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Generation of marker-free transgenic maize by regular two-border *Agrobacterium* transformation vectors

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Abstract

By introducing additional T-DNA borders into a binary plasmid used in *Agrobacterium*-mediated plant transformation, previous studies have demonstrated that the marker gene and the gene of interest (GOI) can be carried by independent T-strands, which sometimes integrate in unlinked loci in the plant genome. This allows the recovery of marker-free transgenic plants through genetic segregation in the next generation. In this study, we have found that by repositioning the selectable marker gene in the backbone and leaving only the GOI in the T-DNA region, a regular two-border binary plasmid was able to generate marker-free transgenic maize plants more efficiently than a conventional single binary plasmid with multiple T-DNA borders. These results also provide evidence that both the right and left borders can initiate and terminate T-strands. Such non-canonical initiation and termination of T-strands may be the basis for the elevated frequencies of cotransformation and unlinked insertions.

Introduction

Agrobacterium tumefaciens genetically transforms plants by transferring a T-strand from a section of DNA, the T-DNA, from a Ti plasmid or a binary plasmid, into plant cells. The transfer process is dependent upon many trans-acting virulence (vir) proteins, as well as DNA elements known as border sequences. The border sequences define the limits of the T-DNA. The T-DNA is processed into a single-stranded T-strand through the action of vir proteins, including VirD1 and VirD2 (reviewed by Tzfira et al., 2000; Zupan et al., 2000). By convention, one of the two borders flanking the T-DNA is defined as the right border, and the other as the left border. T-strand production is generally considered to begin at the right border and terminate at the left border, and most binary vectors used in *Agrobacterium*-mediated transformation are designed to contain a right and left border flanking the T-DNA.

In most applications, T-DNA transformation vectors utilize border regions that vary in size, but usually exceed the minimal 24 bp size required for T-strand production. Although there are differences in the 24 bp DNA sequence of left and right borders for any given *Agrobacterium* strain, a comparison of right border and left border sequences from a number of *A. tumefaciens* and *A. vitis* species, reveals that the consensus sequence is the same for the right border as for the left border within the 24 bp region (Canaday et al., 1992). Despite the apparent similarity of the basic sequences of the right and left borders,

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there is evidence that the right and left borders differ in their T-strand initiation and termination properties. Although right borders can terminate T-strands (Lu et al., 2001) and left borders can initiate T-strands (Horsch & Klee, 1986; Jen & Chilton, 1986a, b), right borders seem to enhance initiation, while left borders appear to attenuate initiation and/or enhance termination (Wang et al., 1987). Since the consensus 24 bp sequences of the right and left borders are identical, flanking sequences probably account for the different properties of the borders. Numerous studies have focused on the enhanced initiation properties of the right border, resulting in the identification of 'overdrive' sequences that boost the ability of the 24 bp right border sequence to initiate T-strand formation (van Haaren et al., 1987; Toro et al., 1988; Veluthambi et al., 1988; Hansen et al., 1992). Less is known about the sequences flanking the left border, and its ability to terminate the T-strand. For most applications, T-strand termination at the left border is desired to prevent vector backbone sequences from being transferred to the plant cell. Despite the presence of the left border, vector backbone sequences are frequently transferred along with the T-DNA region (De Buck et al., 2000; Kononov et al., 1997). This could be due to either the failure to terminate the T-strand at the left border ('readthrough'), or the initiation of a T-strand at the left border. It seems likely that both left border readthrough and initiation contribute to the presence of backbone sequences in transformed plant cells, but the extent to which one occurs more than the other is not known.

