Agrobacterium. A Memoir (In Part Reprinted from Plant Physiology Vol. 125, 2001)

Mary-Dell Chilton

Prologue

It was very safe and comfortable being the wife of an Assistant Professor of Chemistry at the University of Washington in Seattle. Being a postdoc in the Department of Biochemistry at the University of Washington was also comfortable. My place in the academic scene was clear. The discomfort came when the postdoc ended, my second baby arrived, and there I was, a straight A student with state of the art training in DNA manipulation, wishing that I had interesting work but not wanting to miss this fleeting time when my children were small. I discussed the situation with my husband and sometime collaborator Scott Chilton, a solid gold husband and father if ever there was one, and he kindly and repeatedly gave the same counsel: "Do what you want. You can decide." My qualifications looked promising. There were no longer nepotism rules. But the problem was that I lacked geographical mobility. We loved Seattle, and Scott was advancing in his academic career. We had no desire to move. If my scope had been the entire country, at that time surely I could have found a suitable academic appointment that would allow me to work on DNA. In the 1970s, this was such an arcane interest that there was no hope of pursuing it outside of academia. Besides, I wanted to teach. I wanted to do research. And I wanted to spend time with my children. What should I do? I am human. I dealt with the issue by dithering.

M.-D. Chilton (✉)
Syngenta Biotechnology Inc., P.O. Box 12257, Research Triangle Park, NC 27709, USA
e-mail: marydell.chilton@syngenta.com

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A Promising Call

It was a chilly wet December day in Seattle. The year was 1970, which I mark by the birth of my second baby, Mark, who would grow to become the best friend of his two year old brother, Andrew. The phone was ringing insistently as I, with infant Mark in one arm, sprinted to catch what turned out to be the most important call of my career. It was Prof. Helen R. Whiteley, Department of Microbiology and Immunology, University of Washington, Seattle. She had been given a teaching assignment that she would like to pass along to me. It would be part time. Would I be interested? (Interested? I would be ecstatic!) The course was a laboratory on methods in DNA manipulation. This job would be a temporary instructor's position, with no hope of extension. Professionally it was not what I wanted, but at least it would get me out of the house. This job would not be a millstone, I told myself. It would be a stepping stone. I would have teaching experience! I said yes immediately. It was not necessary to discuss it with Scott, who would predictably say, "Do whatever you want!"

How I Met Agrobacterium

I taught the entering class of graduate students much of the DNA methodology I had learned over the previous three years as a postdoctoral fellow. As a final exercise, I had each student present, for the class to evaluate, a paper from the recent literature that employed one or more of the techniques that they had learned in my course. Tom Currier presented a paper from the State University of Leiden in the Netherlands, authored by Robb Schilperoort and collaborators. The paper was intended to test an astounding model for how *Agrobacterium* causes crown gall tumors on plants. The bacteria supposedly transferred DNA to the plant cells, and the transferred genes supposedly triggered growth of the plant cells into a gall. The basis for this model will be discussed below. It piqued my interest because it contradicted all the genetics I had learned and my own research results: Bacteria would only incorporate donor DNA if it matched their own DNA. Mismatch of a few base pairs could be tolerated, but overall the DNA must be homologous in order for recombination to occur. *Agrobacterium* DNA in plant cells sounded like science fiction!

The Schilperoort Paper

Tom Currier described for my class the way Schilperoort et al. measured how much "hot" (labeled) *Agrobacterium* DNA "hybridized" to filter-bound crown gall tumor DNA. As positive control, they measured how much hot *Agrobacterium* DNA hybridized to filter-bound *Agrobacterium* DNA. Surprisingly, their tumor DNA filters "hybridized" with far more of the hot *Agrobacterium* DNA than their

Agrobacterium DNA filters. This result was kinetically impossible. Tom Currier had chosen an excellent paper for my class to discuss and analyze. The students readily noted the need for further controls, especially because the authors were trying to test such an astounding model of bacterial gene transfer to plant cells. My fingers were itching to work on this DNA project!

