Generation of Backbone-Free, Low Transgene Copy Plants by Launching T-DNA from the *Agrobacterium* Chromosome[1][W][OA]

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In both applied and basic research, *Agrobacterium*-mediated transformation is commonly used to introduce genes into plants. We investigated the effect of three *Agrobacterium tumefaciens* strains and five transferred (T)-DNA origins of replication on transformation frequency, transgene copy number, and the frequency of integration of non-T-DNA portions of the T-DNA-containing vector (backbone) into the genome of Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*). Launching T-DNA from the *picA* locus of the *Agrobacterium* chromosome increases the frequency of single transgene integration events and almost eliminates the presence of vector backbone sequences in transgenic plants. Along with novel *Agrobacterium* strains we have developed, our findings are useful for improving the quality of T-DNA integration events.

Since the generation of transgenic plants approximately 25 years ago, *Agrobacterium tumefaciens* has been widely used for introducing genes into plants for purposes of basic research as well as for generation of commercially used transgenic crops. For plant transformation, the gene of interest is placed between the left and right border repeats of *Agrobacterium* transferred (T)-DNA (Gelvin, 2003). The T-DNA region harboring the transgene is stably integrated into the plant genome by using an appropriate plant transformation protocol. T-DNA originates from the *Agrobacterium* tumor-inducing (Ti) plasmid. Because Ti plasmids are large and difficult to manipulate, smaller T-DNA binary vectors are currently predominately used for generation of transgenic plants (de Framond et al., 1983; Lee and Gelvin, 2008).

Although *Agrobacterium* has been used for plant transformation for more than two decades, problems using this bacterium remain. *Agrobacterium*-mediated transformation generally results in lower transgene copy numbers than do other transformation methods such as particle bombardment or polyethylene glycol-mediated transformation (Kohli et al., 1998; Shou et al., 2004). On the other hand, transformation frequently results in unwanted high copy number T-DNA integration events (Jorgensen et al., 1987; Deroles and Gardner, 1988; Shou et al., 2004; De Buck et al., 2009). Multiple integration events, often coupled with inverted repeat T-DNA integration patterns, may affect the stability of transgene expression by silencing mechanisms (Jorgensen et al., 1996). An additional problem with *Agrobacterium*-mediated transformation is the propensity for DNA sequences outside the T-DNA region to integrate into the plant genome (Kononov et al., 1997; Wenck et al., 1997; Shou et al., 2004). Integration of such vector backbone sequences can occur with high frequency. For example, Kononov et al. (1997) detected backbone sequences in 75% of tested transgenic tobacco (*Nicotiana tabacum*) plants, and very often the entire vector backbone is introduced into the plant genome (De Buck et al., 2000). T-DNA vector backbones usually harbor bacterial antibiotic resistance genes that can create governmental regulatory concerns.

Here we show that launching T-DNA from the *A. tumefaciens* chromosome reduces integrated transgene copy number and almost eliminates the presence of T-DNA backbone sequences. We describe several plasmids and bacterial strains to facilitate use of this methodology.

RESULTS

A. tumefaciens Strains and T-DNA Constructions

Our investigation utilized various combinations of the commonly used *A. tumefaciens* strains EHA101, GV3101, and LBA4404 with five different T-DNA binary systems. These *Agrobacterium* strains are nononcogenic (disarmed) and have been used for transformation of a large variety of plants. EHA101

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(Hood et al., 1987) harbors a derivative of the agropine/L,L-succinamopine-type Ti-plasmid pTiBo542, GV3101 (Koncz and Schell, 1986), a derivative of the nopaline-type Ti-plasmid pTiC58, and LBA4404 (Ooms et al., 1981), a derivative of the octopine-type Ti-plasmid pTiAch5. The tested T-DNA vectors contain an identical T-DNA region plus an *aadA* gene for bacterial selection for spectinomycin resistance. However, they contain different origins of replication (*ori*): the pVS *ori*, the pSa *ori*, the RK2 *ori*, and the pRiA4b *ori*. We furthermore analyzed the effect of launching T-DNA from the *Agrobacterium* C58 chromosome at the *picA* locus in strains EHA101 and GV3101. Disruption of this locus does not affect transformation (Rong et al., 1990) and we have generated vectors specifically designed to integrate genes into this locus (Lee et al., 2001).

We analyzed 14 different *A. tumefaciens* strain by replication origin combinations. The T-DNA region, derived from the binary vector pTF101.1 (Paz et al., 2004), harbors a *bar* gene as a plant selectable marker under the control of a cauliflower mosaic virus (CaMV) double 35S promoter (Fig. 1). Utilizing identical T-DNA regions with the same plant selectable marker and the identical non-T-DNA sequence proximal to the T-DNA left border in all constructions enabled us directly to compare results obtained for transformation frequencies, integrated transgene copy numbers, and backbone integration for all strain by construct combinations. All plasmids and *A. tumefaciens* strains used in this study are listed in Supplemental Table S1.

Effect of Binary Vector Replication Origin on Binary Vector Copy Number in *Agrobacterium*

We determined the copy number in *A. tumefaciens* of the four binary vectors used in our study. We placed a *bar* gene into the EHA105 chromosome and separately introduced each of the four T-DNA binary vectors into this strain. The resulting strains were incubated with or without acetosyringone to induce *vir* gene expression, and total bacterial DNA was extracted and subjected to DNA-blot analysis using the *bar* gene as a probe. T-DNA binary vector copy numbers were determined by comparison of the signal intensity of the chromosomal band (one per cell) to the T-DNA binary vector band. Figure 2 shows that plasmids containing the pSa origin are maintained at approximately four copies per cell. The copy numbers of plasmids containing the RK2 and pVS origins are seven to 10 per cell, and plasmids containing the pRi origin replicate to 15 to 20 copies per cell. No significant differences were seen when the strains were cultured under inducing or noninducing conditions.

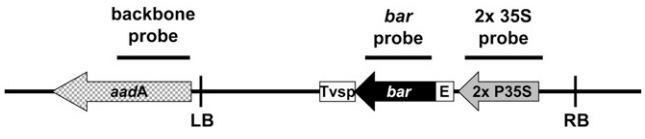

Figure 1. Schematic map of the T-DNA and neighboring regions of the base vector pTF101.1 (Paz et al., 2004) used in constructing the various vectors. Black bars represent fragments used for DNA-blot hybridizations experiments. LB and RB, T-DNA left and right borders, respectively; *aadA*, gene encoding spectinomycin resistance; E, translational leader from *Tobacco etch virus*; Tvsp, VSP (soybean [*Glycine max*] vegetative storage protein) terminator sequence; *bar*, gene conferring resistance to Basta/Bialophos/phosphinothricin; 2x P35S, CaMV double 35S promoter sequence.

Effect of *A. tumefaciens* Strain and T-DNA *ori* on Transformation Frequency

We determined the effect of 14 *Agrobacterium* strain-by-construct combinations on transformation frequency of Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*). Arabidopsis was transformed using a floral-dip protocol (Clough and Bent, 1998). At least four independent transformation experiments were conducted for each vector-by-strain combination, and transformation frequencies were determined by analyzing 1,500 to 4,500 seeds per experiment (Fig. 3A). Transformation frequency was highly dependent upon the *A. tumefaciens* strain utilized. GV3101 resulted in the highest transformation frequencies (0.97%–2.11%), whereas EHA101 and LBA4404 effected medium (0.09%–0.58%) and low (0.01%–0.12%) transformation frequencies, respectively. T-DNA replication origin had little effect on transformation frequency with one exception: Launching T-DNA from the *Agrobacterium* chromosome of EHA101 or GV3101 resulted in transformation frequencies lower than those of the other four T-DNA binary systems of the respective strain. Because the *picA* sequence of the Ach5 chromosome of LBA4404 does not share sufficient homology with the *picA* sequence of the C58-derived recombination vector we used (Lee et al., 2001), we were unable to integrate the T-DNA region into the *picA* locus of LBA4404.