In nearly all plant transformation processes, a selectable marker gene is required to recover the transformed cells and to regenerate a transgenic plant. The marker gene is normally located on the T-DNA, between the right and left borders, along with the gene(s) of interest. The deployment of marker-free transgenic plants is a desirable option in agricultural biotechnology. Many strategies have been described to produce marker-free transgenic plants, including the use of site-specific recombination, homologous recombination, and co-transformation followed by segregation of unlinked insertions (reviewed by Hare and Chua (2002), Hohn et al. (2001)). For the co-transformation approach, several strategies have been developed using two (or more) T-DNAs such that the genes of interest are located on one T-DNA and the marker gene is on another. This has been accomplished in a number of ways. The most straightforward approach is to co-inoculate plant cells with two separate *Agrobacterium* strains, each containing a simple binary vector with a single T-DNA flanked by a right and a left border (De Block & Debrouwer, 1991; Miller et al., 2002). Another approach is to use a single *Agrobacterium* strain containing two separate plasmids, each with a single T-DNA flanked by a right and a left border (Daley et al., 1998; Jacob & Veluthambi, 2002; Vain et al., 2003). Lastly, transformation has been done using plasmids with two (or more) T-DNA regions (Komari et al., 1996; Xing et al., 2000; Matthews et al., 2001; McCormac et al., 2001; Miller et al., 2002; Stahl et al., 2002). In this latter approach, each T-DNA is flanked by a left and a right border. A variation on this approach utilized a single plasmid with three borders: two right borders and one left border (Lu et al., 2001). The objective in all these two T-DNA approaches is to obtain transgenic plants in which the two T-DNAs have integrated at unlinked positions in the plant genome, such that the marker gene insertion segregates independently of the gene of interest insertion, allowing for the recovery of marker-free plants in the subsequent generation.

We considered the possibility that a single plasmid with just two borders could, in principle, produce two (or more) different T-strands and that this approach could be used to generate marker-free plants. This simple design is shown in Figure 1. In order to use this approach to generate a marker free plant, we positioned the plant selectable marker gene in what would normally be considered to be the backbone of the plasmid, along with the bacterial plasmid maintenance elements. We reasoned that the marker gene would be transferred to the plant cell, either as part of a T-DNA in which the T-strand was initiated at the second border or as a T-strand that was initiated at the first border and failed to terminate at the second border. In the latter case, it would still be possible to obtain a marker-free plant as long as additional T-strands were produced through the initiation at the first border and termination at the second border. In one plasmid design, the second border is a right border. In another plasmid design, the second border is a left border. Here

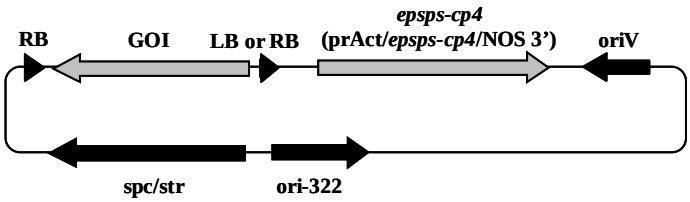

Figure 1. Repositioning of the marker gene in the binary plasmid. The plasmid contains a gene of interest (GOI) within the region between two borders, which is traditionally regarded as the T-DNA. However, the selectable marker, *epsps-cp4*, which confers glyphosate resistance, is positioned outside of the T-DNA along with the bacterial plasmid maintenance elements in what is normally considered the backbone of the plasmid. The designations of the genetic elements are as follows: RB, right border; LB, left border; prAct, promoter of rice actin including the first intron (McElroy & Wu, 1997); *epsps-cp4*, EPSP synthase gene from *Agrobacterium* sp. strain CP4 (Barry et al., 1997); NOS 3′, 3′ signal of nopaline synthase; spc/str, coding region for Tn7 adenylyltransferase conferring resistance to spectinomycin and streptomycin; ori-322, *E. coli* origin of replication; oriV, vegetative origin of replication, functional only when the *trfA* protein is present in the same cells. The genetic elements shown are not drawn to scale.

we describe this tandem T-DNA approach. We show that a simple binary vector with a single right border and a single left border, with the plant selectable marker gene located in the backbone region, can be used to efficiently obtain marker-free plants. Our results also provide additional information about the initiation, termination, and readthrough properties of the right and left borders.

Materials and methods

Construction of binary plasmids

All binary plasmids used contain identical backbone elements shown in Figure 1. The original binary plasmid is similar to pMON65153 (Figure 2A). We replaced, or inserted, additional T-DNA border regions to generate pMON65178, 65179 and 65180 (Figure 3). These T-DNA border regions were previously described by Horsch and Klee (1986). The RB region is a 357 bp DNA sequence that was originally isolated from *A. tumefaciens* plasmid pTiT37 (Depicker et al., 1982). The LB region is a 456 bp DNA sequence that was originally isolated from *A. tumefaciens* plasmid pTi15955 (Barker et al., 1983).

Plant transformation

The binary plasmids were electroporated into *Agrobacterium tumefaciens* ABI strain and introduced into LH198XHiII maize embryos by *Agrobacterium*-mediated transformation (Armstrong & Rout, 2001). LH198 pollen was used to pollinate R0 transgenic plants to produce R1 seeds.

