The 1977 discovery that *Agrobacterium tumefaciens* inserts a specific piece of DNA into the plant cell genome triggered a race towards the first transgenic plant. This race ended in 1983 with four labs publishing their own transgenic plant cell lines. Who won the race? Here's…

A Short History of Plant Transformation

Marc Somssich

Persson Lab, School of BioSciences, the University of Melbourne, Parkville 3010, VIC, Australia

Email: marc.somssich@unimelb.edu.au ; Twitter: @somssichm

The Crown Gall Disease (1892 – 1947)

The history of plant transformation begins in the late 19th century, when fleshy outgrowths were noticed on crown roots of several different fruit trees[1]. In **1892** the name 'crown gall' was chosen to describe these tumor-like outgrowths[1]. At the time it was not clear what causes the disease, but Erwin Smith, an agricultural scientist who was interested in bacterial diseases of plants, already then speculated that bacteria could be the cause[2,3]. This idea that bacteria could infect plants was seen as outrageous by many microbiologists at the time, and when Smith published his review summing up the current state of knowledge in the field in **1896**, he was met with fierce opposition[2,3]. One big opponent to Smith was German microbiologist Alfred Fischer, a highly reputable expert in the field, who published his own '*Lectures on bacteria*' in **1897**, replying to Smith by simply pointing out that bacterial diseases of plants '*do not exist*'[4]. He then went on to attribute Smith's findings to contaminations caused by '*dirty techniques*'[3]. Smith issued a reply to Fischer in the German *Centralblatt für Bakteriologie* in **1901**, in which he not only refuted every single argument Fischer made, but also presented a slew of new evidence for bacterial infections of plants in perfect German, which Smith had learned from his childhood minister[3,5]. This final response ended the debate, but Fischer never forgave Smith for this 'affront'[3]. By that time however, an early scientific description specifically of the crown gall disease was published in **1900** in a bulletin from the Arizona Agricultural Experiment Station[1,6]. In this early paper, crown gall disease is attributed to a slime mold of the Myxomycetes class instead of a bacterium, because the author had isolated that mold from several tumors[6]. So at least for the crown gall, the

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issue was not settled yet. By **1905** crown gall disease was found on over 20 different types of fruit trees, and research into the cause intensified for the first time, mostly in fruit tree nurseries and agricultural research stations[7].

Eventually, in **1907**, a new study published by Erwin Smith demonstrated that it is indeed a bacterium that causes these tumors, even though Smith was careful with this attribution, noting that '*It is too early, perhaps, to say positively that the cause of the wide-spread and destructive crown-gall of the peach has been determined by these inoculations, but it looks that way*'[8]. He proposed *Bacterium tumefaciens* as the name for the bacterium that he isolated from crown galls and successfully used to infect otherwise healthy plants[8]. A subsequent detailed description of the tumor, its growth on and into the plant, as well as a closer description of the bacterium was then published in **1912**, again by Smith, and manifested the idea that *Bacterium tumefaciens* does indeed cause a type of '*plant cancer*'. The use of the word '*cancer*' in a plant context made this another one of Smith's ideas that were not readily accepted within the field[9].

The next major breakthrough in understanding the crown gall disease only came in **1941**, when Philip White and Armin Braun demonstrated that they were able to culture explants from crown gall tumors, and that while these explants retained a tumor-like growth, they were unable to isolate *Phytomonas tumefaciens* bacteria from the cultured tissue[10] (in 1925 *Bacterium tumefaciens* was added to the genus *Phytomonas,* therefore changing its name to *Phytomonas tumefaciens*[11]. It received its final re-classification as *Agrobacterium tumefaciens* in **1942**)[12]. This experiment showed that *Phytomonas tumefaciens* is somehow able to trigger tumor formation in plants, but that these tumor cells then grow autonomously without the bacterium – they appeared to be permanently 'transformed'[10]. To further investigate what causes this event, Armin Braun performed some temperature experiments[13]. Earlier results had indicated that 28° C is the optimal temperature for the crown gall tumors to grow, and in **1947** Braun added to these findings that elevated temperatures of 32° C do inhibit tumor formation, but not tumor growth[13]. The conclusion from these observations was that the bacterium most likely is killed off at these higher temperatures, but that the tumor cells remain just as temperature-resistant as the rest of the plant, thereby supporting his earlier conclusion that the bacterium is only needed initially for the induction of the tumor[13,14]. In the same study he also found that wounding of the plant is required for infection to occur, and that the bacterium must infect the plant cell within a four day window following wounding[14]. When Braun then speculated about the identity of the '*active principle*'

