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A comparison of strategies for transformation with multiple genes via microprojectile-mediated bombardment

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Abstract The stable insertion and expression of multiple transgenes in crops is highly desirable, as the manipulation of complex agronomic traits and the introduction of novel biosynthetic pathways are dependent upon it. This study was performed to explore the frequency and efficiency of introducing multiple genes in soybean by using somatic embryogenesis and microprojectile bombardment transformation. The co-transformation frequency of six selectable marker or reporter genes (*Gus*A, bleomycin resistance, glufosinate resistance, hygromycin resistance, green fluorescent protein, and kanamycin resistance) were followed throughout the T0, T1, and T2 generations. Three bombardment strategies were compared to determine the best method to generate transgenic plants that express the introduced transgenes and have a simple insertion pattern that would facilitate any downstream breeding. The plasmid bombardment treatments were (1) a six-gene-containing plasmid, (2) an equimolar treatment of five individual plasmids that collectively contained the six transgenes of interest (genes of glufosinate and hygromycin resistance were on the same plasmid), and (3) a 1:9 ratio mixture of the five plasmids, in which the plasmid containing the selectable marker used in the regeneration process, hygromycin resistance, was used in ninefold excess to all the other plasmids. Of the six bombardments performed per plasmid treatment, the results of seven independent events for the six-gene plasmid, four events for the 1:9 treatment, and a single regenerated event for the equimolar treatment indicate that containing all the transgenes on one plasmid just had an advantage in terms of frequency of a successful transformation events. Based on Southern analysis, the only events that contained all six transgenes was the one obtained by the equimolar treatment. No event was obtained that expressed all six transgenes, and certain transgenes seem to be non-randomly lost, namely *gusA*, bleomycin resistance, and glufosinate resistance, regardless of treatment. The addition of elements to optimize the expression of each gene cassette when multiple genes are in close proximity needs to be further investigated.

Keywords Co-bombardment · Gene stacking · Meganucleases · Multiple-gene transformation · Soybean

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Introduction

As new generations of transgenic traits get more sophisticated, there will be an ever increasing need to engineer plants with multiple transgenes. Multiple genes are necessary to obtain plants with multiple traits stacked in one plant or to engineer products that are the result of a metabolic pathway.

The challenges and approaches to engineering with multiple genes were addressed in detail by Halpin (2005). To obtain transgenic plants with a phenotype conditioned by multiple genes, plants can be individually transformed and then subsequently crossed together. However, having various independently segregating transgenes complicates the downstream breeding of transgenic cultivars, as each segregating transgene exponentially increases the size of

the segregation population necessary to obtain all the transgenes in one individual. Alternatively, a given plant can be repeatedly transformed with successive transgenes to obtain a multi-gene phenotype. Yet, having several effective selectable marker genes for the necessary rounds of selection is problematic, even when technology that permits removal of genetic markers is used to permit successive transformation cycles (Sugita et al. 2000). This approach is costly and time-consuming, as repeated cycles of transformation are needed to engineer with multiple genes. Hence, there is a need for an efficient method to link multiple gene expression cassettes together before transformation (Shibata and Liu 2000).

The technical difficulties traditionally associated with placing multiple genes in one vector (Shibata and Liu 2000) have left co-transformation with multiple constructs as the main available approach (Francois et al. 2002). Co-transformation is possible with both microprojectile bombardment (Hadi et al. 1996; Chen et al. 1998) and with *Agrobacterium* (Komari et al. 1996), although the number of genes successfully co-transformed with *Agrobacterium* has been far fewer than with microprojectile bombardment.

Microprojectile-mediated transformation simultaneously with multiple independent gene expression cassettes was successfully used to introduce three transgenes in wheat, with a 36% success rate (Campbell et al. 2000), and five genes in rice, with a 16% success rate (Agrawal et al. 2005). Higher numbers have been attempted, with transgenic plants being recovered having 11 of 14 bombarded plasmids (Chen et al. 1998).