A Project Is Launched

Tom Currier told me that his advisor, Prof. Gene Nester of the Department of Microbiology and Immunology, was interested in starting a research project on *Agrobacterium*. It was not difficult to convince Nester that he could benefit from my DNA technology to test definitively the gene transfer hypothesis. We recruited another colleague, Prof. Milt Gordon of the Biochemistry Department, a plant virologist with TMV and tobacco experience, to join our project team. I wrote up the appropriate DNA experiments as a research proposal, and with modest research grant support we founded the Seattle Crown Gall Group. I had my first job. My initial job title was Assistant Biologist, the same as that of the fellow who cleaned mouse cages in the animal room, as I recall.

Amongst our earliest experiments, we repeated the DNA filter hybridization studies in the Schilperoort paper and performed additional controls: kinetics of the reaction and melting curves of the hybridization products. We found that our data did not support the idea of DNA transfer from *Agrobacterium* to the plant cells. The telling control was the use of labeled heterologous DNA. We found that these tumor DNA filters would "hybridize" with any type of labeled DNA we added. Impurities (likely polysaccharides) in the tumor DNA made the DNA-filters sticky. Better DNA purification methods would be essential.

Does Wrong Evidence Prove the Model Wrong?

At this point, it was certainly tempting to throw out the baby with the bath water. If the evidence was wrong, then the astounding conclusion must also be wrong. And, in hindsight, that would actually be the literal truth. The DNA we tested was NOT what was transferred to the plant cells. But if that had led us all to give up and go work on something different, we would have lost the baby, which, alas, had the misfortune of being several years premature. The fact that we continued to study *Agrobacterium* reflects the mix of personalities involved. In our Seattle group, we had some believers and some doubters, and they seemed to switch roles from week to week. If you were to interview the surviving members of our original group today, you would get different stories from different people about who thought what. I think they would all be true, but for different times. Each of us likely has a very human tendency to remember best the versions of our own stories that depict

ourselves as prescient. For the record, I was (intellectually) dragged kicking and screaming to the conclusion that *Agrobacterium* could put DNA into plant cells. Once we had our own direct evidence, I saw the light. But it was a conclusion from careful DNA analysis, not a "belief" based on the indirect clues from earlier work.

In the year 2000, now 16 years ago, I wrote what I called a memoir about the beginnings of the *Agrobacterium* story. With your indulgence, dear reader, I prefer to reprint that part of the story rather than retelling it. I will offer my more current thoughts in the form of an Epilogue. It will not be a review of recent literature, which others are more qualified to produce. Perhaps you could liken it more to some grandmotherly thoughts from an aging, hopefully wise, experimenter who refuses to lay down her pipets because she is still having fun. Stay tuned!

Memoir

This little memoir is not a review; the reader is directed to current authoritative *Agrobacterium* reviews with genetic (Zhu et al. 2000) or cell biology emphasis (Zupan et al. 2000). Likewise, this is not an update on recent advances in plant genetic engineering, which are the subject of a recent book (Hammond et al. 1999). Rather, I invite you to join me on a foray through the story of *Agrobacterium* transformation of plant cells. Our journey will take us back in time about 30 years, and we will note early contributions from laboratories around the globe, including Belgium, the Netherlands, France, Australia, and several in the United States. The scientists in our story represented many disciplines, from traditional ones such as plant pathology, microbiology, and chemistry to younger fields such as molecular biology, plant tissue culture, and plant metabolic chemistry. Many in the course of investigating *Agrobacterium* found intellectual haven in the newly emerging field of plant molecular biology. Beginning at a time when bulk DNA was analyzed as a macromolecule, our story spans the birthing and growth of recombinant DNA technology.

Lest the experiments we revisit seem simple when viewed from the 21st century, our first stop will be a museum of molecular biology research in the time about which I will write, circa 1970. The catalog of restriction endonucleases was unrecognizably thin. What few enzymes were available often were tainted. Kits were unknown. Procedures often did not work. We sized DNA and determined its percentage of G and C in the model E ultracentrifuge. We measured small volumes with 5-, 20-, 50-, or 100-μL glass capillaries. We cultured our plant calli in jelly jars and fleakers. Instead of laminar flow hoods we worked in still air hoods. A few years later when the plasmid came into our lives, we taught ourselves how to do gel electrophoresis, and we designed and built our own gel rigs. (The one with the agarose wicks was known, of course, as the wicked gel.) We made combs from square aluminum rod, using double stick tape to mount teeth that were pieces of glass cut with great difficulty from microscope slides. Each of us hoarded his or her own collection of glass teeth, and it was not uncommon to hear an anguished voice cry "Who took my teeth?" Research in this period presented unique challenges.