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that causes the tumor, he came very close to the correct interpretation, writing that since '*nothing, aside from its biological activity, is known concerning the nature of the active principle it seems reasonable to suppose that in origin it may fall into one of the following four categories. It may be* (…) *(3) a chemical fraction of the bacterial cell* (…) *as in the case of the transforming substance (desoxyribonucleic acid) of the pneumococci* (…)[14]. The '*pneumococci-transforming*' activity of DNA is referring to an experiment, in which a trait of one *Pneumococcus* type could be transferred to another *Pneumococcus* type[15]. This was due to the transfer of DNA from one type to the other, as demonstrated in 1943[16]. It is important to note that this was at a time when little was known about DNA, as the first experimental evidence that DNA could be important for heredity was provided much later, in **1952**, while Crick, Watson and Franklin described its structure only in **1953**[17–19].

Armin Braun continued his research into the nature of crown gall disease over the next 30 years, and, more importantly, he established tumor lines growing on hormone-free medium for decades, which were later used by other groups[20]. His pioneering work in the field earned him the title 'Godfather of Crown Gall Research'[20]. However, while researchers slowly progressed in understanding the biology of *Agrobacterium* over the course of the next 20 years, not much progress was made in figuring out how the bacterium could induce the tumors[3]. To a large degree, this was because the appropriate biological tools were missing at the time. The field of molecular biology only began to develop in the 1950s, and many basic lab techniques, such as *in vitro* polymerase-guided transcription, southern blotting or agarose gel electrophoresis were only developed in the **1960s** and **1970s**[21–24]. Mary Dell-Chilton gave a very nice and visual description of the state of a molecular biology lab around 1970 in the opening paragraphs of her *Agrobacterium memoir*[20].

The Lead-up to the Race (1967 – 1976)

The interest in uncovering the 'tumor-inducing principle', as Braun christened it in 1948, was reignited in **1967**, 20 years after Braun first speculated that DNA might be involved, when Rob Schilperoort and colleagues synthesized a short RNA strand from a complementary *Agrobacterial* DNA-template they had isolated from a cultured *Nicotiana* tumor[25]. This bit of DNA was otherwise only present in *Agrobacterium tumefaciens*, but not in healthy *Nicotiana* plants, indicating that bacterial DNA had indeed been transferred into the plant cell[25]. In two

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follow-up publications it was then shown that the insertion of these bacterial genes into the plant cells actually results in the production of bacterial proteins in infected cells[26,27]. This discovery of bacterial DNA in plant cells got several people interested in working on these crown gall tumors. Among them was Mary-Dell Chilton, a trained chemist with an interest in DNA work and transformation, who initiated a new research project with microbiologist Gene Nester and biochemist Milt Gordon at the University of Washington to figure out how this hypothesized transfer of DNA could be possible[20].

Similarly, in the early 1970s in Belgium, at the University of Gent, bacterial geneticist Jeff Schell and phage geneticist Marc van Montagu also came together to figure out the same thing. By **1970**, Schell and van Montagu were both running their own labs in the phage genetics department of Walter Fiers at the University[28]. The three lab heads regularly got together, when the latest issues of the big journals arrived via mail at the Institute, and eagerly went through them[28,29]. They then sat together and discussed the recent developments in the world of science[28,29]. During one of those sessions, Schell announced that he wanted to get to work on figuring out how *Agrobacterium* causes tumors on plants, and van Montagu immediately decided that he wanted to be part of that project[29]. Schell and van Montagu therefore decided to integrate their labs to form a new group with the aim to figure out how the bacterium transfers its DNA to plants[28]. Schell had previously worked in the lab of microbiologist Jozef de Ley, who in his lab had a huge collection of bacteria, among them several strains of *Agrobacteria*[29,30]. This now came in very handy for the Schell/Montagu lab's new project[29]. However, as neither van Montagu nor Schell had ever worked with plants, they considered cooperating with the Schilperoort lab in Leiden for the plant parts of the project, as they assumed it would be too difficult to get those going in Gent[29]. However, before Schell could contact Schilperoort, van Montagu had already asked a biologist in Gent for advice on how to best infect plant cells who, to their surprise, simply told them to '*buy some carrots in a grocery store, and to surface sterilize and slice them before inoculation*'[28]. '*And so began our first plant experiments. Tumors were obtained without problems on the carrot slices*', Marc van Montagu remembers[28].