Not only does transformation efficiency drop with increasing plasmid number, the use of multiple plasmids does not make it possible to control the stoichiometry between the different genes that get inserted into the genome. Furthermore, they do not always segregate as a unit (Chen et al. 1998; Campbell et al. 2000; Agrawal et al. 2005).

As an alternative, vectors have been designed that can incorporate many genes at once, such as vectors with polycistronic-coding sequences (Lough et al. 1997; Moser et al. 2000; Nakashita et al. 2001), but in the absence of a pre-assembled set of cistrons (Nakashita et al. 2001), the necessary set of coding sequences still has to be assembled together.

The main limitation to ligating multiple genes within one construct is that the multiple cloning sites (MCSs) of the vectors used for plant transformation are based on the standard bacterial type II restriction endonucleases, which recognize six-base or occasionally eight-base sites. The same restriction sites in the MCSs of vectors also occur often in the coding or regulatory sequences of genes inserted into the cloning site because of random chance alone, and the point comes when additional genes cannot be inserted into a vector without also cutting those previously inserted into the MCS (Überlacker and Werr 1996).

To bypass this limitation, vectors have been designed that use site-specific recombinases to incorporate multiple genes together (Lin et al. 2003) or vectors that use rare-cutter enzymes, particularly, homing endonucleases, also known as intron or intein-encoded endonucleases (Goderis et al. 2002; Thomson et al. 2002; Chung et al. 2005). Such vectors have been used to introduce six-gene cassettes via microprojectile bombardment into soybean (Thomson et al. 2002) and via *Agrobacterium* into tobacco (Thomson et al. 2002) and *Arabidopsis* (Goderis et al. 2002).

Thus far, research in the area of multiple gene insertion has seldom analyzed plants past the T0 generation. For example, Chen et al. (1998) cobombarded rice and obtained plants with up to 11 genes in them. However, expression was only tested for four markers.

Only limited information is available on T1 plants from *Agrobacterium*-mediated transformation (Lin et al. 2003). Lin et al. (2003) engineered rice with a seven-gene cassette. Nine T0 and six T1 plants were analyzed for the presence of seven genes, which were also targeted for expression analysis, based on reverse transcriptase polymerase chain reaction (PCR) in four plants—a sample too small to detect any overall trends in behavior of the transgenic gene cluster. Only the *bar* gene was specifically tested for function.

The objective of this study was to compare multiple-gene microprojectile-mediated transformation in soybean using either co-bombardment of multiple plasmids or bombardment with one large plasmid containing all the gene cassettes of interest. Besides comparing a single versus multiple plasmid strategy, this work extends the scope of analyses of T1 plants recovered after microprojectile co-bombardment of multiple gene cassettes, both in terms of number of traits and of number of progeny and events.

Materials and Methods

Plasmid DNAs and particle bombardment. All individual plasmids used and their assembly were described in Thomson et al. (2002). The plasmid pBarHyg (6.9 kb) containing the *bar* and *hph* genes, which encode resistance to the herbicide glufosinate and the antibiotic hygromycin-B, respectively, was used as the source of selectable markers. Four other plasmids were used that each contained a single reporter or selectable marker gene, namely *gusA*, an intron-containing β-glucuronidase (piGUS 6.7 kb); *gfp*, a red-shifted green fluorescent protein (pGFP 4.8 kb); *nptII*, encoding for neomycin phosphotransferase II, which confers resistance to the antibiotic kanamycin (pKan 4.7 kb);

and *ble*, a gene that confers resistance to the antibiotic bleomycin (pBleo, 4.9 kb). A single plasmid (p6-pack, 17.2 kb) containing all six gene-cassettes was constructed through the use of restriction enzymes that have long recognition sites as described by Thomson et al. (2002). The regulatory elements for each gene cassettes used were potato ubiquitin 3 promoter-*hyg*-nos terminator, nos promoter-*bar*-*Arabidopsi*s actin 2 terminator, cauliflower mosaic virus (CaMV) 35S promoter-red shifted *gfp*-nos terminator, actin 2 promoter-intron-containing -*gusA*-actin 2 terminator, nos *promoter-nptII*- actin 2 terminator, and CaMV35S promoter-*ble*-actin 2 terminator.