Using an embryo inoculation protocol (Frame et al., 2002), we conducted at least four independent maize transformation experiments from which relative transformation frequencies for each of 12 strain-by-replication origin combinations (except chromosomal) were established (Fig. 3B). Four experiments were conducted to establish relative transformation frequencies for the two chromosomal replication origin combinations (in EHA101 and GV3101; Fig. 3B). As with Arabidopsis, launching T-DNA from the chromosomes of EHA101 and GV3101 resulted in low transformation frequencies (0.9%), whereas transformation frequencies were considerably higher (5%–15%) when T-DNA was placed on a plasmid binary vector.

Launching T-DNA from the *Agrobacterium* Chromosome Results in a High Percentage of Plants Containing a Single Integrated T-DNA Copy

We investigated the number of copies of integrated T-DNA in transgenic events by DNA dot-blot hybridization. T1 generation (heterozygous for T-DNA)

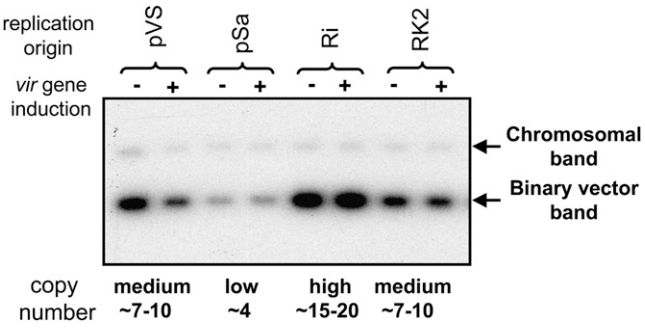

Figure 2. Vector copy number in *Agrobacterium* under inducing and noninducing conditions. *A. tumefaciens* cells harboring a fragment of the *bar* gene in the chromosome and a T-DNA binary vector were grown under *vir* gene inducing and noninducing conditions. DNA-blot analysis was done to determine copy numbers of various T-DNA binary vectors. Membranes were probed with a *bar* gene fragment. The top band represents the chromosomal integrated *bar* gene fragment (copy no. per cell: 1) and the bottom band the T-DNA binary vector.

Arabidopsis leaf samples were analyzed using a *bar* gene-specific fragment (Fig. 1). Figure 4A shows, strikingly, that launching T-DNA from the *Agrobacterium picA* chromosomal locus resulted in 77% to 78% of the events containing a single transgene copy. The percentage of single transgene copy events resulting from use of "conventional" T-DNA binary vectors was much lower. Correspondingly, the average transgene copy number for events generated using conventional T-DNA binary vectors was higher (3.3–4.9 copies/genome; Table I) than was the integrated transgene copy number of events generated using strains with T-DNA launched from the bacterial chromosome (1.3–1.6 copies/genome; Table I).

Maize T-DNA copy number determinations were made using heterozygous T0 generation plants (one plant per event). Because the *bar* gene resulted in background hybridization signals in maize (H. Oltmanns, unpublished data), we used the CaMV double 35S promoter fragment as the probe (Fig. 1). Figure 4B shows that for events generated using conventional T-DNA binary vectors, the percentage of events carrying a single transgene copy ranged from 16% to 48%. Average T-DNA copy numbers resulting from the use of binary vectors ranged from 2.1 to 4.2 copies per cell (Table I). As with Arabidopsis, use of *Agrobacterium* strains containing chromosomal integration of T-DNA resulted in a higher percentage (58%–64%) of single transgene copy number events; the average T-DNA copy number in these transgenic events was 1.7 copies per cell (Table I).

Although antibiotics were used to eradicate *Agrobacterium* after infection, bacterial cells might still contaminate selected transgenic Arabidopsis and maize plants. To eliminate the possibility of contaminating *Agrobacterium* DNA falsely increasing the apparent T-DNA copy number, we hybridized several membranes with the *Agrobacterium* chromosomal *picA* gene. We did not detect a hybridization signal using this probe (data not shown).

Launching T-DNA from the *Agrobacterium* Chromosome Mitigates Integration of T-DNA Backbone Sequences in Transgenic Plants

Integration of T-DNA backbone sequences into the genome of transgenic plants can present regulatory problems, especially when bacterial antibiotic

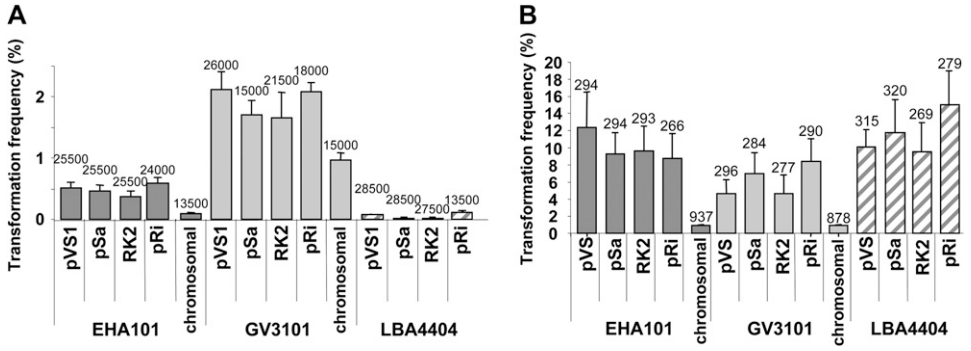

Figure 3. Transformation frequencies of Arabidopsis (A) and maize (B) with different *Agrobacterium* strain by origin of replication combinations. Arabidopsis was transformed by a floral-dip protocol and maize by embryo inoculation. Error bars represent the SE among different transformation experiments. At least four independent transformation experiments were conducted for both Arabidopsis and maize. The total number of seeds screened (A) or embryos infected (B) to calculate transformation frequencies is indicated above each error bar. For maize, five independent experiments were conducted to establish relative transformation frequencies for each of 12 strain-by-replication origin combinations (except chromosomal). Embryos from each of the 22 ears dissected were shared 12 ways at infection. Subsequently, transformation frequencies for the two chromosomal replication origin combinations (in EHA101 and GV3101) were established by inoculating them beside the pTF101.1 replication origin vector in EHA101 and GV3101. In four independent experiments, embryos from 21 dissected ears were shared four ways at infection. Throughout the remainder of this study, additional maize transformation experiments using these 14 strain-by-replication origin combinations were conducted as needed to recover adequate numbers of transgenic events for molecular analysis.

