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ORIGINAL PAPER

Effect of promoter driving selectable marker on corn transformation

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Abstract Identification of an appropriate selection agent and its corresponding selectable marker gene is one of the first steps in establishing a transformation protocol for a given plant species. As the promoter controls expression level of the genes, the promoter driving the selectable marker gene can affect transformation. However, investigations into the direct effect of promoters driving selectable marker on transformation are lacking in the literature though many reports of relative strengths of promoters driving reporter genes like *GUS* or *CAT* or *GFP* are available. In the present study, we have compared rice Actin1 and CaMV.35S (commonly used promoters in monocotyledonous plant transformation) promoters driving *nptII* for their effectiveness in paromomycin selection of transgenic corn events. To enable statistically meaningful analysis of the results, a large sample size of nearly 5,000 immature embryos (explants) was employed producing approximately 1,250 independent events from each of the two constructs in four independent experiments. The rate of appearance of resistant calli and percentage of resistant calli recovered was higher with P-Os.Actin1/*nptII*/nos3′ as compared to P-CaMV.35S/*nptII*/nos3′ in all four experiments. There was no appreciable difference either in the frequency of plant regeneration or in the morphological characteristics of plants recovered from the two constructs. Although the escape rate trended lower with P-Os.Actin1 as compared to P-CaMV.35S, the recovery of low copy events was significantly higher with P-CaMV.35S. The higher transformation frequency with P-Os.Actin1 could be related to the strength of this promoter as compared to P-CaMV.35S in the explants and/or calli. Based on these results, we infer that the promoter driving the selectable marker is an important factor to be considered while establishing a high throughput transformation protocol as it could not only influence the transformation frequency but also the copy number of the transgene in the recovered transgenics.

Keywords Selectable marker · *nptII* · Corn transformation · Promoters · Rice Actin1 · CaMV.35S

Abbreviations

bar/pat	Phosphinothricin *N*-acetyltransferase
CAT	Chloramphenicol acetyl transferase

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EPSPS-CP4	5-Enol-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. CP4
GFP	Green fluorescent protein
GUS	*β*-Glucuronidase
hptII	Hygromycin phosphotransferase II
nos3′	Transcriptional termination signal of *Agrobacterium* nopaline synthase
nptII	Neomycin phosphotransferase II
P-CaMV.35S	Cauliflower mosaic virus 35S promoter
P-Os.Act1	*Oryza sativa* Actin1 promoter

Introduction

Standardization of an effective selection process is critical for establishing efficient transformation protocols. In plant transformation, commonly used selective agents and their corresponding selectable marker genes include kanamycin and *nptII* (Fraley et al. 1983); hygromycin and *hptII* (Waldron et al. 1985); phosphinothricin and *bar/pat* (Wohlleben et al. 1988; De Block et al. 1987, 1989); glyphosate and *EPSPS-CP4* (Zhou et al. 1995). Other selection agents have also been described and are employed in different crop species with the corresponding selectable marker genes (Miki and Mc Hugh 2004). As different plant species and their different tissues/organs vary in their sensitivity to different selective agents, one of the first steps in establishing a transformation protocol is to screen the available selection agents. Several reports on the screening of a variety of selection agents are available (Dennehey et al. 1994; Li et al. 1997; Miki and McHugh 2004). Once identified, the selective agent and its corresponding selectable marker are employed in the actual transformation process.