The first cloning of DNA was out of sight, just over the horizon and of course PCR was not yet conceived.

With this setting in mind, then, let us turn our attention to the crown gall problem and consider what was known at the beginning of the 1970s.

An Idea Born Before Its Time

Dr. Armin Braun of the Rockefeller University (New York), whom many regard as the godfather of the crown gall story, first demonstrated that tumor cells are transformed, i.e. they can be freed from *Agrobacteria* and grown in vitro without the supplemental auxin and cytokinin required by normal plant cells in vitro (Braun 1958). Braun kept tumor lines growing on hormone-free medium quite literally for decades. He reasoned that *Agrobacterium* must give these cells something, and he proposed that this gift must replicate because it is never lost by dilution. He proposed for it the term TIP (tumor inducing principle).

Georges Morel of the Institut National Recherche Agronomique on the grounds of the Palais de Versailles in France discovered copious amounts of new metabolites—octopine and nopaline—in cultured crown gall tumor cells that were free from bacteria (Petit et al. 1970). Morel's group showed that the *Agrobacterium* strain, not the plant, determines the opine made by the tumor. Furthermore, each *Agrobacterium* strain can grow on its own particular opine but not on a different one. He thought Braun's TIP must be or include a gene responsible for opine synthesis in the plant. He proposed that a single enzyme catalyzed opine synthesis in the plant and opine breakdown in *Agrobacterium*, in order to account for the strain specificity of opine catabolism. We now know that part of Morel's model was not correct (the bacteria use a different enzyme for catabolism), but he was certainly on the right track about opine synthesis in tumors. However, the scientific community in 1970 was far from ready to accept the notion of a bacterial gene getting into a plant cell and functioning there. More direct evidence would be needed to support such a radical idea.

Bacterial DNA in Crown Gall Tumors?

Rob Schilperoort at the State University of Leiden, the Netherlands, as part of his Ph.D. research, prepared DNA filters with crown gall DNA and found that they bound radiolabeled *Agrobacterium* DNA amazingly well. The thesis and other publications of Schilperoort (see citations in Chilton et al. 1974) were an important factor in the founding of our Seattle Crown Gall Group. Microbiologist Gene Nester, plant viral RNA biochemist Milt Gordon, and I, an organic-chemist-turned-DNA-hybridizer, all were intrigued by the idea of gene transfer to plants. We realized that we three might collaboratively do a much more definitive type of experiment to identify bacterial DNA in tumors–if it was really there! In 1971 we began our collaboration. Tom Currier, Nester's graduate student, set about giving

cancer to tobacco plants using *Agrobacterium tumefaciens* strains from the American Type Culture Collection (Manassas, VA). He inoculated the bacteria into wound sites in the stems of young plants and observed the development of crown gall tumors that were to make biological history.

The first contribution of our Seattle Crown Gall Group to the problem was a negative one that showed how large a challenge lay ahead. We found that the DNA-filter results reported by Schilperoort were caused by impurities (polysaccharides) in the DNA extracted from tumor cells, and that this technique did not have the sensitivity to detect 1% bacterial DNA in model mixtures (Chilton et al. 1974). (One bacterial genome per plant cell would constitute approximately 0.1%.) We next employed DNA renaturation kinetic analysis, which tested whether a high concentration of tumor DNA ("driver DNA") could make labeled *Agrobacterium* DNA ("labeled probe") renature faster. We showed that this method was sensitive enough to detect one copy of the bacterial genome per three tumor cells, but tumor DNA did not drive our labeled probe (Chilton et al. 1974). It was a clear negative result. We recognized that this method could only detect DNA corresponding to a significant fraction of our labeled probe. The bacterial genome contains perhaps a few thousand genes, so the acceleration of renaturation by even 10 specific bacterial genes in the tumor cells (a fraction of 1% of total bacterial DNA probe) would be below the limit of detection.

