

HISTORICAL PERSPECTIVE ESSAY

The Scientific Roots of Modern Plant Biotechnology

One of the stellar achievements of twentieth century plant biology was the genetic transformation of somatic cells enabling the regeneration of whole plants that were stably transformed and capable of transmitting the inserted genetic material to subsequent generations. This achievement grew out of three independent lines of research initiated early in the twentieth century: plant tissue culture, regeneration of plants from single somatic cells, and the study of crown gall disease. The early discoveries made in these areas represent a combination of basic scientific research and technological innovations and led to the development of genetically transformed crop species expressing traits unobtainable by conventional breeding. Each of these fields can be traced back to a single research publication (Haberlandt, 1902; Smith and Townsend, 1907; White, 1939a) that later came to be considered as the foundation of the field. It is instructive to follow how these three fields were established, progressed, converged, and finally coalesced. Early workers, of course, could not know the ultimate way in which their discoveries would be applied to modern plant biotechnology (Figure 1). Instead, they posed questions that were of interest to them and to the scientific communities of which they were a part, and some of their discoveries led in directions that would later prove to be productive in the progression to biotechnology. This essay is intended to provide a review of the crucial discoveries that ultimately led to modern plant biotechnology and show how they contributed to this progression.

PLANT TISSUE CULTURE

Philip White worked at the Rockefeller Institute for Medical Research in Princeton, New Jersey in the 1930s to develop an experimental system with which to study metabolism in a completely undifferentiated tissue where all cells are identical and hence exert similar influences on one another. White (1939a) defined a plant tissue culture as a system in which cells satisfied two main requirements of remaining “undifferentiated yet capable of unlimited growth” (White, 1939a). Earlier attempts, starting in the late nineteenth century, to grow plant parts in isolation from the organism did not satisfy these requirements and failed for a variety of reasons, including microbial contamination, inadequate nutrient media, and poor selection of tissues to culture (White, 1931; Gautheret, 1983; Höxtermann, 1997).

The most successful of the early attempts involved the culture of maize, pea, and cotton root tips (Kotte, 1922; Robbins, 1922). These could be excised from the plant with minimal trauma and grown aseptically for a few weeks in nutrient media, but ultimately growth ceased. White (1934) succeeded in growing

excised tomato root tips for potentially unlimited periods of time in a liquid medium containing inorganic salts, 2% sucrose, and 0.01% yeast extract. Tips were excised from seedling roots and subcultured at regular intervals. After 52 subcultures over >400 d, the cultured roots showed no diminution of growth rate. White calculated that by this time any nutrients and regulatory substances in the original root tip would have been diluted to $\sim 10^{-40}$, and he concluded that the nutrient medium had supported indefinite growth of the root tips. Thus, while he had demonstrated “potentially unlimited growth,” the second part of his definition of a tissue culture had not been met because the roots clearly were not undifferentiated.

White (1939a) addressed this second question using a hybrid between *Nicotiana glauca* and *N. langsdorffii*. The hybrid plants produced tumor-like calluses and galls on the stem and leaves. Tissue removed aseptically from young stems was cultured on the same nutrient medium used for tomato roots. Proliferated masses from the original explants were divided and subcultured at weekly intervals through 40 subcultures. White calculated that any material from the original explant would have been diluted to at least 10^{-17} by this time and that the cultures therefore satisfied the criterion of a capacity for unlimited growth. The question remained as to whether the cells in the culture were undifferentiated. Histological examination revealed only mature parenchyma cells, regions of dividing meristematic cells, and occasional isolated xylem cells. Despite this level of cellular heterogeneity, White concluded that these tissues approximated an undifferentiated condition and grew for potentially unlimited periods and therefore were true tissue cultures.

Scientific results are greatly strengthened when other workers with appropriate expertise replicate the original findings. Remarkably, this occurred within 6 weeks of the publication of White’s results. Two French workers, Roger Gautheret in Paris and Pierre Nobécourt in Grenoble, both of whom had been attempting to culture plant tissues for several years, reported potentially unlimited growth of cultures derived from carrot tap root tissue when the growth substance indole-3-acetic acid (IAA) was incorporated in the culture medium (Gautheret, 1939; Nobécourt, 1939). Neither cited White (1939a), and presumably they were unaware of his results. Neither study was as detailed as White’s. Gautheret cultured tissue for 13 months making bimonthly subcultures, and Nobécourt cultured tissue for 20 months making seven subcultures.

White’s objective, of studying metabolism in an undifferentiated tissue wherein all the cells are identical and presumably exert similar influences on one another, appears not to have been followed up by him or his contemporaries. It was not until many years later that other scientists began using methods developed by White to study cellular metabolism. Notably, H.E.