In general, the selectable marker genes used in plant transformation are driven by strong constitutive promoters. Among the promoters used to drive selectable markers, P-CaMV.35S (Guilley et al. 1982) is commonly employed during transformation for both monocot and dicot plant species. In fact, a number of plant transformation vector series including pPZP family vectors (Hajdukiewicz et al. 1994), pCAMBIA series, pINDEX1 (Ouwerkerk et al. 2001) and pGreen (Hellens et al. 2000) have plant selectable markers driven by P-CaMV.35S. For monocot transformation, rice Actin1 (McElroy et al. 1990) or maize ubiquitin (Christensen and Quail 1996) and maize derived Emu (Last et al. 1991; Chamberlain et al. 1994) promoters are also being widely used. There are several reports where the above promoters have been indirectly compared based on their strength in terms of their ability to drive reporter genes like *GUS* or *CAT* or *GFP*. Transgene expression driven by the CaMV.35S promoter and maize ubiquitin (*Ubi-1*) promoter (with its first exon and intron) has been studied in rice, using the reporter gene *GUS* (Terada and Shimamoto 1990; Christensen and Quail 1996; Li et al. 1997). Tada et al. (1991) compared LHCP (Light Harvesting Chlorophyll binding Protein) promoter with CaMV.35S promoter using *GUS* as reporter gene. Schledzewski and Mendel (1994) compared maize polyubiquitin, rice Actin1, Emu and CaMV.35 S promoters in cells of barley, maize and tobacco using the reporter gene *GUS*. Similarly, CaMV.35S, maize Adh1 based Emu, rice Actin1 and maize ubiquitin promoters, coupled to the *GUS* gene, were evaluated for transient gene expression in tissues of tobacco, rice, tulip, lily, and leek (Wilmink et al. 1995). Kamo et al. (2000) compared CaMV.35S, rice Actin1 and *Arabidopsis* ubiquitin promoters to drive *GUS* in *Gladiolus* species. Upadhyaya et al. (2000) compared ubiquitin and CaMV.35S in rice. Able et al. (2001) evaluated ubiquitin, rice Actin1, and CaMV.35 S promoters using GUS as the reporter gene in *Sorghum bicolor*. Jang et al. (2002) reported comparative strengths of rice cytochrome c promoter, rice Actin1, and RbcS in rice using *GUS* and *GFP* as reporter genes. In general, in all the above reports, the relative strength of the monocot derived promoters like rice Actin1 or ubiquitin was found to be substantially higher as compared to CaMV.35S in monocotyledonous cells.

Selectable marker gene expression levels are likely to affect transformation. For instance, usage of a weak promoter might result in less than optimal level of expression of the selectable marker gene leading to reduced or non-recovery of transgenic plants. In a report of stably transformed wheat cultures using microprojectile bombardment, Vasil et al. 1991 described that the kanamycin resistance gene driven by CaMV.35S promoter, as being only partially effective owing to the natural resistance of wheat cell lines to kanamycin and less than optimal expression of the kanamycin resistance gene. On the other hand, very

strong promoters driving the selectable marker might result in leakage of the protein conferring resistance to adjacent non-transformed cells leading to cross protection and hence result in production of escapes. However, the impact of the strength of the promoter in front of the selectable gene on transformation frequencies is not well understood (Joersbo 2001).

In-depth studies on the comparative effect of promoters which are employed to drive selectable marker for transformation are lacking. Li et al. (1997) evaluated CaMV.35S, maize ubiquitin, and maize derived Emu promoters for driving hygromycin resistance gene for indica rice transformation. However, the comparison was limited to assessing the size of the GUS staining area of the transformed sectors of calli after 4 weeks of selection. The quantitative data on either the number of transformed sectors or the number of plants recovered with the different promoters driving the selectable marker gene was not reported.

Thus, though it is a common practice to examine different selection agents before standardizing a transformation protocol, the direct effect of promoters driving the selectable markers has not been well studied. In this study, we have compared the rice Actin1 promoter with the CaMV.35S promoter driving *nptII* for its effectiveness in corn transformation. Our results indicate that the promoter driving selectable marker can have a significant effect on transformation frequency as well as on transgene copy number.

Materials and methods

Plant material

Maize H99 plants were grown in greenhouse conditions and ears were pollinated using pollen from H99 donor plants. Ears from these H99 plants were collected 10–12 days after pollination (depending on the temperature and weather conditions) from the greenhouse.