Mary-Dell Chilton in the Nester-lab first set out to confirm and characterize the presence of DNA in the crown gall tumors, as reported by Schilperoort, by using a novel, more specific technique: the renaturation kinetics of isolated DNA[31]. The renaturation of a labeled DNA double-strand following denaturation into single-strands is influenced by the presence of homologous,

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unlabeled DNA, which also binds to the labelled DNA and therefore increases the speed of renaturation[31]. Using this technique, the presence of 0.01 % homologous DNA could be detected[31]. However, when Chilton mixed unlabeled tumor DNA with labelled chromosomal *Agrobacterial* DNA, she could not detect an effect on the renaturation rate[31]. Thus, these experiments questioned the transfer of bacterial DNA to the plant tumors. However, around the same time, new indications supporting the idea came from two other groups, who published their findings in **1971**. First, Hamilton and Fall found that an oncogenic *Agrobacterium* strain could be '*cured*' of its oncogenicity by exposing it to a 37° C heat shock[32]. Then, Allen Kerr observed that when he co-infected plant cells with one oncogenic and one non-oncogenic *Agrobacterium* strain, and then re-isolated them, the non-oncogenic strain had become oncogenic[33]. The interpretation of these two experiments was that oncogenicity could be linked with an extra-chromosomal element, potentially a plasmid or something virus-derived, that could be lost from a bacterial strain or transferred from one to another[32,33].

The Schell/van Montagu lab started their work with a slightly different approach, rooted in their past as phage geneticists[29]. Schell had the idea that the bacteriophage PS8 might be involved in transferring the DNA from the bacterium to the plant[29]. This idea had been around since the late 1960s, and was also shared by Rob Schilperoort[34]. In fact, Schell and Schilperoort at one point thought that they might have found evidence of phage DNA in the crown galls, but this was most likely due to contaminations[31,35]. Nonetheless, in **1971**, Schell assigned the task of finding such a phage in its supercoiled phase in tumor-inducing *Agrobacterium* strains to one of his new lab members, Ivo Zaenen[29]. Zaenen approached this task using alkaline sedimentation ultracentrifugation, which was the state-of-the-art method to separate DNA pieces of different size and molecular weight, but also technically demanding, because the chance of damaging the DNA in the process was very high[29,36]. Zaenen managed to optimize the conditions and technique, however, and eventually got his big break in 1972. What he found was not a supercoiled phage though, but a large supercoiled plasmid[29,36]. This work, published in **1974**, was the first major contribution from the Schell/Montagu lab on the way towards identifying the tumor inducing principle[36]. The large plasmid identified could only be found in tumor-inducing *A. tumefaciens* strains, but not in non-oncogenic strains, and it was found in a 1:1 ratio with the bacterial genome, showing that each bacterium carries exactly one of these plasmids[36]. And since it was only present in oncogenic strains, they concluded that this plasmid '*could be the tumor-*

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inducing principle'[36]. In two follow-up publications they were then able to show that this plasmid is essential for tumor-induction by, first, still in 1974, screening for single bacterial colonies that have lost the plasmid, and demonstrating that this loss correlated with a loss of tumor-inducing capacity of the strain[37]. Then, second, in **1975**, transferring the plasmid to a non-oncogenic *Agrobacterium* strain, and demonstrating that this strain now had indeed gained the ability to induce tumors[38]. Accordingly, they named the plasmid Tumor-inducing (Ti)-plasmid, and in **1976** also published their, by then well established, isolation method[39,40]. Based on these results, Jeff Schell and Marc van Montagu proposed that *Agrobacterium* could be used as a bacterial vector to introduce transgenes into plants - a '*hypothesis met with skepticism from most plant physiologists as a seemingly wild and untestable idea*', as recalled by Marc van Montagu[41,42].

For the Chilton/Nester team, this finding meant that when they performed their renaturation experiments with chromosomal *Agrobacterial* DNA, they had simply used the wrong template. They therefore repeated their experiments using Ti-plasmid DNA[20]. However, to their great surprise, they again were not able to detect the Ti-plasmid in tumor cells[20,43]. At this point, their team was '*disillusioned with the whole project. Some of us were ready to give up*', Chilton remembers[43]. However, they didn't give up. And the one thing that they did not consider up to that point was that maybe not the entire plasmid, but only a part of it could be transferred. This, however, was very hard to test with the methods available at the time. But by involving the entire group over a period of almost three straight days, the lab managed to cut up the Ti-plasmid in several small, but clearly defined pieces, labeled each one of these pieces and test them all for their individual renaturation kinetics in the presence of tumor DNA[20]. Mary-Dell Chilton has described the exact experimental process of their '*brute-force experiment*' as they called it, in her '*Agrobacterium memoir*'[20]. At the end of this process, they indeed succeeded in identifying a specific DNA fragment from the Ti-plasmid, which they labelled the 'Transferred(T)-DNA' that was incorporated into the tumorous plant cells[44]. And while it was not clear how the DNA was incorporated (covalently joined to the plant chromosomes or in another form), their **1977** paper was the first report of bacterial plasmid DNA getting stably integrated into a eukaryotic cell, and demonstrated '*a feat of genetic engineering on the part of A. tumefaciens*'[44]. But most importantly, the larger implications were clear: If the bacterium transfers a specific region of its DNA into plant cells, it must also be possible to replace the genes in this region with other genes