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1990			1 st Maize transformation (Gordon-Kamm et al., 1990)
1980			<ul style="list-style-type: none"> • Gene gun transformation (Klein et al., 1987; Sanford, 2000) • Chimeric genes (Herrera-Estrella et al., 1983b; Fraley et al., 1983; Bevan et al., 1983) • Binary vectors (Hoekema et al. 1983) • Disarmed plasmids (Willmitzer et al., 1983; Joos et al., 1983) • <i>tms, tmr, tm</i> identified (Klee et al., 1984; Barry et al., 1984; Akiyoshi et al., 1984)
1970	•Single cells to somatic embryos (Backs-Hüsemann and Reinert, 1970)		<ul style="list-style-type: none"> • T-DNA nuclear location (Chilton et al., 1980; Willmitzer et al., 1980) • T-DNA in plasmid (Chilton et al., 1977) • Plasmids in <i>Agrobacterium</i> (Zaenen et al., 1974)
1960	•Single cells to plantlets (Vasil and Hildebrandt, 1965a, b)		<ul style="list-style-type: none"> • Bacterial virulence transferred (Kerr, 1969)
	• Controlled Organogenesis (Skoog and Miller, 1957)		<ul style="list-style-type: none"> • TIP named ((Braun and Mandel, 1948) • Autonomous crown gall growth (Braun, 1943) • 1^o vs. 2^o tumors (White and Braun, 1941, 1942)
1940	• First true plant tissue culture (White, 1939a).		
1920			
1900	•Attempted single cell culture (Haberlandt, 1902)		•Bacterial cause of crown gall (Smith and Townsend, 1907)
PLANT TISSUE CULTURE	REGENERATION OF WHOLE PLANTS FROM SINGLE SOMATIC CELLS		CROWN GALL DISEASE

Figure 1. Chronology of Research Leading to Modern Plant Biotechnology.

Some of the major steps and publications are listed. Further details, definitions, and additional citations are provided in the main text.

Street at Manchester University used cultured excised tomato roots in an extensive series of studies to examine the metabolism of inorganic ions, carbohydrates, amino acids, and hormones (Street, 1957). A more direct approach to White’s original intention was followed by F.C. Steward and colleagues at Cornell University. They examined metabolic states in three contrasting carrot tap root cultures: secondary phloem cells as they existed in the intact root, excised tissue maintained on a minimal medium where cells grew predominantly by enlargement, and actively metabolizing cells stimulated by coconut milk in the medium to divide as rapidly as possible. They documented significant differences in rates of accumulation of RNA and DNA under the different culture conditions (Steward et al., 1952, 1964).

With White’s original goal for plant tissue culture having been achieved and confirmed, attention turned in other directions. Gautheret and Nobécourt both reported the occurrence of roots that developed on their carrot tissue cultures. White (1939b) found that shoots were produced from *Nicotiana* tumor tissues submerged in a liquid medium, and Michael Levine (Levine, 1947) reported the spontaneous formation of shoots and roots

on cultured carrot tissue. These observations turned attention to the question of totipotency. That is, did tissue cultured cells retain the full genetic competence of the zygote to form a complete plant?

This question was addressed by Folke Skoog and his collaborators at the University of Wisconsin. Starting from White’s discovery of shoot formation in submerged *Nicotiana* tumor cultures, they found that auxin (IAA) was a potent inhibitor of shoot formation in tobacco tissue cultures (cv Wisconsin 38), but high concentrations of auxin stimulated the formation of roots. The nucleic acid base adenine supplied with low auxin concentrations stimulated shoot formation in these cultured tissues. The amount of shoot and root formation depended on the proportions of auxin and adenine supplied in the culture medium (Skoog and Tsui, 1948). In further work, they isolated from autoclaved DNA an adenine derivative, 6-furfurylaminopurine, that they named kinetin. Using tobacco callus cultures they found that by adjusting the relative concentrations of auxin and kinetin in the culture medium it was possible to induce the formation of shoots and roots or the growth of undifferentiated callus (Skoog and Miller, 1957). Thus, they demonstrated that

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tobacco callus tissues retained the potentialities of the zygote to form both shoots and roots, but their research did not prove that a single cell had these potentialities.

Studies that approached this question were initiated in the laboratory of F.C. Steward at Cornell University. Previously this group had compared metabolism of carrot secondary phloem explants in basal medium or medium supplemented with coconut milk, a source of cell division factors. Explants in the coconut milk medium produced new cells that became detached and proliferated freely in the culture medium, resulting in large numbers of single cells and cell aggregates. Among these aggregates they noted some that formed roots and subsequently shoots to produce whole plants that could later be transferred to agar media and then to soil where they flowered and completed the life cycle (Steward, 1958; Steward et al., 1958a, 1958b).