Constructs used in transformation

The transgene expression cassette, P-Os.Actin1/nptII/nos3′ (2,495 bp) was isolated from the source plasmid pMON99666 by digesting with the restriction enzymes *Nhe*I and *Not*I, while P-CaMV.35S/nptII/nos3′ cassette (1,484 bp) was isolated from the source plasmid pMON78350 by digesting with *Hin*dIII. No vector backbone was present in either of the cassette. The cassettes were purified through gel elution.

Note: The P-Os.Actin1 promoter is the 5′ region of rice actin1 gene, the utility of which was first demonstrated in rice transformation by McElroy et al. (1990). The intron in the 5′ region was shown to be necessary for the higher expression. Hence the rice Actin1 promoter was used in its entirety with the intron.

Surface sterilization of cobs

Cobs were de-husked and sterilized in a 50% dilution of 5.25% sodium hypochlorite (Qualigens 27908) with 0.2% Tween-20 (USB 20605) for 20 min followed by three rinses with sterile water.

Explant preparation

Immature zygotic embryos (1.5–2.0 mm in length) were excised from surface-sterilized cobs and cultured onto the modified N6-basal (Duchefa C0204) medium (Chu et al. 1975) supplemented with 1.0 mg l^{-1} of 2,4-D (Sigma D7299), 100 mg l^{-1} of casein hydrolysate (Sigma C7290), 910 mg l^{-1} L-asparagine monohydrate (Duchefa A0725), 690 mg l^{-1} proline (Duchefa P0717), 16.9 mg l^{-1} silver nitrate (Merck 17528), 20 g l^{-1} sucrose (Qualigens 15955), and 6.0 g l^{-1} agar (Sigma A1296). A total of 4,761 immature embryos were used to transform the two constructs in four independent transformation experiments. Petri plates containing embryos were sealed using Parafilm® and incubated in dark at 25 ± 2°C.

Microprojectile bombardment

Four hours prior to bombardment, 3–5-day-old precultured immature embryos were arranged to form a 2-cm diameter circle at the center of a petri plate on osmotic medium, N6-basal (Duchefa C0204) medium (Chu et al. 1975) supplemented with 1.0 mg l^{-1} 2,4-D

(Sigma D7299), 100 mg l^{-1} casein hydolysate (Sigma C7290), 910 mg l^{-1} L-asparagine monohydrate (Duchefa A0725), 690 mg l^{-1} proline (Duchefa P0717), 16.9 mg l^{-1} silver nitrate (Merck 17528), 120 g l^{-1} sucrose (Qualigens 15955), and 6.0 g l^{-1} agar (Sigma A1296). Pre-cultured high quality embryos were randomly distributed between the two constructs. The immature embryos were bombarded using Bio-Rad (Hercules, California) Biolistic® PDS-1000/He Particle Delivery System. Selectable marker expression cassette DNA containing the *nptII* selectable marker gene driven by either P-Os.Actin1 (pMON99666) or P-CaMV.35S (pMON78350) was precipitated onto gold microprojectiles. For precipitation of DNA onto microprojectiles, 1.8 mg gold particles (0.6 µm Bio-Rad, Hercules, California) was suspended in 50 µl sterile distilled water and constantly vortexed while adding 100 ng of DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. This solution was vortexed for 3 min, briefly centrifuged and the supernatant was removed. The pellets were washed with 250 µl 100% ethanol, re-centrifuged, and finally resuspended in 60 µl 100% ethanol. Aliquots (15 µl) of this solution were spotted onto the center of four macrocarriers. The bombardment conditions were as follows: rupture pressure 1100 psi, vacuum pressure 25 in Hg, travel distance 11 mm, gap distance 0.25 in. Embryos were bombarded once and incubated in a dark chamber at 25 ± 2°C.