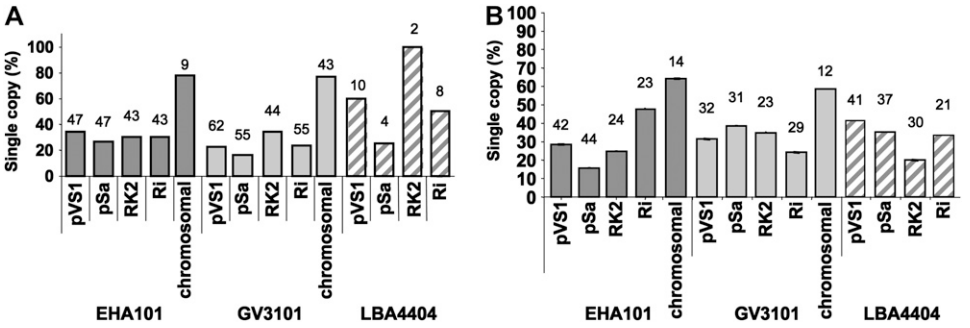

Figure 4. The percentage of events containing a single copy of integrated T-DNA following transformation by the 14 T-DNA replication origin-by-*Agrobacterium* strain combinations for Arabidopsis (A) and maize (B). Copy number reconstructions were done by DNA dot-blot experiments using T-DNA-specific probes (Fig. 1). Analysis was performed on heterozygous T1 generation Arabidopsis plants and heterozygous regenerated T0 generation maize plants. Numbers over the bars represent the number of independent events analyzed.

resistance genes are transferred. We investigated whether the *Agrobacterium* strain or T-DNA replication origin affects the frequency of backbone integration events. We used the spectinomycin resistance (*aadA*) gene immediately outside the T-DNA left border (Fig. 1) as a hybridization probe to detect backbone sequences within the genome of transgenic plants.

Figure 5, A and B show the percentage of transgenic plants with backbone integration events for the 14 strain-by-origin combinations in Arabidopsis and maize, respectively. For Arabidopsis, no plants from 13 EHA101 events (0%) and one plant from 40 GV3101 events (3%), respectively, contained backbone sequences when they were generated by *Agrobacterium* strains in which the T-DNA was launched from the chromosome. For maize, one plant from 14 EHA101 events (7%) and one plant from 12 GV3101 events (8%), respectively, contained the vector backbone. On the other hand, the use of conventional T-DNA binary vectors resulted in a relatively high percentage of plants containing backbone sequences (47%–67% for Arabidopsis; 19%–55% for maize).

Vectors and *A. tumefaciens* Strains to Facilitate Integration of T-DNA into the *Agrobacterium* Chromosome

We generated two systems to facilitate integration of T-DNA into the *Agrobacterium* chromosome (Fig. 6). We first introduced a T-DNA region into the *picA* locus of the C58 chromosome. This T-DNA contains a CaMV 35S-*bar* gene as a plant selection marker, and a small region of pBluescript to provide homology for recombination with a variety of pBluescript-derived plasmids, such as the pSAT series of expression vectors (Chung et al., 2005). Because plasmids harboring the ColE1 *ori* cannot replicate in *A. tumefaciens*, ampicillin/carbenicillin-resistant bacteria can only be selected when the introduced plasmid cointegrates into the homologous T-DNA region (Fig. 6A). This bacterial strain contains an *aadA* gene directly outside the T-DNA left border to detect transfer of non-T-DNA sequences. In addition, the strain can be eliminated from cocultivation reactions by using β-lactam antibiotics containing clavulanate, such as Timentin (L.-Y. Lee, unpublished data).

Table I. *Average transgene copy numbers for T-DNA replication origins-by-A. tumefaciens strain combinations*

A. tumefaciens Strain	T-DNA Vector *ori* (Incompatibility Group)	Average Transgene Copy No.	No. of Events Analyzed
Arabidopsis			
EHA101	pVS1	4.1	47
EHA101	pSa (incW)	4.7	47
EHA101	RK2 (incPα)	4.1	43
EHA101	pRiA4	3.3	43
EHA101	Chromosomal	1.3	9
GV3101	pVS1	4.4	62
GV3101	pSa (incW)	4.9	55
GV3101	RK2 (incPα)	3.3	44
GV3101	pRiA4	4.3	55
GV3101	Chromosomal	1.6	43
LBA4404	pVS1	1.7	10
LBA4404	pSa (incW)	2.0	4
LBA4404	RK2 (incPα)	1.0	2
LBA4404	pRiA4	1.5	8
Maize			
EHA101	pVS1	3.7	42
EHA101	pSa (incW)	3.5	44
EHA101	RK2 (incPα)	4.2	24
EHA101	pRiA4	3.5	23
EHA101	Chromosomal	1.7	14
GV3101	pVS1	3.1	32
GV3101	pSa (incW)	2.6	31
GV3101	RK2 (incPα)	3.3	23
GV3101	pRiA4	3.2	29
GV3101	Chromosomal	1.7	12
LBA4404	pVS1	2.1	41
LBA4404	pSa (incW)	2.9	37
LBA4404	RK2 (incPα)	3.0	30
LBA4404	pRiA4	2.4	21

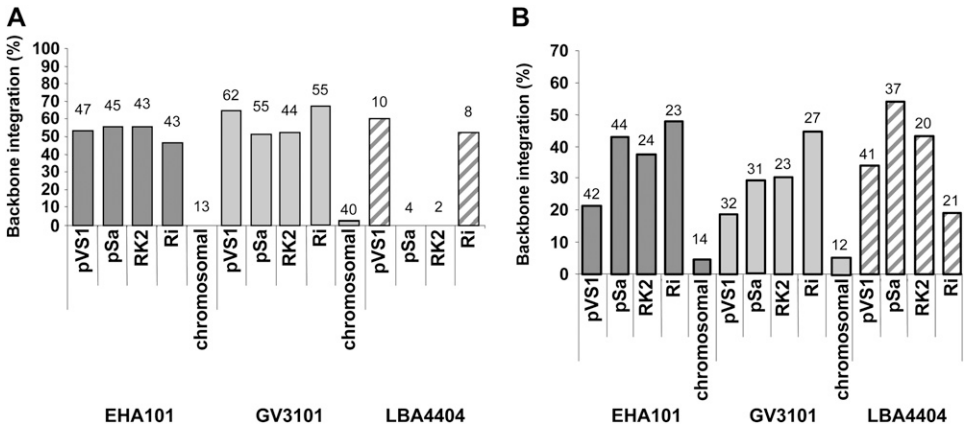

Figure 5. The percentage of independent events containing vector backbone sequences following transformation by the 14 T-DNA replication origin-by-*Agrobacterium* strain combinations for Arabidopsis (A) and maize (B). Blots were hybridized with a backbone-specific probe, the *aadA* gene (Fig. 1). Numbers over the bars represent the number of independent events analyzed.

Although the strain described in Figure 6A is easy to use, it has the potential regulatory disadvantage of introducing a β-lactamase antibiotic resistance gene into the plant when T-DNA integrates. To eliminate such potential regulatory problems for plants destined for field release, we generated a second system to launch T-DNA from the *Agrobacterium* chromosome. A region homologous to the *Agrobacterium pgl/picA* locus was cloned into a plasmid containing a T-DNA. Gene expression cassettes from, for example, pSAT vectors can be cloned into the rare-cutting multiple cloning site of this vector, and the entire vector can be introduced into *A. tumefaciens*. Spectinomycin resistance conferred by this plasmid results from homologous recombination with the *picA/pgl* region of the *A. tumefaciens* C58 chromosome (Fig. 6B). Thus, integration of T-DNA into the *A. tumefaciens* chromosome occurs without introduction of an antibiotic resistance gene into T-DNA.