From these experiments they concluded that “no single parenchyma cell can directly recapitulate the familiar facts of embryology, but, through the formation first of an unorganized tissue culture, which is in fact a colony of dividing cells, the necessary degree of organization is recaptured, first to form roots and then to form shoots” (Steward et al., 1958a). The implication of this statement was that the origin of the regenerated plants was from single cells. If so, cellular totipotency would have been proven for carrot cells, at least. However, other workers (cited in Sussex, 1972) pointed out that there was no incontrovertible proof that single cells rather than cell aggregates that may have contained cells with different genetic potentialities were the source of the new plants that they obtained.

These tissue culture studies that demonstrated the potentially unlimited growth of undifferentiated cells and the production from them of roots, shoots, and entire plants did not contribute further to the questions that we are examining because of their failure to assure the single cell clonal origin of regenerated plants and thus the genetic totipotency of single cells. However, the study of plant tissue and suspension cultures was continued in different directions, including the commercial production of secondary products (Ramawat and Merillon, 2007) and commercial production of trees, crops, and horticultural plants, most notably species of orchids (Arditti and Krikorian, 1996).

REGENERATION OF WHOLE PLANTS FROM SINGLE SOMATIC CELLS

Gottlieb Haberlandt, working in Graz, Austria, was the first to culture isolated somatic cells of higher plants in vitro. He began these investigations in 1898 and published the results in 1902 (Haberlandt, 1902). His intention was to study “the properties and potentialities which the cell as an elementary organism possesses” (Krikorian and Berquam, 1969; translation from Haberlandt’s text). Although Haberlandt failed to induce divisions in any of the cells that he cultured, he is recognized as the founder of plant cell culture because of the novelty of the

methods he proposed and the concluding paragraph in his article “...I believe, in conclusion, that I am not making too bold a prediction if I point to the possibility that, in this way, one could successfully cultivate artificial embryos from vegetative cells.”

Recognizing the lack of knowledge of the nutrient requirements of higher plant cells, he used as a culture medium the seven inorganic elements that had been identified by Knop (1865) as sufficient for the water culture of higher plants, with additions of sucrose, dextrose, glycerine, asparagine, and peptone in various concentrations and combinations.

Haberlandt first attempted to culture green, photosynthetic cells from leaf bract mesophyll of *Lamium purpureum*. Bracts were teased apart in liquid until microscopy examination revealed numerous isolated palisade and spongy mesophyll parenchyma cells. These were then transferred by finely drawn-out pipettes to hanging drops or dishes of culture medium. Microbial sterility was attempted by flaming instruments and glassware but usually failed to eliminate bacterial and fungal contamination completely. Cultures were maintained in lighted rooms at ambient temperature or in darkness. Some cells remained alive for a month in lighted cultures but died soon in darkness. Haberlandt noted several changes in cell structure during the culture period. Cells expanded in length and girth, and cell walls thickened. Plastids remained green in light, photosynthesized, and accumulated starch. However, no cells were observed to divide. He then attempted to culture cells from other species: photosynthetic cells from *Eichhorina crassipes*, glandular hairs of *Pulmonaria mollissima*, stinging hairs of *Urtica dioica*, staminal filament hairs of *Tradescantia virginica*, and stomatal cells of *Ornithogalum umbellatum*, *Erythronium dens-canis*, and *Fuschia magellanica* with equal lack of success (reviewed in Krikorian and Berquam, 1969).

In retrospect, Haberlandt’s failure to obtain dividing cells can be attributed to lack of microbial sterility, culture media that lacked hormones and growth factors that were unknown at that time, and his selection of highly differentiated mature cells. However, he made immense contributions to plant and animal cell culture studies by his technical innovations, including the use of hanging drop culture methods and use of micropipettes to manipulate single cells. Similarly his prediction that cocultivation of vegetative cells with pollen tubes, that were then known to produce chemical stimuli that induced growth of orchid ovules, foreshadowed nurse culture technology, and his prediction that embryo sac fluids might be used as components of the culture medium to induce divisions in isolated vegetative cells foreshadowed the use of coconut milk. Each of these predictions has led to advances in cell culture technology.

Despite continued efforts by Haberlandt’s collaborators and others, no significant progress on cultures derived from single cells was made for 56 years when W.H. Muir, Albert Hildebrandt, and Albert Riker at the University of Wisconsin investigated this question (Muir et al., 1958). They used tissue cultures of tobacco and carrot and crown gall cultures of grape, marigold, periwinkle, and sunflower. By testing the capacity for growth in liquid

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culture media, they identified several that produced large numbers of single cells. Single cells were then transferred by micropipette to filter paper placed on nurse cultures of the same or other species growing on an agar medium. Those of tobacco, marigold, sunflower, and grape divided and produced macroscopic cultures, some of which were transferred through 25 or more agar subcultures without diminution of growth rate. Marigold clones consistently produced roots, and hybrid tobacco clones produced shoots on media containing adenine and kinetin.