Southern analysis

Genomic DNA was isolated from leaves of corn plants using procedures described by Dellaporta (1994). Genomic DNA (15–20 μg) was digested with desired restriction enzymes, separated on a 0.7% (w/v) agarose gel, and transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN). Prehybridization, hybridization, washing, and detection of the membranes were conducted using a non-radioactive DIG-based system (Roche Molecular Biochemicals) following the manufacturer's protocols. DNA fragments corresponding to the desired sequences were labeled by PCR to produce probes.

PCR

Genomic DNA isolation was also similar to the procedures described by Dellaporta (1994), except only 6–10 lyophilized leaf punches were used per plant (~40 mg fresh weight) in a 96-well format. Each PCR reaction used approximately 10 ng of DNA and the reaction was analyzed by a qualitative TaqMan procedure (Applied Biosystems, Foster City, CA) following the manufacturer's protocols. The primers and MGB (minor grove binding) TaqMan probes for each targeted sequence were designed and validated by the manufacturer.

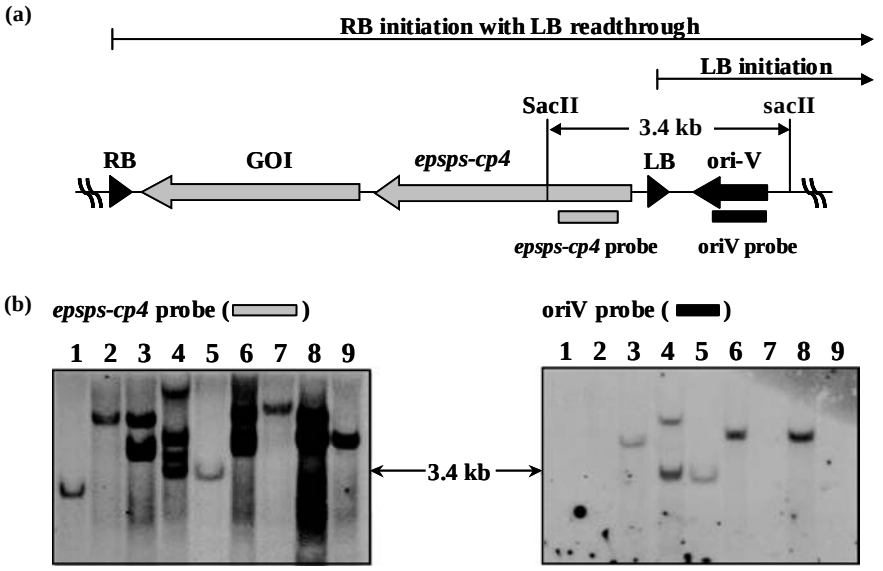

Figure 2. Detection of the left border readthrough and the left border initiated transgene transfer in transgenic plants by Southern analyses. (a) Schematic representation of the approach. Illustrated here is plasmid pMON65153, a typical two border binary plasmid where the GOI along with the marker gene, *epsps-cp4*, are located between two borders. It has *Sac*II restriction sites flanking two sides of the left border at 3.4 kb apart. In the case of the left border readthrough, a 3.4-kb band would be found on a *Sac*II digested genomic DNA gel blot using probes that correspond to either *epsps-cp4* or oriV sequences. On the other hand, an oriV insertion resulted from a left border initiated T-strand would generate bands with various sizes that hybridize to the oriV probe on the same *Sac*II digested DNA gel blot. (b) *Sac*II digested DNA gel blot hybridized with *epsps-cp4* probe (left) and oriV probe (right). Transgenic plants in lane 4 and 5 display a 3.4-kb band and have the left border readthrough insertion, while transgenic plants in lanes 3, 4, 6 and 8 appear to contain left border initiated transgene transfer.