DISCUSSION

We studied the effect of three commonly used *A. tumefaciens* strains and five T-DNA replication origins on transformation frequency and the quality of T-DNA integration events in Arabidopsis and maize. Within a factor of approximately three, there was little difference among tested *Agrobacterium* strains harboring various T-DNA binary vectors with respect to maize transformation frequency. These results contrast with Arabidopsis flower-dip transformation, where we ob-

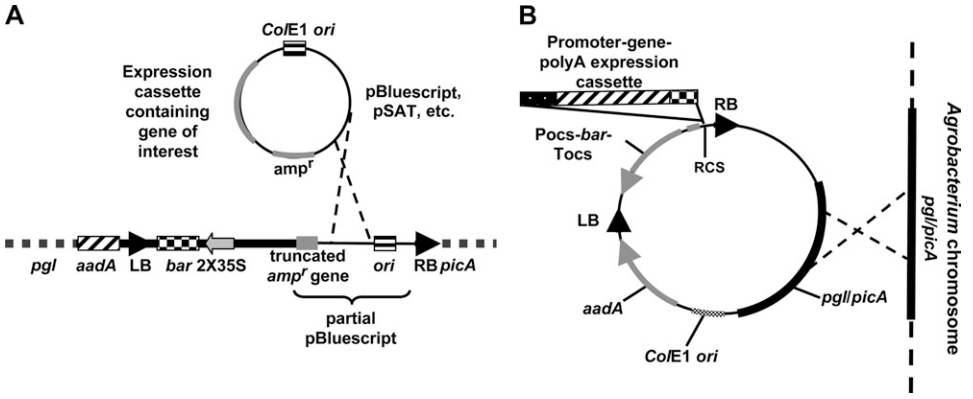

Figure 6. Systems for introducing T-DNA harboring transgene expression cassettes into the *pgl/picA* region of the *A. tumefaciens* chromosome. A, T-DNA containing a CaMV 35S promoter-*bar* gene plant selection marker and a fragment of pBluescript, and an *aadA* gene to the left of the T-DNA left border, has been inserted into the *A. tumefaciens* C58 chromosome. When a pUC-derived plasmid containing an expression cassette is introduced into this strain, carbenicillin-resistant colonies can only be obtained if homologous recombination occurs, resulting in a cointegrate between this plasmid and the pBluescript region of the T-DNA. B, In this system, a transgene expression cassette is first cloned into a T-DNA region located on a plasmid harboring a ColE1 *ori*. The plasmid also contains the *pgl/picA* region of the *A. tumefaciens* C58 chromosome. When this plasmid is introduced into *A. tumefaciens* C58-derived strains, carbenicillin-resistant colonies can only be obtained if homologous recombination occurs between the *pgl/picA* regions of the plasmid and the chromosome. LB, T-DNA left border; RB, T-DNA right border; *ori*, origin of replication; *amp*^{r}, β-lactamase gene conferring ampicillin/carbenicillin resistance upon the bacterium; *aadA*, spectinomycin resistance gene; 2X35S, double CaMV 35S promoter; *bar*, gene conferring resistance to Basta/Bialophos/phosphinothricin; P_{ocs}, octopine synthase promoter; T_{ocs}, octopine synthase terminator; RCS, multiple rare cutting sites flanking various pSAT vectors (*Asc*I, I-*Ppo*I, I-*Sce*I, I-*Ceu*I, PI-*Psp*I, and PI-*Til*I). Broken crossed lines indicate homologous recombination events.

served a strong dependency upon the *Agrobacterium* strain, but not the T-DNA binary vector, used. Interestingly, efficient transformation of *Brassica juncea* (Pandian et al., 2006), *Brassica napus*, and *Brassica oleracea* (De Block et al., 1989; Bhalla and Singh, 2008) was obtained using a variety of *Agrobacterium* strains, including GV3101, LBA4404, and AGL1 (which is similar to EHA101; Lee and Gelvin, 2008). However, none of these studies utilized a flower-dip transformation protocol. It may be that flower-dip transformation is more sensitive to the *Agrobacterium* strain used than is somatic cell transformation.

We decided to integrate T-DNA into the *Agrobacterium* chromosome at the *picA* locus because previous work had indicated that disruption of this site in the bacterial chromosome does not negatively affect transformation frequency (Rong et al., 1990). In addition, we had previously developed plasmids to facilitate integration of DNA into this locus (Lee et al., 2001). Miranda et al. (1992) had previously launched T-DNA from the *picA* locus. It is possible that launching T-DNA from other positions of the *Agrobacterium* chromosome would also be suitable. However, we have not investigated this possibility. We note, however, that Hoekema et al. (1984) launched T-DNA from an uncharacterized position in the *Agrobacterium* chromosome, suggesting that other chromosomal sites could function as T-DNA launching pads. Launching T-DNA from the *Agrobacterium* chromosome results in fewer integrated transgene copies and almost eliminates the presence of T-DNA binary backbone sequences in recovered transgenic events. However, these two advantageous aspects of plant transformation are accompanied by decreased transformation frequency (Fig. 3). In Arabidopsis, this decrease is slight (2- to 4-fold) but it can be greater (approximately 10-fold) in maize. Whether scientists are willing to compensate decreased transformation frequency with a higher quality transformation event will depend upon the ease, time, and cost in generation of multiple transgenic events for different plant species.

Stable and predictable transgene expression is a major objective for both basic and applied research. Multiple integrated T-DNA copies, especially when combined with complex T-DNA integration patterns, can trigger transgene silencing (Jorgensen et al., 1987, 1996; Stam et al., 1997). The routine generation of single-copy transgenic events is therefore a major goal for agricultural biotechnology. Launching T-DNA from the *Agrobacterium* chromosome may provide one approach for achieving this goal.

Several studies have analyzed T-DNA locus and/or copy numbers in transgenic Arabidopsis. Feldmann (1991) concluded that the average number of independently segregating, active transgene loci in his initial library of T-DNA-tagged plants is 1.4. This value is similar to that of other T-DNA-tagged collections in Arabidopsis (McElver et al., 2001; Alonso et al., 2003) and rice (*Oryza sativa*; Barakat et al., 2000; Jeon et al., 2000). However, the number of active loci in these plants is generally less than the number of integrated T-DNA molecules. T-DNA insertions frequently occur as partial or complete multimers in direct or inverted repeat orientation (Jorgensen et al., 1987; Feldmann, 1991). Bechtold et al. (1993) showed that 70% of tested Arabidopsis transformants generated by a vacuum infiltration protocol carried direct or indirect tandem repeat copies of T-DNA. In this study the average T-DNA copy number in Arabidopsis ranged from 1.0 to 4.9, and in maize from 1.3 to 3.9 per diploid genome (Fig. 4; Table I). Our results agree with those of Galbiati et al. (2000) who investigated 38,000 transgenic Arabidopsis plants generated by a floral-dip method. Interestingly, transformation using *A. tumefaciens* LBA4404 resulted in an average transgene copy number (1.0–1.7 in Arabidopsis and 2.1–3.0 in maize; Table I) lower than that resulting from transformation using the other tested strains. Grevelding et al. (1993) investigated whether the transformation method affected transgene copy number in Arabidopsis. Most transgenic plants produced by a leaf-disc inoculation method contained multiple T-DNA insertions, whereas root transformation resulted mostly in single T-DNA insertions. Therefore, the *Agrobacterium* strain, transformation method, and plant target tissue may influence the number of integrated T-DNA molecules (De Buck et al., 2009).

Although T-DNA integration into the plant genome was experimentally shown almost 30 years ago, little is known about how many T-DNA strands are produced in *Agrobacterium* and transferred to the plant cell. It is likely that considerably more T-strands are transferred than are integrated (Virts and Gelvin, 1985; Atmakuri et al., 2007). T-DNA copy number of the chromosomal integration construction in the bacterial cell is one (1; except during replication before cell partition). Low integrated transgene copy numbers in plants may result from a limited number of T-strands transferred to the plant cell. We might therefore have expected to see a correlation between bacterial and plant T-DNA copy number using the different T-DNA replication origins because they replicate to different extents in the bacteria. However, we did not find such a correlation.