Subsequently, Vimla Vasil and Hildebrandt (Vasil and Hildebrandt, 1965a, 1965b) transferred single tobacco hybrid cells to a drop of culture medium on a microscope slide that could be observed and photographed repeatedly under phase contrast microscopy. Cells were observed to divide to form a filament and subsequently a microcallus mass that was transferred to an agar medium for further growth, where roots and leafy shoots were differentiated. Rooted shoots were transferred to soil where they produced buds and flowers. Thus, these studies demonstrated that plantlets derived from single cultured cells had the capacity to produce whole plants. However, they did not prove that the whole plants were the direct product of a single cell, rather than the product of a tissue mass within which somaclonal or other genetic changes might have taken place during growth to produce a chimeric tissue mass.

The final step proving Haberlandt's prediction that "one could successfully cultivate artificial embryos from vegetative cells" came from the research of Dietlinde Backs-Hüsemann and Jakob Reinert in Berlin. They cultured single carrot cells from suspension cultures on microscope slides where they could be observed and photographed repeatedly. Isolated cells divided to form a mass of embryogenic and parenchyma cells, and the embryogenic cells developed into heart-shaped and torpedo-shaped embryos with recognizable cotyledons, hypocotyls, and radicles (Backs-Hüsemann and Reinert, 1970).

This early research in plant tissue culture demonstrated that tissues isolated from plants can be grown in culture for indefinite periods of time, they can produce shoots and roots, and finally, single isolated cells in culture can produce embryos. These studies provided the platform for genetic transformation of plants, as described below.

CROWN GALL DISEASE

In 1907, Erwin Smith, working at the USDA Bureau of Plant Industry on diseases of plants, reported that the cause of crown gall disease of Paris Daisy (*Chrysanthemum frutescens*) was a bacterium that he named *Bacterium tumefaciens* (Smith and Townsend, 1907). This was subsequently reclassified as *Phytomonas tumefaciens* and then as *Agrobacterium tumefaciens* (Conn, 1942). Smith established that this bacterium was the cause of the disease by plating bacteria from galls onto an agar medium, inoculating uninfected plants with subcultures of the bacteria, reisolating bacteria from the galls produced, and

inducing galls on new plants (Koch's postulates). Tumors were also produced on stems of tobacco, tomato, and potato and on roots of sugar beet and peach trees that were inoculated with *B. tumefaciens* (Smith and Townsend, 1907). The latter galls closely resembled peach crown gall disease on which Smith had been working for several years without identifying the cause. In a subsequent publication, Smith et al. (1911) reported that the bacterium isolated from Paris Daisy caused tumors on 24 dicot species but not on nine other dicots or the single monocot (*Allium cepa*) that they tested.

Smith also observed secondary tumors that developed on stems or leaves of infected plants of some species at some distance from the primary gall. Based on histological examination, he concluded that secondary tumors developed from tumor strands that were root-like outgrowths from the primary gall (Smith et al., 1912). Smith believed that tumor strands might be comparable to certain types of metastases that occurred in malignant tumors of animals and humans (Smith, 1916).

In addition to establishing the cause of crown gall disease, Smith and Townsend (1907) suggested that their results might shed light on the origin of cancerous growths in animals. Smith frequently alluded to the similarities between plant and animal tumors (Smith, 1916) for which he received a Certificate of Honor from the American Medical Association in 1913, and in 1925, he was elected to the presidency of the American Association for Cancer Research.

However, little progress was made on the nature of crown gall disease until Armin Braun at the Rockefeller Institute for Medical Research, began an investigation of crown gall disease in the 1940s that lasted for 40 years and that laid the foundation for the molecular studies that were to come. He began this investigation by examining the question of tumor strand connections between primary and secondary tumors in sunflower. By examining tissue sections cut between the primary and secondary tumors, he found no histological support for a tumor strand connection and concluded that the mechanism of formation of secondary tumors may not be identical to that concerned in the formation of the primary tumor (Braun, 1941). Although crown gall tissues did not always yield cultures of the inducing bacterium, it had been assumed that the bacteria had been present at some stage in the development of the tumor. Thus, the discovery that secondary tumors lacked direct cellular continuity with the primary tumor raised questions as to their origin and development, some of which are still unresolved.

Braun collaborated with Philip White of the same institution to investigate these questions. Specifically, they set out to investigate whether host cells under the influence of the bacteria acquire the capacity for autonomous growth. First, they induced secondary tumors in sunflower and showed by culturing tissue from these tumors that those separated by more than one internode from the primary gall were free of crown gall bacteria. Next, they cultured tissue from secondary tumors for 30 subcultures on a basal medium known to support growth of *Phytomonas* (*Agrobacterium*). Secondary tumor-derived tissue

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grew rapidly, whereas tissue from noninfected plants grew very slowly. They did not observe growth of *Phytomonas* in any of these subcultures. To test for the presence of *Phytomonas* contained within the cultured secondary tumor tissues, they tested these serologically with negative results. Secondary tumor tissue when grafted onto healthy sunflower or artichoke (*Helianthus tuberosus*) plants produced typical tumors. From these experiments they concluded that the secondary tumor tissue had acquired the capacity for autonomous growth both in vivo and in vitro and that this permanent change had been induced by the bacteria (White and Braun, 1941, 1942).

