



## Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct

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### Abstract

We describe a variation of the method to generate single-copy transgenic plants by recombinase-mediated resolution of multiple insertions. In this study, a transgene construct flanked by oppositely oriented *lox* sites was co-bombarded into maize cells along with a *cre*-expressing construct. From analysis of the regenerated plants, a high percentage of the primary transformants harbored a single copy of the introduced transgene, and among these, a majority also lacked the *cre* construct. We deduce that the expression of *cre* must have contributed to resolving concatemeric molecules either prior to or after DNA integration into the maize genome.

### Introduction

Transgenic plants that harbor a single copy of the introduced transgene are often preferable to those with multiple copies of the introduced DNA. High transgene copies have been associated with functional and structural instability (for reviews, see Meyer and Saedler, 1996; Matzke and Matzke, 1998). A complex integration pattern also makes structural characterization more difficult. Cereal crops transformed by either *Agrobacterium*-mediated or direct DNA-uptake methods tend to yield a large proportion of single locus insertions. However, there is a high incidence for multiple copies of DNA to cluster at the insertion locus (Register *et al.*, 1994; Cooley *et al.*, 1995). Since the reduction of copy number through genetic segregation is often not an option, the chance of finding single-copy transformants can be as low as a few percent, particularly with plants obtained through direct DNA delivery methods.

Previously, we described a strategy in wheat to generate single-copy transgenic plants through the resolution of complex integration structures (Srivastava *et al.*, 1999). The strategy is based on flanking a transgene with oppositely oriented *lox* recombination

sites. Regardless of the number of insertions at a single locus, or the orientation of the inserted transgene construct, Cre-mediated recombination of the outermost *lox* sites would likely reduce the copy number to a single unit. The Cre recombinase was provided through a sexual cross with a plant line that expresses *cre*. The *cre* locus was segregated out in the following generation. This strategy is generally applicable for most plant transformation systems. In some instances, however, an alternative strategy may be necessary. For instance, the need to cross in and then segregate out the recombinase gene might not be compatible with breeding programs for vegetatively propagated crops. Moreover, the introduction and removal of the recombinase gene requires additional generation time.

To bypass the need to cross in and out the recombinase gene, one approach is to place the recombinase gene under an inducible promoter such that induced expression of the recombinase gene deletes its own coding sequence from the genome, as was demonstrated with the R/R<sub>S</sub> system and the Cre-*lox* system (Sugita *et al.*, 2000; Zuo *et al.*, 2001). Alternatively, the recombinase can be introduced transiently. Transient cre expression would resolve concatemeric transgene copies either prior to or after DNA integra-

tion into the genome. In this paper, we report that the co-introduction of a recombinase gene enriches the number of simple integrations patterns obtained from biolistic transformation of maize. In this study, 85% of the transgenic plants contain 1 to 2 copies of the introduced DNA, with 38% harboring a single copy. Among the single copy plants, 60% lack the recombinase gene, yielding an overall efficiency of 23% for plants that harbor only the transformation construct.

## Materials and methods

### *Transformation vector*

Plasmid pVS62 is essentially the same as pVS11 (Srivastava *et al.*, 1999), except that the *loxP* site between the phosphinothricin acetyl transferase gene *bar* and the maize ubiquitin promoter (designated *P2*) has been removed. This was done by ligating a 6 kb *HindIII*-cleaved, Klenow-filled fragment derived from pVS11 that lacks the *loxP-bar-loxP* segment, with a *HindIII*+*EcoRI*-cleaved, Klenow-filled *loxP-bar* fragment from the same plasmid. The resulting plasmid has a *lox511*-flanked DNA fragment that consists of a *bar* gene driven by the rice actin promoter (designated *P1*) followed by *P2*. A *loxP* site is situated between *P1* and the *bar*-coding sequence. A *FRT* site from the FLP-*FRT* recombination system is located upstream of *P1*. Plasmid pP2-cre, with the *cre* coding region under the control of *P2*, has been described previously (Srivastava *et al.*, 1999). Both constructs use a pUC18 backbone.

### *Maize line*

Callus was induced from F<sub>1</sub> immature embryos of a Hi-II line (Hi-II parent A × Hi-II parent B, callus line was provided by Monsanto). Each of these parents is derived from tissue culture selection of an A188 × B73 line. Callus induction and regeneration were conducted as described by Armstrong (1993).