Tumor-Inducing Genes Are on an Extra-Chromosomal Element

Indirect genetic evidence that *Agrobacterium* might carry a virus or plasmid with tumor-inducing genes emerged from two kinds of experiments published in 1971. Hamilton and Fall at the University of Pennsylvania (Philadelphia) discovered that strain C58, when grown at 37 °C (28 °C is optimal), lost virulence irreversibly. They proposed that tumor induction must be a plasmid- or virus-born trait because of its susceptibility to "curing" (Hamilton and Fall 1971). At the same time, plant pathologist Allen Kerr at the Waite Institute in Adelaide, South Australia, was attempting to develop a biocontrol microbe to protect plants against crown gall disease. He co-inoculated avirulent and virulent *Agrobacteria* into the same sunflower plant. When he re-isolated the "avirulent" strain from the gall, it had become virulent! This transfer of virulence suggested to Kerr the existence of an extra-chromosomal element as vector for tumor induction (Kerr 1971).

Back in Seattle, Gene Nester read these reports and became convinced that there must be a plasmid in *Agrobacterium*. He and Alice Montoya reproduced the transfer of virulence with our own strains. Bruce Watson, a student in Milt Gordon's lab, reproduced the C58 curing experiment also. (The reproduction of published claims was clearly an important activity for our group, cast as we found ourselves in the role of iconoclasts. It was essential to know what could be believed.) Nevertheless, Bruce Watson repeatedly had no luck when he looked for plasmids in *Agrobacterium* using established methods (i.e. methods that were established for small plasmids).

Fig. 1 Photograph of Ivo Zaenen, who discovered the Ti plasmid of *Agrobacterium*

Key Discovery in Ghent: Ti Plasmid is Gigantic

In 1974, Ivo Zaenen (Fig. 1) at the University of Ghent (Belgium) cracked the crown gall problem wide open for everyone. Working in the laboratory of Jeff Schell and Marc van Montagu, Ivo Zaenen was the first to lay eyes on the megaplasmids of *Agrobacterium*. I asked him recently how he succeeded where others had failed. He replied that at first he did not recognize what he had found. He was using alkaline sucrose gradients to look for something else: a replicating form of an *Agrobacterium* phage called PS8 (whose DNA was once claimed to be in tumor DNA). He eventually found plasmids ranging from 96×10^6 to 156 to 10^6 M_r in 11 virulent strains and not in eight avirulent strains (Zaenen et al. 1974). His publication in the prestigious *Journal of Molecular Biology* is a landmark.

When news of this discovery came to us in Seattle, it set off a flurry of experiments and launched a vigorous competition between the Seattle and Ghent groups. We quickly isolated plasmid DNA from several *Agrobacterium* strains by Zaenen's method. Both groups found that strain C58 lost a megaplasmid when grown at 37°. Transfer of virulence was mediated by transfer of a plasmid. It quickly emerged that the genes for catabolism of octopine and nopaline were located on their respective giant plasmids, which the Ghent group christened Ti (tumor-inducing) plasmids.

Is There Ti Plasmid DNA in Tumor Cells?

At last with the Ti plasmid of our *Agrobacterium* strain in hand, we felt confident that we had the right probe to look for TIP in crown gall tumors. But when we performed renaturation kinetic analysis with the whole plasmid as probe, we got the

Fig. 2 Photograph of collaborators in the "brute force" experiment that first demonstrated the presence of T-DNA in crown gall tumor DNA. *Left to right* Don Merlo, Martin Drummond, Gene Nester, Daniela Sciaky, Mary-Dell Chilton (author of this article), Alice Montoya (deceased 1989), and Milt Gordon

by now too familiar result: It was not there. Our experiment ruled out the presence of the entire plasmid, but just as before, we recognized that a few genes could be there without our noticing any kinetic change. In order to settle the issue, we decided to cut the Ti plasmid into specific fragments and test each piece by renaturation kinetic analysis.

