vector backbone DNA fragment (Table 2). The extent and length of the tagged backbone DNA varied from event to event. Events A4-7, A4-13, A4-18, and A4-19 were PCR positive on all of the screened regions suggesting that the entire backbone region was transferred during transformation (A4-18 in Figure 5B). The other 16 events carried partial backbone regions (A4-3 and A4-8 in Figure 5B). Events A4-1, A4-5, A4-10, A4-16, A4-17, and A4-29 were free of backbone DNA (A4-1 in Figure 5B). PCR using primers 1, 2, and 3 that covered the region flanking the left border were positive on all of the 20 events that contained backbone DNA, indicating that the backbone DNA may be transferred by the incomplete cleavage of the left-border of the T-DNA. PCR product of fragment 8 in event A4-18 was smaller than that of the plasmid DNA control. This may be caused by rearrangement of the inserted DNA.

Discussion

Our study indicated that *Agrobacterium*-mediated maize transformation showed a number of advantages over the bombardment method including higher proportions of transgenic events with low copy number and high expression of these transgenes, as well as more stable transgene expression over generations. Although transformation efficiency is sacrificed (3.3% for Agro vs 7.1% for gun in this study) using this *Agrobacterium*-mediated method, we have shown that the resulting transformants have overall better qualities than their bombardment-derived counterparts.

The results we obtained from real-time PCR or real time RT-PCR technology for quantifying transgene copy number and expression level correlated well with those obtained using the traditional Southern and northern analyses. In addition, the high sensitivity and efficiency of the new technology allowed us to analyze more samples and quantify the transgene expression more accurately.

Our observations are in agreement with the current limited public information regarding transgenic cereal plants in which these two transformation methods are compared (Dai et al. 2001; Zhao et al. 1998). Dai et al. (2001) compared transgene copy number and GUS expression in transgenic rice events using Southern Blot analysis and a GUS activity assay, respectively.

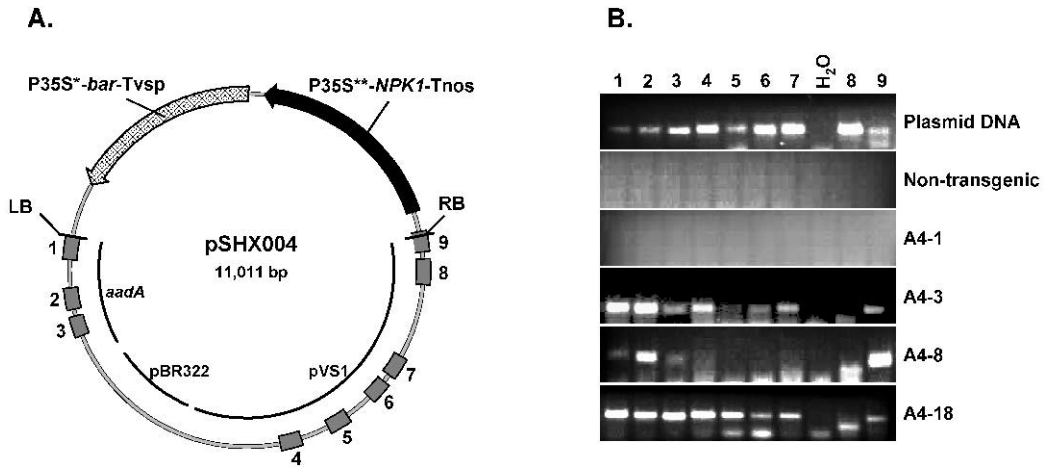

Figure 5. Integration of the *Agrobacterium* binary vector backbone DNA into transgenic maize events. A. Map of construct used for *Agrobacterium*-mediated transformation. B. PCR amplifications of backbone DNA fragments; lanes 1 to 9 correspond to the fragment 1 to 9 labeled on Figure 5A. P35S*-bar-Tvsp, bar gene with its double 35S promoter and *VSP* terminator; P35S**-NPK1-Tnos, NPK1 gene with its modified 35S promoter and *nos* terminator; LB, 25 bp direct repeat of left border; RB, 25 bp direct repeat of left border; 1 to 9: binary vector fragments outside the T-DNA region; coordinates of primers designed from these regions for PCR amplifications shown in Table 2. H_2O, negative water control.

Low copy number transgene integration using the super binary *Agrobacterium* system for maize transformation has been described in publications by Ishida et al. (1996) and Zhao et al. (1998). However, the stability of transgene expression over generations in the transgenic events produced by the two methods was not assessed in either publication.