### *Particle bombardment and selection of callus*

A thin layer of Hi-II callus was bombarded with 1 μm gold particles coated with either pVS62 alone, or a mixture of a molar ratio of 3 pVS62 to 1 pP2-cre. The particle bombardment procedure was as described for wheat by Altpeter *et al.* (1996). Bombarded calluses were selected for resistance to bialaphos and

then regenerated into plants. Bialaphos was used in the culture media at a concentration of 1 mg/l.

### *Molecular analysis*

Maize genomic DNA was isolated by the CTAB method. About 5 μg of genomic DNA was cleaved with *Bam*HI, fractionated on a 0.7% agarose gel, transferred onto a positively charged nylon membrane and hybridized with a <sup>32</sup>P-labeled DNA probe.

## Results

### *Experimental design*

We used a transformation construct with the fragment of interest flanked by a pair of inverted recombination sites. Plasmid pVS62 is derived from pVS11, a construct used previously for wheat transformation (Srivastava *et al.*, 1999). As the presence of a selectable marker is needed to detect the transformation event, a *loxP* site downstream of *bar* was removed from pVS11 to generate pVS62 (Figure 1A). This modification permits maintenance of the *bar* gene. If the desired final product were a marker-free transgenic plant, this alternative strategy would not be suitable as depicted, but downstream steps can be devised to remove the marker gene (see Discussion). The components in pVS62 are the rice actin promoter (*P1*), a *loxP* site, the phosphinothricin acetyl transferase gene (*bar*), and a maize ubiquitin promoter (*P2*). The *P1-bar* linkage provides selection for bialaphos resistance. The entire *P1-loxP-bar-P2* fragment is flanked by a pair of inverted *lox511* sites that do not recombine with *loxP*. If this fragment were to integrate in multiple copies, regardless of their relative orientation, Cre-mediated recombination between the outermost *lox* sites would reduce the copy number. As long as the outermost copies have functional *lox* sites, the resolution process should reduce the integrated structure to a unit copy.

In pP2-cre, the *cre* gene is transcribed by the maize ubiquitin promoter (*P2*). If pP2-cre were introduced at the same time as the transformation construct, expression of *cre* may provide sufficient Cre recombinase to resolve extrachromosomal or integrated concatamers of pVS62. If pP2-cre also integrates into the genome, it may be possible that it will insert into a genetically unlinked locus. In that case, the pP2-cre DNA can be segregated away. Most likely, however,

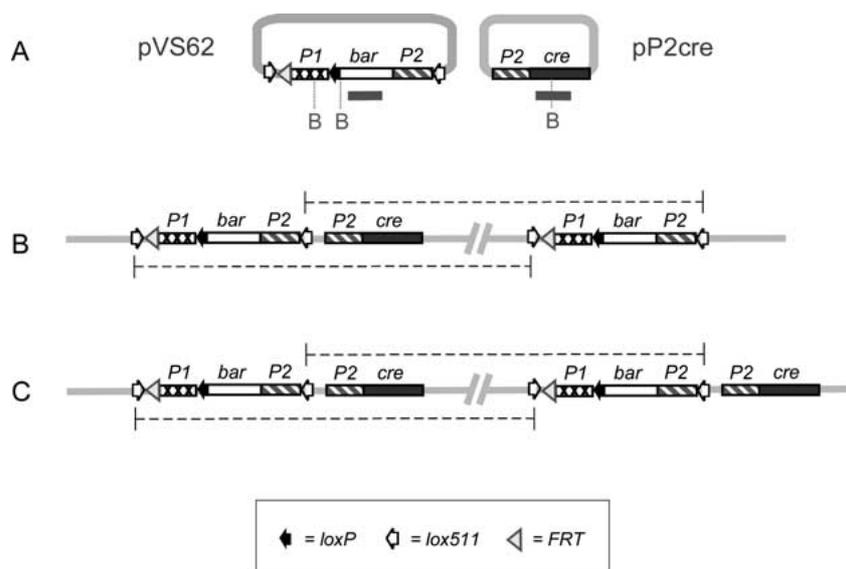


Figure 1. A. Transformation constructs used in maize transformation. Recombination sites are shown by arrowheads as indicated. Plasmid pVS62 contains a *bar* gene driven by the rice actin promoter (*P1*) followed by the maize ubiquitin promoter (*P2*). Plasmid pP2-cre contains a *cre* gene driven by *P2*. Shown are the relevant *Bam*HI (*B*) sites. The extent of DNA fragments used as *bar* and *cre* hybridization probes are indicated below the respective genes. B. Recombination between outermost *lox511* sites, indicated by the dashed line, resolves pVS62 concatamers and removes internal copies of *cre*. C. The same process as in B but the *cre* DNA that lie outside of the outermost *lox511* sites is retained.

pP2-cre will be a part of the pVS62 integration locus, as the co-integration frequency of co-introduced DNA in biolistics transformation has been known to occur at high frequency (reviewed by Pawlowski and Somers, 1996). For this latter possibility, if all of the pP2-cre molecules lie within the outermost pVS62 inserts, the recombination-mediated resolution process would also eliminate the *cre* DNA (Figure 1B). Thus, it should be possible to obtain a primary transformant with a single copy of pVS62 without incorporation of pP2-cre.

