Improving the Quality of Plant Transformation Events

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Agrobacterium-mediated plant genetic transformation is a fundamental technology for both basic science research and agricultural biotechnology. Ever since the generation of the first transgenic plants in 1983,[1] scientists have broadened the range of transgenic plant species and the traits imparted by introduced transgenes. Transgenic crop species have undergone several "generations" of technology improvement. In the first generation, scientists were content merely to generate plants that would constitutively express a transgene. The next generation of technology produced plants with highly regulated transgene expression at particular developmental stages, in particular cell types, or following biotic or abiotic stresses. Current and future generation technologies include selection marker-free crop species, perhaps generated by selection-free processes. In addition, scientists desire stable and predictable transgene expression under a variety of field conditions. In order to achieve this latter aim, transgene introduction technologies need improvement.

Problems with *Agrobacterium* transformation

Agrobacterium-mediated genetic transformation tends to result in a simpler "package" of integrated transgenes than do other transformation technologies such as direct DNA (uptake using PEG or electroporation) or particle bombardment.[2] Although improvements in particle bombardment technology have produced low transgene copy number integration events containing relatively non-rearranged transgene sequences,[3] most laboratories prefer to use *Agrobacterium*-mediated transformation because of its simplicity, lack of requirement for specialized equipment, and propensity to generate low copy number, intact transgene events. However, multi-copy (but still single locus) T-(transferred) DNA insertions, especially head-to-head dimers surrounding T-DNA right border sequences, are common using *Agrobacterium*. These multi-copy transgene organizations have a penchant for transgene silencing,[4] a characteristic unfavorable for field-release plant lines. In addition, integrated T-DNAs frequently are linked to binary vector "backbone" sequences.[5] These additional sequences, from regions of the binary vector outside the T-DNA borders, can generate regulatory concerns.

T-DNA binary vectors and systems

For many years, scientists have used binary vector systems for *Agrobacterium*-mediated plant transformation. These systems, first devised more than 25 years ago,[6] are composed of two parts: a T-DNA (flanked by "border repeat" sequences) on a relatively small plasmid that can replicate both in *E. coli* and in *A. tumefaciens*; and a "disarmed" Ti-plasmid from which the T-DNA has been deleted. Because this latter plasmid contains the virulence (*vir*) genes essential for T-DNA processing and transfer to the plant, it is also termed the "vir helper" plasmid. The essence of binary systems is that T-DNA and *vir* genes are on separate replicons. Although these replicons are usually plasmids, it is possible to integrate and "launch" T-DNA from the *Agrobacterium* chromosome.[7] Several recent articles discuss various binary vector systems (see, e.g., [8]). However, only recently have scientists compared various binary vector systems to determine the quality of resulting transformation events.

Comparison of T-DNA binary systems

Oltmanns et al.[9] recently compared numerous T-DNA binary vector replicons and disarmed *Agrobacterium* strains to determine both the frequency of transformation of two plant species (*Arabidopsis thaliana* and *Zea mays*) and the quality of the transformation event. The authors defined high quality events as those plants containing a single copy integrated transgene lacking binary vector backbone sequences. They mixed and matched binary vectors based on four commonly used T-DNA binary vector plasmid replicons (pRK2, pVS, pSa, and pRi) and three commonly used disarmed *Agrobacterium* strains (LBA4404, GV3101, and EHA101). In addition, T-DNA was integrated into and launched from the *Agrobacterium* chromosomes of strains GV3101 and EHA101. Crucial to their experimental design was the use of identical T-DNA and flanking regions in each construction (**Fig. 1**). Thus, they could directly compare various vector-by-strain combinations. Commonly used flower-dip (for