Callus selection and shoot regeneration

After 16–20 h of post-bombardment, pre-cultured embryos were transferred onto selection medium (SM), N6-basal (Duchefa C0204) medium (Chu et al. 1975) supplemented with 1.0 mg l^{-1} 2,4-D (Sigma D7299), 100 mg l^{-1} casein hydolysate (Sigma C7290), 910 mg l^{-1} L-asparagine monohydrate (Duchefa A0725), 690 mg l^{-1} proline (Duchefa P0717), 500 mg l^{-1} paromomycin (Sigma P8692), 20 g l^{-1} sucrose (Qualigens 15955) and 6.0 g l^{-1} agar (Sigma A1296). Embryogenic callus was selected and expanded by sub-culturing four times at 1–2 week intervals. At each subculture the callus was subdivided into small pieces (approximately 2–4 mm in diameter) and transferred to fresh selection medium. Calli produced from each embryo were kept separate from the calli produced from other embryos throughout different sub-cultures. After each transfer, plates were sealed using Parafilm® and incubated in a dark chamber at 25 ± 2°C. After 40–45 days on the selection medium, paromomycin tolerant callus was transferred onto shoot induction medium (SIM), a modified N6 (Duchefa C0204) medium (Chu et al. 1975) supplemented with 3.52 mg l^{-1} BAP (Sigma B3408), 100 mg l^{-1} casein hydolysate (Sigma C7290), 910 mg l^{-1} L-asparagine monohydrate (Duchefa A0725), 690 mg l^{-1} proline (Duchefa P0717), 250 mg l^{-1} paromomycin (Sigma P8692), 20 g l^{-1} sucrose (Qualigens 15955), and 6.0 g l^{-1} agar (Sigma A1296). The calli were incubated in dark at 25 ± 2°C for 7–9 days. Complete plantlets were regenerated after transferring the calli from SIM media onto regeneration and rooting medium (RRM), a modified Murashige and Skoog (1962) basal medium (Hi Media PT0018) supplemented with 100 mg l^{-1} paromomycin (Sigma P8692), 150 mg l^{-1} L-asparagine monohydrate (Duchefa A0725), 100 mg l^{-1} myo-inositol, 20 g l^{-1} sucrose (Qualigens 15955), 20 g l^{-1} maltose (Wartex SE) and 6.0 g l^{-1} agar (Sigma A1296) and incubation under 16 h light (General Electric cool white 110 W fluorescent bulbs, 50–100 µmol s^{-1} m^{-2}) at 25 ± 2°C in a growth room. Plantlets that developed in tissue culture bottles after 2–3 weeks of culture were then transferred to greenhouse for acclimatization. Although in many instances multiple plants were regenerated from callus out of a single embryo, only one plant was transferred to soil and considered one independent event for molecular analysis.

Transgene copy number analysis

DNA extraction

Genomic DNA was isolated from 100 mg transgenic corn leaf material at V3 stage using Qiagen DNeasy 96 plant kit (Cat. No: 69181). Total DNA was quantified using Nano Drop (ND-100 spectrophotometer) and used for Invader assay.

Invader assay

Invader assay was performed to determine the *nptII* transgene copy number in transgenic corn plants

using '*nptII* for genomic corn DNA detection kit' developed by Third Wave AGBIO (Cat. No. 2328-31). Approximately 20 ng corn genomic DNA in a volume of 2.5 µl was denatured at 94°C for 5 min and immediately chilled on ice. The master mix was prepared by mixing 1.16 µl cleavase mix, 1.0 µl nptII probe mix and 0.3 µl $MgCl_2$ mix. The denatured DNA (2.5 µl) was mixed with the master mix (2.5 µl) and the reaction mixture (5 µl) was then incubated at 63°C for 4 h in Peltier Thermal Cycler (PTC200, M J Research). Assay plates were read in a Tecan plate reader and the copy number were estimated with reference to the Southern validated copy control plants.

Statistical analysis

The individual treatment means were analyzed using a generalized linear mixed model with fixed treatment effects and random experiment effects were used and proportions were analyzed with logit link. Estimates for this model were obtained using PROC GLIMMIX in SAS Version 9.1.3 (SAS Institute Inc., Cary, North Carolina, USA).