A biolistic-mediated transformation experiment was conducted on maize embryogenic calluses. In one set of calluses, pVS62 was the transforming DNA. In a second set, pP2-cre was included along with pVS62. Bialaphos-resistant calluses were transferred to regeneration medium and transgenic plants were obtained and analyzed by Southern blotting.

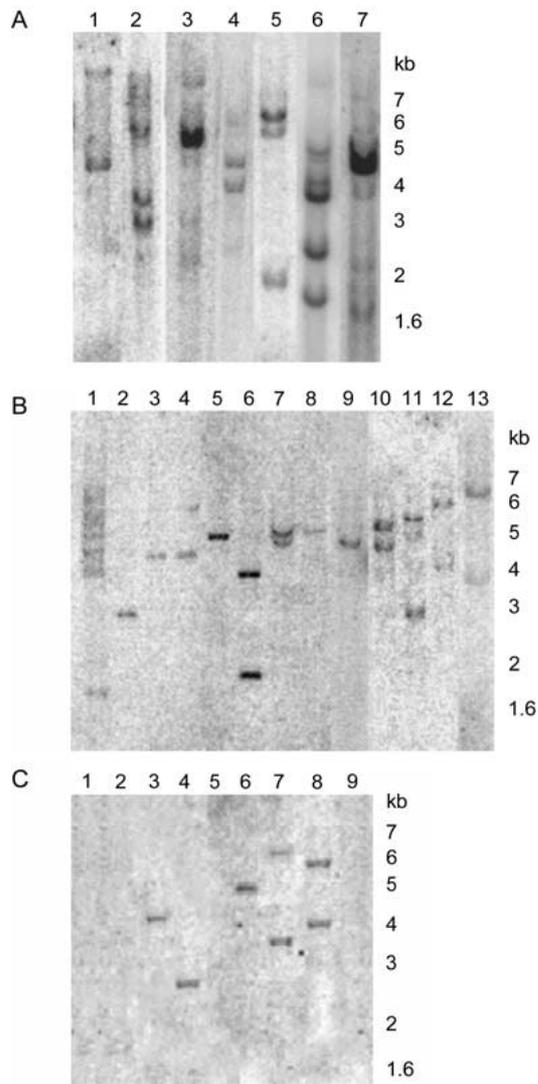
#### Transformation with pVS62 alone

At a frequency of about one transformation event per bombarded plate, ten bialaphos-resistant callus lines were obtained by the bombardment of pVS62 DNA. Seven of the calluses regenerated transgenic plants. The genomic DNA of these 7 lines were cleaved with *Bam*HI and hybridized with a *bar* DNA probe (see

Figure 1A). Plasmid pVS62 contains a *Bam*HI site upstream of the *bar*-coding sequence. For a single-copy insertion, the *bar* probe should only detect a single-border band in *Bam*HI-cleaved genomic DNA. All 7 plants displayed multiple *bar*-hybridizing bands (Figure 2A). Therefore, every plant harbors multiple copies of pVS62 DNA. Some lines show a banding pattern indicative of complex integration. Since *bar* is ca. 1 kb and *P2* is 2 kb, a band size smaller than 3 kb would indicate the insertion of a partial *bar*-*P2* fragment of pVS62 (Figure 2A, lanes 5–7). Tandem repeats of pVS62, appearing as intensively hybridizing bands, are seen in 3 lines (Figure 2A, lanes 3, 6, 7).