Three bombardment treatments were used, all with the final gold particle concentration of 1 μg DNA μl^{-1}, as follows: (a) six-gene-containing plasmid (p 6-pack); (b) 1:9 molar ratio of the hygromycin-containing plasmid to a mixture of the remaining four plasmids (p1:9), such that the hygromycin consisted of 10% of the total DNA weight, the remaining plasmids contributed 90% of the DNA weight; and (c) equimolar mixture of all five plasmids (p1:1). A diagram of p6-pack is in Fig. 1. Each preparation was enough for three separate shots.

The induction, maintenance, and transformation of soybean somatic embryos, cv Jack, using the microprojectile bombardment method were as described (Trick et al. 1997). Eight plates were bombarded per treatment, with each plate bombarded twice at 7,584 kPa (1,100 psi) and 914 mbar (27 in.) of Hg vacuum. Five days post-bombardment, tissue was placed in liquid Finer and Nagasawa lite medium (Samoylov et al. 1998) containing 20 μg ml^{-1} hygromycin-B (http://www.calbiochem.com). The medium was replaced weekly for 8 wk, at which time lines of hygromycin-resistant tissue were isolated. Transgenic tissues were transferred to soybean histodifferentiation and regeneration medium as described by Schmidt et al. (2005). Tissues from these primary cultures, as well as their progeny (T_1 generation), were analyzed for the presence of the six transgenes.

Analysis of transgenic tissue and plants. The presence of the transgenes was preliminarily determined by PCR analysis. Genomic DNA was isolated according to Doyle and Doyle (1990) and quantified by a TKO 100 mini-fluorometer (http://www.hoeferinc.com). The primer sequences are listed in Table 1. PCR conditions were as recommended by the DNA polymerase manufacturer's instructions (http://www.stratagene.com). PCR was also used to detect the presence of the transgenes in the T0, T1, and a limited sample of the T2 generation.

For Southern blot analysis, 10 μg DNA were digested with *Eco*RI and, subsequently, run on a 0.8% agarose gel and, in turn, transferred to a Hybond-N+membrane (http://www.amershambiosciences.com). General hybridization and washing was performed as described in Sambrook et al. (1989). Blots were hybridized in a modified Church's buffer (Church and Gilbert 1984). Gene-specific DNA probes, each less than 1 kb, consisted of gel-purified PCR amplicons, which are indicated in Fig. 1. Probes were labeled using Rediprime II random priming labeling kit (Amersham) along with ^{32}P-dCTP (http://www.appliedbiosystems.com). Unincorporated nucleotides were removed using a MicroBio Spin 6 chromatography column (http://www.bio-rad.com). After washes, membranes were exposed to BioMax™ autoradiography film (http://www.kodak.com) at −80°C until banding patterns were visible.

Expression of transgenes. In vitro-grown material was used to assess transgene expression in primary transformants and in 20 progeny obtained from each primary transgenic. Specifically, GFP was detected using a Nikon TE inverted microscope equipped with the appropriate filter set and a mounted charge-coupled device camera, in conjunction with IP Lab Spectrum imaging software (http://www.scanalytics.com). GUS activity was determined by application of X-Gluc (www.biosynth.com) and assessed according to Jefferson (1987). NPTII activity was measured by enzyme-linked immunosorbant assay using the Agdia kit