Integration of binary vector backbone sequences in transgenic plants is a common phenomenon (Kononov et al., 1997; Wenck et al., 1997; De Buck et al., 2000). Launching T-DNA from the *Agrobacterium* chromosome almost eliminates the presence of integrated T-DNA backbone sequences (Fig. 5). In contrast, 47% to 67% of the Arabidopsis plants generated by *Agrobacterium* strains harboring a T-DNA binary vector contained integrated vector backbone sequences. Although elimination of these sequences from transgenic plants is a major goal for agricultural biotechnology, only one previous report described a methodology to effect this result. By incorporating a lethal *barnase* gene into the non-T-DNA region of the binary vector, Hanson et al. (1999) reduced the number of plants harboring backbone sequences. However, up to 18% of the transgenic plants still carried backbone sequences.

Transfer of binary vector backbone sequences can occur when the T-DNA left border repeat is not recognized by the VirD2 endonuclease during processing of the T-DNA strand. It can also occur as a result of VirD2 linkage to the 5′ end of the vector DNA directly outside the T-DNA left border, followed by transfer of the backbone in a manner analogous to that of T-DNA transfer (Durrenberger et al., 1989; Kononov et al., 1997; De Buck et al., 2000). If the T-DNA strand were derived from a binary plasmid and during T-DNA processing the left border repeat is skipped, T-DNA processing will either end at a sequence in the backbone that resembles a T-DNA border or, due to the circular nature of binary vectors, when the right border repeat is reached. However, if T-DNA were integrated into the bacterial chromosome, read through at the T-DNA left border repeat could result in very long T-DNAs, theoretically as long as the *Agrobacterium* chromosome itself if no adequate termination site were present. Although transfer of long T-DNA molecules is possible (Miranda et al., 1992; Hamilton et al., 1996), it is less frequent than transfer of small T-DNAs. The observation that large T-DNAs only integrate into the plant genome very rarely offers a possible explanation for why chromosomal integration of T-DNA results in transgenic plants lacking backbone sequences. If the T-DNA left border were skipped during T-DNA strand processing (or if DNA transfer initiates from sequences directly to the left of the left T-DNA border), the resulting T-DNA would be too long for efficient transfer to the plant or integration into the plant genome. Although there might be concern that sequences from the bacterial chromosome next to the T-DNA right border could be integrated into the plant genome, probing of the DNA membranes with an *Agrobacterium picA* fragment, located immediately to the right of the right T-DNA border, failed to detect its presence (data not shown).

It is tempting to speculate whether integration of vector backbone sequences into plants is a consequence of simplifying *Agrobacterium*-mediated plant transformation by using small T-DNA binary vectors. Transfer of non-T-DNA portions of a large Ti plasmid to plants is possible but rare: On average only one out of 80 transgenic tobacco calli contained a *nptII* gene positioned outside the T-DNA left border (Ramanathan and Veluthambi, 1995). In contrast, Kononov et al. (1997) detected vector backbone sequences in approximately 75% of transgenic tobacco plants generated using an *Agrobacterium* strain carrying a small T-DNA binary vector. These results suggest that backbone integration occurs more frequently when a small T-DNA binary vector is used.

Although T-DNA binary vectors are ubiquitously used because of their ease of handling, we present here two vector systems to simplify launching T-DNA from the *Agrobacterium* chromosome. The first vector system (Fig. 6A) is easier to use because it requires only one cloning step. This is followed by introduction of the resulting expression plasmid into *Agrobacterium* for homologous recombination into a T-DNA already positioned at the *picA* locus. However, this system has the potential regulatory disadvantage of transferring a β-lactamase antibiotic resistance gene into the plant genome. We thus developed a second system (Fig. 6B) to integrate a T-DNA, containing a gene expression cassette but lacking an antibiotic resistance gene, into the *A. tumefaciens picA* locus. This latter system will not transfer an antibiotic resistance gene to plants. However, it requires two cloning steps before introducing the final plasmid into *Agrobacterium* for recombination into the *picA* locus. Together, these two vector systems will facilitate generation of single T-DNA copy number transgenic plants lacking vector backbone sequences.

MATERIALS AND METHODS

Agrobacterium tumefaciens Growth Conditions

Agrobacterium tumefaciens strains were grown on solidified or liquid AB Suc or yeast extract peptone medium (Lichtenstein and Draper, 1986) supplemented with appropriate antibiotics (rifampicin, 10 μg/mL; spectinomycin, 100 μg/mL; kanamycin, 25 μg/mL; gentamicin, 25 μg/mL).

T-DNA Constructions

The T-DNA region and the bacterial *aadA* (spectinomycin resistance) gene (Fig. 1) used in all T-DNA binary constructions derives from pTF101.1 (Paz et al., 2004). pTF101.1 contains a pVS1 origin of replication. To generate the various binary vectors, we replaced the pVS1 replication origin (*ori*) with those from other plasmids. For introducing the RK2 *ori*, we removed the pVS1 origin from pTF101.1 using *Sca*I and *Not*I and replaced it with a *Not*I/*Nru*I fragment from pBIN19 (Bevan, 1984), generating pTF::Bin19. For introducing the pSa *ori*, we removed the pVS1 *ori* from pTF101.1 using *Sca*I and *Nsi*I and replaced it with a *Pst*I/*Sac*II fragment from pUCD2 (Close et al., 1984). All overhanging ends were made blunt using T4 DNA polymerase (New England Biolabs) to enable ligation. The resulting plasmid was designated pTF::UCD2. The pRiA4b origin isolated from *Agrobacterium rhizogenes* A4 (Jouanin et al., 1986) was cloned as a *Bam*HI-*Hin*dIII fragment into pBluescriptII KS$^+$, generating pBluescript::Ri. To confirm that the cloned pRi replication origin effects replication in *Agrobacterium*, pBluescript::Ri was transformed into *A. tumefaciens* by electroporation and plasmid DNA was isolated from carbenicillin-resistant colonies. pBluescript::pRi was digested with *Cla*I and the overhanging ends were made blunt using T4 DNA polymerase. The product was subsequently digested with *Not*I and cloned into pTF101.1 prior digested with *Sca*I and *Not*I to remove the pVS1 replication origin. The resulting plasmid was designated pTF::Ri.

The four T-DNA vectors pTF101.1, pTF::Bin19, pTF::UCD2, and pTF::Ri (Supplemental Fig. S1) were separately transformed into *A. tumefaciens* EHA101 (Hood et al., 1987), GV3101 (Koncz and Schell, 1986), and LBA4404 (Ooms et al., 1981) by electroporation.

Construction of *Agrobacterium* Strains Containing T-DNA Integrated into the *pgl/picA* Locus of the C58 Chromosome

A 4.2 kb *Sca*I-*Nsi*I fragment containing the T-DNA region plus the *aadA* gene of pTF101.1 was inserted into the blunted *Spe*I and *Pst*I sites of the integration vector pE1931, generating pE2759 (Supplemental Fig. S1). pE2759 was separately introduced into *A. tumefaciens* EHA101 and GV3101, generating *A. tumefaciens* At1586 and At1588, respectively. The eviction plasmid pPH1JI or pVK102 was introduced into *A. tumefaciens* At1586 and At1588, respectively, and colonies were selected on gentamicin and carbenicillin. Tetracycline-sensitive colonies (that had lost pE2759) were selected, generating *A. tumefaciens* At1589 and At1591, respectively, and recombination of the T-DNA region into the *pgl/picA* locus of the *Agrobacterium* chromosome was confirmed by DNA-blot hybridization (Lee et al., 2001).