#### Co-bombardment of pVS62 and pP2cre

Twenty callus lines were obtained by the co-bombardment of pVS62 and pP2-cre. These calluses arose at about the same frequency of one transformation event per bombarded callus plate. Thirteen of the calluses regenerated plants. The integration pattern in *Bam*HI-cleaved DNA was analyzed by hybridization to *bar* DNA (Figure 2B). One line contains numerous hybridizing bands (Figure 2B, lane 1) and another line reveals 3 hybridizing bands (Figure 2B, lane 11). Six lines showed 2 bands (Figure 2B, lanes 4, 6, 7, 10, 12, 13), and the remaining 5 lines showed a single



**Figure 2.** Southern hybridization of primary transgenic maize lines. A. *Bam*HI-cleaved transgenic lines generated by bombardment of pVS62 DNA hybridized with the *bar* probe. B. *Bam*HI-cleaved transgenic lines generated by co-bombardment of pVS62 and pP2-cre DNA and hybridized with the *bar* probe. Lanes 1 to 13 correspond to lines 1 to 13 in Table 1. C. The blot shown in Figure 2B (lanes 1–9) was re-hybridized with the *cre* probe.

band (Figure 2B, lanes 2, 3, 5, 8, 9). These 5 lines therefore represent single-copy insertions. The Southern blot with the 5 single-copy plants was hybridized to *cre* DNA (Figure 2B and C, lanes 1–9). Figure 2C shows that 3 out of the 5 single-copy lines (Figure 2B and C, lanes 2, 5, and 9) and one multi-copy line (Figures 2B and C, lane 1) lack a *cre*-hybridizing band. Since a full-length copy of the *cre* gene should generate two unique hybridizing fragments (see Figure 1A),

**Table 1.** Maize pVS62 transgenic lines regenerated from the co-bombardment with a *cre*-expression construct.

Line	<i>bar</i> copy number	<i>cre</i> insertion	Cre activity in callus
1	multiple	none	–
2	1	none	–
3	1	partial copy	–
4	2	partial copy	–
5	1	none	–
6	2	partial copy	–
7	2	full-length copy	+
8	1	full-length copy	+
9	1	none	–
10	2	ND	ND
11	3	ND	ND
12	2	ND	ND
13	2	ND	ND

ND = not determined; – = not detected; + = detected.

3 transgenic lines (Figure 2B and C, lanes 3, 4, 6) must contain only a partial copy of the *cre* gene, while 2 transgenic lines (Figures 2B and C, lanes 7 and 8) may have retained a full copy of *cre*.

The presence of *cre* was also examined at the level of activity. The calluses of the 9 lines (Figure 2B, lanes 1–9) were bombarded with a tester construct. This tester construct has an antisense *gus* gene driven by *P2* (Srivastava *et al.*, 1999). The *gus*-coding region is flanked by oppositely oriented *loxP* sites such that Cre-mediated inversion of the *gus* fragment would place the coding region under the transcription of *P2*. The expression of *gus* would be detected as blue spots in GUS activity staining of the calluses. Based on this assay, only 2 out of 9 lines showed functional *cre* activity (Table 1). These two lines, lines 7 and 8, correlate to the ones that showed two bands in the hybridization analysis.

Inheritance of pVS62 integration was monitored in the three single-copy lines that lack *cre* DNA (Table 1, lines 2, 5, and 9). The primary transformants were crossed with wild-type pollen. Line 5 failed to set seeds and hence appears to be female-sterile. Lines 2 and 9, however, successfully transmitted the transgene to the F<sub>1</sub> generation (data not shown).

## Discussion

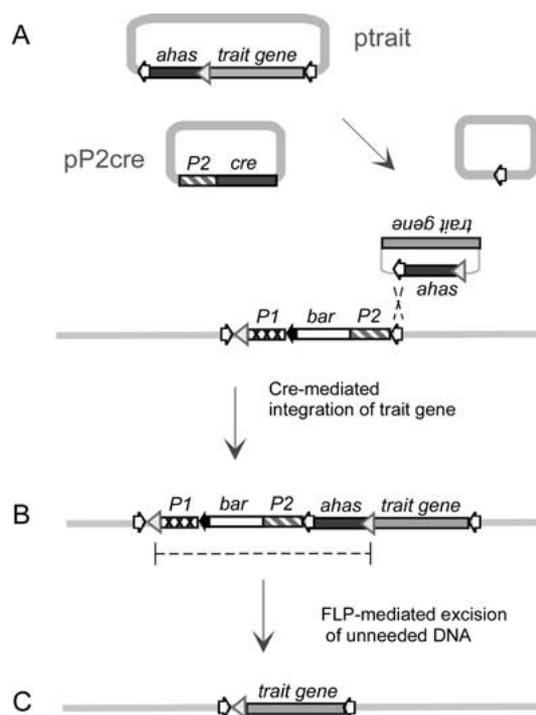
As multiple-copy transgenic plant lines are susceptible to expression and structural variability, single-

copy lines are desired for long-term propagation. The introduction of DNA through current delivery methods tends to generate a high proportion of insertions with complex integration patterns. The integration of foreign DNA into the plant genome is not well understood. Some studies have suggested that multiple copies of the introduced DNA may be ligated extrachromosomally prior to integration into a chromosomal location. Alternatively, a foreign DNA may integrate into a chromosomal location and then copied into the same or a different chromosomal location (reviewed by Pawlowski and Somers, 1996). A third possibility is that the first integrated molecule attracts the integration of additional molecules to the same site (Kohli *et al.*, 1998).