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of interest, and get the *Agrobacterium* to transfer these genes into the plant as well. And so, the race towards the first transgenic plant was officially on.

The Race towards the first Transgenic Plant (1977 – 1983)

At this stage, three teams were involved in the race: Rob Schilperoort's lab in Leiden (Netherlands), the Marc van Montagu/Jeff Schell labs, which were now split between Ghent (Belgium) and Cologne (Germany), as Jeff Schell became Director of the Max-Planck Institute in Cologne in 1978, and Mary-Dell Chilton's lab at Washington University in St. Louis (USA). Following the publication identifying the T-DNA, Chilton moved on from Nesters lab in Seattle and started her own group in St. Louis. So at the start of the race, her team consisted of only herself and one student in an empty lab[20,43]. She remembers in 2018: '*I was starting from scratch! Meanwhile, my competitors, including my former collaborators, were busily galloping on ahead of me. My reaction to this challenge was to seize a box cutter and get to work*'[43]. However, as St. Louis was also home to Monsanto, and the Company had also realized the potential the research into *Agrobacterium*-mediated plant transformation held, a partnership between the Chilton lab and Monsanto was quickly established, immediately bringing the Chilton lab up to speed[43]. The ensuing race between the three groups was competitive and fierce, but as Marc van Montagu recalls, it was '*conducted on amicable terms, with information being exchanged and synchronized publication of many of the notable papers*'[41]. And it proved successful for all involved, with loads of high-impact publications for the different labs over the course of the next 7 years.

To start things off, the Schell/van Montagu lab found in **1978** that a specific region appeared to be highly conserved between all Ti-plasmids compared, even if the rest of the plasmid did not exhibit high sequence-similarity[45]. They concluded that this region, which appeared to be flanking the known genes on the plasmid, might be involved in determining the oncogenicity of the plasmid[45]. They then followed this up with an analysis of the bacterial DNA in infected plant cells in **1980**, and could indeed show that these regions were always located at the flanks of the integrated T-DNA[46]. Furthermore, they found in this experiment that in some cases, the region was flanked by bacterial DNA on one, but plant DNA on the other side, a first real indication that the transferred bacterial DNA was actually integrated into the plant's genome[46]. In **1982** Patricia Zambryski and colleagues then described in closer detail what is now known as the Left and

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Right Borders, the regions essential for the transfer of the T-DNA, and also determined that the integration of the T-DNA into the plant's genome is not a site-specific event[47]. In between these publications, in **1980**, the Chilton and Schell/van Montagu labs both published a paper each showing that the bacterial DNA in infected plant cells is indeed part of the nuclear, not the mitochondrial or plastidial DNA fraction, the next step towards clear evidence that the DNA is actually integrated into the plant's genome[48,49]. Still in **1980**, the Schell/van Montagu lab managed to insert a piece of foreign DNA, the *Transposon 7* (*Tn7*) of *Escherichia coli* (*E. coli*), into the T-DNA of *Agrobacterium* and demonstrated that this piece was then transferred into plant cells together with the rest of the T-DNA[50]. At this stage it was clear that foreign DNA could be inserted into the T-DNA, and that this foreign DNA would be transferred to plant cells upon infection of the plant with the bacterium. However, because of the tumorous character of the tissue, it was not possible to regenerate a healthy plant from these transformed tissues. In earlier attempts, getting rid of cells with tumorous character after the transformation procedure, was always accompanied by a loss of the T-DNA[51]. And another problem was that it was not yet clear if a transferred gene would be transcribed in the host cell. So these were the next major hurdles to tackle.

The year **1980** brought another major change to the race. Monsanto had been involved in the race indirectly since 1978[29]. They funded researchers working in the Chilton lab, and Chilton, Schell and van Montagu all functioned as advisors or consultants for the company at one point[29]. However, things changed following the conclusion of the Diamond v. Chakrabarty United States Supreme Court case dealings on June 16, 1980[29]. The question in front of the judges was if living genetically modified organisms can be patented, and the ruling was a 5-4 in favor of patenting[52]. This prompted Monsanto to start their own in-house work on producing the first genetically modified plants, and so they had entered into the race, even though did not declare that openly[29].