Construction of an *Agrobacterium* Strain to Facilitate Integration of Expression Cassettes into the T-DNA Region on the C58 Chromosome

A 1.549 kbp *Pvu*II-*Sca*I fragment from pBluescript was cloned into the *Sma*I site of pTF101.1, generating pE3265. A 5.8 kbp *Sca*I-*Nsi*I fragment from pE3265 containing T-DNA, part of pBluescript, and the *aadA* gene was cloned into blunted *Spe*I and *Pst*I sites of pE1770, generating pE3349. pE3349 was introduced into *A. tumefaciens* EHA105, generating *A. tumefaciens* At1687. *Escherichia coli* strains containing pVK102 and pRK230, respectively, were used to conjugate with *A. tumefaciens* At1687. The recombinant *Agrobacterium* strain containing the T-DNA borders, *aadA* gene, and a portion of pBluescript sequence was named *A. tumefaciens* At1702.

Construction of an Integration Binary Vector to Facilitate Launching T-DNA from the *A. tumefaciens* C58 Chromosome

A blunted *Eco*RI fragment containing the *pgl/picA* locus was cloned into the blunted *Nde*I site of pE3055. Removal of the *Nde*I fragment from pE3055 resulted in the loss of the pVS *ori* to create pE3361, a binary vector containing rare cloning sites (*Asc*I, I-*Ppo*I, I-*Sce*I, I-*Ceu*I, PI-*Psp*I, and PI-*Til*I), a plant selection marker (*bar* gene), a bacterial selection marker (*aadA* gene), and a ColE1 *ori*. This plasmid cannot replicate in *Agrobacterium*.

Determination of Binary Vector Copy Number in *Agrobacterium*

The four T-DNA vectors pTF101.1, pTF::Bin19, pTF::UCD2, and pTF::Ri were separately transformed into *A. tumefaciens* EHA105 (Hood et al., 1993) containing a *bar* gene introduced into the *picA* locus, grown under *vir* gene inducing or noninducing conditions (Gelvin, 2006), and total DNA isolated by phenol:chloroform extraction. Equal amounts of DNA were digested with *Bgl*II and *Eco*RI and separated by electrophroesis through 0.9% agarose gels. DNA was transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech) and hybridized with a *bar* gene PCR fragment using conditions described below. Hybridization signal intensity was determined by scanning autoradiograms and using Labworks 4.6 Image Acquisition and Analysis Software (UVP).

Agrobacterium-Mediated Transformation of Arabidopsis and Maize

Arabidopsis (*Arabidopsis thaliana*; ecotype Wassilewskija-2) was transformed by a floral-dip protocol (Clough and Bent, 1998). Phosphinothricin-resistant plants were selected on Gamborg's B5 medium (Caisson Laboratories) supplemented with 10 μg/mL phosphinothricin and 100 μg/mL Timentin. Transformation frequencies were calculated as follows: (no. of phosphinothricin-resistant plants/no. of seeds tested) $\times$ 100. Ten milligrams of seeds correspond to approximately 500 seeds. Maize (*Zea mays*) transformation was as previously described (Frame et al., 2002).

Genomic DNA Extraction and DNA Dot-Blot Hybridization

Genomic DNA was extracted from three to five leaves of 3- to 4-week-old Arabidopsis plants or approximately 100 mg maize leaf tissue according to Murray and Thompson (1980). Genomic DNA was quantified using a Gemini XPS microplate spectrofluorometer (Molecular Devices, excitation: 488 nm, emission: 525 nm) using a Quant-iT PicoGreen dsDNA assay kit (Invitrogen).

DNA dot blots were prepared as follows: Arabidopsis (75 ng) or maize (1.5 μg) genomic DNA samples were denatured by adding NaOH and EDTA to final concentrations of 0.4 M and 10 mM, respectively, followed by 10 min incubation in boiling water. A Hybond N+ nylon membrane (Amersham Pharmacia Biotech) was prewetted with water and placed between the layers of the dot-blot apparatus. Samples were applied to the wells of the dot-blot apparatus, incubated for 30 min, and then drawn onto the nylon membrane using a gentle vacuum. DNA was cross-linked to the membrane by using a CL-1000 UV crosslinker (UVP). The membranes were incubated in 2$\times$ SSC for 10 min and dried.

Probes for dot-blot hybridizations were generated using random prime Ready-To-Go DNA labeling beads and ^{32}P-dCTP (both Amersham Pharmacia Biosciences). Unincorporated radioactive nucleotides were removed by Sephadex G-100 gel filtration. Membranes were prehybridized in 7% (w/v) SDS, 0.5 M sodium phosphate (pH 7.2), and 10 mM EDTA (pH 8.0) at 65°C for 2 h. Hybridization was conducted overnight at 65°C. After hybridization, membranes were washed two times with 2$\times$ SSC, 0.1% (w/v) SDS, 10 mM EDTA (pH 8.0), then two times with 1$\times$ SSC, 0.1% (w/v) SDS, 10 mM EDTA, and finally two times with 0.1$\times$ SSC, 0.1% (w/v) SDS, 10 mM EDTA at 65°C. Membranes were exposed at $-$80°C for autoradiography. Integrated dot density was determined using Labworks 4.6 Image Acquisition and Analysis Software (UVP). For reprobing, membranes were stripped using boiling 0.1% (w/v) SDS twice. Calculations for T-DNA copy number standards were based on an Arabidopsis genome size of 125 Mb (Arabidopsis Genome Initiative, 2000) and a maize genome size of 2,500 Mb (Arumuganathan and Earle, 1991). The size of pTF101.1 is 9,189 bp (Paz et al., 2004).

DNA dot-blot membranes were hybridized sequentially with various probes. For determining T-DNA copy numbers of Arabidopsis plants, dot-blot membranes were hybridized with a *bar* gene PCR fragment (Fig. 1). To determine T-DNA copy numbers of maize plants, membranes were probed with a 759 bp *Pst*I/*Xho*I fragment from pTF101.1 harboring the double *CaMV 35S* promoter (Fig. 1). To detect vector backbone sequences in both Arabidopsis and maize DNA, a 656 bp PCR fragment derived from the non-T-DNA region next to the T-DNA left border of pTF101.1 (Fig. 1) was used as a probe. The fragment was amplified using pTF101.1 as a template and 5′-TCACCGTAACCAGCAAATCA-3′ and 5′-CTCGGCACAAAATCACCACT-3′ as primers. A 3.1 kb *Eco*RI fragment containing the *pgl/picA* locus (Rong et al., 1990) was used as a probe to check for the presence of contaminating *Agrobacterium* DNA in plant genomic DNA. All DNA fragments were gel purified prior to labeling using a QIAEX II gel extraction kit (Qiagen). To normalize amounts of DNA in each dot, membranes were hybridized with genomic Arabidopsis or maize DNA.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Schematic representation of the various T-DNA binary vectors and integration plasmid used in this study.

Supplemental Table S1. Plasmids and strains.