It was a brute force experiment involving everyone in the lab (Fig. 2). In order to label our probe to maximum specific activity, Martin Drummond seized the fresh ^{32}P-dCTP the moment we received it from New England Nuclear and labeled our plasmid DNA by nick translation. Daniela Sciaky digested the labeled DNA with *Sma*I (purified by Alice Montoya—the enzyme was not for sale). Daniela and I ran the preparative gel, made an autoradiogram, and decided whether the fragments looked good enough. (Too much nicking in the nick translation reaction could lead to breakage of the largest *Sma*I fragments, which then ran too fast during electrophoresis and contaminated the smaller fragments.) If the autoradiogram looked good, we all canceled plans for the weekend: the experiment had to be completed within 48 h, before radiation damage to the DNA began to affect the kinetics. I excised the 15 resolvable plasmid bands, which were passed to Don Merlo for electroelution of DNA from the gel slices. (He used a device involving many small dialysis bags that he designed for the purpose. He called it "The Cow" for reasons that I will leave to the reader's imagination.) We set up 75 renaturation kinetic assays (5 unlabeled "driver" DNAs $\times$ 15 labeled probes) and worked around the clock to sample the reactions and assay the percentage of renatured probe DNA in

525 (7 × 75) samples. Milt held the stopwatch and called out time points. We all did whatever had to be done next. I have never experienced such completely committed teamwork in my entire career, before or since. Although it is now nearly 25 years ago, I can clearly remember the moment of truth. While calculating and plotting the results amid a sprawl of printer tape from the scintillation counter, I suddenly saw that the T-DNA (as it would soon be known) was there in the tumor cells. Labeled probes of band 3AB, and later on, triplet band 10ABC, renatured faster in the presence of tumor DNA and no other part of the plasmid did.

A reviewer of the manuscript describing our finding required that we separate the doublet 3AB and determine which fragment was in the tumor. Although initial cloning experiments were just beginning in our group, we had no idea how to clone these blunt-ended *Sma*I fragments, and we found no enzyme that would cut one member of the doublet and spare the other. In desperation I finally managed to separate fragment 3A from 3B by a heroic serial electrophoresis of 4 days duration. We found that 3B was the fragment in the plant cells, and the paper was accepted (Chilton et al. 1977). Resolution of the band 10 triplet showed us that 10C was the member in T-DNA, and when we subsequently determined the fragment map of our Ti plasmid, fragments 3B and 10C were contiguous, showing that T-DNA was a single segment of the Ti plasmid.

Where Is T-DNA and What Defines It?

By this time, genomic Southern blots had been developed and were clean enough to show T-DNA bands; renaturation kinetic analysis was a dying art that nobody mourned. The Southern blots showed recognizable intact Ti plasmid fragments and in addition "border fragments" that were different in different tumor lines, suggesting attachment of T-DNA to plant genomic DNA. By analysis of Southerns of nuclear DNA, chloroplast DNA and mitochondrial DNA, the T-DNA of several tumor lines was proven to be located in the nuclear fraction (Chilton et al. 1980; Willmitzer et al. 1980).

In 1979, I moved from the University of Washington to Washington University in St. Louis, and focused on nopaline Ti plasmids, while the founding group in Seattle continued with the octopine strain. My new group at Washington University, the Seattle group, and Patti Zambryski in the Ghent group (Fig. 3) all succeeded in cloning T-DNA fragments from tumor DNA. When we sequenced through the junctions of T-DNA and plant DNA, comparing plasmid DNA with T-DNA, we found a 25-bp imperfect direct repeat on the Ti plasmid at the edges of what is incorporated into the plant genome. These border sequences define T-DNA on the plasmid but not in the plant: they are not transferred intact to the plant cell (reviewed in Binns and Thomashow 1988).

Fig. 3 Photograph of research group in Ghent, Belgium, 1984. *Left to right* Jeff Schell, a visiting scientist from China, Marc van Montagu, Patricia Zambryski, and Ken Wang (a student)

Genetic Picture of the Ti Plasmid

vir *Genes*

Transposon mutagenesis of the Ti plasmid in Leiden, in Seattle, and in Ghent showed that all mutations affecting tumor induction mapped to a sector of approximately 42 kb, separate from T-DNA, called the virulence (*vir*) region. The *vir* genes constitute a regulon inducible by acetosyringone and other phenolics that are found in plant wound juice (Stachel et al. 1985). These compounds, directly or indirectly, affect the "antenna" protein VirA, which autophosphorylates, then phosphorylates VirG, a transcriptional activator for all of the *vir* genes.

