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# It Is a Long Way to GM Agriculture

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## Abstract

When we discovered that crown gall induction on plants by *Agrobacterium tumefaciens* is a natural event of genetic engineering, we were convinced that this was the dawn of a new era for plant science. Now, more than 30 years later, I remain overawed by how far and how rapidly we progressed with our knowledge of the molecular basis of plant growth, development, stress resistance, flowering, and ecological adaptation, thanks to the gene engineering technology. I am impressed, but also frustrated by the difficulties of applying this knowledge to improve crops and globally develop a sustainable and improved high-yielding agriculture. Now that gene engineering has become so efficient, I had hoped that thousands of teams, all over the world, would work on improving our major food crops, help domesticate new ones, and succeed in doubling or tripling biomass yields in industrial crops. We live in a world where more than a billion people are hungry or starving, while the last areas of tropical forest and wild nature are disappearing. We urgently need a better supply of raw material for our chemical industry because petroleum-based products pollute the environment and are limited in supply. Why could this new technology not bring the solutions to these challenges? Why has this not happened yet; what did we do wrong?

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## THE EARLY YEARS

Born in 1933 in Ghent, Belgium, in a period of great economic recession and raised in a working-class neighborhood, I was an unlikely candidate ever to do higher studies, let alone make contributions of importance to society.

My mother died during my delivery. Death of either the mother or the newborn was very common in those days. My mother was the only survivor of my grandmother's nine pregnancies. For the first three years I was raised by my maternal grandmother and her sisters, surrounded by lots of love and attention, being the only child of my generation in the whole family. When my father remarried, I went to live with him and my stepmother, who took care of me like a real mother. They had decided not to have another child for fear of treating us differently. The contact with my maternal grandmother remained close, and during the next ten years, I spent several days a week with her and her sister.

The neighborhood I grew up in was typical for a city relying on a flourishing textile industry: large factories surrounded by a network of dead-end alleys with small working-class houses. Most houses did not have running water; there was a central tap in the street. Some even had common toilets in the middle of the street. Light came from petroleum or gas lamps; few houses had electricity. Heating was done mostly with a coal stove, which also served for the cooking. Since the bedrooms were not heated, there were fascinating ice flowers on the windows during winter.

The factories were dark and very noisy, and clouds of cotton dust would be floating around the spinning machines. They were so frightening and convincingly repulsive that I felt I never wanted to be obliged to work there. At noon-time, when it was not raining, many workers would sit in the street on the sidewalk, eating their bread and drinking from a gourde they had brought from home. They were completely covered by the white dust, which made them look like ghosts. I often made remarks about these disturbing working conditions to my grandmother. She insisted that this was so much better than before, when she was a child. Now they were working only a good 8 hours; before it was 10, sometimes even up to 12 hours a day. In my grandmother's youth 50% of the workers were children, many younger than 10 years of age. They were important for sliding under the machines and knotting the broken

treads. Her father-in-law had been one of the founders of the workers movement in Ghent in the 1870s. My stepmother also came from a militant socialist family, from Bruges. That meant that during all of my youth I was solidly embedded in a politically conscious environment. May 1st (our labor day) was the most important holiday of the year. Never would we miss the parade, and the whole day we would sing militant songs. Of course that was before the War and later again after 1945.

Political activities remained very central until my graduation, after which science took over, although the striving for more social justice and better living conditions remained a basic motivation. Did not the Enlightenment teach us that knowledge would set us free?

## PRIMARY AND HIGH SCHOOL

A brother-in-law of my grandmother was the only family member with three years of secondary education. He became a school teacher and retired as a director of a primary school. He insisted that I go to the best primary school within walking distance. That certainly gave me a good head start.

The daily school routine was disturbed in early 1940. Part of the school building was turned into army barracks. Family members were drafted into the army. As the German troops were approaching, many bridges of the city were blown up, and the barges on the canals were sunk with explosives. I was puzzled by the view of hundreds of Belgian soldiers waving white handkerchiefs above their heads, marching in surrender toward two German soldiers. School continued, although during the following years the atmosphere in class changed. Learning French was now optional, and the lesson was moved to the late afternoon. During music lesson we had to learn songs expressing our devotion to Flanders. Nobody dared make remarks. Intuitively we felt these were dangerous times. Great excitement arose during the cloudy nights: The blackout made streets very dark; one could really bump into somebody. Also, starting from 1943, bomb alarms were fre-

quent at night. The sky would be lit by searchlights and exploding anti-aircraft shells, a beautiful *son et lumière* for a 10-year-old.

I had the good fortune that the teacher of the last year in primary school insisted, as I was among the top pupils of the class, that I should go on to high school—preferably to the Latin section of the Atheneum, a state school with an outstanding reputation. In a city where 80% of schools were run by the Catholic Church, the ranking of the best went to some Jesuit colleges, but these were for children of the middle or upper class, not of the working class. As nobody in the family, not even friends or acquaintances, was religious, it was self-evident that if I continued with the schooling system, I would go to the Atheneum. Going on with my education was a big decision, but the family concluded that it was worth trying because everybody noticed that the only thing I seemed able to do was to read books all day long.

The first years were not brilliant. Educated with the strong imprint never to disturb or try to draw attention, I was isolated and lonely. I always managed to sit on a bench in the first row, but that was the most daring thing I ever did; I never went any further and did not attract the attention of the teachers.

A complete change occurred in the third year. The mathematics teacher was an outstanding pedagogue who really made an effort to have the whole class follow him. Also, the courses of physics and chemistry fascinated me. This was the first year of a new section called Latin/Science. The best teachers had made an effort to obtain an assignment to this section, and the program was novel and experimental.

I became most captivated by chemistry and decided to start a small laboratory in the attic of our house. In winter, as there was only a coal stove on the ground floor, conditions were a bit harsh. But the heat of the Bunsen burner was sufficient, and, yes, I could convince the parents to extend the gas line to the attic.

From then on I made my first class friend, Hubert Sion; he too had installed a lab in his house. His parents were clearly financially much better off than mine, but interactions

between us went very well. At the end of the sixth year of Athenaeum I was again in the top group of the class. The great uncle, the schoolmaster, suggested that I should try for University. It was financially possible because the inscription fee for the State University Ghent, as it was then called, was minimal and I would have few other costs because I lived and ate at home. Walking for 40 minutes or taking my bicycle cut the transportation costs. The minimal tuition fee was 5000 Belgian francs (today 120€ or \$165) and represented a one-month salary for my father, which was a lot of money. If I had started working as an office clerk, I might have earned that amount of money. But the parents were so proud with the idea of their son entering university, a level never attained by anybody in the family, that without much discussion they accepted that I should try a year. By then I was already resolute to study the chemistry of living organisms, biochemistry as it seemed to be called.

I found out that there was indeed a Professor of Biochemistry at the University of Ghent, teaching in the Veterinary School and in the School of Agriculture. Upon asking him what studies to start, he answered that pharmacy was the best mix between chemistry and biology.

I did not feel like following his advice. I was afraid that I would end up in a chemist shop. I had made good friends with a pharmacist in my neighborhood; it was the place where I bought my chemicals. I did not see myself being satisfied with this profession. So I decided to choose chemistry, a choice I have never regretted.

## STATE UNIVERSITY GHENT

In those days, the Ghent University was a small university (5000 students in the Fifties, now 36,000) with a good tradition in chemistry. The university was founded in 1817, after the Napoleonic wars, when Belgium was united with The Netherlands. The courses were first given in Latin. Later, when Belgium became independent in 1830, the courses were given in French, but in the 1930s it switched to Flemish, the language of the region.