Results

Detection of the left border readthrough and left border initiation T-DNA insertion in transgenic plants

For consistency, all the binary plasmids used in the study contain an identical gene of interest (GOI). It consists of the coding region of a lysine-insensitive dihydrodipicolinic acid synthase (DHDPS) from *Corynebacterium*, *cordap*A (Falco et al., 1998) with a maize DHDPS leader peptide, flanked by maize *glb*1 promoter and terminator (GenBank Accession L22344, Belanger and Kriz, 1991). A typical binary plasmid is shown in Figure 2a, where the GOI is inserted adjacent to the selectable marker, 5-enolpyruvylshikimate-3-phosphate synthase gene from *Agrobacterium tumefaciens* strain CP4 (*epsps-cp4*), and both are bordered by T-DNA border regions. In all vectors used in this work, the right border region is a 357 bp fragment derived from the nopaline plasmid pTiT37 (Depicker et al., 1982), and the left border region is a 456 bp fragment from the octopine plasmid pTi15955 (Barker et al., 1983). The T-DNA region of this plasmid encodes both *epsps-cp4* and GOI. This allows them to be co-integrated when transferred into plant cells and therefore the glyphosate selection during regeneration greatly enhances the recovery of transgenic plants that contain the GOI. Vector backbone sequences, i.e. those sequences outside the T-DNA region, are also stably integrated in approximately 30% of transgenic plants generated, as detected by PCR for the oriV replication origin. The transfer of backbone sequences could be due to the failure to terminate the T-strand at the left border ('readthrough'), or due to the initiation of a T-strand at the left border. By performing Southern analysis of the transgenic plants, we have investigated the likelihood of either of scenario during transformation.

The plasmid pMON65153 (Figure 2a) has two *Sac*II restriction sites flanking the left border region, approximately 3.4 kb apart. This 3.4-kb *Sac*II fragment spans from the 3′ end of

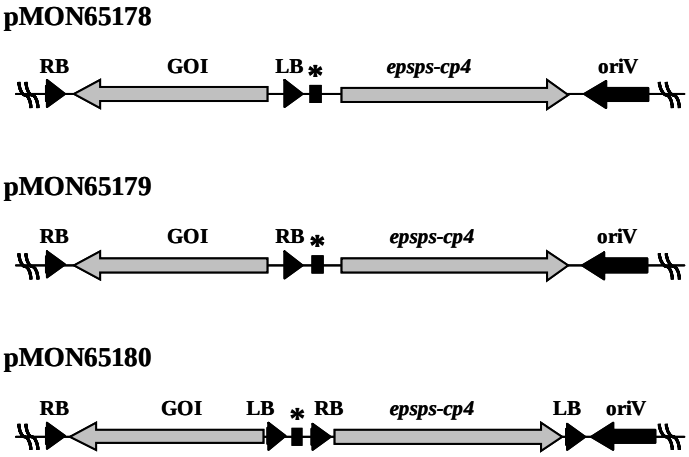

Figure 3. Plasmid maps of pMON65178, 65179 and 65180. The genetic elements are identical to those described in Figure 1. The region designated by (*) and oriV are the targeted sequences for the PCR assay determining the presence of vector backbone in Table 1, row (e).

EPSPS-CP4 gene cassette through the left border and into the vector backbone, including oriV. If left border readthrough occurs and the T-strand proceeds through oriV, T-DNA sequences and backbone sequences would be inserted into transgenic plants as a single contiguous molecule, and a 3.4-kb band would be found on a *Sac*II digested genomic DNA gel blot using probes that correspond to both *epsps-cp4* and oriV sequences. This was the case for transgenic plants in lanes 4 and 5 shown in Figure 2b. On the other hand, if the transfer and insertion of oriV sequences resulted from a left border initiated T-strand, it would probably generate bands with various sizes that hybridize to the oriV probe on the same *Sac*II digested DNA gel blot. This is because the left border initiated insertion contains only one *Sac*II restriction site in the inserted backbone DNA and the other unspecified *Sac*II site must be present in a genomic sequence near the site of insertion to produce a *Sac*II fragment in Southern analysis. The results of Southern analysis on transgenic plants in lanes 3, 4, 6 and 8 (Figure 2b) are consistent with the possibility that left border initiated transfer did occur in these plants during transformation. It is also possible that left border readthrough occurred, followed by truncation, partial transfer, or rearrangement of sequences in the *Sac*II fragment. Note that, of the six bands hybridizing to the oriV probe, five appear to overlap with the same size band revealed by the *epsps-cp4* probe. Thus, if left border initiation occurred, the left border initiated fragment inserted in the chromosome in such a way as to be contiguous with the *epsps-cp4* fragment. Among 165 transgenic maize plants analyzed in this manner, we detected the 3.4 kb band expected by left border readthrough in 40 (24.2%) plants, and other sized bands which may result from left border initiation in 42 (25.5%) plants, respectively, while both were detected in 28 plants. Overall, 54 (32.7%) plants were identified positive for the presence of oriV.

