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Fundamental discoveries and simple recombination between circular plasmid DNAs led to widespread use of *Agrobacterium tumefaciens* as a generalized vector for plant genetic engineering

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ABSTRACT Fundamental research aimed to determine the limits of the *Agrobacterium* transfer DNA (T-DNA) element that stably inserted into plant nuclear DNA to cause crown gall tumor formation. The T-DNA borders were discovered to be exceedingly precise, revealing that T-DNA insertion into the plant genome was reproducible and exact. Deletion of the internal regions of the T-DNA, to remove the tumor forming genes, while retaining the T-DNA borders, resulted again in efficient DNA transfer to plant cells, but now such cells were capable of completely normal growth and differentiation. Thus, the internal region of the T-DNA was not needed for DNA transfer, and one could envisage insertion of any DNA of interest in between the T-DNA borders. Thus began plant genetic engineering.

KEY WORDS: *plant transformation, genetic engineering, tumor forming gene, crown gall, T-DNA border*

Early experiments toward plant genetic engineering

It was late 1977 and the tumor inducing (Ti) plasmid of *Agrobacterium tumefaciens* was beginning to be uncloaked. A particular segment of the Ti plasmid, thereafter designated the transferred (T)-DNA, had been found integrated into plant cell DNA in tumors induced following infection of plant cells with Agrobacterium. I was invited by Marc Van Montagu to the University of Ghent to use two-dimensional electrophoresis of proteins extracted from Agrobacterium with and without the Ti plasmid in the hope of discovering what types of proteins encoded by the Ti were responsible for causing the tumorous phenotype in transformed plants. Alas the experiment was unsuccessful, because at the time it was not known that the genes encoded by the T-DNA element were only expressed in plant cells. Nevertheless, I was hooked on trying to figure out how this soil bacterium managed to carry out trans-kingdom gene transfer to a eukaryotic host cell. The next project, to determine the limits of the T-DNA element, was more successful.

I returned to the University of California in San Francisco (UCSF) where I was a continuing postdoc in Howard Goodman's laboratory, loaded with carefully wrapped sterile tissue culture plates carrying Agrobacterium induced plant tumors. As Howard's laboratory was adept at new techniques for molecular cloning, the idea was to prepare DNA from the tumor tissue, and then clone the ends of the T-DNA element in tumor DNA to determine which sequences define the T-DNA "borders" on the Ti plasmid. While current polymerase chain reaction (PCR) cloning is swift and simple, the most efficient cloning method in the late 1970s involved making a genomic library in lambda phage. Southern blotting of tumor DNA probed with fragments across the T-DNA region of the Ti plasmid defined which fragments were closest to the T-DNA borders, and these fragments were used to probe the tumor DNA phage library. DNA sequencing of border fragment clones then revealed the junctions between the ends of the T-DNA and the plant DNA integration sites. Remarkably, T-DNA homologous sequences ended precisely within identical base pairs in independent DNA clones prepared from different tumor lines. A comparison of these "ends" with the contiguous sequences on the Ti plasmid revealed that they mapped to 25-bp direct repeats on the left and right side of the T-DNA element, thus defining the T-DNA borders (Fig. 1) (Zambryski *et al.,* 1980, 1982).

After these initial discoveries, I moved from UCSF to the Uni-

Abbreviations used in this paper: GOI, gene of interest; T-DNA, transfer DNA; Ti, tumor-inducing.

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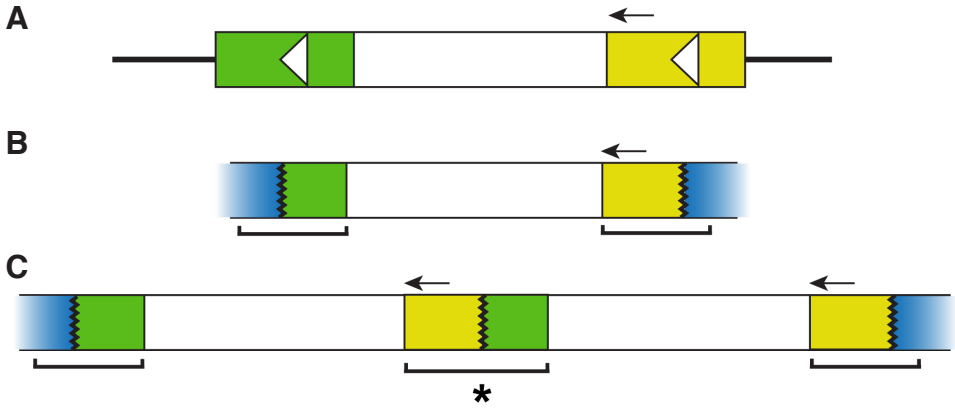