In the middle of the nineteenth century the organic chemistry section of Ghent University attracted international reputation with August Kekulé (structure of benzene), Adolf von Bayer (the synthesis of Aspirin, barbiturates), and Leo Baekeland (the invention of Bakelite). From the first year of university onward, I was very active in the student movements and the political, philosophical, and even the natural sciences circles. This was a great worry for my parents, because several days a week I came home after 2AM. One rule was strict: Be ready for breakfast at 7AM and leave the house at 7:45 for the classes. The second year was even worse: I had already become either president or a member of the directory council of several student organizations, so all evenings were taken. The courses, however, became more interesting since there was organic chemistry. A very positive point was an introduction to biology by a fascinating man, Lucien De Coninck. He talked about evolution but also about DNA and RNA, cell biology, and the compartmentalization of biochemical reactions. That was in 1952, a year before the *Nature* publication on the work of Watson and Crick. He was in close contact with the team of Jean Brachet (1909–1988), Raymond Jeener (1904–1995), and Hubert Chantrenne (1918–2007) at the Free University of Brussels (ULB). During the war the ULB had been closed by the occupation forces because quite a fraction of the professors were either Jewish, freemasons, or left-wing militants. Jean Brachet went underground in the forests of the Ardennes, where he installed a small lab in which he developed the Unna-Brachet staining for DNA and RNA. He demonstrated that DNA was concentrated all in the nucleus and that RNA was present all over each cell but highly concentrated in the areas of intense protein synthesis, called ergastoplasma. This was also what we learned in De Coninck's course. Other professors still mentioned that the DNA was for animal cells (Thymo Nucleic Acid) and the RNA (Zymo Nucleic Acid) was for bacteria, yeast, fungi, and plants. In the botany course, it was mentioned that in a plant cell, besides the chloroplast, one could often

observe strange dot-like structures. They had received many different names, varying from chondriokont to mitochondria. However, we were told not to worry too much because it was not sure whether they really were important. This was my first fascination for what we now call cell biology and was further stimulated several years later, when Jean Brachet came to Ghent for a lecture series (Francqui chair) on “The Cell” and when I learned how Christian De Duve discovered the lysosomes.

Only in the fourth year did I again have contact with the life sciences, by choosing biochemistry as an optional course and by convincing the organic chemistry professor to be the mentor of a masters (then called licentiate) thesis in that discipline. My study results were not brilliant, as I had become the National President of the socialist students. I often had to travel to Brussels and Liège. It was also too hard to refuse an invitation to fly to Warsaw, my first flight ever, and join a student group that was to visit Auschwitz. I managed to convince the physical chemistry professor that this was important and that I could skip his practical courses. Anyway, I passed each year and finished with “distinction,” essential for being accepted for PhD research.

## THE FASCINATION FOR RESEARCH

My parents had probably hoped that after these four years I would start to work. However, during my research thesis in biochemistry, I was asked by the Professor of Physiological Chemistry of the Medical School, Laurent Vandendriessche, to become research assistant.

As this position yielded a salary equivalent to that of a high-school teacher, I did not hesitate. Above all, should not my parents be glad that I enjoyed the prospect of doing a PhD? They were happy, but they remained convinced that I would go into politics since I continued to go to so many meetings.

The research topic was immediately frontier research: resolve the structure of the phosphodiester bond in RNA by asking whether it is

a 2'- or a 3'-5' diester bond. The experimental approach was enzyme kinetics measured by dilatometry, when a (2',3') ribonucleotide ester was digested by pancreatic RNase.

Walter Fiers had started this work six months earlier. He taught me the dedication to careful experimentation and the fascination for research. He was my real mentor. We had first met in the Biochemistry lab, during my last year as an undergraduate. He was an introvert who knew immediately what was important and relevant. We were the first to open the packages with science journals that the mailman brought and to discuss what intrigued us. A new science called molecular biology was emerging and there were so many topics to follow.

The real revelation came when, during another Francqui lecture, Raymond Jeener explained how, in the United States, molecular genetics had developed thanks to Salvatore Luria, Max Delbruck, and the Cold Spring Harbor Phage School. Both Walter and I decided that this was the topic we wanted to study. That was also the opinion of an undergraduate zoology student attending these lectures, Jeff Schell (1935–2003) (**Figure 1**). However, there was no phage research in Ghent, so we asked ourselves if we should move to the ULB. This would be expensive and would imply a change of language, so the phage research had to wait. Jeff started his PhD in the laboratory of Microbiology. The research topic, taxonomy of *Acetobacter*, did not entuse him much, but stimulated him to apply for summer grants in the United Kingdom.

Bill Hayes, with whom Jeff stayed with a short-term fellowship at the Hammersmith Hospital in London, confirmed that Jeff's curiosity in bacterial genetics and phages was clearly timely. So Jeff entered in the mysterious world of restriction and modification, a topic that would keep him interested during all of the Sixties. Meanwhile, Walter decided for a big change and left the Physiological Chemistry lab for CalTech and the lab of Robert Sinsheimer. His research topic was the genome of the *Escherichia coli* phage,  $\phi$ X-174. Sinsheimer had shown that this phage was very UV sensitive





**Figure 1**

Marc Van Montagu, Walter Fiers, and Jeff Schell during the 1974 EMBO workshop in Ghent (Drongen), Belgium.

and had proposed that it had a single-stranded DNA genome. Walter could demonstrate that it was a single-stranded circle. Previously, Bill Hayes had predicted from recombination frequencies that the genetic map of *E. coli* would be a circle. This was the first proof that a circular genome existed as a physical entity. This result made the front page of the *New York Times*, but remained unnoticed in Belgium.

In the meantime, I followed a completely different path, and after one year of research, mixed with political activities, I received the remarkable proposition to become deputy director of a novel institute for training technicians and technical engineers for the nuclear industry. My task would be to discuss the content of the future courses with the scientists of the nuclear reactor center in Mol and to identify the possible teaching staff. Three and a half days on the spot was sufficient during the week, so I could still continue with my PhD research. An offer difficult to refuse, especially when one is barely 23 years old. I was even able to select as teaching staff well-qualified and appreciated colleagues from around my

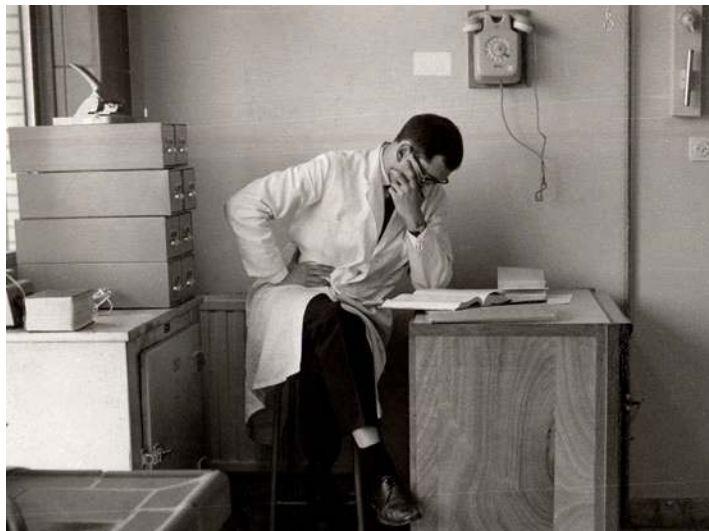
very age group. This experience became a unique learning process that lasted several years of working out teaching programs and setting up laboratories where all organic and analytical chemistry was done on a microscale and, with a dedicated team, where solidarity and mutual friendship were high. In those days the students in this area of northern Belgium often did not have the economic possibility to start university studies, so the creation of this kind of higher institute was a real opportunity for them. For us it was a reward to have such high-quality students. Later, several students, among whom Ivo Zaenen, the first author on our paper describing the isolation of the Ti-plasmid (54), joined our research team in Ghent and made important contributions.

After some years and a change of government, however, it became clear that we would never obtain the quality research laboratories that were promised. In this context, a deputy director who takes care of the science quality of the courses made no sense. So I abandoned the position, did my military service, still compulsory in those days, and again joined the

laboratory of Physiological Chemistry of the Ghent University, now on a full-time basis. Walter had just left for CalTech, and the research group was looking for novel nucleases and RNases in plants. As an organic chemist I was supposed to synthesize original substrates to facilitate the identification of such nucleases. The early Sixties was the period when Gobind Khorana was very active in the chemical synthesis of the ribo- and deoxyribo-oligonucleotides. The game of using protective groups that could be selectively removed was mesmerizing.

Some defeatists wondered what was the use of synthesizing some milligrams of di- or trinucleotides or some microgram of a longer oligonucleotide. I admired Khorana's work and followed his papers closely. I also started to make new derivatives and protective groups. The head of the laboratory, Laurent Vandendriessche, was appreciative when somebody was dedicated to a subject and did not interfere but instead tried to help. He was already very involved in the International Science Organisation and traveled regularly to the Soviet Union and other Eastern European countries. One day he announced that a whole team in a lab of the Academy of Sciences in Prague was specializing in the chemical synthesis of nucleotide derivatives and said that I should join it for a short term. He took care of a grant and the invitation by the director of the Institute of Organic Chemistry and Biochemistry, František Šorm. A superior personality, and former Minister of Education, he tried to democratize the system. When the situation became difficult, particularly for Jewish scientists, he helped them escape to the West. Later, in 1968, he was too openly active against the Soviet intervention, which caused his destitution and banishment into forced labor. He died a year later.