The finding that the plant cells had been permanently altered after interaction with the bacteria led Braun to begin experiments to determine the time when this interaction took place. These experiments depended on observations that *Phytomonas* was killed at a temperature of 46°C, whereas periwinkle plants (*Vinca rosea*) tolerated this temperature for several days. Plants were inoculated with bacteria via needle punctures to the stem, placed at 25°C for varying periods, then transferred to 46 to 47°C for 5 d and returned to 25°C for continued growth. Plants transferred to the heat treatment 1 d after inoculation failed to form tumors when returned to 25°C. Those transferred after 1.5 to 3 d produced small tumors, and those transferred after 4 to 5 d produced tumors comparable to those on control plants that had not been subjected to the heat treatment. These large tumors were bacteria free. Thus, the plant–bacteria interaction must have occurred between 1 and 4 d, after which time tumor growth became autonomous and independent of the continued presence of bacteria (Braun, 1943). Additional temperature shift experiments narrowed the time of maximal tumor formation to 24 to 48 h after wounding in plants maintained at 25°C. These experiments suggested that an active principle resulting from the plant–bacterium interaction was responsible for the transformation, and Braun suggested four possible categories for it: (1) a metabolic product of the crown-gall bacterium; (2) a host constituent converted by the bacterium to a tumor-inducing substance; (3) a chemical fraction of the bacterial cell that is capable of initiating in the host cell a permanent developmental alteration; and (4) a viral or other agent present in the crown-gall organism (Braun, 1947). This active principle whose chemical nature was still unknown was named the tumor inducing principle (TIP) (Braun and Mandle, 1948).

Initial understanding of the biochemical nature of the TIP came from research conducted by Georges Morel at the Centre National de Recherches Agronomiques, Versailles, France, who was studying amino acid changes during tuber development in Jerusalem artichoke (*Solanum tuberosum*), paying particular attention to arginine metabolism (Duranton and Morel, 1958). When they compared tuber tissue cultures with crown gall cultures of this species, they found that the latter contained a conjugate of arginine with pyruvate that had previously been identified from octopus muscle and named octopine (Ménagé and Morel, 1964). Subsequently, when investigating crown gall cultures of the cactus *Opuntia vulgaris*, they discovered another

arginine derivative, a conjugate with α -ketoglutarate, which they named nopaline, from Nopal, the French common name for *Opuntia* (Goldmann et al., 1969). They performed a systematic study of these arginine derivatives, named guanidines or opines, in crown gall cultures induced by 43 different strains of *A. tumefaciens* and showed that with few exceptions they contained either nopaline or octopine and that the opine produced was specific to the inducing bacterial strain (Petit et al., 1970). Opines could not be isolated from normal, habituated, or genetic tumor tissue cultures of the same species. Since the opine-specifying information was proposed to move with the TIP from the bacterial cell to the plant cell where it was maintained in a functional state in bacteria-free crown gall tissue cultures, it represented a useful marker for the transformed state.

Further information on the nature of the transforming agent TIP came from two studies. First, Alan Kerr working at the Waite Agricultural Research Institute in South Australia found that tomato plants inoculated with *Agrobacterium* sp, a virulent strain, became contaminated with water-splashed soil containing *A. radiobacter*, an avirulent strain. Approximately 50% of the *A. radiobacter* contaminants were then found to be virulent and indistinguishable from *A. tumefaciens*. Kerr (1969) concluded that this was “the first unequivocal evidence for transfer of virulence from a plant pathogenic to a saprophytic species of bacterium” and suggested that this may have resulted from DNA transfer. Second, Hamilton and Fall (1971) working at Pennsylvania State University found that the tumor-initiating ability of *A. tumefaciens* was lost after incubation at high temperatures. They incubated cultures of *A. tumefaciens* C-58, an extremely virulent strain, at 36°C for different lengths of time and found a progressive decline in the number of virulent colonies, so that after 120 h at 36°C no virulent colonies could be recovered. They concluded that this result could be explained by a loss of the virulence factor (Hamilton and Fall, 1971).

Suggestions that crown gall transformation resulted from transfer of genetic material from the bacterium to plant cells generated several studies to investigate this possibility. However, these studies, based on filter or solution hybridization using the whole *Agrobacterium* genome, yielded either negative results or results that could not be substantiated (Chilton, 2001).