Fig. 1. Determination of the T-DNA borders. **(A)** *Left (green) and right (gold) T-DNA border regions of the Ti plasmid.* **(B)** *T-DNA inserted into plant DNA (shown in blue). The left (green) and right (gold) ends of the T-DNA element adjacent to plant DNA are shown as zig-zag lines. The square brackets below indicate clones of T-DNA–plant DNA junctions. Comparison of the nucleotide sequences of these left and right T-DNA junctions (zig-zag) with the nucleotide sequence of homologous regions on the Ti plasmid revealed that the junctions occurred within 25 bp repeated sequences on the Ti plasmid. These 25-bp repeats define the T-DNA borders (triangles) within the left and right T-DNA border regions* **(A)**. *The direction of the triangles (from right to left) shows the direction of T-DNA transfer determined by genetic studies (Wang* et al.*, 1984) and the direction of synthesis of the single-stranded T-DNA intermediate, called the T-strand (Stachel* et al.*, 1986).* **(C)** *A tandem double T-DNA insert into plant DNA results in left and right T-DNA–plant DNA junctions, as in* **(B)** *and another T-DNA–T-DNA junction between the two T-DNAs. The asterisked square bracket below indicates the clone containing this right-left T-DNA junction; this clone was used to generate pGV3850 (Fig. 2). The leftward pointing arrows adjacent to the right T-DNA borders* **(A-C)** *marks the transcription position and orientation of the* NOPALINE SYNTHASE *gene.*

versity of Ghent to immerse myself in *Agrobacterium* biology. The Ghent laboratory had recently developed tools that enabled genetic analyses to create deletion mutants by double homologous recombination between specific areas of interest on the T-DNA element. These studies aimed to determine what genes were encoded on the 22-kb T-DNA element that might cause the tumorous phenotype in plant cells. Following up on my analyses of T-DNA borders, my immediate question was to ask whether the T-DNA itself encoded products that were essential for its transfer from *Agrobacterium* to the plant cell?

Thus, I set out to use these genetic tools to delete the internal region of the T-DNA, while retaining the T-DNA borders to determine if T-DNA transfer still occurred. I was able to jump in and immediately perform this major genetic alteration as one clone of T-DNA borders from tumor DNA turned out to be remarkably useful. This clone (Fig. 2B) contained sequences of a junction between the left and right borders of the T-DNA resulting from an integration event that produced a directly repeated tandem insertion of two T-DNAs (Fig. 1C) (Zambryski *et al.,* 1980, 1982). Moreover, this clone provided sequences homologous to the left and right T-DNA border regions so that a double recombination between these partial border regions and the T-DNA border regions of the Ti plasmid (Fig. 2A) would precisely remove the entire internal region of the T-DNA element (Fig. 2 D,E) (Zambryski *et al.,* 1983).

The *Agrobacterium* strain carrying the deletion of the internal T-DNA sequences of the Ti plasmid was named pGV3850 (Zambryski *et al.,* 1983). Next we applied pGV3850 to decapitated tobacco (*Nicotiana tabacum*) seedlings and waited. Usually, tumors form after 2-3 weeks following inoculation with *Agrobacterium* carrying the wild-type Ti plasmid. However, no tumors formed following inoculation with pGV3850. Thus, part of the experiment was a success, i.e., we had eliminated tumor-forming genes. But how could we know if DNA had actually been transferred to the plant cells? Luckily the region just adjacent to the right T-DNA border carries the *NOPALINE SYNTHASE* (*NOS*) gene (Fig. 1). The *NOS* gene is only expressed in plant cells following T-DNA transfer and integration and results in the synthesis of a specific sugar-amino acid derivative called nopaline. In fact, the *NOS* promoter and terminator were some of the first plant specific transcriptional regulatory elements to be discovered and they promptly became widespread tools for engineering expression of genes of interest in plant cells.

The seemingly unaffected plant cell surface inoculated with pGV3850 potentially contained T-DNA transformed cells expressing *NOS*, thereby producing nopaline that could be detected easily by thin-layer chromatography. We gingerly scraped the surface of the pGV3850 inoculated cut tobacco stem and tested for nopaline. The results were positive and demonstrated that deletion of the internal region of the T-DNA element did not interfere with T-DNA transfer! We then grew the nopaline-positive tissue with the plant growth hormones auxin and cytokinin to generate callus tissue. Removal of callus tissue onto media containing only cytokinin produced numerous shoots sprouting from the callus. Shoots were tested for nopaline and nopaline positive shoots were grown into mature plants to establish the stable genetic transmission of the pGV3850 T-DNA element (Zambryski *et al.,* 1983). Fig. 3 compares the tumors that form on a tobacco seedling inoculated with *Agrobacterium* carrying a wild type T-DNA region versus a completely normal transgenic pGV3850 plant.