Construction and transformation of alternative binary plasmids

Given that backbone sequences are frequently transferred to plant cells, and that this sometimes appears to occur via independent T-strand production, we designed and constructed a series of vectors to test the transfer of marker genes positioned in the backbone of the vectors, and tested the ability to derive marker-free plants from these vectors. pMON65178 and pMON65179 contain two border regions, RB–LB and RB–RB respectively (Figure 3). The EPSPS-CP4 gene has been placed in what would normally be considered to be the 'backbone' of the vector. The third vector, pMON65180, has four border regions, with the GOI and *epsps-cp4* each flanked by the separate right and left borders (Figure 3).

All vectors were used in a series of transformation experiments of maize immature embryos. The transformation results are summarized in Table 1. In total, more than 260 individual R0 events were created for each binary plasmid. Although highly variable, the transformation frequencies of the modified vectors pMON65179 ($11.3 \pm 6.4\%$) and pMON65180 ($13.4 \pm 6.5\%$) were comparable to the efficiency of the conventional vector, pMON65153 ($11.0 \pm 5.3\%$). pMON65178, however, had a much lower transformation frequency ($3.0 \pm 3.2\%$). All R0 plants were assayed for the presence of the GOI by PCR. The percentages of R0 plants that were positive for the GOI for pMON65178 (91.2%) and pMON65179 (88.9%) suggested that almost all the transgenic events transformed by these two constructs had co-integrated the GOI with the selectable marker. This was similar to events produced by pMON65153 (93.3%) where the marker gene and the GOI were on the same T-DNA. However, the percentage of plants that were positive for the GOI for pMON65180, in which the GOI and the selectable marker gene were on separate T-DNAs, was lower (67.3%), indicating that some transgenic events did not contain the GOI.

Identification of R0 events that carry an unlinked GOI

All R0 plants that were identified as positive for the GOI were then assayed by Southern blot to determine the frequency of plants that contain an unlinked insertion of the GOI (Figure 4a). These plants would be predicted to be able to segregate marker-free, GOI positive progeny plants in the next generation. Southern analysis to assess linkage was accomplished by digesting the plant genomic DNA with a restriction enzyme with a 6 bp recognition sequence that does not occur in any part of the binary plasmids. The use of a 6 bp cutter was not only sufficient to resolve the linkage, but also produced DNA fragments that could be easily separated on a 0.7% agarose gel. An example of the results of the Southern analysis is shown in Figure 5, where bands representing unlinked copies of the GOI are designated by the arrows. R0 transgenic plants that produced such bands in Southern analysis were identified as plants with unlinked insertions that should be able to segregate marker-free progeny. We found that pMON65178 generated more unlinked events (35.6%) than pMON65179 (12.4%) and pMON65180 (16.0%) (Table 1, row c). Southern analysis also revealed that most events with an unlinked insertion had only a single unlinked insertion of the GOI (Table 1, row f).

Recovery of marker-free transgenic plants in R1 generation

Figure 4b illustrates the process of recovering marker-free transgenic plants. R1 seedlings derived from R0 plants with unlinked insertions were tested for the segregation of *epsps-cp4* and the GOI by PCR. Marker-free segregants (GOI (+) and *epsps-cp4* (−)) were identified from 93.0, 72.0 and 72.0% of unlinked events transformed by pMON65178, 65179 and 65180, respectively (Table 1, row d), indicating that the Southern analysis on R0 plants was reasonably accurate at predicting the ability to recover marker-free plants. We also performed PCR to confirm the absence of the oriV sequence in the marker-free events and that the sequence between the first left border and the second right border in pMON65180 (Figure 3) was also absent. It was possible that these two regions, both flanked by two borders in pMON65180, were capable of producing T-DNAs insertions unlinked to *epsps-cp4* and the GOI. As predicted, we detected these backbone sequences in R1 plants that segregated GOI (+) and *epsps-cp4* (−), in 10 of 18 pMON65180 events. For two of these events, we were not able to obtain GOI (+), *epsps-cp4* (−) R1 plants that were also backbone-free. In contrast, all GOI (+), *epsps-cp4* (−) R1 plants derived from pMON65178 and pMON65179 events tested negative for the backbone sequences (Table 1, row e). In pMON65178 and pMON 65179, the backbone sequences are contiguous with *epsps-cp4,* i.e. not separated by T-DNA borders, and all these sequences were likely included together in a single T-strand. Therefore, the selection of *epsps-cp4* (−) F1 plants by PCR also resulted in backbone negative R1 plants.