In the summer of 1963, I went to Prague for three months. It was a definitive step toward my decision to remain in fundamental research. Whatever the problems of the country were, whatever the shortage of equipment and chemicals the Institute had, the unit was hard working and enthusiastically dedicated to progress in



**Figure 2**

Marc Van Montagu, 1965.

science. Uridylic and cytidylic acids were prepared in kilogram amounts and bartered for other chemicals with U.S. companies. As the preparation started with an acid hydrolysis of RNA, a method that destroyed the purine analogs, I worked only with pyrimidine derivatives and synthesized the corresponding triplets UCU, CUC, etc. The protecting group reagent was rather original; it was unstable and had to be synthesized as a condensation intermediate between HCN and ketene, a reaction I was not welcome to use once back home. Upon my return I learned that Khorana's team had already synthesized the 64 different triplets. But that was not a major problem for my PhD defense (**Figure 2**).

## RNA PHAGES

Meanwhile Walter had returned from the United States and decided to work on an RNA phage that was capable of infecting *E. coli* bacteria that carried an F plasmid. This phage had just been described by David Baltimore in Norton Zinder's lab at Rockefeller University and the only bacterial messenger easy to purify, because the viral particles could be prepared in gram amounts. In the Fifties we had



tried to work with tobacco mosaic virus (TMV) RNA, a plant virus that we also prepared in gram amounts, but in vitro protein synthesis was not yet ready to exploit the molecular biology knowledge that this mRNA could generate. Walter had decided to develop methods to sequence MS2 RNA, quite a daring decision, which generated many smiles. Nevertheless, 10 years later he succeeded and his team was the first to sequence a viral genome (19).

I was given the possibility to start my own research unit in the department of Histology of the Medical Faculty. I had access to an electron microscope and could teach cell biology. For research I decided to join Walter, but focused on the genetics of the phage. This was the period when the genetic code was unraveled, when Brenner and Crick identified the UAG and UAA stop codons with their sophisticated mutagenesis of the phage T4rII locus, and when the one gene–one enzyme theory flourished. As recombination with RNA phages was unknown, it became clear that the only way to map the mutants would be via the sequencing of the mutant proteins—a challenge, because amino acid sequenators existed but they were prohibitively expensive for a beginning team. So I convinced a bright beginning PhD student named Joel Vandekerckhove to join me and to use the Fred Sanger method of separating peptides by paper chromatography and electrophoresis to elute the spots, hydrolyze them, and determine their composition.

I had the good luck that this was really frontier research at the time and many labs like those of Fred Sanger (Cambridge, U.K.) and Jim Watson (Cambridge, U.S.) had taken RNA phages as model systems for establishing the discipline of molecular genetics. As a consequence, I was accepted when I applied for the first European Molecular Biology Organization (EMBO) courses and for the first Spetsai meeting in August 1966.

This was a very essential step because it introduced me to many young U.S. postdocs, who remained friends for life, and the topics presented opened new horizons. Indeed, we never had formal graduate schools where the

emerging science was presented; we were expected to acquire this knowledge through appropriate reading.

Evolving knowledge about tRNAs and ribosomes and the universality of the genetic code were stimulating discussion points. The phages  $\lambda$  T4 were well represented, and I realized their importance as model systems for molecular genetics. Back home, I intensified my contact with René Thomas at the ULB. His team was already the world leader in the genetics of phage  $\lambda$ . The whole molecular biology department of the ULB had just moved to a nice new suburban location in St-Genesius-Rhode. Every week leading scientists from all over the world gave seminars, something entirely nonexistent in Ghent. The fact that in 1963 I had moved from Ghent to Brussels helped me to develop better social contact with the Brussels group and the visiting seminar speakers. I became part of the world community of phage geneticists. For visualizing the phages and their anatomic structure, electron microscopists had worked out nice enhancing methods (negative stains). An EMBO course in Geneva, organized by the Kellenbergers and Werner Arber, was very helpful for getting acquainted with these techniques. I also learned the Kleinschmidt techniques for visualizing DNA. Spreading heteroduplexes between wild-type and phage  $\lambda$  DNA containing insertions or deletions allowed the physical localization of the alteration in the phage genome. Later we used this method for localizing IS sequences, transposons, and phage Mu insertions in plasmids and for positioning cloned restriction fragments on the Ti plasmid.

Meanwhile, Walter had become professor in molecular biology and had started his own unit in the Faculty of Science. Jeff Schell was still located in the Veterinary School where biochemistry and microbiology were initiated. At the end of the Sixties he also moved to the *Lede-ganckstraat* and introduced us to the *E. coli* K and B restriction and modification system.

Our understanding of the RNA phages had meanwhile progressed well, but we rapidly realized that we should start considering eukaryotic

model systems. Cancer research became more molecular, and tumor viruses became fashionable model systems. Walter decided that, in addition to his efforts to sequence phage MS2, he would start SV40 research, including sequencing. Jeff and I, with our limited budgets, thought it could be worthwhile to start plant tumors. Plant cell culture had recently been developed that did not need expensive media, CO<sub>2</sub> incubators, or special deep freezers. Using live animals as test systems was also not appealing to either of us. The microbiology lab where Jeff did his PhD kept a collection of 150 *Agrobacterium* strains of which some were called *tumefaciens* because they were able to induce crown galls on a large variety of plants, and others, seemingly very related, that could not, were called *radiobacter*.

## PLANT TUMORS

Let me first give some milestones on plant tumors studies. In the late 1940s plant pathologists, microbiologists, and chemists had developed a keen interest in crown-gall-inducing strains of the bacterium *Agrobacterium tumefaciens* (47) because the interaction between the bacteria and their hosts displayed many unusual features. Unlike other pathogenic bacteria that are known to cause plant tissues to die, wilt, or become discolored, *A. tumefaciens* had the unusual ability to cause infected plant cells to proliferate and form a tumor. The bacterium could not be detected intracellularly, either in plant cells that had been transformed or in the cells of sterile crown-gall tumors grown in vitro even in the absence of growth hormones for many years. This observation led Braun to introduce, in 1947, the concept of a tumor-inducing principle (TIP), a postulation that implied that this principle was transferred by the bacteria to the plant cell to induce transformation (5). Importantly, Braun proposed that the TIP must be capable of replication, because it was never lost by dilution. Much work ensued to identify the TIP, but only in the late 1960s the first indications were forthcoming that bacterial DNA may somehow be involved

in tumor induction. DNA sequences from *A. tumefaciens* were claimed to be present in sterile crown-gall tumor DNA preparations, suggesting that bacterial DNA had been transferred to the plant cells (46), and later, expression of some bacterial genes was demonstrated by the detection of bacterial antigens in sterile crown-gall tissue. Ever since the discovery of lysogeny in *A. tumefaciens* (2), much attention has been focused on the possible relationship between lysogeny and pathogenicity. However, efforts to determine the causative role of temperate *A. tumefaciens* phages in tumorigenicity were inconclusive and contradictory. One of the most important indications of phage involvement in tumor induction by *A. tumefaciens* was the detection of  $\Omega$ -group temperate phage DNA sequences in strain A6-induced sterile crown-gall tissue (45). Nevertheless, no phage could be induced from strain A6, and no  $\Omega$ -phage DNA sequence could be detected in DNA extracted from A6. Furthermore, presumably cured strains derived from lysogenic strains were no less pathogenic than the parental strain (28, 29). These results still did not rule out the possibility that supposedly cured strains contain a defective or cryptic prophage that may be present as a plasmid or may be part of a bacterial plasmid.

## THE GREAT SHIFT: DISCOVERY OF TI PLASMID AND T-DNA

To explore the plasmid hypothesis, a systematic search was conducted for the presence of a plasmid DNA in a number of pathogenic and non-pathogenic strains of agrobacteria. This work culminated in the landmark discovery of a large supercoiled plasmid in all virulent strains, but interestingly, this plasmid was not detected in any of eight avirulent strains tested (54).