Results

Number of calli at different stages of selection

Paromomycin resistant calli were observed after 7–8 days of culture on the selection medium with either of the nptII constructs. Non-responding bombarded embryos and non-bombarded control embryos turned white during the same period of time. Healthy looking yellow Type-I calli were sub-cultured on to fresh medium every 1–2 weeks. There was no apparent difference in the appearance or in the vigor of the calli derived from the two constructs.

The percentage of embryos producing paromomycin resistant calli was determined at each subculture (Fig. 1). The reduction in the number of calli was sharper between SM1 and SM2 as compared to the reduction between any other selection stages. This reduction in the number of calli between SM1 and SM2 was steeper with P-CaMV.35S/*nptII*/nos3′ with about 42.6% of explants forming calli as against 73.5% observed with P-Os.Actin1/*nptII*/nos3′ in

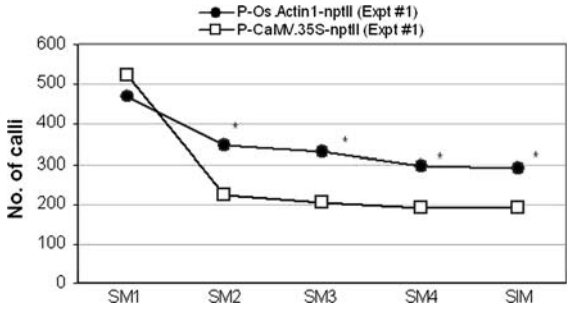

Fig. 1 Number of calli at successive selection steps following bombardment with P-Os.Actin1/*nptII*/nos3′ or P-CaMV.35S/*nptII*/nos3′. Value at SM1 represents the number of embryos used for bombardment, while values at SM2, SM3, SM4, and SIM represent number of calli at 2nd, 3rd, 4th selection steps and shoot induction medium, respectively. Data from only one experiment are represented. Values from the other two experiments showed a similar trend. Log-rank test was used to compare the two curves. The symbol * indicates highly significant difference between the treatments at the respective time point ($P < 0.01$)

experiment #1. A similar trend was observed in the other three experiments.

The number of calli recovered after four rounds of selections (approximately after 40–45 days on selection medium) is shown in Fig. 2. The percent recovery of resistant calli varied from 36 to 55% with P-CaMV.35S/*nptII*/nos3′ and from 42 to 62% with P-Os.Actin1/*nptII*/nos3′. The percentage recovery of

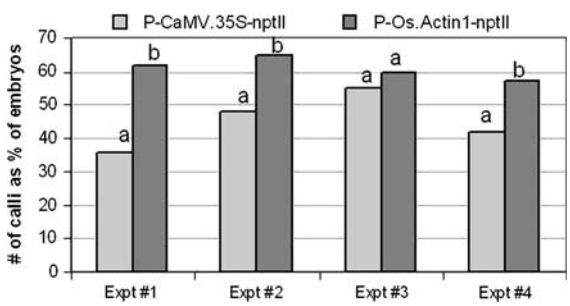

Fig. 2 Percentage of bombarded pre-cultured embryos forming callus on selection medium. Standard chi-square tests were done to see whether the two treatments were significantly different in each experiment. Different letters on each pair of bars indicates highly significant difference between the treatments within each experiment ($P < 0.01$). The predicted proportions of embryos forming calli in a large number of experiments are shown in the above figure as "Overall". The mean and 95% CI (confidence interval) for P-CaMV.35S was 45.9% (37.9%, 54.1%) and for P-Os.Actin1 was 60.6% (52.6%, 68.1%). The difference between the two overall means was significant ($P = 0.002$)