The resolution of these diverse conflicting results came from a study by Ivo Zaenen working with Marc Van Montagu and Jeff Schell at the University of Ghent, Belgium. Alkaline or neutral lysis of *Agrobacterium* B6-S3 cells followed by sucrose density gradient centrifugation or dye-buoyant density centrifugation and electron microscopy examination revealed the presence of a large supercoiled circular plasmid in this crown gall–inducing bacterial strain. The plasmid was present as one or a few copies per cell. Examination of other crown gall–inducing *Agrobacterium* strains belonging to seven different groups revealed the presence of plasmids of lengths comparable to those in B6-S3 in all of them, whereas plasmids were not detected in eight different nonpathogenic strains. They proposed the hypothesis that “the tumor-inducing principle (Braun, 1947) in crown-gall

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inducing *Agrobacterium* strains is carried by one or several large plasmids of various lengths” (Zaenen et al., 1974). Confirmation of this hypothesis came within a year when three reports of the essentiality of the *A. tumefaciens* plasmid for crown gall induction were published (Van Larebeke et al., 1974, 1975; Watson et al., 1975).

These results stimulated further attempts to demonstrate the presence of whole plasmid genomes in plant tumor cells, but these were not successful and raised the possibility that only part of the plasmid genome was transferred to the plant. This was demonstrated soon after by Mary-Dell Chilton and her colleagues Milton Gordon and Eugene Nester at the University of Washington, Seattle. They digested radioactively labeled plasmid DNA from *A. tumefaciens* strain A277 with the restriction endonuclease *Sma*I and electrophoresed the digest. Nineteen bands were resolved, the 17 largest of which were then hybridized to tobacco tumor DNA or to control DNAs. Renaturation kinetics indicated that bands 3B and 10 showed homology to tumor DNA (Chilton et al., 1977), indicating that only part of the plasmid DNA was present in the tumor DNA. By 1978, terminology for the plasmid, called Ti plasmid, and the transferred DNA, called T-DNA, had become established (Chilton et al., 1978; Depicker et al., 1978). The experiments of Chilton et al. (1977) did not address questions of the location of the plasmid DNA in the plant cells, whether covalently integrated, or located in the nuclear or plastid genomes.

Three years later, research groups in Washington and Europe (Belgium and Germany) reported that Ti plasmid DNA was present in the crown gall cell nucleus and not in plastids or mitochondria (Chilton et al., 1980; Willmitzer et al., 1980). Whether the DNA was integrated into plant chromosomes or functioned as an independent replicon was not yet established. However, almost simultaneously with these reports two studies showed by molecular cloning and sequencing of a border fragment of T-DNA and flanking plant DNA that the T-DNA was covalently integrated into the plant nuclear genome in tobacco teratoma cell lines (Yadav et al., 1980; Zambryski et al., 1980).

The next step in elucidating the role of T-DNA in plant tumorigenesis was to determine the function of the genes that were presumed to be located in it. This was done by disrupting T-DNA genes by insertion of foreign DNA or by deletion of T-DNA sequences. The first studies using this approach were those of Peter Klapwijk and Gert Ooms in the lab of Robbert Schilperoort in Leiden (Klapwijk et al., 1980; Ooms et al., 1981). A Ti plasmid insertion mutant containing a Tn904 transposon in the center of the T-DNA region induced tumors on tobacco plants that gave rise to roots. Insertion of an IS element, IS60, encoding streptomycin resistance into the left arm of the T region of an octopine Ti plasmid gave rise to tumors that produced shoots. Shoots were also produced on in vitro-cultured tissue of the IS60 mutant tumors, and Ooms concluded that “the tumor phenotype should depend on relative concentrations of various phytohormones present in a tumor” (Ooms et al., 1981).

At about the same time, David Garfinkel and collaborators working with Milt Gordon and Gene Nester made a detailed genetic analysis of the T-DNA of the octopine plasmid pTiA6NC using Tn3 and Tn5 transposon inserts. Twenty-five insertions defined three distinct loci affecting tumor morphology: *tms*, *tmr*, and *tml*, mutations in which caused shooty tumors, rooty tumors, and abnormally large tumors, respectively. These three loci were all located in the core T-DNA segment present in all octopine-type tumors and were found to encode enzymes involved in auxin or cytokinin biosynthesis. *tms1* codes for tryptophan monooxygenase, and *tms2* codes for indoleacetamide hydrolase, both of which specify steps in auxin (IAA) biosynthesis (Klee et al., 1984), and *tmr* codes for isopentenyl transferase, which catalyzes the first step in cytokinin biosynthesis (Akiyoshi et al., 1984; Barry et al., 1984). A fourth locus, *ocs*, was located outside this core region and codes for octopine synthase (Garfinkel et al., 1981).