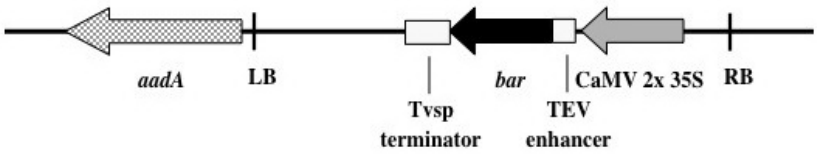

Figure 1. Map of the common T-DNA used in all binary vector and chromosomal integration constructions. *aadA* gene sequences (encoding spectinomycin resistance) were used as a probe for transfer of vector backbone sequences beyond the left T-DNA border. LB/RB, T-DNA left and right borders, respectively; *bar*, gene encoding Basta/bialophos/phosphinothricin resistance; CaMV 2x 35S, Cauliflower Mosaic Virus double 35S promoter; TEV enhancer, translational enhancer from Tobacco Etch Virus; Tvsp terminator, polyA addition signal from a soybean vegetative storage protein gene.

Arabidopsis) and embryo inoculation (for maize) protocols resulted in varying transformation frequencies using the different vector-by-strain combinations, as determined by phosphinothricin-resistant plants (T-DNA contained a plant-active *bar* gene). DNA dot-blot analyses revealed integrated T-DNA copy number and the presence or absence of vector backbone sequences.

The results of these experiments uncovered differences amongst the examined vector-by-strain combinations. With regard to transformation frequency, the authors confirmed the previous observation that *A. tumefaciens* GV3101 was the most efficient for *Arabidopsis* flower-dip transformation. However, all tested disarmed *Agrobacterium* strains effected approximately the same frequency of maize transformation. There was little effect of binary vector replicon on the frequency of plant transformation with one major exception: launching T-DNA from the *Agrobacterium* chromosome resulted in decreased transformation frequency (~2-fold for *Arabidopsis*, but ~10-fold for maize).

Although launching T-DNA from the *Agrobacterium* chromosome resulted in decreased transformation frequency, this method led to a greatly increased quality of transformation event. In both species, the frequency of single-copy insertions was substantially (2- to 4-fold) higher using the "chromosomal launch" technology. In addition, launching T-DNA from the *Agrobacterium* chromosome almost completely eliminated transgenic events containing vector backbone sequences (or sequences flanking T-DNA on the bacterial chromosome).

Systems to use chromosomal launch technology

Integrating T-DNA (containing a gene of interest) into *Agrobacterium* normally involves sophisticated microbial genetic methodologies that may be beyond the capabilities of many plant biotechnology laboratories. Oltmanns et al. thus developed two systems to facilitate integration of T-DNA into the bacterial chromosome. The first system, although easier, results in the introduction of a bacterial antibiotic resistance gene into T-DNA and, thus, introduction of this additional marker into plants. Whereas introducing antibiotic resistance genes in transgenic plants may be common for research purposes, it is not desirable for field release of transgenic crops. This group therefore developed a second protocol that, while slightly more cumbersome, results in a T-DNA lacking the bacterial selection marker.

Did making things easier make things worse?

T-DNA binary vectors were developed to facilitate introduction of genes-of-interest into T-DNA regions. Specialized binary vectors are commonly used in plant biology laboratories to create transgene expression cassettes for numerous purposes, including regulated transgene expression and production of proteins with "tags" to aid in purification.[8] However, the results of several studies suggest that the convenience of T-DNA binary vectors comes at a price: a higher likelihood of integrating multiple T-DNA copies and vector backbone sequences.[5,9] Although transfer of DNA sequences outside T-DNA borders can occur when derived from large Ti-plasmids, such transfer is relatively rare compared to the higher frequencies associated