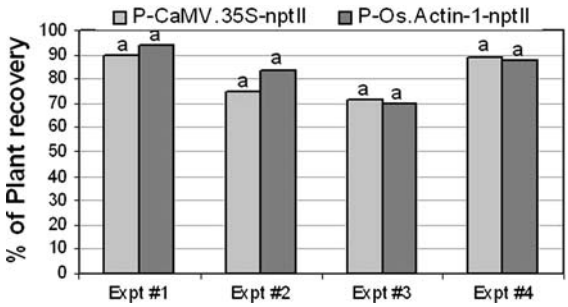

Fig. 3 Percentage of plants recovered from paromomycin resistant calli. Standard chi-square tests were done to see whether the two treatments were significantly different in each experiment. Different letters on each pair of bars indicates highly significant difference between the treatments within each experiment ($P < 0.01$). The predicted proportions for % Plant Recovery that would be obtained in a large number of experiments are included in the above figure as "Overall". The mean and 95% CI (confidence interval) for P-CaMV.35S was 82.4% (59.5%, 93.8%) and for P-Os.Actin1 was 85.1% (64.0%, 94.9%). The difference between the two overall means was not significant ($P = 0.37$)

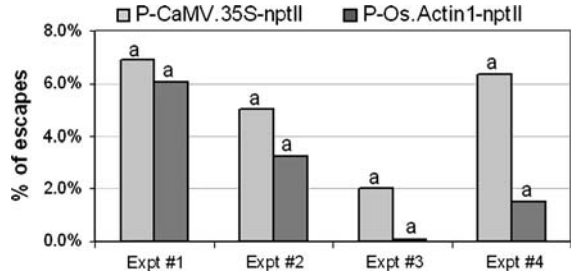

Fig. 4 Frequency of escapes (Invader-negative events). Standard chi-square tests were done to see whether the two treatments were significantly different in each experiment. Different letters on each pair of bars indicates highly significant difference between the treatments within each experiment ($P < 0.01$). The predicted proportions for % of Escapes that would be obtained in a large number of experiments are included in the above figure as "Overall". The mean and 95% CI (confidence interval) for P-CaMV.35S was 5.4% (3.2%, 9.1%) and for P-Os.Actin1 was 2.8% (1.3%, 5.7%). The difference between the two overall means was not significant ($P = 0.20$)

resistant calli with P-Os.Actin1/*nptII*/nos3′ selection was higher in all the four experiments as compared to P-CaMV.35S/*nptII*/nos3′ (Fig. 2).

Plant recovery after regeneration

Upon transfer to the RRM, shoot and root initials appeared after 7–10 days of culture under light. After 20–28 days of culture on the same medium, formation of plantlets with well-developed shoot and roots was observed. The plant regeneration frequency from the calli selected on paromomycin supplemented regeneration medium is depicted in Fig. 3. There was no appreciable difference between P-Os.Actin1/*nptII*/nos3′ and P-CaMV.35S/*nptII*/nos3′ in the percentage of plants recovered across the four independent experiments. Also, there was no visible difference in the quality of the plants produced (vigor or morphology) or in the regeneration time frame between the two constructs.

Frequency of escapes

The regenerated plants were tested for the presence of the transgene using Invader technology with probes designed for the *nptII* gene. The frequency of escapes (plants without the transgene) is shown in Fig. 4. The escape frequency ranged between 2 and 7% with P-CaMV.35S/*nptII*/nos3′, while it ranged between 0 and 6% with P-Os.Actin1/*nptII*/nos3′ across the four experiments. Though the escape frequency was lower with P-Os.Actin1/*nptII*/nos3′ as compared to P-CaMV.35S/*nptII*/nos3′ in the four transformation experiments performed, the difference was not statistically significant.

Transformation frequency (TF) was superior with P-Os.Actin1/*nptII*/nos3′

The comparative transformation frequency [(total number of independent events/number of precultured immature embryos bombarded) × 100], is shown in Fig. 5. The transformation frequency ranged from 42 to 54% with P-Os.Actin1/*nptII*/nos3′ while for P-CaMV.35S/*nptII*/nos3′ it ranged from 30 to 38%. The transformation frequency was consistently higher with Actin1/*nptII* as compared with CaMV.35S/*nptII*.