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LITERATURE CITED

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796–815

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep **9:** 229–233

Atmakuri K, Cascales E, Burton OT, Banta LM, Christie PJ (2007) *Agrobacterium* ParA/Mind-like VirC1 spatially coordinates early conjugative DNA transfer reactions. EMBO J **26:** 2540–2551

Barakat A, Gallois P, Raynal M, Mestre-Ortega D, Sallaud C, Guiderdoni E, Delseny M, Bernardi G (2000) The distribution of T-DNA in the genomes of transgenic *Arabidopsis* and rice. FEBS Lett **471:** 161–164

Bechtold N, Ellis J, Pelletier G (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C R Acad Sci III **316:** 1194–1199

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res **12:** 8711–8721

Bhalla PL, Singh MB (2008) *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. Nat Protoc **3:** 181–189

Chung SM, Frankman EL, Tzfira T (2005) A versatile vector system for multiple gene expression in plants. Trends Plant Sci **10:** 357–361

Close TJ, Zaitlin C, Kado CI (1984) Design and development of amplifiable broad-host-range cloning vectors: analysis of the *vir* region of *Agrobacterium tumefaciens* plasmid pTiC58. Plasmid **12:** 111–118

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J **16:** 735–743

De Block M, De Brouwer D, Tenning P (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and new genes in the transgenic plants. Plant Physiol **91:** 694–701

De Buck S, de Wilde C, van Montagu M, Depicker A (2000) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. Mol Breed **6:** 459–468

De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in *Arabidopsis* transformants is highly determined by the transformed target cell. Plant J **60:** 134–145

de Framond AJ, Barton KA, Chilton MD (1983) Mini-Ti: a new vector strategy for plant genetic engineering. Biotechnology (N Y) **1:** 262–269

Deroles SC, Gardner RC (1988) Analysis of the T-DNA structure in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. Plant Mol Biol **11:** 365–377

Durrenberger F, Crameri A, Hohn B, Koukolikova-Nicola Z (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc Natl Acad Sci USA **86:** 9154–9158

Feldmann KA (1991) T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. Plant J **1:** 71–82

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SEK, Li B, Nettleton DS, Pei D, et al (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol **129:** 13–22

Galbiati M, Moreno MA, Nadzan G, Zourelidou M, Dellaporta SL (2000) Large-scale T-DNA mutagenesis in *Arabidopsis* for functional genomic analysis. Funct Integr Genomics **1:** 25–34

Gelvin SB (2003) *Agrobacterium* and plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol Biol Rev **67:** 16–37

Gelvin SB (2006) *Agrobacterium* virulence gene induction. *In* K Wang, ed, Methods in Molecular Biology: *Agrobacterium* Protocols, Vol 44. Humana Press, Totowa, NJ, pp 77–84

Grevelding C, Fantes V, Kemper E, Schell J, Masterson R (1993) Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. Plant Mol Biol **23:** 847–860

Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc Natl Acad Sci USA **93:** 9975–9979

Hanson B, Engler D, Moy Y, Newman B, Ralston E, Gutterson N (1999) A simple method to enrich an *Agrobacterium*-transformed population for plants containing only T-DNA sequences. Plant J **19:** 727–734

Hoekema A, Roelvink PW, Hooykaas PJJ, Schilperoort RA (1984) Delivery of T-DNA from the *Agrobacterium tumefaciens* chromosome into plant cells. EMBO J **3:** 2485–2490

Hood E, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res **2:** 208–218

Hood EE, Fraley RT, Chilton MD (1987) Virulence of *Agrobacterium tumefaciens* strain A281 on legumes. Plant Physiol **83:** 529–534

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Lee S, Yang K, et al (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J **22:** 561–570

Jorgensen R, Snyder C, Jones JDG (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet **207:** 471–477

Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol Biol **31:** 957–973

Jouanin L, Tourneur J, Tourneur C, Casse-Delbart F (1986) Restriction maps and homologies of the three plasmids of *Agrobacterium rhizogenes* strain A4. Plasmid **16:** 124–134

Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci USA **95:** 7203–7208

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet **204:** 383–396

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector "backbone" sequences into the tobacco genome: evidence for multiple complex patterns of integration. Plant J **11:** 945–957

Lee LY, Gelvin SB (2008) T-DNA binary vectors and systems. Plant Physiol **146:** 325–332

Lee LY, Humara JM, Gelvin SB (2001) Novel constructions to enable the integration of genes into the *Agrobacterium tumefaciens* C58 chromosome. Mol Plant Microbe Interact **14:** 577–579

Lichtenstein C, Draper J (1986) DNA cloning: a practical approach. *In* DM Glover, ed, Genetic Engineering of Plants, Vol 2. IRL Press, Washington, DC, pp 67–119

McElver J, Tzafrir I, Aux G, Rogers R, Ashby C, Smith K, Thomas C, Schetter A, Zhou Q, Cushman MA, et al (2001) Insertional mutagenesis of genes required for seed development in *Arabidopsis thaliana*. Genetics **159:** 1751–1763

Miranda A, Janssen G, Hodges L, Peralta EG, Ream W (1992) *Agrobacterium tumefaciens* transfers extremely long T-DNAs by a unidirectional mechanism. J Bacteriol **174:** 2288–2297

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res **8:** 4321–4325

Ooms G, Hooykaas PJJ, Moolenaar G, Schilperoort RA (1981) Crown gall plant tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying mutated octopine Ti plasmids; analysis of T-DNA functions. Gene **14:** 33–50

Pandian A, Hurlstone C, Liu Q, Singh S, Salisbury P, Green A (2006) *Agrobacterium*-mediated transformation protocol to overcome necrosis in elite Australian *Brassica juncea* lines. Plant Mol Biol Rep **24:** 103a–103i

Paz MM, Shou H, Guo Z, Zhang Z, Banerjee AK, Wang K (2004) Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant. Euphytica **136:** 167–179

Ramanathan V, Veluthambi K (1995) Transfer of non-T-DNA portions of the *Agrobacterium tumefaciens* Ti plasmid pTiA6 from the left terminus of TL-DNA. Plant Mol Biol **28:** 1149–1154

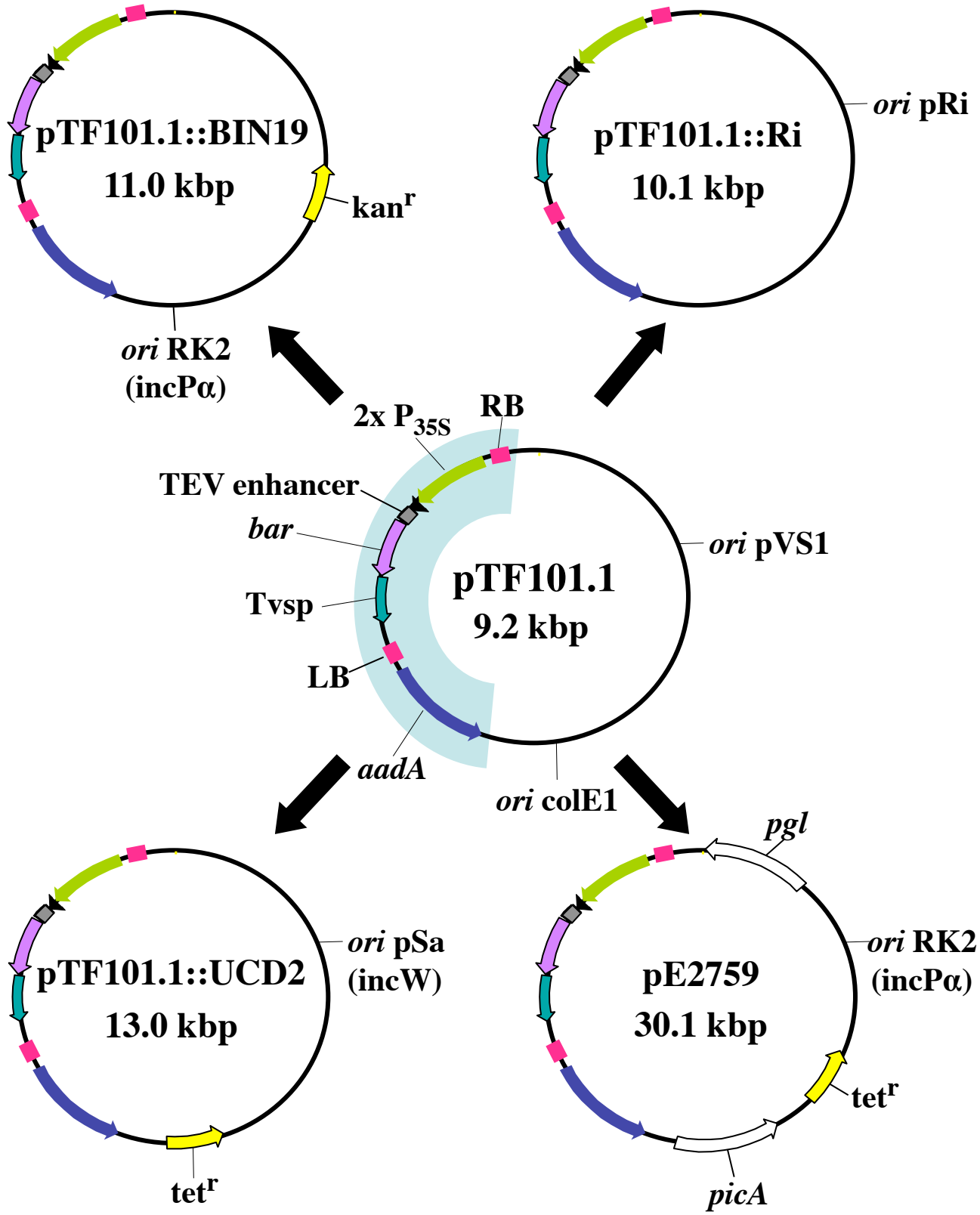
Rong L, Karcher SJ, O'Neal K, Hawes MC, Yerkes CD, Jayaswal RK, Hallberg CA, Gelvin SB (1990) *picA*, a novel plant-inducible locus on the *Agrobacterium tumefaciens* chromosome. J Bacteriol **172:** 5828–5836