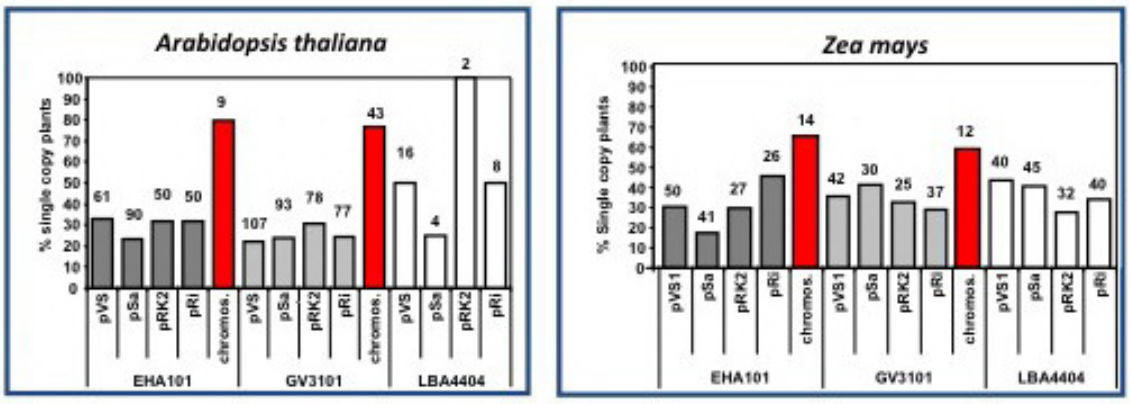

Figure 2. Percentage of transgenic plants containing a single T-DNA insertion. Transgenic Arabidopsis thaliana plants (left panel) were generated using a flower-dip protocol; transgenic Zea mays plants (right panel) were generated using an embryo inoculation protocol. DNA was extracted from independent transgenic events and examined for T-DNA copy number by quantitative dot blot analysis. The three Agrobacterium strains used are listed at the bottom of the graph. Bars represent T-DNA binary vectors, or T-DNA chromosomal insertion constructions (chromos.), for each Agrobacterium strain. Numbers above each bar indicate the number of transgenic events analyzed for each vector-by-strain combination. Very few transgenic Arabidopsis events were generated using A. tumefaciens LBA4404. Therefore, the importance of these few events for this analysis is limited. Note that the transgenic events generated using the "chromosomal launch" technology were most likely to contain a single copy T-DNA insertion (red bars).

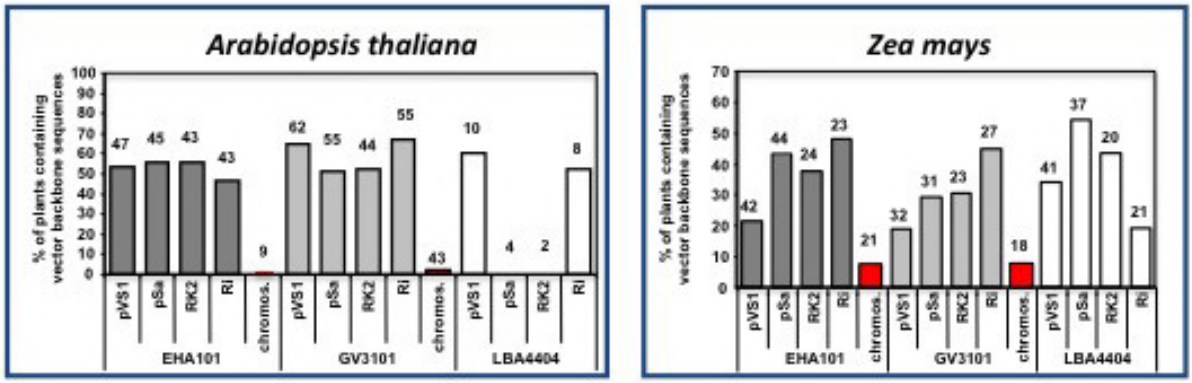

Figure 3. Percentage of transgenic plants containing vector backbone sequences. Transgenic Arabidopsis thaliana plants (left panel) were generated using a flower-dip protocol; transgenic Zea mays plants (right panel) were generated using an embryo inoculation protocol. DNA was extracted from independent transgenic events and examined for the presence of the aadA gene by dot blot analysis. The three Agrobacterium strains used are listed at the bottom of the graph. Bars represent T-DNA binary vectors, or T-DNA chromosomal insertion constructions (chromos.), for each Agrobacterium strain. Numbers above each bar indicate the number of transgenic events analyzed for each vector-by-strain combination. Very few transgenic Arabidopsis events were generated using A. tumefaciens LBA4404. Therefore, the importance of these few events for this analysis is limited. Note that the transgenic events generated using the "chromosomal launch" technology were least likely to contain vector backbone sequences (red bars).