Careful experimental design was crucial to the success of these experiments because it was essential to distinguish between possible supercoiled plasmids and covalently closed circular forms of phage DNA, which was not easy with such large (200,000 base pairs) plasmids. Also at that time, only 20 years after Lederberg's discovery of plasmids, the plasmid

isolation methodology was in its infancy. To avoid possible contamination with phage DNA, strain B6S3 was chosen because it could not be induced to produce phage particles. Detection of a large 54.1- $\mu$ m plasmid in alkaline and neutral sucrose sedimentation gradients and in cesium chloride gradients of B6S3 was confirmed by electron microscopy. Via comparison of a large number of pathogenic, virulent, and avirulent strains using this methodology, conclusive insights into the role of the plasmid in tumorigenicity were obtained. All pathogenic *Agrobacterium* strains tested harbored a large plasmid, including all cured but still pathogenic derivatives, but none of the avirulent strains tested. Furthermore, whether a tumor-inducing strain of *Agrobacterium* was lysogenic for an inducible prophage did not interfere with the presence of a plasmid in that strain. Conversely, an *Agrobacterium* strain could be lysogenic without harboring a plasmid because two of the avirulent strains included in the study produced phage particles upon induction but did not contain plasmid DNA. So, Braun's TIP had been identified 27 years after it had first been proposed. We designated these plasmids tumor-inducing (Ti) plasmids. This landmark discovery prompted a worldwide intensification of efforts to prove directly that these plasmids were responsible for the tumor-inducing capacity of their host strains.

The evidence for association of an extra chromosomal plasmid with virulence was irrefutable. Direct proof that the Ti plasmids were responsible for the tumor-inducing capacity of their host strains came shortly thereafter through isolation of plasmid-free derivatives from tumor-inducing strains. For some time, strain C58 of *A. tumefaciens* had been known to be possibly "cured" of its virulence by growth at 37°C and not at the normal growth temperature of 28°C, and, predictably, strain C58 was found to have lost its plasmid when grown at 37°C. A further landmark was achieved in 1975 when nononcogenic *A. tumefaciens* strains were demonstrated to acquire virulence as a result of plasmid transfer (48), in confirmation of Kerr's experiments (37) that first pointed out the pos-

sibility of mobile genetic elements as the basis for virulence. A year after the initial discovery of the Ti plasmid, the first genetic marker, Agrocin 84 resistance, was shown to be encoded on the Ti plasmid, thereby opening the way for more refined genetic analysis (17). With increased focus on the Ti plasmid, a worldwide effort was launched to unravel the molecular basis for tumorigenicity, and our group continued to dominate the field. The finding that oncogenicity and virulence were determined by a mobile, extrachromosomal element was revolutionary and opened up hitherto unforeseen scenarios for initiating the molecular genetics of a plant-microbe interaction.

### Functional Mapping of the Ti Plasmid

In the late 1960s Morel's group in France demonstrated that crown galls generate copious amounts of novel metabolites, octopine, and nopaline and that crown-gall cells that were free from bacteria were still able to produce them (42). They showed also that the *Agrobacterium* strain, not the plant, determines the type of opine made by the tumor and that each *Agrobacterium* strain can catabolize solely its own particular type of opine. Morel proposed that Braun's TIP must be the cause, or it must include a gene responsible for opine synthesis in the plant. To account for the strain specificity of the opine catabolism, he proposed that a single enzyme catalyzes opine synthesis in the plant and opine breakdown in the bacterium. At that time it was difficult to accept the notion that a bacterial gene could enter a plant cell and function there. Five years later, with the Ti plasmid in hand, we were in a position to test the idea and to prove it by demonstrating integration of T-DNA into the plant nuclear genome. Building on expertise in bacterial genetics and the use of phage mutagenesis in functional mapping (20, 50), we set about dissecting the genetic structure of the Ti plasmid and the molecular basis of the metabolic interaction between pathogen and host. Transposon-insertion mutagenesis of the Ti plasmid revealed the functional organization

of the nopaline Ti plasmid pTiC58, and a year later the functional organization of the octopine pTiB6S3 had been established (12, 30), thereby confirming the strain dependency of the opine synthesis observations of Morel. Transposon hits in T-DNA were found to eliminate opine production, to alter tumor morphology, or to have no phenotypic effect at all. The morphological mutations were used to demonstrate that genes encoding a pathway for the synthesis of the plant-growth regulators auxin and cytokinin mapped to the T-DNA. The same mutagenesis approach showed that another group of mutations affecting tumor induction mapped to a sector of the Ti plasmid, separate from T-DNA, the *virulence* (*vir*) region (16, 23, 30).

As soon as the structure and function of the Ti plasmid were understood, the concept that the Ti plasmid could be used to deliver novel genes into plants was born, raising the possibility that the molecular dissection of plant physiology through target manipulation of gene expression was in sight. The further possibility of introducing, at will, genes that could confer new desirable properties to a plant and the potential for revolutionizing plant breeding and crop production was obvious. Galvanized by the realization of this outstanding opportunity in plant science, research progressed rapidly, both in analysis of the Ti plasmid and in understanding the process by which the plasmid DNA is integrated into the plant genome. The race for the development of a workable plant gene vector was tight, particularly between Chilton's lab in St. Louis and our lab in Ghent. In 1977, both labs had access to the novel technique of Southern blot and we could demonstrate that only certain parts of the Ti plasmid, the T-DNA, are integrated into the plant genome. Unfortunately, we lost the battle to publish first (6), and the only record of our result is a talk given at a Cold Spring Harbor Symposium in 1978 and in Angers (8). The fact that between different Ti plasmids sequences are conserved pointed toward their central role and essentiality for oncogenicity (15). Adding quickly to this discovery, at a time when little was known about DNA sequence determinants for heterologous

integration, a 25-basepair direct repeat on the Ti plasmid was identified at the borders of what is incorporated into the plant genome (39, 56). It was shown that these sequences define T-DNA on the plasmid. At the same time, elegant cell fractionation analyses revealed that the T-DNA integrated into the nuclear, and not the chloroplastic or mitochondrial, genome of plants (7, 53).

## THE WAY TO PLANT TRANSFORMATION

### Plant Gene Vectors

Conversion of the Ti plasmid of *Agrobacterium* into a gene vector progressed in multiple stages. Unraveling the functional organization of the Ti plasmid was central to our efforts to use the integrative properties of T-DNA for the genetic modification of plants. Methods were developed for site direct mutagenesis and for insertion of DNA into any part of the Ti plasmid by homologous recombination by means of a co-integrate plasmid, which is the product of homologous recombination through a single crossover between a small plasmid of bacterial origin and an *Agrobacterium* Ti plasmid. Integration of the two plasmids requires a region of homology present in both plasmids introduced into *A. tumefaciens* (41, 49). We gained understanding that no mutation in the T-DNA could block T-DNA transfer and that all the genes affecting the process of T-DNA export to the plant cell mapped in the so-called *vir* region, outside the T-DNA borders.

Knowing which sequences on the Ti plasmid were required for integration of the bacterial DNA and, importantly, what needed to be removed from the T-DNA to avoid tumor development and to produce transformed plants, we designed the first nononcogenic Ti plasmid (pGV3850) capable of transforming plants without tumor formation (55). Besides demonstrating the feasibility of creating transgenic plants, this work was the first demonstration that the T-DNA borders is all you need as T-DNA elements to integrate

foreign DNA into the plant genome. Such co-integrated vector system was used extensively as a gene vector for plant transformation (10).

The functional organization of the Ti plasmid opened the way for disarming the T-DNA completely and for the construction of *vir* region-containing plasmids lacking even the T-DNA borders. A versatile vector system was developed, now known as binary vectors, in which the T-DNA element and the *vir* genes were located on separate replicons (27). The strategy to clone transgenes in their T-DNA was to take advantage of rare restriction endonuclease sites that were introduced into T-DNA sequences. More recently, the T-DNA binary vectors were redesigned to benefit from novel in vitro recombinational cloning schemes, mainly based on the site-specific Gateway<sup>®</sup> system (Invitrogen, Carlsbad, CA) (22, 36). Gateway-compatible binary destination vectors have been developed for a wide range of gene-function analyses, including overexpression, promoter fusion, protein fusion, and silencing, and they have been optimized for various plant species (35). This technology even facilitates the recombination of multiple segments of DNA contiguously or in independent expression units (18, 33, 34).