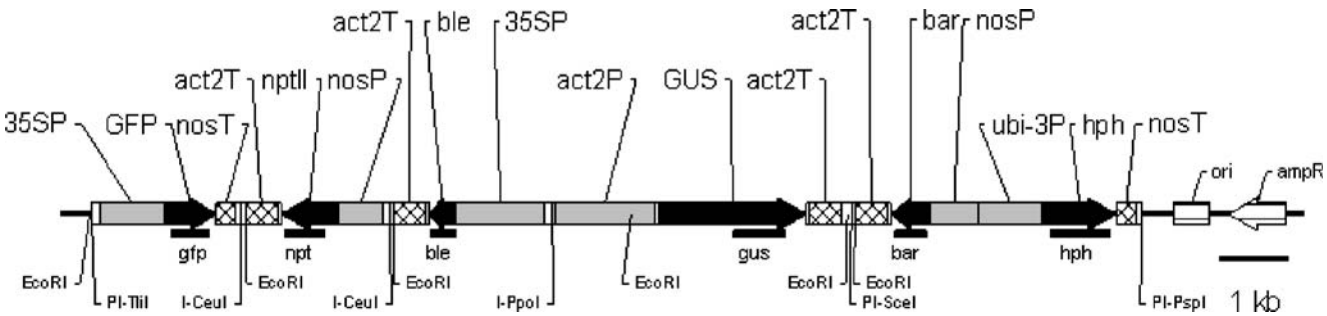

Figure 1. The six-gene-containing p6-pack vector. The plasmid is 17,236 bp long. *Lines* underneath each gene indicate the areas analyzed by PCR, using the primers and conditions described in Table 1. These amplicons were also used as probes for Southern blot analysis. *Eco*RI sites are indicated, as *Eco*RI was used to digest the genomic DNA of the transformants. *Open rectangles* are MCSs carried in from the original cloning vectors.

Table 1. Primer sequences and PCR conditions used

Gene	Forward primer-3′	5′-Reverse primer-3′	Annealing temperature
Bar	ACCATCGTCAACCACTACATCG	AAACCCACGTCATGCCAGT	55°C
Ble	GCAAGCAATTCGTTCTGTATCAGG	CATGAGATGCCTGCAAGC	58°C
Hph	CGATGTAGGAGGGCGTGGATA	CTTCTGCGGGCGATTTGTG	60°C
gusA	GTGGCAGTGAAGGGCCAACAG	GCAGCCCGGCTAACGTATCC	60°C
nptII	AGAGGCTATTCGGCTATGACT	CCATTTTCCACCATGATATTCG	55°C
Gfp	TGGAGAGGGTGAAGGTGATGC	CGAAAGGGCAGATTGTGTGG	58°C

Thermal cycling consisted of one cycle at 94°C for 4 min, 40 cycles at 94°C for 45 s, annealing for 45 s, 72°C for 60 s, and a final extension at 72°C for 7 min.

(Elkhart, IN) as per manufacturer's instructions. For primary transformants, embryogenic clusters were tested for resistance to hygromycin, glufosinate, and bleomycin by growing tissue on MSD20 medium (Trick et al. 1997) supplemented with 20, 8, or 100 mg l^{-1} of the respective selection agent for 2 mo. For progeny analysis, the expression of hygromycin, glufosinate, and bleomycin was determined by placement of sterilized leaf discs (70% isopropanol, 1 min; 1% sodium hypochlorite, 10 min; three washes of sterile water, each for 2 min) on soybean callus-induction medium (Luo et al. 1994) containing the appropriate selectable agent (as mentioned previously) for approximately 2–3 mo.

Results and Discussion

Although *Agrobacterium*-mediated transformation with six genes has been successful in tobacco (Thomson et al. 2002), arabidopsis (Goderis et al. 2002), and seven genes in rice (Lin et al. 2003), ours is the first study that has tested the expression of all genes used in a multi-gene transformation attempt in a crop plant and followed some segregation to the T2 generation, using samples large enough to start examining general patterns. The previous record was a study that monitored expression of four genes in rice plants co-transformed with 9 to 11 genes (Chen et al. 1998). Although DNA instability in *Agrobacterium* has been reported (Song et al. 2003), our six-gene vector used in this study was stable in two commonly used strains of *Agrobacterium*, although a nine-gene version of it was no longer stable (data not shown). These uncertainties associated with *Agrobacterium*, along with the widespread use of microprojectile-mediated bombardment, merited the examination of multiple gene transformation studies via microprojectile-mediated bombardment.