The final step of the characterization was determining the copy number of GOI by Southern analysis (Table 1, row f). We selected only transgenic events with a single inserted copy of the GOI for further analyses in order for

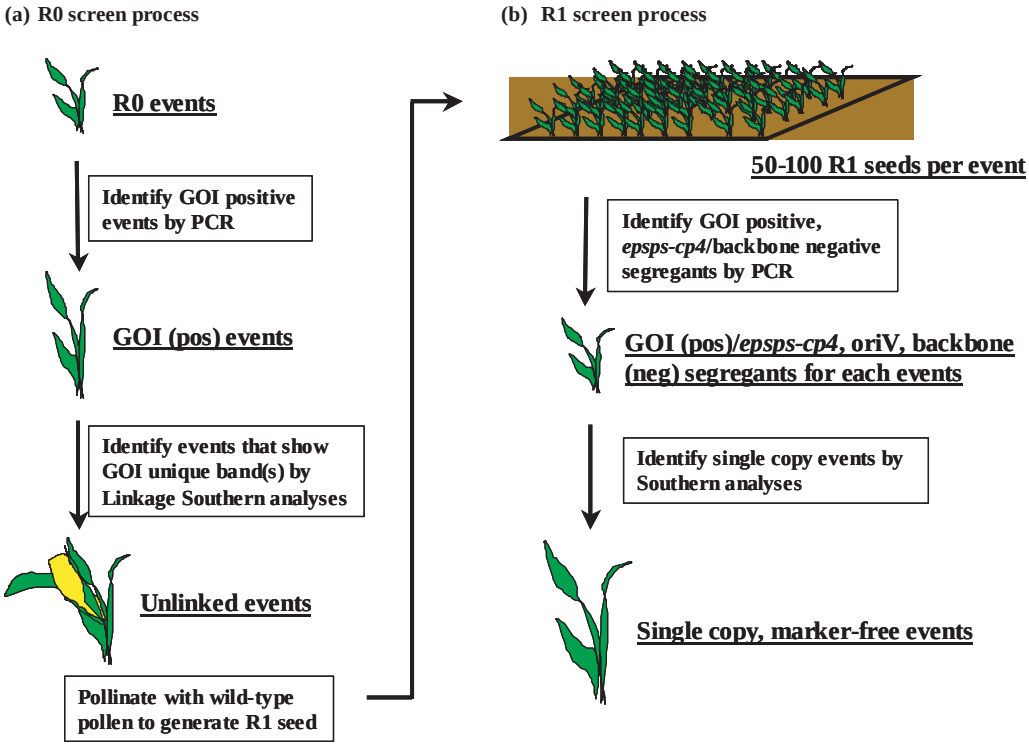

Figure 4. Screening process to recover single copy marker-free transgenic plants. The screening process required two generations, R0 (a) and R1 (b), to complete. (a) R0 screen process. R0 plants were first tested for the presence of GOI by PCR and followed by Linkage Southern analysis on GOI positive plants. The determined unlinked events were advanced to set R1 seeds. (b) R1 screen process. For each unlinked event, 50–100 seeds were planted. DNA extracted from seedlings was tested by PCR to identify GOI (+) and *epsps-cp4* (−) seedlings. Additional PCR, as described in Figure 3, was performed on GOI (+), *epsps-cp4* (−) seedlings to exclude those that contained backbone sequences. Finally, only single-copy events, resolved by a second Southern analysis, were advanced for further analyses.

equivalent transgene dosage across events. Interestingly, all three modified binary plasmids had similar overall efficiencies of generating single-copy, marker-free transgenic plants (Table 1, row g). Nevertheless, when calculations were based on R0 events generated, the efficiency of pMON65178 was 4-fold greater than that of pMON65179 and pMON65180 (Table 1, row h). With pMON65178, the majority of unwanted events were eliminated at the earliest possible stage, transformation, which in turn, reduced the resources required for the subsequent laborious molecular characterization.