### **Selection and Regeneration of Transformed Plants**

An ongoing problem was how to regenerate viable plants from cells that had been transformed with the T-DNA. Of great help was the availability of a large bank of T-DNA mutants. Thanks to this bank, gene vectors could be constructed in which the cytokinin synthesis genes had been disrupted. By screening rooty tumor cells for nopaline production, researchers showed that these, unlike crown-gall tumor cells, were able to regenerate into whole plants that passed the T-DNA copies to their progeny as Mendelian traits (1, 38).

However, screening the transformants for their ability to synthesize nopaline or their inability to grow in the absence of exogenous cytokinin was cumbersome and was

incompatible with the main goal of selecting few transformants from a large background of false positives. Sequencing the nopaline synthase (*NOS*) and the octopine (*OCS*) genes (3, 13, 14) and fusing their promoter and terminator sequences to a kanamycin-resistance encoding gene enabled the creation of a selectable marker for plant cells. The bacterial regulatory sequences that were known to function in planta would drive the expression of a gene that would allow survival of transformants under antibiotic-selection conditions.

### **The First Genetically Modified Plants**

The way to obtain the first transgenic plants had been paved, and almost simultaneously, our lab, Monsanto's, and Chilton's reported success in the use of Ti-derived plant gene vectors, antibiotic selection of transformants, and regeneration of fertile plants that passed on the chimeric gene in a stable and Mendelian manner to their progeny (4, 21, 25).

Only nine years after the discovery of the Ti plasmid, the "Golden Era" of plant molecular genetics had begun, and the development of plant transgenic technologies would expand dramatically in the 1980s and 1990s.

### **THE IMPACT OF THE DISCOVERY**

Funding agencies always make a difference between fundamental and applied research when in fact there is no dichotomy in science. The discovery of the Ti plasmid is a classic case of curiosity-driven research that ultimately led to major scientific breakthroughs both in the proliferation of new research tools and quantum leaps in fundamental and applied knowledge about control circuits in biological systems. Indeed, *Agrobacterium* is an extremely interesting biological system, and its study has led to a feast of fundamental insights (58).

For example, (a) the recognition that bacterial genes could integrate into the plant genome and direct the synthesis of plant growth regulators, thereby creating the microenvironment



(the tumor) for their growth, was revolutionary and formed the basis for much of what we know about horizontal gene transfer. The discovery that the T-DNA intermediate is a single-stranded molecule (44) elicited the hypothesis that T-strand transfer occurs by a conjugative mechanism, where the recipient is a eukaryotic plant cell (24, 52). This interkingdom DNA conjugation stimulated basic research in bacterial conjugation. In the postgenomics era, we now know that horizontal DNA transfer is responsible for a great deal of adaptive responses and invasive behavior and that the genomes of living organisms are much more fluid than previously imagined. (b) The fact that T-DNA genes trigger plant growth regulator synthesis not only explained why crown-gall calli can be grown on minimal media without addition of exogenous cytokinin or auxin, but also, and more importantly, paved the way for a more rational understanding of how plant growth regulators function in plants and for plant gene regulation. It must be kept in mind that in these days “hormonal control of plant growth and development” theories were rampant in the absence of hard gene function data, and research was largely in the grasp of “spray and pray” approaches that to a large extent obscured what was really going on. (c) The induction of *vir* genes by plant phenolics was one of the first examples of communication between microbes and plants in the soil environment (43). Today, the importance of many more developmental regulatory circuits has been identified.

In contrast, the demonstration that *Agrobacterium* could be used as a vehicle to transfer and integrate any foreign gene into the plant genome undoubtedly opened the door to a complete new area of plant sciences and plant engineering. For the academic world, this technology allowed researchers to study plant processes through gain of function and enabled a systematic and refined analysis of the impact of single genes on all aspects of plant biology. In the early years, the technology was applied in model plants, such as tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* and was the basis to identify and study fundamental

principles, such as the *cis*-regulatory elements in plant genes (e.g., promoters), the translocation of proteins in the plant cell (e.g., the signal peptides to transport a protein to the chloroplast) (26), and transcriptional regulation in plants (e.g., the signals needed to induce expression of genes by light). These initial successes convinced an ever-increasing number of labs throughout the world to rapidly adopt the novel genetic engineering technology. Much of today’s detailed knowledge of how plants grow and develop as well as phenomena such as the reaction of plants toward pathogens or abiotic stress (such as drought and salt) have been unraveled by creating transgenic plants through *Agrobacterium*-mediated transformation.

Some of the first efforts to understand the function of all genes in a genome have been launched in plants, to a large extent, thanks to the availability of the efficient gene-transfer technology offered by *Agrobacterium*. The possibility of using T-DNA as an insertional mutagen had been realized early on, and the publicly available populations of T-DNA-tagged mutants in *Arabidopsis* are now so large that nearly every gene in the genome is tagged. In addition, reverse-genetic strategies on DNA from populations of T-DNA mutants allow researchers to start with one gene sequence and to identify a mutant line. The use of these mutant lines in labs throughout the world has led to a thorough understanding of the physiology, biochemistry, and developmental programs of plants.

Much of our current understanding of how plants grow and develop; how primary and secondary metabolic pathways are controlled; as well as how plants defend themselves against pests, diseases, and harsh environments has been obtained by using transgenic plants. Gene inactivation by T-DNA insertion, RNA interference, and the introduction of new genes and ectopically altered transgenes have been invaluable tools for plant molecular biology.

Many interesting fundamental questions remain regarding the information encoded by the T-DNA and the interaction of the T-DNA with the chromatin. Techniques for an in-depth study of the functional genomics of different

T-DNAs are available. The phenotype of the crown-gall tissues induced by these different T-DNAs can vary spectacularly, from shooty teratoma tissues to smooth, seemingly undifferentiated galls. The demonstrated differences in the cytokinin/auxin ratio might not be the only cause of these differences. Our present knowledge of regulatory small RNAs and the emerging understanding of hundreds of plant-encoded peptides suggest that it can be rewarding to analyze the T-DNAs for the possible presence of small RNA and peptide-encoding sequences.

## THE INNOVATION CHALLENGE

We were quite aware that genetic engineering of crop plants would transform the agricultural world. Until then, new plant varieties could be obtained only by classical breeding. But we had no clue of the legal steps for seeking patent protection, and there was no technology-transfer officer at the Ghent University. In those days, our universities were centers of knowledge with little or no contact with the industrial world. Luckily, in 1978, Jeff Schell had accepted a position as director at the Max Planck Institute in Cologne where the expertise of filing patent applications was available. This is the reason why the patent to engineer plants via *A. tumefaciens* belonged to the Max Planck Institute, even though the research had been performed in Ghent. A few years later a new start-up company in Ghent, Plant Genetic Systems (PGS) (see below), had the funds to buy the patent as an important asset. Today all transformations of the major crops, including maize, soybean, cotton, rice, and cereals are done primarily with *Agrobacterium*.

## ENTREPRENEURSHIP

With the first gene-cloning experiments, it became evident that this technology had enormous economic potential for producing novel peptides and proteins for the pharmaceutical industry. Particularly in the United States, a climate developed for start-up companies that

were often directed by a business person and a leading scientist and were established at a technology campus close to a leading university.

When our *Agrobacterium* story started, entrepreneurs decided to try out plant biotechnology start-ups. One of these, Advanced Genetic Sciences (AGS), with Laurence Bogorad (1921–2003) as Chair of the Science Board, asked Jeff and me to join its scientific board.

AGS had raised an—for us—impressive amount of money, had set up in a record time excellent laboratories, and had attracted outstanding scientists. Why not try and start a similar venture in Belgium? As a good civil servant I approached our Minister of Education and Research. He was a law professor but had the good sense to see that it was not the task of the university to establish a start-up company. He directed us to a just-established regional investment company (GIMV) and a board of industrialists (Innovi). The latter appointed a CEO and contacted some private investors, among which were the Belgian Sugar Factory of Tienen and a Swedish equivalent called Hilleshög. Laboratory space was rented from the School of Engineers at the Ghent University. Marc Zabeau, who did his PhD with us on restriction alleviation of phage  $\lambda$ , was hired as laboratory director, and the research started in 1983. Many scientists and technicians from my laboratory wanted to join this exciting novelty, PGS. Quite a number of the original team remained after PGS was sold to Hoechst/Aventis in 1996 and are still there after the transfer to Bayer in 2001.