T-DNA is excised from the Ti plasmid by endonuclease VirD2, with facilitation by VirD1 and VirC1. VirD2 nicks the bottom strand of the right border sequence after the third base and attaches to the 5′ end of the nick, forming the "leading" end of the T-strand to be delivered to the plant. The details of left border scission are not clear, but VirD2 produces a similar nick there. The *vir* E2 gene encodes a single strand binding protein essential for tumor induction, that can alternatively be expressed in the plant with equal effect. The VirB operon consists of 11 open reading frames, which encode the T-DNA conduit from bacterium to plant. The structural and functional similarity of many of these to proteins involved in plasmid transfer to other bacteria has led to the view that T-DNA transfer has evolved from plasmid conjugation (reviewed in Zhu et al. 2000 and Zupan et al. 2000).

T-DNA Genes

Transposon hits in T-DNA were found to eliminate opine production or to alter tumor morphology or to have no recognizable effect at all. The morphology mutations were eventually shown to eliminate cytokinin autonomy ("rooty" tumors) or auxin autonomy ("shooty" tumors). T-DNA genes were shown to encode a two-step pathway to the plant auxin indoleacetic acid and an enzyme producing the cytokinin isopentenyladenosine 5′monophosphate (reviewed in Binns and Thomashow 1988). Most importantly, no mutation in T-DNA blocked T-DNA transfer. All of the genes affecting the process of T-DNA export to the plant cell mapped in the *vir* region. This fact would greatly simplify the disarming of T-DNA and construction of *vir* region-containing helper plasmids lacking any T-DNA.

From Pathogen to Gene Vector

In order to use the Ti plasmid as a vector, we needed a method of putting genes into T-DNA (and knocking some out, as well). In Ghent and in St. Louis, methods were developed for inserting DNA into any specific part of the Ti plasmid. The DNA to be inserted was cloned between pieces of T-DNA on a plasmid, introduced into the bacterium by conjugation or by transformation, and subjected to "forced recombination" (Matzke and Chilton 1981; Van Haute et al. 1983). A simpler approach to engineering T-DNA was to make a small separate T-DNA plasmid that could be manipulated directly. Although *Agrobacterium*, in nature, keeps *vir* genes and T-DNA on the same replicon, there is no requirement for this arrangement. If you place T-DNA on a separate replicon in *Agrobacterium* (a binary vector, as it is now called), the process of T-DNA transfer to the plant cell still occurs with good efficiency (De Framond et al. 1983; Hoekema et al. 1983). Thus, the T-DNA of a binary vector could be engineered directly in *Escherichia coli* and then transformed into *Agrobacterium*.

Another problem for the genetic engineer was plant regeneration. All efforts to regenerate a plant from transformed cells were initially rewarded with only rare deletion mutants that had lost practically all of their T-DNA, a strong indication that at least part of T-DNA was inimical to plant regeneration. We discovered the critical part almost serendipitously. Tony Matzke and Ken Barton, post-docs in my group, introduced a yeast gene into T-DNA in a position that we thought might hit an oncogene (Matzke and Chilton 1981). It turned out indeed to inactivate the cytokinin production gene. In collaboration with Andrew Binns at the University of Pennsylvania, we discovered that this single insertion event produced an engineered T-DNA that was completely disarmed. It produced transformants that synthesized nopaline but that could not grow autonomously without hormones. Binns identified the transformed plant cells by screening for nopaline production. In contrast to crown gall tumor cells, the tobacco cells transformed by multiple copies of this

T-DNA were able to regenerate into normal plants that passed the T-DNA copies to progeny plants as Mendelian traits (Barton et al. 1983). By 1982 we had the first evidence that foreign DNA engineered between T-DNA borders and transformed into the plant nuclear DNA could be stably maintained in the plant genome and passed intact to progeny.

Starting in about 1980, a formidable new group was assembled by Ernie Jaworski at Monsanto (our neighbor in St. Louis) to harness the T-DNA transfer technology for crop improvement. At this time Michael Bevan in my laboratory found himself in a race with Patti Zambryski's team in an effort to sequence the nopaline synthase (*nos*) gene and map its promoter and terminator by S1 nuclease protection (Bevan et al. 1983; Depicker et al. 1982). Then a second race ensued amongst Bevan, Zambryski and her Ghent collaborators, and the Monsanto group to isolate the *nos* gene promoter and splice it to a kanamycin resistance coding region in order to create a selectable marker that might work in plant cells. If this scheme worked, then one would no longer have to screen for nopaline production to find transformed plant cells: one could select the cells with T-DNA inserts on kanamycin agar.