Shou H, Frame BR, Whitham SA, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Mol Breed **13:** 201–208

Stam M, de Bruijn R, Kenter S, van der Hoorn RAL, van Blokland R, Mol JNM, Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. Plant J **12:** 63–82

Virts EL, Gelvin SB (1985) Analysis of transfer of tumor-inducing plasmids from *Agrobacterium tumefaciens* to Petunia protoplasts. J Bacteriol **162:** 1030–1038

Wenck A, Czako M, Kanevski I, Marton L (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. Plant Mol Biol **34:** 913–922



Supplemental Figure 1. Schematic representation of the various T-DNA binary vectors and integration plasmid used in this study. The parent plasmid was pTF101.1. The region highlighted in blue indicates the T-DNA region plus the *aadA* gene incorporated into all other plasmids. LB/RB, T-DNA left/right border regions; *ori*, vegetative origin of replication; $2xP_{35S}$, cauliflower mosaic virus double 35S promoter; TEV, tobacco etch virus; *bar*, phoshinothricin-resistance gene; Tvsp, vsp polyA addition signal; *aadA*, spectinomycin-resistance gene; tet/kan^r, tetracycline/kanamycin resistance gene; *pgl*/*picA*, *pgl*/*picA* genes from the C58 chromosome.

Supplemental Table 1. Plasmids and Strains

Plasmid	Description	Origin of replication	*E. coli* stock #	Antibiotic Resistance[a]	Reference
pTF101.1	Binary vector, T-DNA and *aadA* gene donor	pVS1	E2302	Spe	Paz et al., 2004
pBIN19	Binary vector, incPα ori donor	incPα	E330	Kan	Bevan, 1984
pUCD2	Replicating plasmid, incW ori donor	incW	E347	Amp, Kan, Spe	Close et al., 1984
pBluescript::Ri	Replicating plasmid, pRiA4b ori donor	pRiA4b	E2776	Amp	This study
pTF::Bin19	T-DNA and *aadA* gene with pBin19 ori	incPα	E2707	Spe	This study
pTF::UCD2	T-DNA and *aadA* gene with pUCD2 incW ori	incW	E2706	Spe	This study
pTF::Ri	T-DNA and *aadA* gene with pRiA4b ori	pRiA4b	E2777	Spe	This study
pE1931	*picA* recombination vector	incPα	E1931	Amp, Tet	Lee et al., 2001
pE2759	T-DNA and *aadA* gene of pTF101.1 cloned into *picA* recombination vector pE1931	incPα	E2759	Amp, Spe, Tet	This study
pE3265	1.549 kbp *Pvu*II-*Sca*I fragment from pBluescript cloned into the *Sma*I site of pTF101.1	pVS1	E3265	Spe	This study
pE1770	*lacZ* gene and mcs from pBluescript cloned between the *pgl* and *picA* genes in pLAFR1	incPα	E1770	Tet	Lee et al., 2001
pE3349	5.8 kbp *Sca*I-*Nsi*I fragment from pE3265 containing T-DNA, part of pBluescript, and *aadA* gene cloned into blunted *Spe*I and *Pst*I sites of pE1770	incPα	E3349	Spe, Tet	This study
pE3055	Binary vector with *bar* gene in pPZP-RCS2	pVS1, colE1	E3055	Spe	Gelvin lab
pE3361	pE3055 lacking the pVS1 *ori*	colE1	E3361	Spe	This study

***A. tumefaciens* strain**	**Description**	**Antibiotic Resistance[a]**	**Reference**
EHA101	Disarmed agropine/L,L-succinamopine-type super-virulent strain	Kan, Rif	Hood et al.,1987
EHA105	Disarmed agropine/L,L-succinamopine-type super-virulent strain	Rif	Hood et al., 1993
GV3101	Disarmed nopaline-type strain	Gen, Rif	Koncz and Schell, 1986
LBA4404	Disarmed octopine-type strain	Rif	Ooms et al., 1981
At1472	pTF101.1 in EHA101	Kan, Rif, Spe	This study
At1473	pTF101.1 in GV3101	Gen, Rif, Spe	This study
At1474	pTF101.1 in LBA4404	Rif, Spe	This study
At1475	pTF::UCD2 in EHA101	Kan, Rif, Spe, Tet	This study
At1476	pTF::UCD2 in GV3101	Gen, Rif, Spe, Tet	This study
At1477	pTF::UCD2 in LBA4404	Rif, Spe, Tet	This study
At1478	pTF::BIN19 in EHA101	Kan, Rif, Spe	This study
At1479	pTF::BIN19 in GV3101	Gen, Kan, Rif, Spe	This study
At1480	pTF::BIN19 in LBA4404	Kan, Rif, Spe	This study
At1494	pTF::Ri in EHA101	Kan, Rif, Spe	This study
At1495	pTF::Ri in GV3101	Gen, Rif, Spe	This study
At1496	pTF:Ri in LBA4404	Rif, Spe	This study
At1589	T-DNA and *aadA* gene from E2759 integrated into the *picA* locus of EHA101	Car, Kan, Rif, Spe	This study
At1590	T-DNA and *aadA* gene from E2759 integrated into the *picA* locus of EHA105	Car, Rif, Spe	This study
At1591	T-DNA and *aadA* gene from E2759 integrated into the *picA* locus of GV3101	Car, Gen, Rif, Spe	This study
At1687	pE3349 in EHA105	Rif, Spe, Tet	This study
At1702	T-DNA of pTF101.1 and *aadA* gene integrated into the *picA* locus of At1687	Rif, Spe	This study

[a] Amp, ampicillin; Car, carbenicillin; Gen, gentamicin; Kan, kanamycin; Rif, rifampicin; Spe, spectinomycin; Tet, tetracycline