As cofounder, member of the Board, and initial Scientific Director of PGS, I remained a 100% state employee as professor at Ghent University and part time at the Vrije Universiteit Brussels (VUB) (**Figure 3**). PGS had the good sense to focus on some early successes: the cloning of a *Bacillus thuringiensis* insecticidal protein and the engineering of tobacco plants that expressed this gene at a level to convey resistance to *Manduca sexta* (tobacco hornworm) (51). The other breakthrough was the expression in plants of a bacterial gene that detoxified a herbicide, produced by the companies Hoechst and Meiji, termed Basta (9) in cooperation with

Biogen, then established in Geneva, and initiated by their laboratory director Julian Davies. We operated with the same Science Board as that of AGS, with, in addition to Lawrence Bogorad, Dick Flavell, Bob Goldberg, Howard Goodman, and Ingo Potrykus.

PGS grew rapidly. Jan Leemans replaced Marc Zabeau as lab director when Marc left to start Keygene (Wageningen, The Netherlands), and Walter De Logi became CEO after Jozef Bouckaert had joined AGS in California. We also realized that to demonstrate that our novel plants had commercial value we had to introduce plant breeding. Willy De Greef, who had started as an oil palm breeder with Unilever in Africa, immediately appreciated the opportunities offered by the tapetum-specific genes cloned in Bob Goldberg's lab at UCLA. Expression of an RNase from *Bacillus amyloliquefaciens*, Barnase, under control of a tapetum-specific promoter, was so tight that it inhibited pollen production but did not interfere with the normal development of the genetically modified (GM) plant. Barnase was chosen because the gene was available that encoded an inhibitor protein, Barstar, which the *Bacillus* produces to protect itself against the toxicity of Barnase. Expression of Barstar under the same tapetum-specific promoter restored the pollen production inhibited by Barnase. Meanwhile we had developed a nuclear male sterility/restorer system that could be the basis of hybrid vigor in quite some crop plants (40). Around that time we convinced Suri Sehgal, a veteran of the seed industry, to join PGS as chief operating officer. He immediately streamlined the organization, stopped several ongoing projects where value recovery was questionable, brought crop-trait focus in select crops, and convinced everybody to focus on a crop with high commercial value—hybrid canola (*Brassica napus*). These moves created the products and identified the real value of PGS.

While AGS was under the direction of John Bedbrook, it was sold to Dupont. The takeovers became a trend in the 1990s. All the start-ups in plant biotechnology were acquired by big multinational firms because they were, and still



**Figure 3**

Marc Van Montagu, 1984.

are, the only companies that can afford the huge costs of the full product-development chain.

## GENETICALLY MODIFIED AGRICULTURE TODAY AND BEYOND

I feel warmly rewarded in that GM crops are having a durable and unprecedented effect on improving the quality of life and on sustainable food, feed, and fiber production. From the first commercial launch in 1996, the global GM crop area had grown by 2009 to some 130 million hectares, representing more than 9% of the total agricultural land. GM crops are cultivated primarily in the Americas and in China. The global seed value of GM crops is over \$10 billion. Notably, 90% (13 million) of the beneficiary farmers are resource-poor farmers in developing countries (31).

The data clearly show that the current GM crops achieve higher yields in a more sustainable way. At the same time, novel applications that provide environmental benefits appear as technologies mature and are more widely adopted. Remarkable progress in genomics and functional genomics has



**Figure 4**

Marc Van Montagu and Jeff Schell, 1993.

brought the first insights into the gene pool and transcriptional regulation of model plants and of some important crop species.

The adaptation of plants to biotic and abiotic stress conditions is now open to molecular analysis and manipulation. Through these approaches, the next wave of crops will be resistant to biotic and abiotic stresses and will be able to grow productively on more marginal land. Yield gains are important for food security and land conservation, particularly in a changing climate. Our planet requires the prompt and widespread adoption of more efficient and sustainable agricultural practices to improve food security and to reduce the negative effects of intensive agriculture on the global environment. To close the yield gap between productivity in the field and what can be achieved best will require further innovation, be it in providing information and infrastructure or in generating new crop varieties that are better adapted to specific local environments.

In this context, the potentials of biotechnology are manifold. GM crops will help to maintain sufficient availability of food, but also

to “domesticate” crops for biomass and bioenergy production. Renewable raw materials offer an alternative to the chemical industry and can play a role in rural income growth and poverty alleviation in developing countries. Nutritionally enhanced GM crops can improve peoples’ health, and, last but not least, GM crops can bring about environmental benefits, for example, by decreasing pesticide use or by reducing soil erosion.

### **GENETICALLY MODIFIED ORGANISM POLEMIC**

In spite of the above-mentioned benefits, GM crops have aroused passionate opposition. That one can engineer a gene from a species belonging to a certain kingdom into a species from another kingdom has struck the imagination of many and frightened the public at large. The fears for health and environmental risks have been disproportionate and could not be reassured by science-based risk-assessment analysis. Anti-GMO (genetically modified organism) activists bombarded the media with

unfounded claims that GM foods are not safe for human and animal consumption, that GM crops would become super weeds, and that the involuntary spread of transgenes into the environment would be irreversible and devastating for our habitat. On top of that, some groups are concerned about adverse social implications of GM crops because they are in the hands of powerful multinationals. Rather than tackling these issues, they use the precautionary principle as a firm philosophical basis for saying “no” to GM crops, arguing that there is remote chance of irreparable damage.

Personally, I admit that it was a surprise to us, plant molecular geneticists, to realize that society did not follow our scientific rationality, because we see our planet as one large natural genetic laboratory where all the living organisms continuously activate and silence part of their genomes in response to environmental changes. It is essential for adaptation, and hence for evolution, that plants can alter their genomes through transposition of movable elements, accumulation of deletions, insertions, gene amplifications, and point mutations. Genomic studies during the past decade have clearly documented that a genome is not a static entity but a dynamic structure continuously refining its gene pool. So, for a geneticist, generating a transgenic organism is a surgical alteration compared with the genomic changes induced during crossing and breeding events exploited in agriculture and animal husbandry. The tools of molecular biology offer precision, speed, and ability to reach this invaluable endeavor of species domestication.

It is not simple to answer the following concern: “Give proof that GM crops are safe and pose no risk.” It seems a scientific question but it is not. Science can prove the presence of danger, but not its absolute absence. The experts’ claim that a GM variety is not more or less a health or an environmental risk than the non-GM parent crop does not answer the question. Scientists thought that it was sufficient to show that intensive agriculture causes environmental damage, independent of whether it is

GM or not, and that, on the contrary, we can make novel GM varieties that are more environmentally friendly. Society wanted step-by-step examples of studies to support such statements. Now, after 25 years of field releases and 15 years of commercial use without evidence of harm, fears continue to trigger the precautionary principle in Europe. It is important for the sake of humanity and our planet to abandon this deliberately one-sided position and determine the advantages and disadvantages of this technology on the basis of scientifically sound risk assessment.

The regulatory requirements to get a GM crop into the market are costly and constitute a major obstacle that adds to the chronic underinvestment in science and technology. Scientists from the public sector cannot afford such regulatory compliance costs, which range from tens of thousands to millions of dollars (32). The result is that, although the present generation of GM crops can be traced back to discoveries made in the public sector, there is a misperception that biotechnology is the exclusive domain of a handful of multinationals.

We, scientists of the public sector involved in biotechnology for public good, have taken steps to fight for a regulatory framework that is less counterproductive. In 2004 we started a worldwide initiative, the Public Research and Regulation Initiative (PRRI) (<http://www.pubresreg.org/>) (11), to offer public researchers involved in modern biotechnology a forum through which they can participate in and are informed about relevant international discussions and agreements that influence national regulations. The goals are to advise negotiators about the objectives and progress of public research in modern biotechnology, to bring science to the negotiations, and to inform the negotiators about concerns public researchers may have. We hope in this way to curb the prohibitive costs and length of the regulatory requirements and that technologies that are pro-poor, pro-environment, and pro-economy find their way to those who need them the most.



## **BIOTECHNOLOGY FOR DEVELOPMENT**

### **Institute of Plant Biotechnology for Developing Countries (IPBO)**

The pro-poor biotechnology has always been a goal for me. While Director of the Lab of Genetics at Ghent University I was keen to receive trainees from all over the world. Hundreds of scientists were trained during the 1980s and 1990s, many from developing countries. Together we contributed to major advances in plant sciences, most notably in the fields of plant growth, development, and flowering as well as biotic and abiotic stress. Most of them returned to their home countries where they have continued their plant biotechnology research.