Effect of promoter driving *nptII* gene on copy number

Among the transgenic events produced, one and two copy events are the most useful for studying the transgene effect as multicopy events more often show

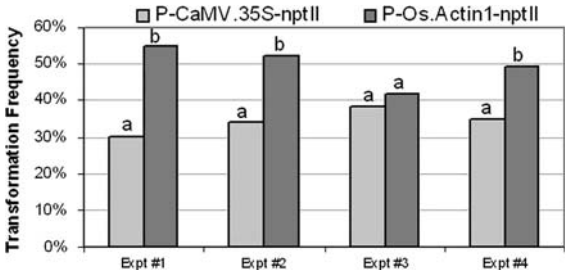

Fig. 5 Transformation frequency from four promoter comparison experiments. Transformation frequency was calculated as the number of Invader positive independent events divided by the number of precultured embryos bombarded then ×100. Standard chi-square tests were done to see whether the two treatments were significantly different in each experiment. Different letters on each pair of bars indicates highly significant difference between the treatments within each experiment ($P < 0.01$). The predicted proportions for % Plant Recovery that would be obtained in a large number of experiments are included in the figure ("Overall"). The mean and 95% CI (confidence interval) for P-CaMV.35S was 34.8% (31.7%, 38.1%) and for P-Os.Actin1 was 49.2% (46.0%, 52.3%). The difference between the two overall means was not significant ($P = 0.002$)

gene silencing or complex heritability pattern (Iyer et al. 2000; Altpeter et al. 2005). Therefore, in this study, the frequency of one and two copy events was examined (Table 1). The occurrence of transgenic plants with one and two copies of the transgene was higher with P-CaMV.35S driving *nptII* as compared to P-Os.Actin1. Conversely, the frequency of multi-copy events was higher in the transgenic events produced with P-Os.Actin1 driving *nptII* (Table 1).

Discussion

Surprisingly few reports exist in the literature describing direct comparisons of different promoters driving selectable markers, and these reports vary widely in their conclusions. Several early reports focused on increasing the level of selectable marker gene expression, following the hypothesis that more expression is better (Sanders et al. 1987; Maliga et al. 1988; Yenofsky et al. 1990). More recent reports have shown that the relationship between selectable marker promoter strength and transformation frequency varies. Mengiste et al. (1997) compared the 35S promoter to the 1′ promoter from *Agrobacterium* T-DNA and demonstrated that the weaker 1′ promoter gave significantly higher transformation rates in the *Arabidopsis* floral dip transformation system, using *bar* as the selectable marker. Li et al. (1997) compared three different promoters (35S, Emu, and Ubi) driving selectable markers in Indica rice and found that, of the promoters tested, the strongest promoter (Ubi) yielded more and faster growing callus sectors using the *hph* gene and hygromycin selection. An intermediate level of selectable marker gene expression was shown to be optimal using phosphomannose isomerase (PMI) as the selectable marker for sugar beet transformation (Joersbo et al. 2000). In this study, five promoters were compared, and a modified mannopine synthase promoter with an intermediate expression level, as compared to the other promoters, demonstrated the highest transformation frequency (Joersbo et al. 2000).

Table 1 Copy number distribution among the transgenic plants obtained after transformation with P-Os.Actin1/*nptII*/nos3′ or P-CaMV.35S/*nptII*/nos3′ (as determined by Invader)

Promoter driving NPTII	P-CaMV.35S	P-Os.Actin1
Low copy	134^{a} (56%)	110^{b} (44%)
Multiple copy	93^{a} (39%)	134^{b} (53%)
Escapes	13^{a} (5%)	7^{a} (3%)
Total	240 (100%)	251 (100%)

Different letters on each pair of values indicate highly significant difference between the treatments within each experiment ($P < 0.01$)