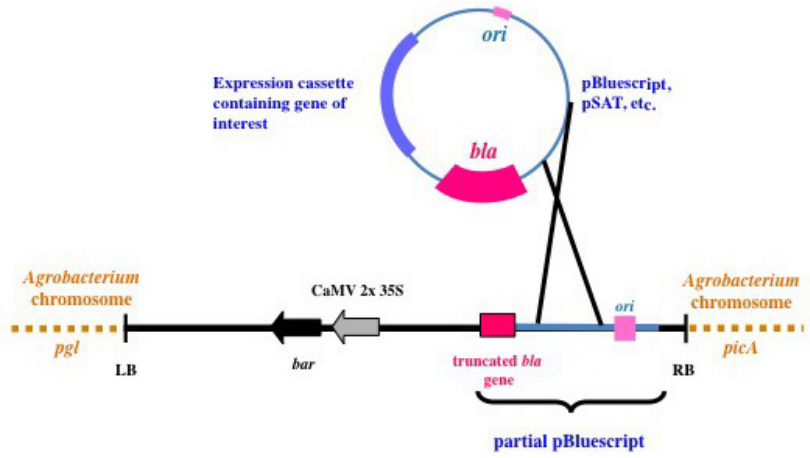

Figure 4. Schematic representation of one of the systems developed to integrate T-DNA into the Agrobacterium chromosome. This system utilizes an Agrobacterium strain with a T-DNA already integrated into the bacterial chromosome (between the pgl and picA genes). The T-DNA contains a plant-active bar gene selectable marker and a portion of pBluescript. pBluescript-derived plasmids containing transgene expression cassettes can be co-integrated into the T-DNA by homologous recombination. However, the resulting T-DNA contains, in addition to the transgene expression cassette, a b-lactamase gene conferring ampicillin/carbenicillin resistance upon the bacterium. LB/RB, T-DNA left and right borders, respectively; bar, gene encoding Basta/bialophos/phosphinothricin resistance; CaMV 2x 35S, Cauliflower Mosaic Virus double 35S promoter; ori, ColE1 origin of replication; bla, b-lactamase gene.

with T-DNA binary vectors.[10] Thus, it may be that these easy-to-use binary vectors may have engendered unforeseen technical and regulatory difficulties.

Chromosomal launch: to use or not to use?

Although chromosomal launch technology would appear to mitigate problems with high integrated transgene copy number and vector backbone integration, it also comes with a price: lower transformation frequency. This frequency decrease can be trivial (~2-fold for *Arabidopsis* flower dip transformation) or substantial (~10-fold for maize embryo inoculation). Whether or not to use the chromosomal launch technology may depend upon the relative costs of having to generate, screen, and discard multiple "low quality" transformation events (including the costs of greenhouse space to generate plant tissue for transformation, personnel to conduct the transformation process and to carry the cultures until they can be screened for single-copy/no vector backbone events, and supplies to generate large numbers of transgenic events) versus the costs to generate multiple rare but "high quality" events. To a great extent, this may depend upon the transformation frequency of particular genotypes. If generation of large numbers of events is not a great problem, generating 10-fold fewer but higher quality events may still be feasible. However, for difficult-to-transform genotypes, generating even low numbers of high quality events may not be feasible. Thus, the costs associated with this technology may depend upon the susceptibility of particular genotypes to *Agrobacterium*-mediated genetic transformation.

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