In our study, the majority ($>$ 90%) of the transgenic events produced using *Agrobacterium*-mediated transformation contained fewer than 3 copies of the transgene. In contrast, most of the transgenic events obtained from particle bombardment had more than 3 copies with some having as many as 100 copies of the transgene. Furthermore, the *Agrobacterium* events in this study generally showed higher transgene expression than the bombardment events. The inverse correlation between transgene copy number and expression level that we observed supports the argument that multiple copies of a transgene may lead to co-suppression and silencing (Vaucheret et al. 1998; Fagard and Vaucheret 2000; Dai et al. 2001). Transgene silencing may explain the discrepancy between the *NPK1* and *bar* copy number. During the tissue culture process associated with transformation, transformed events were selected on bialaphos-containing medium. Thus, we expect that only the events properly expressing the *bar* gene survived selection and were regenerated to produce transgenic plants. It is possible that those events with *bar* gene copy numbers as high as their corresponding *NPK1* copy numbers simply did not survive selection due to silencing induced by excessive transgene copy numbers in such events. Because a direct correlation between copy number and expression of the transgene was not detected on an individual event basis in this study, it is likely that additional factors may influence transgene silencing, including methylation, DNA rearrangement, and chromatin structure of the surrounding area of the transgene insertion (Matzke 1994, 1996; Kumpatla 1998; Muskens et al. 2000; Vaucheret and Frgard 2001).

In this study, we used twice the amount of gene of interest (GOI) DNA (molar ratio) than selectable marker DNA in bombardment in order to achieve higher rates of co-transformation (*bar* gene and GOI). This excessive quantity of GOI DNA, however, may also have contributed to the phenomenon of high transgene copy number in the bombardment-derived events produced by the protocol. Using less GOI DNA or less total DNA approach in particle bombardment transformation may aid in reducing transgene copy number and improve transgene expression (Duncan and Spencer 2003).

Although *Agrobacterium* was believed to deliver only the DNA fragment in between the T-DNA border sequences, integration of non-T-DNA binary vector backbone sequences into the genome of transgenic plants frequently occurs (Martineau et al. 1994; Cluster et al. 1996; Kononov et al. 1997; Wenck et al. 1997). Kononov et al. (1997) demonstrated that about

75% of these transgenic tobacco plants contained various lengths of a *gusA* gene fragment placed outside of T-DNA region. Wenck (1997) showed that the frequency of vector backbone co-transfer ranges from 30-60% depending on plant species, *Agrobacterium* strain and transformation method. In our case, we observed that as high as 75% of R_2 transgenic maize events contained the backbone DNA sequence of the binary vector. These sequences could be the result of one large extended T-DNA segment that initiated from the right border, or the result of two separated T-DNA segments initiated from the right and the left border sequences, respectively.

It is known that the 25 bp direct repeats at the right and the left border regions delimit the transferred T-DNA segment and the transfer process occurs in a polar, right to left, fashion (Gelvin 2000). While the 25 bp repeats alone can promote T-DNA transfer, their flanking sequences on the wild-type Ti-plasmid enhance (on the right border) or attenuate (on the left border) their activity (Wang et al. 1987). This selective use of the T-DNA borders would lead to more effective T-DNA transfer events for *Agrobacterium* in nature by preventing the DNA transfer away from the tumor genes that reside on the T-DNA element. The vector used in this study is the derivative of pPZP vector (Hajdukiewicz et al. 1994) that contains the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens*. A Blast search showed that most of the commonly used binary vectors, including Bin19 based vectors (used by Kononov et al. 1997), pCAMBIAs, pPZPs (used in this study), pINDEXs contained a similar left border fragment of 320 to 550 bp in size (data not shown). Thus, if the flanking sequence of the left border is not strong enough to attenuate the transfer of vector backbone sequence during transformation, it is likely that backbone DNA contamination might exist more commonly in *Agrobacterium*-derived transgenic plants than previously thought when using these binary vectors.

Using transgenic maize plants carrying the gene of interest *NPK1*, we compared copy numbers and the expression levels of the GOI over two generations. Our comparative study was not perfectly parallel. In the *Agrobacterium* method, we placed both GOI and the *bar* gene cassette on one binary vector, while for bombardment, the GOI and the *bar* gene were on two separate plasmids and co-bombarded into maize tissue. However, our object for this comparative study was the *NPK1* gene cassette, which was identical in both systems.

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