In **1981**, the Schilperoort, Schell/van Montagu and Nester labs all published on Ti-plasmid mutants carrying insertions in different regions of the T-DNA[53–55]. Bacteria carrying these plasmid variants only induced smaller tumors and, more importantly, some of the tumor cultures were able to form shoots or/and roots[53–55]. Furthermore, these experiments provided a first genetic map of the Ti-plasmid[53–55]. Following this work, one of these mutant Ti-plasmids, carrying the previously used *Tn7* transgene, was again used in the Schell/van Montagu lab to regenerate a *Nicotiana tabacum* plant from tumor tissue, which still carried the bacterial T-DNA

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and passed it on to the next generation in a Mendelian fashion[56]. So one may consider this as the first engineered transgenic plant, but it did not express a new gene or carry a new trait, and it still expressed some unwanted *Agrobacterial* genes and produced octopine or nopaline, markers for *Agrobacterium*-induced tumor tissue[56].

Then came the big year **1983**, and already in mid-January at the Miami Winter Symposium it became clear to the world that the race would end[57]. In the morning of January 18th, the '*Genetic manipulation of plants*' session was held[29]. Mary-Dell Chilton and Jeff Schell were both scheduled to speak in that session, with another researcher from Yale University holding the third spot between these two[20,29,57]. However, shortly before that day, Chilton and Schell were informed that there had been a last minute change in the schedule, and that a different speaker would take the third spot in their session[29]. This last minute replacement was Bob Horsch, the head of Monsanto's in-house plant culture team[20,29,57]. And so, all three labs announced the successful transformation of plant cells with an antibiotic resistance gene within one session at the Symposium[20,57]. The only difference was that Monsanto had also brought a public relations expert to the meeting, and so *The Wall Street Journal* subsequently announced that Monsanto had reported a major breakthrough at the Symposium[29]. But in the following months, high-impact publications came in one after another:

First, in April, the Chilton lab published the successful regeneration of healthy *Nicotiana tabacum* plants carrying a full-length engineered *Agrobacterial* T-DNA, including a yeast *ALCOHOL DEHYDROGENASE I* gene[58]. However, as this gene was inserted into the Ti-plasmid without any plant-active regulatory sequences, it was not expressed in the transformed plants[58]. This paper was quickly followed by a publication from the Schilperoort lab on May 12th, who created the first binary plant vector set to use for plant transformation[59]. This meant splitting up the two parts of a Ti-plasmid, the transferred T-DNA and the *virulence* (*vir*) region, which confers the bacterial ability to infect the plant[59]. By moving the T-DNA region to a separate plasmid, this plasmid could readily be maintained due to its small size, which made the cloning work to insert a gene of interest a lot easier[59]. The engineered T-DNA plasmid (the '*binary plasmid*') then has to be transformed into a *vir*-plasmid (the '*helper plasmid*')-carrying *Agrobacterium* strain[59].

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One week after that, on May 19th, the Schell/van Montagu lab published their first transgenic plant cell line, expressing a foreign gene, and conferring a novel trait to the plant[60]. They used the *chloramphenicol acetyltransferase* (*cat*) gene from *E. coli*, conferring antibiotic resistance, and to allow expression from the T-DNA cloned it downstream of the *nopaline synthase* (*nos*) promoter[60]. This promoter had not been published at the time, but the Schell/van Montagu lab had a manuscript in preparation describing both, the *nos* and *ocotpine synthase* promoters, which was one of the next big publications of that year[61]. This paper was not just important because this promoter allowed the expression of the *cat* transgene, and therefore the first publication of a transgenic plant cell line, but also because it was the first plant-active promoter described in detail[61]. With their paper, the Schell/van Montagu lab had won this scientific race, but certainly it was a photo finish, as the Chilton lab had their transgenic plant cell line ready as well. Published just two months later, on July 14th, the Chilton lab described their transformed *Nicotiana* cells carrying a *G418* transgene that had been inserted into a nopaline Ti-plasmid at the position of the *nos* coding region, thereby also exploiting the *nos* regulatory sequences[62]. They showed that they could then select transformed cells by growing them on G418-containing medium[62]. Just another half month following the publication from the Chilton lab, Robert Fraley and colleagues from the Monsanto lab published their transgenic Petunia lines, carrying the bacterial *Aminoglycoside-3'-phosphotransferase* (*npt*) gene, again under control of the *nos* regulatory sequences[63]. As the *npt* gene confers resistance to aminoglycoside antibiotics, they used kanamycin-resistance to select their transgenic cell lines[63]. Finally, to end the year in style, the lab of Timothy Hall in Madison, Wisconsin, also published a paper describing their transgenic cell lines[64]. They transformed sunflower cells with constructs carrying the *Phaseolin* gene from *Phaseolus vulgaris* under control of, first, the *octopine synthase* promoter, but then also using a large genomic fragment of *Phaseolin*, including ~ 1000 bp upstream of the coding region, and therefore the putative endogenous regulatory sequences[64]. And indeed, this second construct also resulted in expression of the *Phaseolin* gene in the sunflower cell lines[64].