When I reached the “emeritus age,” the choice to dedicate myself full time to activities to promote biotechnological applications that meet the needs of poor farmers was obvious. With the support of the S.M. Sehgal Foundation and the Flemish Government, I started in 2000 at Ghent University the Institute of Plant Biotechnology for Developing Countries (IPBO), dedicated to fostering plant biotechnology in low- and middle-income countries.

The challenge of IPBO is to help find biotechnological solutions to improve the life of the rural poor, who make up 80% of the world’s 1.4 billion hungry people. No segment of humanity depends more directly on environmental resources and services than the rural poor. Their lives are interwoven with the surrounding environment in ways that make them both particularly valuable as custodians of environmental resources and particularly vulnerable to the impact of environmental degradation. When population pressure grows and food is scarce, hunger can drive them to plough under or overgraze fragile rangelands and forest margins, threatening the very resources upon which they depend.

I am well aware that science and technology alone will not have the power to overcome the challenges. Solutions must come from concerted actions of different segments of society. It will require political will and strong

commitments of the nations, as it will lead to a full revision of the way we perceive our society and our interaction with the environment. I maintain, however, that in the near future, as the technology matures further and the full impact of the postgenomics era is felt, we will be able to tackle some of the most intractable problems in plant productivity. Then the benefits will be more obvious, particularly in the developing world, where economies are largely dependent on the health of the agricultural workforce and where much of the impact of the Green Revolution was not felt. How can IPBO contribute? The strategy I propose is to take advantage of the extensive network of cooperation that I have established during my years as director of the Lab of Genetics and to promote international actions to respond to the needs of developing countries. The aim is not only to transfer technology, but also to stimulate competitiveness and independent biotechnological research for the development of locally relevant crop improvements. IPBO helps partners in developing countries to identify agricultural needs that cannot be worked out by conventional means and then contact research groups from the network to develop strategies for solving the problems. This plan includes providing basic training. The explicit goal is to create a network of experts to help establish independent research capacities in the least-developed countries and to stimulate the interest of highly qualified institutes in the richer economies to investigate the constraints imposed on poor farmers. In addition to its role of facilitator, IPBO carries out its own research addressing the agricultural productivity needs of small-holder farmers and capturing the value of biodiversity in developing countries.

### **International Industrial Biotechnology Network (IIBN)**

Beyond the traditional agricultural products of food, feed, and fiber, the remarkable breakthroughs in the fundamental plant sciences are fueling new opportunities in agriculture and transforming the bio-based economy. Faced

with a global energy crisis and concerns over climate change, we may find a reasonable fraction of the energy demand to be met through the exploitation of plant-based resources. Modification of lignin biosynthesis, increased biomass production and yield, resistance to abiotic stress, and metabolic engineering to improve oil content and composition for biodiesel as well as sugar and starch for ethanol are examples of the biotechnology solutions for bioenergy.

Metabolic engineering will also become an important approach for increasing nonfuel bioproducts. Plants are being used more and more in industrial approaches that are not dependent on petroleum, such as biodegradable plastic or intermediates for the chemical industries. Indeed, advanced bioproducts might be the greatest long-term benefit of the current biofuels research race. There is significant room for growth of this sector given that 60% of the chemical industry is carbon based. It is highly likely that a large number of presently underutilized plant commodities will emerge in the coming years as sources of raw material for the carbon-based chemical industry.

Plants also are being manipulated for use as vehicles for development and manufacture of high-value pharmaceuticals. The production of pharmaceutical proteins in plants has several potential advantages over current systems such as mammalian and bacterial cell cultures, including the lower costs and scalability of agricultural production and the absence of human pathogens. Another interesting aspect is that in some cases crops, e.g., fruit, leafy vegetables, or grains, can also serve as delivery systems of these high-value proteins to humans and animals. Research and development in plant-made pharmaceuticals include a number of vaccines already progressing to clinical trials, antibodies, and nutraceuticals.

The sustainable use of plants as feedstock for industry and energy has already attracted significant investments in the technologically proficient countries, but much needs to be done to promote an enabling environment for the development of a plant-based industry in the least-developed countries. It is urgent to

develop mechanisms to empower developing countries so that, for once, they will not be marginalized and will be able to participate in—and contribute to—the emerging global knowledge-based bioeconomy.

Aware of the importance of applying science and technology to agricultural production processes, and of the need to create strong linkages between agriculture and industry, the United Nations Industrial Development Organization (UNIDO), together with IPBO, launched in March of 2010 the International Industrial Biotech Net (IIBN) aimed at advocating, promoting, and facilitating access to industrial biotechnology for the generation of value-added products from genetic resources of developing countries. The IIBN will create a unique platform for participatory, proactive, and pre-competitive sharing of new technologies, knowledge, and best practices for product development. Through this network we want to seize and exploit the new opportunities that biotechnology offers to utilize biomass and biodiversity for the production of bioenergy and added-value products derived from feed stocks that are renewable and environmentally friendly.

## FINAL THOUGHTS

Like most scientific innovations that impact our society, the field of plant biotechnology did not emerge from targeted research efforts to increase agronomic productivity. Rather, it is the by-product of curiosity and basic scientific questioning. Plant biotechnology is now expected to contribute to strategies for meeting the U.N. Millennium Development Goals the fact that it is even on the agenda for such a noteworthy cause is the result of the scientific discovery of the Ti plasmid and the story that spans the birth and growth of recombinant DNA technology. This discovery and the revolution that swept science in its wake are dramatic examples of how science works best and how basic research can lead to practical results that were unimaginable when the research began. The story is also a significant

example of how inquisitive scientific thought freed from preconceptions can create an entire scientific culture and philosophical framework able to comprehend an increasingly complex larger picture through attention to the minutest detail.

Despite the fact that such enormous progress has been made in fundamental science, the expected innovation boom did not happen. The molecular plant community is still small compared with other life science disciplines. Still, I believe that it will continue to grow once the value of the tremendously wide range of possible applications for humankind has been recognized.

Genetically engineered plants and plant biotechnology have the potential to revolutionize agriculture in a sustainable manner; improve environmental quality; yield new medicines; act as biofactories for the production of pharmaceutical proteins (molecular pharming); generate biofuels; contribute to a less-polluting industry; and profoundly improve the health, quality of life, and livelihood of mankind.

The economic and environmental benefits of GM plants should, however, not be the sole privilege of the developed world. Much work has to be done to lessen and simplify the regulatory burden and to make the technology freely available for those who need it the most.

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## LITERATURE CITED

1. Barton KA, Binns AN, Matzke AJM, Chilton M-D. 1983. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. *Cell* 32:1033-43
2. Beardsley RE. 1960. Lysogenicity in *Agrobacterium tumefaciens*. *J. Bacteriol.* 80:180-87
3. Bevan M, Barnes WM, Chilton M-D. 1983. Structure and transcription of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res.* 11:369-85
4. Bevan MW, Flavell RB, Chilton M-D. 1983. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* 304:184-87
5. Braun AC. 1947. Thermal studies on the factors responsible for tumor initiation in crown gall. *Am. J. Bot.* 34:234-40

6. Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, et al. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 11:263–71
7. Chilton M-D, Saiki RK, Yadav N, Gordon MP, Quetier F. 1980. T-DNA from *Agrobacterium* Ti plasmids is in the nuclear DNA fraction of crown gall tumor cells. *Proc. Natl. Acad. Sci. USA* 77:4060–64
8. De Beuckeleer M, De Block M, De Greve H, Depicker A, De Vos G, et al. 1978. The use of the Ti-plasmid as a vector for the introduction of foreign DNA into plants. In *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*, ed. M Ridé, pp. 115–26. Angers: Institut National de la Recherche Agronomique
9. De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, et al. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J.* 6:2513–18
10. De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P. 1984. Expression of foreign genes in regenerated plants and their progeny. *EMBO J.* 3:1681–89
11. De Greef W. 2004. The Cartagena Protocol and the future of agbiotech. *Nat. Biotechnol.* 22:811–12
12. De Greve H, Decraemer H, Seurinck J, Van Montagu M, Schell J. 1981. The functional organization of the octopine *Agrobacterium tumefaciens* plasmid pTiB6S3. *Plasmid* 6:235–48
13. De Greve H, Dhaese P, Seurinck J, Lemmers M, Van Montagu M, et al. 1982. Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. *J. Mol. Appl. Genet.* 1:499–512
14. Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM. 1982. Nopaline synthase: transcript mapping and DNA sequence. *J. Mol. Appl. Genet.* 1:561–73
15. Depicker A, Van Montagu M, Schell J. 1978. Homologous DNA sequences in different Ti-plasmids are essential for oncogenicity. *Nature* 275:150–53
16. Dhaese P, De Greve H, Decraemer H, Schell J, Van Montagu M. 1979. Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of *Agrobacterium tumefaciens*. *Nucleic Acids Res.* 7:1837–49
17. Engler G, Holsters M, Van Montagu M, Schell J, Hernalsteens J-P, et al. 1975. Agrocin 84 sensitivity: a plasmid determined property in *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* 138:345–49
18. Fernandez AI, Viron N, Alhaghdow M, Karimi M, Jones M, et al. 2009. Flexible tools for gene expression and silencing in tomato. *Plant Physiol.* 151:1729–40
19. Fiers W, Contreras R, Duerinck F, Haegeman G, Iserentant D, et al. 1976. Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. *Nature* 260:500–507
20. Fiers W, Van Montagu M, De Wachter R, Haegeman G, Min Jou W, et al. 1969. Studies on the primary structure and the replication mechanism of bacteriophage RNA. *Cold Spring Harbor Symp. Quant. Biol.* 34:697–705
21. Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, et al. 1983. Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA* 80:4803–7
22. Hartley JL, Temple GF, Brasch MA. 2000. DNA cloning using in vitro site-specific recombination. *Genome Res.* 10:1788–95
23. Hernalsteens J-P, De Greve H, Van Montagu M, Schell J. 1978. Mutagenesis by insertion of the drug resistance transposon Tn7 applied to the Ti-plasmid of *Agrobacterium tumefaciens*. *Plasmid* 1:218–25
24. Herrera-Estrella A, Chen Z-M, Van Montagu M, Wang K. 1988. VirD proteins of *Agrobacterium tumefaciens* are required for the formation of a covalent DNA-protein complex at the 5' terminus of T-strand molecules. *EMBO J.* 7:4055–62
25. Herrera-Estrella L, Depicker A, Van Montagu M, Schell J. 1983. Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. *Nature* 303:209–13
26. Herrera-Estrella L, Van den Broeck G, Maenhaut R, Van Montagu M, Schell J, et al. 1984. Light-inducible and chloroplast-associated expression of a chimaeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. *Nature* 310:115–20
27. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA. 1983. A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303:179–80
28. Holsters M. 1970. *Een onderzoek over getemperde bacteriofagen bij Agrobacterium*. Undergraduate thesis. Ghent Univ., Belgium

29. Holsters M. 1975. *Lysogenic in bet genus Agrobacterium tumefaciens*. PhD thesis. Ghent Univ., Belgium
30. Holsters M, Silva B, Van Vliet F, Genetello C, De Block M, et al. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. *Plasmid* 3:212–30
31. James C. 2007. *Global status of commercialized biotech/GM crops: 2007* (ISAAA Brief No. 37). Ithaca, NY, International Service for the Acquisition of Agri-Biotech Applications (<http://www.isaaa.org/resources/publications/briefs/37/executivesummary/default.htm>)
32. Kalaitzandonakes N, Alston JM, Bradford KJ. 2007. Compliance costs for regulatory approval of new biotech crops. *Nat. Biotechnol.* 25:509–11
33. Karimi M, Bleys A, Vanderhaeghen R, Hilson P. 2007. Building blocks for plant gene assembly. *Plant Physiol.* 145:1183–91
34. Karimi M, De Meyer B, Hilson P. 2005. Modular cloning in plant cells. *Trends Plant Sci.* 10:103–105
35. Karimi M, Depicker A, Hilson P. 2007a. Recombinational cloning with plant Gateway vectors. *Plant Physiol.* 145:1144–54
36. Karimi M, Inzé D, Depicker A. 2002. GATEWAY<sup>TM</sup> vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7:193–95
37. Kerr A. 1971. Acquisition of virulence by non-pathogenic isolates of *Agrobacterium radiobacter*. *Physiol. Plant Pathol.* 1:241–46.
38. Leemans J, Shaw C, Deblaere R, De Greve H, Hernalsteens J-P, et al. 1981. Site-specific mutagenesis of *Agrobacterium* Ti plasmids and transfer of genes to plant cells. *J. Mol. Appl. Genet.* 1:149–64
39. Lemmers M, De Beuckeleer M, Holsters M, Zambryski P, Depicker A, et al. 1980. Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline crown gall tumours. *J. Mol. Biol.* 144:353–76
40. Mariani C, Gossele V, De Beuckeleer M, De Block M, Goldberg RB, et al. 1992. A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. *Nature* 357:384–87
41. Matzke AJM, Bevan MW, Yadav NS, Chilton M-D. 1981. *Agrobacterium* Ti plasmid as a vector for introducing novel DNA into higher plants. *Abstr. Pap. Am. Chem. Soc.* 181:79–AGFD
42. Petit A, Delhaye S, Tempé J, Morel G. 1970. Recherches sur les guanidines des tissus de crown gall. Mise en évidence d'une relation biochimique spécifique entre les souches d'*Agrobacterium* et les tumeurs qu'elles induisent. *Physiol. Vég.* 8:205–13
43. Stachel SE, Messens E, Van Montagu M, Zambryski P. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318:624–29
44. Stachel SE, Timmerman B, Zambryski P. 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature* 322:706–12
45. Schilperoort RA, van Sittert NJ, Schell J. 1973. The presence of both phage PS8 and *Agrobacterium tumefaciens* A<sub>6</sub> DNA base sequences in A<sub>6</sub>-induced sterile crown-gall tissue cultured *in vitro*. *Eur. J. Biochem.* 33:1–7
46. Schilperoort RA, Veldstra H, Wamaar SO, Mulder G, Cohen JA. 1967. Formation of complexes between DNA isolated from tobacco crown gall tumours and RNA complementary to *Agrobacterium tumefaciens* DNA. *Biochim. Biophys. Acta* 145:523–25
47. Smith EF, Townsend CO. 1907. A plant-tumor of bacterial origin. *Science* 25:671–73
48. Van Larebeke N, Genetello C, Schell J, Schilperoort RA, Hermans AK, et al. 1975. Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. *Nature* 255:742–43
49. Van Haute E, Joos H, Maes M, Warren G, Van Montagu M, et al. 1983. Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of Ti plasmids of *Agrobacterium tumefaciens*. *EMBO J.* 2:411–18
50. Van Montagu M, Leurs C, Brachet P, Thomas R. 1967. A set of amber mutants of bacteriophages λ and MS2 suitable for the identification of suppressors. *Mutat. Res.* 4:698–700
51. Vaeck M, Reynaerts A, Höfte H, Jansens S, De Beuckeleer M, et al. 1987. Insect resistance in transgenic plants expressing modified *Bacillus thuringiensis* toxin genes. *Nature* 328:33–37
52. Wang K, Stachel S, Timmerman B, Van Montagu M, Zambryski P. 1987. Site-specific nick in the T-DNA border sequence following *vir* gene expression in *Agrobacterium*. *Science* 235:587–91
53. Willmitzer L, De Beuckeleer M, Lemmers M, Van Montagu M, Schell J. 1980. The Ti-plasmid derived T-DNA is present in the nucleus and absent from plastids of plant crown-gall cells. *Nature* 287:359–61



54. Zaenen I, Van Larebeke N, Teuchy H, Van Montagu M, Schell J. 1974. Supercoiled circular DNA in crown gall inducing *Agrobacterium* strains. *J. Mol. Biol.* 86:109–27
55. Zambryski P, Depicker A, Kruger K, Goodman H. 1982. Tumor induction of *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *J. Mol. Appl. Genet.* 1:361–70
56. Zambryski P, Holsters M, Kruger K, Depicker A, Schell J, et al. 1980. Tumor DNA structure in plant cells transformed by *A. tumefaciens*. *Science* 209:1385–91
57. Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M, et al. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* 2:2143–50
58. Zupan J, Muth TR, Draper O, Zambryski P. 2000. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant J.* 23:11–28



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