Discussion

One of the advantages of *Agrobacterium*-mediated transformation resides in its ability to produce relatively simple transgenic insertions in transgenic plants. This is because the gene transfer mechanism employed by *Agrobacterium* is generally precise, which involves sequence recognitions at border sequences, resulting in specific sites of T-strand initiation and termination. Although it has been known for some time that sequences outside the T-DNA region are sometimes transferred to the plant cell, the intention is usually such that the T-DNA, initiated at the right border and terminated at the left border, is the only DNA introduced into plant cells with a minimal sequence disruption to both the T-DNA and plant genome. We have taken advantage of the occasional transfer of backbone sequences that occurs during *Agrobacterium*-mediated transformation to efficiently generate marker-free, backbone-free, R1 transgenic plants containing a single copy of the GOI despite the initial complications in R2. The vector designs used to produce these transgenic plants in the

Table 1. Characterization of transgenic plants harboring various binary plasmids

Screen procedures	pMON65153	pMON65178	pMON65179	pMON65180
(a) Transformation efficiency (TE)	11.0 ± 5.3%	3.0 ± 3.2%	11.3 ± 6.4%	13.4 ± 6.5%
(b) % GOI (+) from (a)	93.3% (485/520)	91.2% (239/262)	88.9% (232/261)	67.3% (177/263)
(c) % unlinked events from (b)	–	35.6% (85/239)	12.4% (27/218)	16.0% (28/175)
(d) % events with GOI (+), *epsps-cp4* (-) R1s from (c)	–	93.0% (66/71)	72.0% (18/25)	72.0% (18/25)
(e) % events confirmed backbone (-) R1 from (d)	70.7% (343/485)	100% (66/66)	100% (18/18)	88.9% (16/18)
(f) % events with single-copy GOI from (e)	69.3% (196/283)	81.8% (54/66)	77.8% (14/18)	93.8% (15/16)
(g) Overall efficiency	5.03%	0.74%	0.70%	0.87%
(h) Overall efficiency excluding TE	45.7%	24.7%	6.2%	6.5%

(a) Events generated/embryos treated ± standard deviation.
(b) GOI (+) events were determined by PCR.
(c) Unlinked events were identified by Linkage Southern analysis.
(d) PCR was performed on R1s of unlinked events to isolate GOI (+), *epsps-cp4* (−) plants.
(e) GOI (+), *epsps-cp4* (−) plants were further confirmed to be backbone-free by PCR.
(f) Single-copy, backbone-free (marker-free) events isolated/embryos transformed.
(g) Single-copy, backbone-free (marker-free) events isolated/events generated.

study also provide new insights into the T-DNA initiation and termination.

Left border readthrough and initiation

We analyzed a large number of pMON65153 events by PCR and Southern analysis. Approximately half of them were single-copy events, free of the oriV segment of the vector backbone (Table 1). Another 20% of events were also backbone-free, but contained multiple copies of the GOI. The remaining events, about 30%, contained the oriV sequence in addition to the GOI. In some of these events, we determined that the oriV sequence appeared to be contiguous with T-DNA sequences through the T-DNA left border. This could be the result of a left border initiated T-DNA ligating back to a regular right border initiated T-DNA before the insertion, or simply from failure to terminate a right border initiated T-strand at the left border. In other events, the transfer of backbone sequences was possibly due to the initiation of T-strand production at the left border. We cannot conclude, however, that left border initiation occurred, since the Southern blot results could also be explained by left border readthrough, followed by rearrangement or truncation of the sequences flanking the left border. Although it is clear that left border can initiate T-strand production and transfer (Horsch & Klee, 1986; Jen & Chilton, 1986a, b; Ramanathan & Veluthambi, 1995), it appears to be a less efficient process, due either to the action of an attenuator or the lack of an overdrive element.

From the frequency observed in pMON65153 events, left border readthrough and/or initiation are not the most common occurrences in the production of T-DNA. During transformation, the majority of T-strands produced are still initiated by the right border and terminated by the left border. By positioning the marker downstream of the left border in the vector backbone and leaving only the GOI between the two borders, transgenic events are selected based on left border readthrough and/or initiation. However, these transgenic plants are likely to have received more copies of the T-strands that initiated at the right border and terminated at the left border than those that had readthrough and/or initiation of the left border, and thus may have a higher probability of having independently inserted regular T-DNA(s) containing the GOI. Indeed, pMON65178 has lower transformation efficiency, but a higher percentage of unlinked events.