The results for transgene expression analysis of the T0 plants are seen in Table 2. There was a clear advantage to having all the genes in one plasmid only in terms of the number of transgenic plants recovered. Given that six bombardments were done per treatment, seven T0 plants were recovered with the six-gene-containing plasmid (p6-pack) treatment, as compared to four from six bombardments when an excess of the selectable marker plasmid was used (p1:9), and only one line when all plasmids were included in equimolar ratios (p1:1). Thus, when multiple plasmids are used, there appears to be an advantage to using an excess of the selectable marker in terms of the number of lines recovered. Only two plants were recovered with all six genes, one from the 1:9 ratio bombardment and one from the equimolar ratio bombardment (Fig. 2).

In the case of the six-pack multigene construct, one T0 plant had five genes, and the remaining ones only had three. As expected, the selectable marker gene (*hph*) was present every time, as resistance to hygromycin was used to select transgenic cell lines before regeneration of plants. In contrast, *bar* was always missing in the p6-pack lines, although it was adjacent to *hph* on the vector, and was part of the same cassette shuttled between the PI-*Psp*I and PI-*Sce*I sites of pUGA, the master docking vector. The other frequently missing genes from the p6-pack lines were *gus*A and *ble*.

Of the four plants recovered from the p1:9 shots, three were missing *ble*, one was also missing *gus*A and *bar,* and another was missing *nptII*. As before, *bar* and *hph* were on the same plasmid, whereas all the other transgenes, *gus*A, *gfp*, *ble*, and *nptII*, were on separate plasmids for these bombardments (Table 3). It is obvious that a much greater number of transgenics would be needed with this approach so as to obtain a plant transgenic for all the genes.

Regardless of plasmid treatment, the results show a clear bias against the integration of some genes, particularly, *gusA*, *ble*, and *bar*. These results are perplexing. Although the transgenes were on separate plasmids in the p1:9 and the p1:1 treatments, these genes were adjacent to each other in the six-pack vector used for transformation. A feature in common with the frequently lost transgenes, *gus*A, *ble*, and *bar*, was the *act2* terminator, which may have provided homologous regions for recombination and excision of the intervening DNA during the integration process. Yet, *nptII* was adjacent to those three genes, and it also has the *act2*