The Aftermath of the Race (1984 – 1986)

The four 'transgenic plants' published in 1983 were actually just 'transgenic plant cell cultures' that held the potential to be regenerated into a full plant. The final problem that needed to be solved to obtain healthy regenerated plants carrying the transgene of interest was to get completely rid of the tumorous character of the cells, without losing the T-DNA as well. To

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overcome this problem, the Schell/van Montagu lab published another important paper at the tail-end of 1983, describing the first non-oncogenic Ti-plasmid that is still able to transfer the T-DNA into plant cells[65]. They then used this plasmid in **1984** to transform *Nicotiana* calli and regenerate fully healthy transgenic *Nicotiana* plants[66]. These cells and plants were resistant to kanamycin, methotrexate or chloramphenicol (depending on the transgene used) and passed this trait on to the next generation in a Mendelian fashion – demonstrating that the transgene was indeed stably integrated into the plants genome[66].

The group around van Montagu and Patricia Zambryski furthermore were then able to determine that a 25 bp sequence at the right border is essential for transfer of the T-DNA, and that this is also providing a direction for the transfer[67]. In their model, the Ti-Plasmid would be cut at or near that site, then a copy of the T-DNA is synthesized from that position up until the left border, and this copy is then transferred right border first into the plant cell[67]. In **1985**, they followed this up with another publication demonstrating that the *vir*-genes required to facilitate the transfer of the T-DNA, are activated by the chemical signal acetosyringone, which is derived from wounded plant tissue[68]. In nature, this chemical is secreted into the soil from a wound, and is exploited as chemotactic signal by the *Agrobacteria*[68]. For this reason, acetosyringone is still part of many plant transformation protocols today.

Michael Bevan from the Chilton lab, the first author on their 1983 paper, went on to create the pBIN19 binary vector in **1984**, which became the most widely used T-DNA vector in the following years, until Roger Hellens' pGreen vector set took over in the year 2000[69,70]. In **1986,** the Shell lab then added the pMP90 helper-plasmid to the GV3101 *A. tumefaciens* strain – thereby creating another standard to use for transformations to this day[71].

Also in **1984**, the year after the first *Agrobacterium*-transformed plants were published, the first cauliflower mosaic virus (CaMV)-transformed plant was published[72]. Since scientists had noticed that CaMV inserts DNA into plant cells, and that these genes are then expressed in the plant, they worked on establishing the virus as a vector for plant transformation[73,74]. However, by the time the CaMV-transformed methotrexate-tolerant turnip plant was published, it had already been shown that CaMV would only tolerate the insertion of DNA fragments of up to ~250 bp[72,75]. So this upper size-limit to the genes that could be transferred via CaMV, together with the successful establishment of *Agrobacterium*-mediated plant transformation in 1983, effectively put an end to

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the work on CaMV-mediated plant transformation (see also 'A Short History of the CaMV 35S promoter'[73]).

In **1985**, microinjection of DNA into protoplasts was established as another alternative plant transformation method[76]. Using this technique, DNA is injected into immobilized plant cells using a glass capillary[76]. These cells are then used to regenerate a transformed plant[76]. As such, microinjection is very laborious, but it holds the advantage that large bits of DNA, even whole chromosomes can be transferred[77]. Another transformation method first established that year was electrophoresis, first for transient transformation, and then, in **1986**, also to achieve stable transformation of maize plants[78,79]. Transient transformation was performed for protoplasts, and a suspension culture for the stable transformation, as this could be used for the regeneration of calli, and then healthy plants[78,79]. This feat was important, as it was assumed at the time that most monocots were insensitive to *Agrobacterium*-mediated transformation[80].