A number of approaches have been described to enrich for single-copy transgenic lines, including the 'Agrolistic' method that has been shown to generate reduced gene copy integration in tobacco and maize (Hansen and Chilton, 1996; Hansen *et al.*, 1997), and the use of niacinamide to reduce recombination of extrachromosomal molecules (De Block *et al.*, 1997).

In the present work, we revised the original strategy (Srivastava *et al.*, 1999) by co-transforming the recombinase gene along with the introduced DNA. When two DNA molecules are co-bombarded, they often integrate at a single locus. To minimize the integration of *cre* DNA, we used a molar ratio of 3 pVS62 to 1 pP2-cre. This ratio was sufficient to obtain a 23% of single-copy pVS62 lines that also lacked *cre* DNA, but we do not know whether this is the optimal ratio. As the frequency of 23% single-copy plants seems too high to be fortuitous, we believe that Cre-mediated resolution of multiple-copy molecules had played a vital role.

How much of this is due to resolution of DNA prior to integration or after integration into the genome is not known. In the case of post-integration resolution, chromosomal deletions are possible, if the transgene copies contain interspersed chromosomal DNA. However, based on a number of reports on generating deletions or inversions in plant chromosomes (Medberry *et al.*, 1995; Osborne *et al.*, 1995; Stuurman *et al.*, 1998; Vergunst *et al.*, 2000), the efficiency of recombination is much reduced for recombination sites that are far apart. Compounding the fact that the recombinase is only transiently available, this further reduces the probability of recombination for interspersed *lox* sites, and this may account for some of the transgenic lines with greater than a single-copy insertion. In a resolution-based strategy, the outermost



**Figure 3.** A strategy for generating transgenic plants that carry only the trait gene. Recombination sites are depicted as arrowheads defined in Figure 1. **A.** The single-copy pVS62 locus serves as the target site for site-specific insertion of plasmid ptrait, which contains a promoterless *ahas* gene (encoding resistance to imidazolinone herbicides) followed by a *FRT* site and the trait gene. The *ahas*-trait gene fragment is flanked by *lox511* in direct orientation. Plasmids ptrait and pP2-cre are co-introduced in cells containing pVS62 locus. Cre-mediated resolution of ptrait produces two circular molecules. **B.** Cre-mediated recombination between the genomic *lox511* site and the corresponding site in the circular molecule that contains *ahas* and the trait gene. The integration event results in a *P2-ahas* linkage to confer resistance to the imidazolinone. **C.** Excision of unneeded DNA (indicated by the dashed line) from the integration locus by a second recombination system such as FLP-*FRT*.

recombination sites are the most important. If the integration results in having the two outermost *lox* sites in the same orientation, the entire transgenic locus would be excised. However, this is not likely to be a frequent event, given that the transformation efficiency in the co-bombardment experiment was not significantly different from that of bombardment of pVS62 only.

The co-transformation strategy requires that a selectable marker be retained in the primary transformants. However, that does not mean a selectable marker must be retained in subsequent generations. In Figure 3, we outline a strategy for the delivery of a trait gene into the single-copy pVS62 target site. The co-transformation of pP2-cre with the 'ptrait' plasmid would most likely resolve ptrait into

the two molecules, one with the plasmid backbone, and the other with the trait gene and a promoterless marker gene, *ahas* (encoding acetohydroxyacid synthase) (Figure 3A). A functional *P2-ahas* linkage would be the selection for insertion of the trait gene into the chromosomal target (Figure 3B). As the introduced DNA brings along a *FRT* sites downstream of *ahas*, if desired, the unneeded marker DNA can be removed subsequently through FLP-mediated site-specific recombination (Figure 3C). In short, with the use of two different recombination systems, and two different selectable markers, it is possible to engineer a trait gene at a chromosome target without incorporating additional unneeded DNA in the final plant product.

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