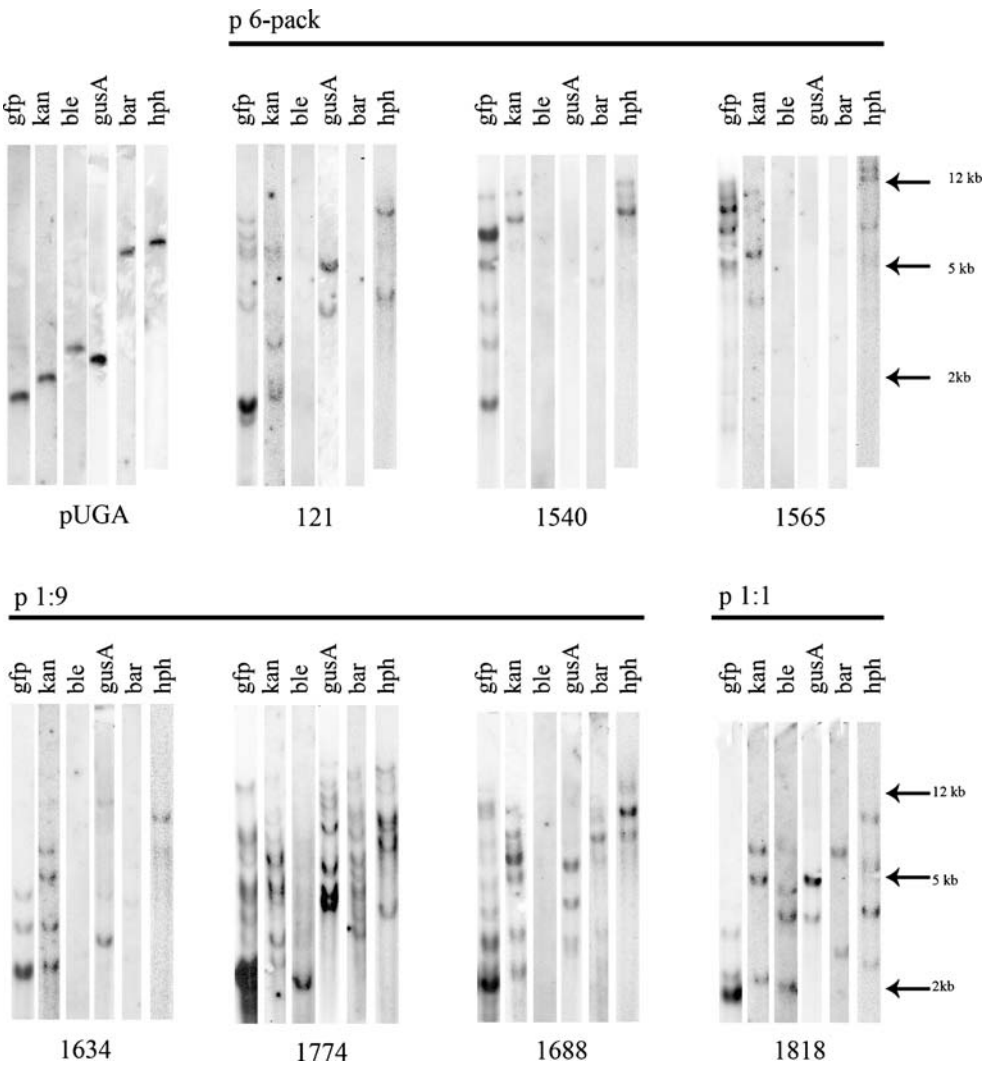

Figure 2. Southern blot analysis of randomly chosen T0 plants recovered after bombarding embryogenic tissue with six genes, either in one plasmid (p6-pack) or a cocktail of plasmids (p1:9 and p1:1). The first panel (*top*, *left*) is with the plasmid vector itself.

terminator but was always integrated. An additional explanation for the absence of certain genes integrating could be that each cassette had residual DNA sequences from the MCS used, and these residual sequences may also have acted as a site for homologous recombination while the plasmid was still in *E. coli* or even during the integration process. The nonrandomness of gene loss is suggestive that, perhaps, the structure of the plasmid itself, such as secondary DNA associations, can lead to instabilities. If so, proper plasmid design will be a major component of single-vector, multiple-gene transformation strategies.

Another possible explanation for the low frequency of transgenic plant recovery might be the *ble* gene itself. Looking across the three strategies, 12 plants were recovered, and only three of these contained *ble*. No plant showed any expression of *ble*, suggesting that, perhaps, *ble* expression is detrimental to soybean. In hindsight, the literature is devoid of successful reports using *ble* for plant transformation. In the work with *Artemesia*, bleomycin-resistant callus could be obtained, but plants could not be obtained from that callus (Vergauwe et al. 1998), while callus resistant to other selection agents did regenerate. Even the initial report on the use of bleomycin for the selection of transgenic plants only obtained *ble*-resistant callus but no plants (Hille et al. 1986).