Kick-starting the Biotech-Industry – and blocking it with patents (1980 – 2005)

Also in **1986**, the lab of Robert Fraley at Monsanto published two more breakthrough papers, both capitalizing on recent major developments in plant science. First, they leveraged the identification of the CaMV 35S promoter to engineer the first herbicide resistant plant, a glyphosate-tolerant *Petunia* line[81,82]. Then, they capitalized on the recent adoption of *Arabidopsis thaliana* as a plant model organism, by publishing a transgenic *Arabidopsis* plant carrying a hygromycin-resistance gene, together with the transformation protocol (see also 'A Short History of *Arabidopsis thaliana* (L.) Heynh. Columbia-0'[83])[84].

In Europe, Marc van Montagu and Jeff Shell had founded their own biotech company, Plant Genetic Systems (PGS), already in 1982, when it was apparent that they would be able to produce their first transgenic plant. The company was Europe's first biotech company and the first company to produce an insect resistant plant in **1987**. The PGS *Nicotiana tabacum* plant expressed a fragment of the *Bacillus thuringiensis (Bt) berliner 1715 Bt2*-gene[85]. The protein product of Bt2 was a known toxin to larvae of insect crop-pests, and had at that point already been approved for use in insecticides[29]. Now, the transgenic tobacco plants expressed this toxin inside their cells, thereby killing only the larvae that started to actually feed on the plant, and without contaminating the environment as spraying insecticides would[86]. While this plant never

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made it to the market, other crops with a *Bt*-based pesticide have been used widely since 1995, when a *Bt*-potato was the first *Bt*-crop to be approved for the food market in the US[87].

The development of new varieties and of the biotech industry as a whole was hampered from the start, however, due to the obscure patent situation concerning this 'invention'[88]. While *Agrobacterium*-mediated plant transformation is now used routinely in acdemic research institutes, not many transgenic crop plants have been transformed using this technique. **1983**, immediately following the first successful transformation of plant cells, Monsanto filed a patent for the invention of *Agrobacterium*-mediated dicotyledonous plant transformation using an integrated (not binary) vector[88]. Jeff Schell and the Max-Planck Society quickly countered this with their own application, and so did Mary-Dell Chilton and Washington University[88]. This led to an interference, meaning that no patent was granted, resulting in a situation of legal uncertainty[88]. This interference was only resolved in **2005**, with a settlement between Monsanto, the Max-Planck Society and Bayer CropScience, who worked out a scheme to share their licenses[88]. In the meantime, several more patents had been granted on different aspects of *Agrobacterium*-mediated plant transformation, making the situation even less transparent[88]. For example, Mogen Syngenta was granted a patent on the use of binary vectors to transform dicotyledonous plants, Japan Tobacco on the transformation of calli from monocotyledonous plants, Washington University on the transformation of dicotyledonous plants using an *Agrobacterium* strain carrying a disarmed plasmid, Monsanto on the transformation of dicotyledonous cells when using an antibiotic during the inoculation phase, and Rob Schilperoort and Leiden University for the transformation of plants from the *Liliaceae* or *Amaryllidaceae* families, if the T-DNA and *vir*-genes are integrated into the genome of the bacterium[88]. Curiously, this means that every scientists, even in an academic research environment, is infringing on these patents, despite many academics believing in a '*myth of the "experimental use exception"*', meaning that they are somehow exempt, due to the non-commercial nature of their work[88]. But this is not actually the case[88]. Following the 2005 settlement, the Max-Planck Society used the back royalties they received to fund the Jeff Schell Professorship at the University of Cologne.

Years of Expansion and Simplification (1987 - today)

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The first improvement of the actual transformation method came in **1987**. Kenneth Feldman and David Marks moved away from tissue culturing, and published a seed-transformation method[89]. On top of that, the particle gun was introduced in the same year, to deliver DNA into plant cells using ballistics[90]. At first, this technique was limited to achieve transient transformation of small cell populations within tissues, but in 1998 also stable transformants were acquired by particle bombardment of the plant stem cells in the meristem[91]. Biolistic transformation remains a standard technique, mainly for plants that are resistant to *Agrobacterium*-mediated transformation. However, over the past decades, protocols have been developed for more and more plants that were initially thought to be resistant to the bacterium, among them wheat, maize and rice in the 1990s, and also the patent issue around *Agrobacterium* could be resolved[92–94].

During the 1990s, several of the established transformation methods were optimized for *Nicotiana* plants, and with ultrasonication in **1991**, a new technique was added to the already available toolbox[95]. But more importantly, transient transformation systems for *Nicotiana* leaves were developed[96]. In its simplest incarnation, *Agrobacterium*-solution is injected directly into the leave cells through their stomata using a syringe[96]. The cells are transformed by the bacterium, allowing the expression of a transgene for, e.g., intracellular gene-localization or subsequent protein extraction[96].