Using a similar approach of DNA deletions or transposon insertions, genes that were present in a common core of both octopine and nopaline tumor T-DNA were identified by Willmitzer et al. (1983) and Joos et al. (1983), working with Marc Van Montagu and Jeff Schell. Two of these genes inhibited shoot formation and ensured tumorous growth. The third gene inhibited root formation. Mutants missing all three genes did not induce tumors, nor shoot or root formation, although the mutant T-DNA was transferred to plant cells. This last fact supported the earlier conjecture of Garfinkel et al. (1981) for the “eventual use of the Ti plasmid as a vehicle for introducing genes of choice into the genomes of higher plants.” Plasmids lacking all oncogenic genes would be of use for such introductions and were said to be “disarmed” (Binns, 2002).

If the Ti plasmid was to be the vehicle for introduction of genes of choice into plant genomes, three molecular requirements had to be satisfied. First, a promoter that functioned in plant cells had to be identified; second, a dominant selectable marker that indicated the functioning presence of the introduced DNA and that would replace the opine synthesis locus (which was a screenable, but not selectable, marker); and finally, polyadenylation termination signals that would function in plant cells. Surprisingly, at the Miami Winter Symposium, January 1983, three research groups (Jeff Schell, Rob Horsch, and Mary-Dell Chilton) all reported success in producing chimeric genes that satisfied these criteria and that functioned in transformed plant cells (Downey et al., 1983). All three groups used the NOS (nopaline synthetase) promoter spliced to the bacterial NPT II (neomycin phosphotransferase) coding sequence as a dominant selectable marker and NOS polyadenylation signals or a variation of this strategy. The first peer-reviewed publication reporting similar results was that of Herrera-Estrella et al. (1983a). The technological innovation that underlay these reports was the development of binary plant vector systems in which the *vir* region (virulence region) and the T-region of the *A. tumefaciens* Ti plasmid were located on different plasmids. With this approach, the T-DNA, located on a wide host-range replicon,

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could be easily genetically manipulated and modified in *Escherichia coli* then reintroduced into *A. tumefaciens* cells that harbored the *vir*-containing plasmid (Hoekema et al., 1983).

Using this binary vector approach, Herrera-Estrella et al. (1983b), working with Marc Van Montagu and Jeff Schell, used the NOS promoter sequence and the dominant selectable markers APH(3')II of Tn5 or DHFR Mtx of the R67 plasmid. APH(3')II inactivates a number of aminoglycoside antibiotics, such as kanamycin, neomycin, and G418. Kanamycin, G418, and methotrexate are very toxic to plant cells and thus function as selectable markers for transformed cells. The chimeric genes transferred to tobacco cells via the Ti plasmid as a vector were expressed and conferred resistance to the antibiotics. Fraley et al. (1983) at Monsanto produced chimeric genes containing the NOS promoter and regulatory regions linked to NPT type I or II from bacterial transposons Tn5 or Tn601. Cocultivation of *A. tumefaciens* containing these chimeric genes with cells derived from protoplasts of petunia, tobacco, sunflower, and carrot resulted in transformed cells that expressed the chimeric genes and were resistant to inhibitory levels of the antibiotic. Mike Bevan, Richard Flavell, and Mary-Dell Chilton (Bevan et al., 1983) combined the NOS promoter and regulatory sequences with NPTII from transposon Tn5 to transform sterile stem explants of tobacco. The successes of these three research groups heralded the way for the genetic transformation of crop plants.

However, it remained to be shown that such transformed cells could be regenerated into intact normal plants and that the inserted DNA would be inherited in subsequent generations in a stable manner. Proof of these requirements came quickly in several reports. Ken Barton and colleagues, working with Mary-Dell Chilton, regenerated tobacco plants that contained full-length copies of genetically engineered T-DNA that were transmitted to the R1 progeny (Barton et al., 1983). Patty Zambryski, working with Marc Van Montagu and Jeff Schell, inoculated decapitated tobacco plants and regenerated transformed plants from callus that developed at the inoculation sites (Zambryski et al., 1983). Finally, Marc De Block, working with Luis Herrera-Estrella, Marc Van Montagu, Jeff Schell, and Patty Zambryski, infected single protoplasts of tobacco with *Agrobacterium*-containing chimeric genes and regenerated plants from the resulting calli (De Block et al., 1984). These plants developed normally, flowered, and set seed. F1 seedlings when grown on an antibiotic-containing medium segregated in a Mendelian manner. These three studies all reported successful production of transformed tobacco plants from transformed cells.

The broader application of gene transfer into plants was reported by Rob Horsch and colleagues of Monsanto, who transformed tobacco, petunia, and tomato (several cultivars) with genetically engineered T-DNA (Horsch et al., 1985). They cocultivated *A. tumefaciens* containing a plasmid-encoded NOS/NPTII/NOS chimeric gene with leaf discs of these species. The leaf discs were subsequently transferred to callus-inducing medium containing carbenicillin and kanamycin. Shoots that developed from the callus were rooted and transferred to soil for

further growth. F1 generation plants of all species expressed kanamycin resistance in a simple Mendelian fashion. This fact suggests that the regenerated shoots had originated from single transformed cells and were not chimeric in origin.