Shortly thereafter the beauty and resourcefulness of the non-tumor forming pGV3850 was quickly revealed. A pBR322 *Escherichia coli* plasmid carrying the T-DNA borders had been used to generate pGV3850 and the double cross over event that deleted the internal T-DNA genes placed the sequences of the bacterial plasmid pBR322 between the T-DNA borders (Fig. 2E). Here comes the “aha” moment. Basically any gene of interest (GOI) to be expressed in plant cells could be cloned first into pBR322 in *E. coli.* Second, this plasmid could then be transferred into *Agrobacterium* cells carrying pGV3850. Third, as pBR322 did not contain an origin capable of replication in *Agrobacterium*, the only way that this exogenous pBR322 plasmid could be maintained stably in *Agrobacterium* was by homologous recombination with the pBR322 sequences of pGV3850 (Fig. 2F). To make this simple single homologous recombination detectable and selectable, one simply added a bacterial selectable marker gene to the pBR322 plasmid carrying the plant GOI.

Figure 2F illustrates the first proof of principle of the generalized plant transformation vector potential of pGV3850 (De Block *et al.,*

1984). Basically, the *NOS* promoter and terminator sequences were placed upstream and downstream of bacterial sequences to allow expression of antibiotic resistance proteins in plant cells. Bacterial transposons, Tn5 or Tn9, were used as the sources of antibiotic resistance to kanamycin or chloramphenicol, respectively (Hererra-Estrella *et al.,* 1983). For example, a chimeric (containing plant regulatory and bacterial coding sequences) kanamycin resistance GOI was cloned into pBR322 carrying a separate bacterial specific antibiotic resistance gene to allow selection of the recombination between the GOI plasmid and pGV3850; this co-integrate plasmid was designated pGV3850::Km (De Block *et al.,* 1984). Co-cultivation of single plant cell protoplasts with Agrobacterium carrying pGV3850::Km resulted in transformation frequencies comparable to those using the wild-type Ti plasmid. Thus, the nononcogenic pGV3850 plasmid retained full capacity for DNA transfer of even an exogenous recombinant GOI to plant cells!

The use of bacterial resistance genes to demonstrate the first plant genetic engineering experiments was exceptionally valuable for an additional reason. The selectable marker gene conferred a dominant phenotype (De Block *et al.,* 1984). Any plant cells that were not transformed would die on media containing kanamycin, while those cells transformed by pGV3850::Km could persist. The use of single plant cells in the original transformation event ensured that the resulting plant tissues were clonal. After growth on medium with appropriate plant hormones, entire plants could be regenerated, where each cell carried a stably integrated copy of DNA capable of expressing kanamycin resistance. Finally, such transformed plants produced viable seeds that segregated kanamycin resistance in the expected Mendelian ratio for a single dominant trait (De Block *et al.,* 1984).

These straightforward experiments laid the foundation for the ensuing *Agrobacterium*-mediated DNA transfer to plants. Basically, one simply placed a dominant selectable marker gene immediately adjacent to a GOI. GOIs were (and continue to be) of two general varieties, either endogenous plant genes or exogenous genes that might provide useful and novel plant traits. It was the middle 1980s and plant genetic engineering had begun! In 1987, the first transgenic tobacco plants that produced insect toxins were created, paving the way for engineering of transgenic corn (*Zea mays*) and other crops that synthesized such toxins, thereby reducing pesticide applications that harm our environment (Vaeck *et al.,* 1987).

One experiment from the middle 1980s contributed substantially to our understanding of fundamental plant biology. *Agrobacterium* pGV3850 carrying a plant selectable kanamycin resistance gene between its T-DNA borders was cultivated with thousands of *Arabidopsis Thaliana* seeds germinating in liquid media; plating the seeds on kanamycin media produced hundreds of seedlings that were transformed and able to germinate (Feldmann and Marks, 1987). Repeating this simple experiment again and again led to the isolation of 20,000 independent transformants that could be screened for genetic defects (Azpiroz-Leehan and Feld-