Termination of T-DNA by the right border

The ability to recover marker-free events from pMON65179 transformed plants is an indication of right border termination. We are now working on isolating the sequences at the T-DNA insertion junctions of these marker-free events to understand the nature of the T-DNA termination by the right border.

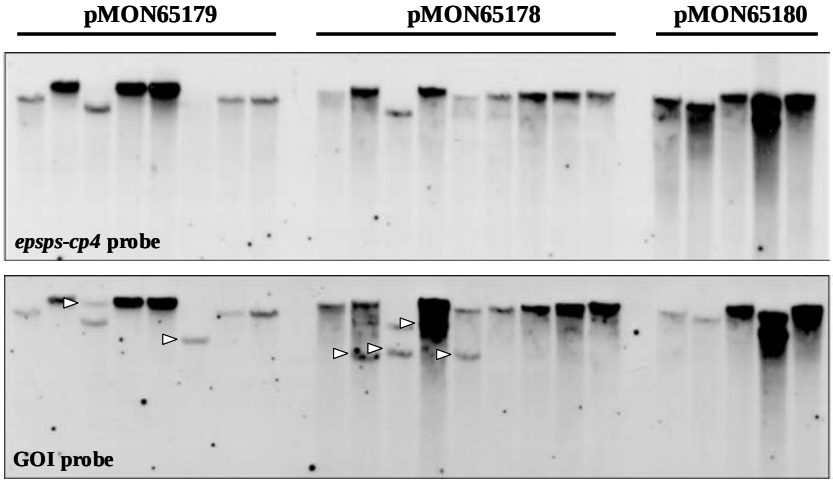

Figure 5. Examples of Linkage Southern analysis. Genomic DNA isolated from transgenic plants harboring pMON65178, pMON65179 and pMON65180 was digested with a six base pair restriction enzyme that did not recognize any sequence of the binary plasmids. The DNA gel blot was first hybridized with the GOI probe and rehybridized once more with the *epsps-cp4* probe after removal of the GOI probe. The arrows indicate bands that are specific to the GOI probe and transgenic plants that contained such a band were determined to be unlinked events.

Similar to the regular binary plasmid, pMON65153, pMON65179 and pMON65180 have a right border initiated marker-containing T-DNA. It is not surprising, therefore, that the transformation efficiencies of these two vectors are also comparable to the efficiency of pMON65153. On the other hand, comparing the percentages of GOI positive (or oriV positive in the case of pMON65153) plants among R0 events for each construct indicates a more complicated explanation for the co-integration of GOI/oriV. In both pMON65179 and pMON65180, the co-integration of GOI primarily relies on the production of right border initiated T-DNA (containing the GOI), whereas in pMON65153, the co-integration of oriV primarily relies on the production of left border initiated T-DNA (containing oriV). Because the right border has enhanced T-DNA initiation, the GOI co-integration efficiency in pMON65179 (88.9%, Table 1, row b) and pMON65180 (67.3%, Table 1, row b) are higher than the oriV co-integration efficiency in pMON65153 (27.3%). The later number is calculated by dividing oriV positives (142, in Table 1, row e) with total pMON65153 events (520, Table 1, row a). Furthermore, the difference observed between pMON65179 and pMON65180 can be explained by the less effective T-DNA termination by the right border. In pMON65179, the marker-containing T-DNA, which was selected during transformation, can also be generated from GOI-containing T-DNA with right border readthrough. This, in turn, gives pMON65179 a higher percentage of GOI positives over pMON65180. When the marker-linked GOI positives are removed by linkage Southern analysis, pMON65179 and pMON65180 show almost identical efficiency of generating unlinked events, 11.0 and 10.8%, respectively (calculated by multiplying rows b and c in Table 1).

Two T-DNAs vs. four T-DNAs

Binary plasmids similar to pMON65180 have been used to generate marker-free transgenic plants in various species (Komari et al., 1996; Xing et al., 2000; Matthews et al., 2001; McCormac et al., 2001; Miller et al., 2002). These binary plasmids contain GOI and a marker gene each flanked by a separated set of T-DNA borders, which are usually referred as 'two' or 'twin' T-DNA plasmids. As the data presented in this study suggest, it is possible, through left border initiation that these vectors can actually produce four T-strands. By simply positioning the marker gene into the backbone of a regular two-border binary plasmid, we have shown that a T-DNA carrying only the GOI can be independently inserted and segregated from the marker contain-

ing T-DNA to generate marker-free transgenic maize plants.

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