The recalcitrance of some species, especially monocotyledons, to transformation by *Agrobacterium* led to searches for other methods for introducing DNA into plant cells. These included electroporation, microinjection, floral dipping, and particle bombardment/biolistics (Peña, 2005). The most successful and widely used of such methods is particle bombardment by the gene gun, developed by John Sanford and colleagues at Cornell University in 1984 (Klein et al., 1987; Sanford, 2000). This was the method used in the first successful transformation of maize cells and the regeneration of fertile transgenic plants from them that transmitted the introduced genes to the R1 generation (Gordon-Kamm et al., 1990).

These workers at DEKALB Plant Genetics (now Monsanto) bombarded cells from maize embryogenic suspension cultures with tungsten particles coated with plasmids containing the selectable marker gene *bar*. This gene confers resistance to the herbicide bialaphos, which was used to select transformed callus cells. Transformed calli were shown to contain the integrated *bar* gene and to express the enzyme phosphinothricin acetyltransferase encoded by *bar*. Fertile transformed plants were produced from the calli, and of 53 progeny tested, 29 had phosphinothricin acetyltransferase activity. In other experiments, they cotransformed embryogenic suspension culture cells with a mixture of two plasmids, one containing the *bar* gene and the other containing the gene encoding β -glucuronidase. Regenerated plants expressed both genes. The authors concluded that "this system provides a new, powerful tool for both the study of basic plant biology and the introduction of important agronomic traits into one of the world's major crops" (Gordon-Kamm et al., 1990).

CONCLUDING REMARKS

Modern plant biotechnology, defined as the genetic modification of plants, resulted from a century-long combination of basic research findings and technological innovations. The basic scientific findings that underlay this include *in vitro* tissue culture, auxin/cytokinin regulation of organogenesis, single cell culture, discovery of cellular totipotency, the bacterial cause of crown gall disease, the TIP, opines as markers of transformed cells, transfer of virulence between *Agrobacterium* strains, T-DNA, the genes that determine tumor morphology (*tms1*, *tms2*, and *tmr*), disarmed plasmids, and regeneration of transformed cells. The technological innovations include aseptic tissue/cell culture, hanging drop culture, micropipettes, nurse cultures, binary plant vectors, and gene gun transformation. Of course, some basic discoveries and technological innovations were adopted from other disciplines, such as plant culture medium requirements and plasmid genetic manipulation in *E. coli*, but many originated as the research that led to the genetic transformation of plants.

HISTORICAL PERSPECTIVE ESSAY

A remarkable feature of this research is the changing aspect of publication frequency and author contribution. From 1900 to 1949, 20 articles that are here regarded as crucial were published by 29 authors. This is a frequency of 0.4 articles per year and 1.5 authors per article. From 1950 to 1969, 13 articles were published by 30 authors with a frequency of 0.7 articles per year and 2.3 authors per article. From 1970 to 1990, 34 articles were published by 185 authors with a frequency of 1.7 articles per year and 5.4 authors per article. These numbers reflect the changing pace of plant biology and the increasing attractiveness of it as a scientific career where large laboratory groups have come to characterize the field.

This story emphasizes the relationship between basic scientific research and technological developments and the necessity for both. In retrospect, we can trace the sequence of research that ultimately led to the genetic modification of plants, but could we have predicted it? Obviously not in 1902, 1907, or 1939, when the three founding articles in crown gall disease causation, single plant cell culture, and plant tissue culture, respectively, were published. It was not until the early 1980s that research articles were predicting the use of the Ti plasmid as a vehicle for transfer of genes of choice into plants.

POSTSCRIPT

The first transgenic food crop to be commercialized was Flavr Savr, a delayed ripening tomato, in 1994 (Martineau, 2001). In 2006, transgenic crops were planted on 102 million hectares (252 million acres) in 22 countries (11 industrial countries and 11 developing countries) by 10.3 million farmers: 9.3 million of these farmers were resource-poor with small farms in developing countries. Soybean was the principal transgenic crop in 2006, occupying 58.6 million hectares, followed by maize (25.2 million hectares), cotton (13.4 million hectares), and canola (4.8 million hectares).

The first field trials of transgenic crops were conducted in 1986 to test herbicide tolerance in tobacco. By 2005, 3647 field trials had been conducted at >15,000 sites in 34 countries on 56 crop species. The eight most frequently tested species were maize, canola, potato, tomato, tobacco, soybean, cotton, and melon (James, 2006). In 2007, it was estimated that ~140 species of angiosperms had been genetically transformed (James, 2007).

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