The symbolic coming of age of genetic engineering occurred at the Miami Winter Symposium, January 18, 1983. During one session, Jeff Schell, Rob Horsch from Monsanto, and I all gave talks about *Agrobacterium* and its adaptation as a gene vector for plants. All three of us reported success with chimeric kanamycin resistance genes as a selectable marker for plant cells (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983). I described initial success in transforming tobacco cells with binary vectors (which we called MiniTi at that time). In addition, I described our tobacco plants engineered with a disarmed Ti plasmid, and Southern blots proving that they passed their T-DNA insert to progeny intact. It was clear from the progress in all three groups that crop improvement by genetic engineering would become a reality.

Reflections from 2000

Finding that T-DNA can integrate into a plant genome without benefit of homology was a real intellectual shock to me. The bacterial transformation studies I had made as a student and again as postdoc taught me the absolute need for good homology in those systems. Now illegitimate recombination seems the rule not only for T-DNA but also for foreign DNA integration in animal cells and indeed naked DNA delivery to plant protoplasts or bombardment of plant cells with DNA-coated microprojectiles. Incorporation of foreign DNA is clearly a process that cells carry out efficiently, perhaps in the course of repairing genomic damage. It is not a trade secret of *Agrobacterium*, although we may yet discover some secret details of the process. *Agrobacterium* acted as an inspiration to others who have developed various means of DNA delivery. It gave us our first selectable marker (tumor induction). It gave us the promoters and terminators for the next generation of

selectable markers (octopine/nopaline synthase promoters and *nos* terminator, bounded as they were by T-DNA borders and neighboring genes in this highly compact T-DNA). The wide host range of *Agrobacterium* (even wider now that monocot transformation is facile) inspired the idea that DNA incorporation may be a universal phenomenon. But perhaps the most important legacy from *Agrobacterium* has been its inspiration of confidence that foreign gene integration, even though DNA is sometimes delivered artificially, is a perfectly natural process. My most fervent wish is that well-meaning environmental proponents will come to recognize this and embrace the technology based on it.

Epilogue

Will This Really Work in Crops?

The memoir reprinted above concluded in 1983, at the beginning rather than the end of a chapter of history. We could see that the method of introducing new genes into plants was going to work. It was crude, but it could be improved. What was less clear, to me at least, was how to improve real crop plants. What pests—viruses, bacteria, fungi, viroids—are a problem for which crops and what genes would be useful to combat them? What environmental conditions affect yield in which crops? How could we find genes to make the crop tolerant to drought, flood, cold, heat, famine, plague or pestilence? Could we teach *Agrobacterium,* whose host range was known to include dicots and gymnosperms, how to deliver genes to monocots such as corn, wheat, rice and other cereals, the most important food crops? I never even considered the idea that the public might fear these improved plants! All we had at that point was a sound and promising start.

A Visitation

One additional aspect was not clear to me: what role would I have in harnessing this new technology. I realized that if I were to carry on, I would need to develop a strong collaboration with scientists experienced in agriculture. I remained a consultant for the genetic engineering group at Monsanto, and their support of my university research program continued, but they seemed to show little interest in my longer term career. This matter seemed to be of greater interest to three businessmen from Swiss multinational CIBA-Geigy, who visited me at Washington University in mid-1982. They disclosed, after a lengthy discussion, that they had been tasked with identifying a leader for their projected new agricultural biotechnology group to be established in North Carolina. They wanted to know whether I would be interested in being a candidate. Suppressing my excitement was difficult. I asked for

some time to think it over. Scott, as was his custom, advised: do whatever you want! Both sets of aging parents would be nearby. Scott was from Virginia and I grew up in North Carolina.

CIBA-Geigy, which today after two mergers, spinoffs and name changes is called Syngenta, offered me the position in early 1983, and we moved to Raleigh in the summer of that year. It was a real adventure for each of us. Scott had the challenge of finding a new academic position, and this time it was he who lacked geographical mobility. He turned out to be quite successful at this, and before long the Botany Department at North Carolina State University was both happy and fortunate to hire him as a visiting professor. It turned out to be a lengthy visit.