The next major improvement of the *Agrobacterium*-mediated plant transformation protocol came exactly 10 years after the race toward the first transgenic plant had ended. Nicole Bechtold, Jeff Ellis and Georges Pelletier published their 'vacuum infiltration' plant transformation protocol in **1993**[97]. This method of immersing the whole adult plant in *Agrobacterium*-solution under vacuum meant a giant step forward as it not only simplified the procedure, it also improved the efficiency immensely[97]. It is important to note though, that this protocol is mostly specific for *Arabidopsis* transformation. This was then followed by another major simplification protocol another five years later, again facilitated by the ease of transforming *Arabidopsis*. Steven Clough and Andrew Bent published their 'floral-dip' method in *The Plant Journal* in **1998**, fittingly the journal plant transformation pioneer Jeff Schell helped to establish in 1991[98]. The floral-dip method eliminated the uprooting and replanting of the plants, as well as the vacuum-step from the protocol, by simply dipping the above-ground part of the plant into *Agrobacterium*-solution for a few seconds, and then keeping the plants in a humid environment for a day, and repeating this step once after roughly 6 days[98]. This floral-dip method is one of the most cited papers in plant

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science history. Finally, in **2006**, the last somewhat critical step in the protocol, the handling of large volume liquid cultures, was removed by simply scraping *Agrobacteria* from a plate, resuspending them in infiltration medium and then dipping the *Arabidopsis* plant into such solutions[99].

In **2003**, *Agrobacterium*-mediated plant transformation was at the center of another big advancement of plant science: The creation of the SALK T-DNA mutant collection[100]. Here, *Agrobacterium* was used to create a library of over 220.000 *Arabidopsis* plant lines, each carrying an independent T-DNA insertion in a random position of its genome[100]. Due to this high number, a T-DNA was inserted into almost every single one of the ~ 30.000 *Arabidopsis* genes, providing researchers with ready-to-order mutants for almost all their genes of interest[100].

Curiously, in **2008**, a quarter century after the first transgenic plant lines were published, Bekir Ülker and colleagues found that several of the commonly used *A. tumefaciens* strains do not just transfer the clearly defined T-DNA into plant cells, but occasionally also large fragments of their chromosomal DNA[101]. These transferred fragments can be up to ~ 18 kb in size, and may be present in as many as 0.4 % of transgenic lines[101]. Similarly, when the team of Joe Ecker at the SALK Institute revisited their SALK T-DNA insertion lines (as well as lines from two more T-DNA collections, SAIL[102] and WISC[103]) in **2019**, they found that these insertions had caused a wide range of changes in the plant's genome, such as rearrangements, exchanges of chromosome arm ends, enrichments of siRNAs, or changes in the methylome[104]. So even though *Agrobacterium*-mediated DNA transfer is well understood today, these findings indicate that there still remains a lot to learn by studying this bacterium and its interaction with plants[101,104,105].

In retrospect, it would appear somewhat strange that people would go through intensive callus- and plant-regeneration stages, when one could just dip the flowers of the plant into a solution of bacteria that were scraped off a plate to obtain transformed plants. But particularly these later advances were only possible because of the adoption of *Arabidopsis thaliana* as a plant model in the mid-1980s, as these simplified methods appear not to work on most other plants. On the other hand, the initial assumption that *Agrobacterium* can transform most dicotyledonous plants, but only very few monocots also proved to be wrong: Modifications and tweaks to the transformation procedure and the generation of more efficient strains eventually produced transformation protocols for most of the commonly used monocot plants, and even transformation of woody

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tissue on trees[106,107]. Even more impressively, it became clear that *A. tumefaciens* can not only transform plants, but also yeast, fungi and even human cells[106]. This wide applicability demonstrates the huge impact that the development of *Agrobacterium*-mediated plant transformation had not just for the plant field. In summary, it is certainly fair to say that *Agrobacterium*-mediated plant transformation is one of the most important achievements in plant science history and has helped to kick-start the plant biotech industry.

Further Reading:

- Armin C. Braun - A History of the Crown Gall Problem[3]
- Mary Dell-Chilton – Agrobacterium. A Memoir[20]
- Geert Angenon et al. - From the tumor-inducing principle to plant biotechnology and its importance for society[28]
- John Zupan et al. - The transfer of DNA from agrobacterium tumefaciens into plants: a feast of fundamental insights[108]
- Mary Dell-Chilton - My Secret Life[43]
- Judith M. Heimann - Using Nature's Shuttle[29]

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