In the study described here, we used a large sample size of about 5,000 explants to produce approximately 2,500 independent transgenic events to test the effect of two different promoters driving the selectable marker. In this study, a sharp decrease in the number of surviving and proliferating calli was observed after first selection (SM2) with both of the promoters examined (Fig. 1). Following the first selection, very few proliferating calli ceased growing (Fig. 1), indicating that most of the selection takes place within the first 1–2 weeks of culture on selection medium. Hence, in this system it is likely important to use promoters which drive adequate expression of the selectable marker in the explant and the initial callus formation stage of the transformation process. P-Os.Actin1/*nptII*/nos3′ provided a statistically significant higher transformation frequency as compared to P-CaMV.35S/*nptII*/nos3′ (Fig. 5). Higher transformation frequency may be attributable to higher expression of *nptII*; P-Os.Actin1 is generally more

active than P-CaMV.35S in monocots (McElroy et al. 1990, 1991; Zhang et al. 1991; Christensen et al. 1992; McElroy and Brettel 1994; Schledzewski and Mendel 1994; Wilmink et al. 1995; Li et al. 1997; Kamo et al. 2000; Able et al. 2001; Jang et al. 2002). The frequency of shoot regeneration from the paromomycin tolerant calli did not influence the transformation frequency as the plant recovery on the paromomycin supplemented regeneration medium was similar for the two promoters (Fig. 3).

Theoretically, higher levels of expression of the selectable marker gene could lead to cross protection of neighboring non-transgenic cells in the explant or subsequent callus. This would result in survival and multiplication of non-transformed cells leading to higher plant escape rate. However, in the present study, the escape frequency was low for both the promoters tested and there was no significant difference between them (Fig. 4).

While transformation frequency was higher using P-Os.Actin1/*nptII*/nos3′, a significantly higher frequency of multiple copy events was observed with P-Os.Actin1/*nptII*/nos3′ as compared to P-CaMV.35S/*nptII*/nos3′ (Table 1). The increased occurrence of multiple copy events with P-Os.Actin1/*nptII*/nos3′ is difficult to explain. One possible explanation could be multi-copy P-CaMV.35S/*nptII*/nos3′ events may be more susceptible to gene silencing than multi-copy P-Os.Actin1/*nptII*/nos3′ events; however, this possibility needs further experimental verification. It should be noted that the two cassettes used in the investigation differ in their size. The P-Os.Actin1/*nptII*/nos3′ cassette is 2,495 bp while the P-CaMV.35S/*nptII*/nos3′ cassette is 1,484 bp. Hence, one might expect more (1.7 times) P-CaMV.35S/*nptII*/nos3′ cassette molecules delivered as compared to P-Os.Actin1/*nptII*/nos3′ cassette when the same quantity of DNA (100 ng) was used for bombardment. We have recently demonstrated that the transgene copy number is reduced by employing limited quantity of cassette DNA delivered (manuscript under preparation). Conversely, a higher number of DNA molecules delivered is expected to increase the frequency of multi-copy events. Hence, in the absence of a promoter effect, recovery of more multi-copy events with P-CaMV.35S cassette as compared to P-Os.Actin1/*nptII*/nos3′ cassette might be expected because the number of molecules delivered with P-CaMV.35S/*nptII*/nos3′ cassette was 1.7 times higher than that of P-Os.Actin1/*nptII*/nos3′ cassette. To the contrary, the recovery of more low copy events with P-CaMV.35S/*nptII*/nos3′ cassette in spite of it being delivered in higher numbers, implicates the effect of the promoter driving the selection marker on the resulting copy number of the transgenics.

In the present study, we show that the choice of promoter driving *nptII* has a significant effect on transformation frequency as well as on the copy number of the transgenic plants recovered using the H99-based corn transformation system. While P-Os.Actin1 provides higher transformation frequency, it was coupled with a higher frequency of multi-copy events. The data from this study as well as other studies cited above indicate that optimal results in a given transformation system are dependent upon the choice of promoter, but usage of a strong promoter does not always correlate with higher transformation frequency. It is likely that choice of an optimal promoter to drive a selectable marker is dependent on the plant species, the choice of explant, and the selectable marker system used.

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