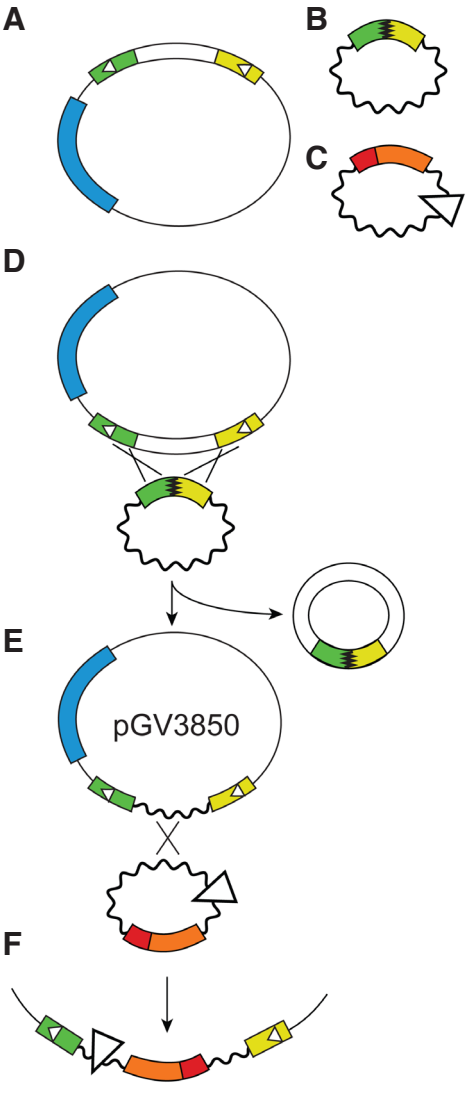

Fig. 2 (Left). Generation and utility of the non-oncogenic Ti plasmid derivative pGV3850. (A) *Wild-type Ti plasmid. As in Fig. 1, the left and right border regions are shown in green and gold and the T-DNA borders are white triangles inside the border regions. The white area between the T-DNA borders represents the genes encoding for tumor formation in plants. The blue region represents the virulence (*vir*) region.* **(B)** *A pBR322 plasmid (wavy line) containing the left-right border junction clone (asterisk in Fig. 1C).* **(D)** *Double recombination event between the Ti plasmid and the junction clone* **(B),** *resulting in the deletion of the oncogenic T-DNA genes (circular DNA that is displaced and shown to the right of panels D and E) and their replacement with the plasmid sequences of pBR322 to generate pGV3850 shown in (E).* **(C)** *GOI (orange) adjacent to a plant selectable marker gene (red). The triangle in the pBR22 sequences represents a bacterial selectable marker gene used to select for recombination between this plasmid and the pBR322 sequences of pGV3850* **(F***). See text for further details.*

Fig. 3 (Right). From tumors to fully normal plants. *Inset, tumors (indicated by arrowhead) that form on decapitated tobacco seedlings following inoculation of the cut surface with* Agrobacterium *carrying the wild-type Ti plasmid containing the "tumor"-forming genes. pGV3850 transgenic plant, in which all the cells carry the nononcogenic T-DNA region of pGV3850 and thus are capable of normal growth and differentiation.*

mann, 1997) facilitating the identification of any T-DNA tagged gene that when knocked out produced a visible phenotype. Notably, the identification of genes in *Arabidopsis* has been used extensively to discover homologous genes in numerous other plant species.

In the last 30 years, numerous variations of the fundamental ability of *Agrobacterium* to transfer DNA to plants have been engineered. Many of these newer tools are described by authors in this volume. Today, the most commonly used vectors are binary vectors instead of the original cointegrating vectors, such as pGV3850. Binary vectors essentially carry a plant selectable marker gene and multiple cloning sites for insertion of GOIs in between T-DNA borders in a plasmid with an *Agrobacterium* compatible origin of replication. Binary vectors then are introduced into *Agrobacterium* carrying Ti-derived plasmids with deletions of the entire T-DNA region, while retaining the virulence genes (blue region in Fig. 2A) essential for generating a single stranded transferable DNA (T-strand) (Stachel *et al.,* 1986) and facilitating transfer of the T-strand through a specialized type IV secretion system that spans the bacterial envelope (Aguilar *et al.,* 2011).

Although today *Agrobacterium* is relegated to the generic "toolbox" in most plant molecular biology laboratories, my own research group continues to study the fundamental biology of how *Agrobacterium* goes about its trans-kingdom gene transfer (Aguilar *et al.,* 2011), and *Agrobacterium* persistently reveals novel pathways and signaling mechanisms (Zupan *et al.,* 2000) that enrich our understanding of important processes in the microbial world.

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