Beginnings

Meanwhile, I had many new responsibilities but was given plenty of help. I had both a US boss and a Swiss boss, and our operation was managed by the Biotech Executive Committee, consisting of the three of us plus two additional Swiss executives. We had to invent the Agricultural Biotechnology Research Unit (ABRU) from the ground up. Working in leased facilities for the first year and a half while building our own new laboratory in Research Triangle Park, N. C., we developed a project portfolio and began recruiting scientists with relevant skills. CIBA-Geigy was in part an agricultural chemical business, and many of the chemists were not pleased at the prospect of biotechnology solving problems that they viewed as their purview. The KL (top Swiss Executive Committee) subscribed to the longer term view that if biotech could capture in part the value of the agricultural chemicals business, it was prudent for CIBA-Geigy to enter this new biotechnology business. Our Biotech Executive Committee had to decide how to do that. Would we enter the seeds business more extensively, or would we license biotech to others, or both?

The Overall Lesson

Choosing project objectives was a very different process from what I had used in the university setting. We had an expert from Basel headquarters who helped us to figure out, for each project idea, what financial value would be added to the genetically modified seed by our new gene(s), assuming that the science worked well. What would the improved seed save the grower in input cost? What would be the value of any increased yield (or prevented loss of yield)? Would the yield have higher intrinsic value because of nutritional improvement, improved processing attributes, etc.? Could it be segregated from bulk crop yield and sold at higher price, or perhaps at the same price but ballooning market share? Next, my scientists and I estimated how long the project would take and what was the probability that it

would work as hoped. Additional experts were consulted to consider other business aspects—were we in the right business with the appropriate customer base, in the right geographical region and country? Were there regulations in place for testing and eventually selling our genetically modified seeds (and for the grower to sell his crop, of course)? And then there was the vital question of whether our product could be blocked from the market by patents of competitors. The overall lesson of this exercise was that the seed business, and even more so the genetically modified seed business, was going to be a challenge.

The Big Picture

Since the dawn of the technology in January 1983 (date of the Miami Winter Symposium), 33 years have now passed. *Agrobacterium* has kept its promise. The bacteria deliver DNA to monocots, soybeans, and (I suppose) any plant it wants, or indeed we want, not to mention yeast, fungi and probably robots and iPads. Inspired by this clever microbe no doubt, additional methods of DNA delivery have been perfected. While these methods scatter donor DNA to random locations on host chromosomes, newer methods have been developed for directing the donor DNA to a desired location (gene targeting), and those same methods can be used to edit the plant (or other) genome quite precisely. Technology for manipulating DNA available today is mind-boggling!

In 1983 the main limitation was technology. The technology that my postdocs Tony Matzke and Ken Barton and I developed in collaboration with Andrew Binns, primitive though it appears today, was prized at that time because it provided the proof of concept. At that time, the company that could transform the highest value crop with greatest efficiency promised to be the biggest winner. Currently that aspect is not such a problem and the big challenge is to identify the best genes to address the plant's problems. We are clever and I believe we can do that. But today we face a challenge that goes beyond the science: the societal part of the picture, which had hardly crossed our minds in 1983: The product must be attractive to the consumer or at least to the end-user (who must convince the consumer of its value), if genetically modified seed is to sell.

You will read about many of the advances as you enjoy the stories in the chapters assembled here. This book will contain a collection of personal histories as much as the story of scientific advances in agriculture. I hope that it may project for the reader more than what we and others have done, but also how and why we came to do it, a part of the story that is usually interesting but rarely told.

The Message

If the prophets of global warming are correct (as I fear they are), climate change in addition to population growth will bring an urgent need for accelerating the rate of plant breeding advances. With changing climate, our food and fiber crops will face new challenges. New weather patterns will bring new insect pests that damage plants, and pests that act as carriers of new diseases. The task of the plant breeder will be even more challenging than it is today. Breeders will need the best technology available, and genetic modification of plants promises to be an important tool. It is a safe procedure that, like the traditional plant breeding of the past, we have learned from nature.

Therefore I need to leave you with one closing message, and you will not be surprised to note that it bears a strong resemblance to my plea of 16 years ago. I hope to see the technology for producing genetically modified plants accepted, even embraced, by the public in my lifetime. We must hasten if we are to succeed at this, for I am not getting any younger. If you agree with my concerns, do not keep it a secret. Spread the word when you have an opportunity. Believe me, I will do my part by continuing as long as I am able.

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