In the case of expression, the only gene consistently expressed was the *hph* gene, which is not surprising, as it was the selectable marker. Other than that, there was no clear pattern to gene expression, and in no case were all six genes expressed. Expression might be a function of the way the multiple genes were assembled. In the case of p6-pack, the use of the same promoter multiple times in the same construct could have contributed to lack of expression of one gene. Besides avoiding the repeated use of the same promoter, it is possible that additional design features, such as the use of additional polyadenylation sites (Breyne et al. 1992; Ingelbrecht et al. 1991) or other transcription blockers (Padidam and Cao 2001), can stabilize the expression of all genes placed within a tight cluster. Likewise, the orientation of genes relative to each other within the

Table 2. Transgene expression in T0 plants

Event	*hph*	*gfp*	*gusA*	*Ble*	*Bar*	*nptII*
P6-pack						
1210	p	p	n	n		p
1521	p	n				p
1529	p	p				n
1530	p	p				n
1540	p	p				p
1541	p	p				p
1565	p	n				n
P1:9						
1634	p	p				p
1667	p	p	p		p	p
1668	p	p	n		p	p
1774	p	n	p	p	n	
P1:1						
1818	p	p	p	n	p	p

Transgene expression was tested in plants found by PCR to have the gene being tested. A "p" indicates positive gene expression, an "n" indicates negative expression. A *blank square* indicates the gene was not present in the plant.

transformation cassette has been shown to affect transient expression (Padidam and Cao 2001). Because four of the five homing endonuclease recognition sites are not symmetrical, the shuttles come in two versions, with the homing endonuclease sites added in either the forward or the reverse orientation, thus, making it easier to control the orientation of each gene within the cluster (Thomson et al. 2002).

All the events recovered, except one from the p6-pack and one from the p1:9 treatment, fit a 3:1 ratio, as determined by the segregation of *hph*, the selectable marker, indicating integration of the transgene(s) into a single locus. However, for those events that segregated 3:1, there were always T1 plants that did not contain all the genes present in its corresponding T0 plant. One interpretation is that this is an indication that not all transgenes integrated into one locus, or additional copies of the transgene integrated into a separate locus. In this regard, there was no clear advantage to using one plasmid versus multiple plasmids, although it had been anticipated that having multiple transgenes on a single vector would facilitate downstream breeding efforts.

Two T1 plants from event 1818 (the p1:1 treatment) having all six genes were selected at random. Thirteen T2 plants from one and 14 from the other were tested for the

Table 3. Number of T1 plants containing the specified transgene found in its corresponding T0 plant, as determined by PCR

Event	*n*[a]	$\chi 2$ for hph[b]	Hph	Gfp	gusA	Ble	Bar	*nptII*	No. with all[c]
P6-pack									
1210	18	5.14*	18	18	18	18		18	18
1521	20	1.07	13	15				12	12
1529	17	0.18	12	13				14	10
1530	20	2.40	12	14				13	2
1540	20	0.27	16	15				14	13
1541	20	0.27	14	15				15	14
1565	17	0.02	13	10				10	7
P1:9									
1634	20	1.07	17	17				17	17
1667	19	0.44	13	13	11		13	14	6
1668	20	4.26*	11	12	8		11	11	9
1774	19	0.16	15	18	16	17	16	16	15
P1:1									
1818	11	0.27	9	8	8	8	9	9	8

[a] Number of T1 plants tested

[b] Goodness of fit to a 3:1 ratio was tested for *hph*, the selectable marker. The *asterisk* indicates a significant ($p=0.05$) deviation from a 3:1 (single insert site) ratio.

[c] Number of T1 plants that had all the genes present in its corresponding T0 plant.

presence of the transgenes. All 27 plants had all six transgenes, suggesting insertion into too many loci to obtain null segregants in the sample tested.

In the end, the combination of microprojectile-mediated transformation and multiple-gene cassettes is promising, although more work is needed to determine the stability of the construct and determine proper gene placement to ensure proper expression of all transgenes in the vector. As mentioned in the introduction, there are reasons to expect that a single plasmid strategy might give more desirable results than the use of multiple plasmids for transformation. However, based on the results presented here, it is difficult to state that one strategy is superior to the other. All three strategies had shortcomings when it came to providing single-site integrations with a high frequency of co-transformation of all the genes. Thus, the major issue that must be resolved for multiple gene transformation is the design of vectors amenable to (a) integration of all the desired genes and (b) expression of all the desired genes.

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