A Really Useful Pathogen, *Agrobacterium tumefaciens*

Bacteria of the genus *Agrobacterium* are very useful and unusual plant pathogens. Through a rare interkingdom DNA transfer, the bacteria move some of their genes into their host's genome, thereby inducing the host cells to proliferate. The result is uncontrolled cell growth leading to a tumor (as induced by *Agrobacterium tumefaciens* and *Agrobacterium vitis*) or excessive production of roots (as induced by *Agrobacterium rhizogenes*). The proliferating plant tissues produce opines, which are compounds that few organisms other than *Agrobacterium* can metabolize. *Agrobacterium*'s ability to transfer DNA makes it extraordinarily useful because it can be modified to introduce other genes not involved in pathogenesis, such as those encoding useful traits, into plant genomes. Furthermore, it can be used as a model with which to study plant responses to pathogenic bacteria. The development of *Agrobacterium* as a tool to transform plants is a landmark event in the development of modern plant biology, and, as with most great advances, involved the cooperation and competition of many labs and individuals. This article provides an introduction to *A. tumefaciens* and its related species, focusing on their modes of pathogenicity and their usefulness as tools for plant transformation, as well as their use as a model for the study of plant–pathogen interactions.

A. TUMEFACIENS, CROWN GALL DISEASE, AND THE TUMOR-INDUCING PRINCIPLE

Initial interest in the genus *Agrobacterium* followed from the plant diseases this genus causes; these include crown gall, cane gall, and hairy root. The economic impact of the genus arises from its effects on long-lived woody plants, including grapevines, fruit, and nut trees, and the uncontrolled root growth of cultivated plants, such as tomato (*Solanum lycopersicum*), although the potential host range is much broader.

The first written record of crown gall disease is a description of the disease on grapevine (*Vitis vinifera*) that dates from 1853. Bacteria were isolated from crown galls in the late 19th century, and in 1907, *A. tumefaciens* was unambiguously shown to be the causal agent of crown gall through the work of Smith and Townsend, two scientists at the USDA. Following Koch's postulates, they reported that they had isolated and purified the bacterium from galls, induced the same disease symptoms in plants inoculated with their purified bacteria, and reisolated the bacteria from the inoculated plants.

Between 1907 and 1977, several generations of scientists contributed toward an understanding of how *A. tumefaciens* induces crown galls in plants. An examination of some of the key experiments from this period reveals the rapid advances in understanding of the principles of genetics and the importance of the tools developed through the nascent field of molecular biology.

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AGROBACTERIUM-INDUCED TUMORS ARE METABOLICALLY UNUSUAL

It quickly became clear that the neoplastic (derived from new growth) tumor-like growths induced by *A. tumefaciens* were unlike other wound- or pathogen-induced neoplastic growths. The proliferation of neoplastic growths induced by some other types of pathogenic bacteria requires continued bacterial presence, whereas tumors induced by *A. tumefaciens* can persist even when the bacteria are eliminated. Furthermore, *A. tumefaciens*–induced tumors are capable of persistent growth in tissue culture, independent of the presence of exogenous growth hormones, whereas most uninfected tissues require externally supplied auxin and cytokinin to proliferate in culture. Exposure to *Agrobacterium* fundamentally changed the nature of the infected plant cells.

In 1958, Armin Braun, working at the Rockefeller Institute for Medical Research, proposed that the autonomous growth of a plant tumor resulted from the permanent activation of growth substance–synthesizing systems. This suggestion was consistent with elevated levels of the plant hormones auxin and cytokinin found in crown gall tissues but the uncertainty remained of how the bacteria induced elevated hormone production. Another clue as to how *Agrobacterium* alters plant cells came from later studies that showed that tumors produce opines and that the type of opine produced is determined by the genotype of the bacterium, not that of the plant.

The Tumor-Inducing Principle

The unusual phenotypes of gall tissues generated significant interest in this disease, including the unsubstantiated hypothesis that it might provide a system with which to study the origins of human cancers. In pursuit of this idea, Braun set out to identify the tumor-inducing principle. He found that "The active principle that is responsible for the conversion of normal cells to neoplastic cells…has the capacity of bringing about the cellular alteration as early as 36 to 48 h after the bacteria are brought in contact with susceptible tissues." On the nature of this principle, he proposed that it might be a metabolic product of the bacterium, a host constituent that is converted by the bacterium into a tumor-inducing substance, a virus or other agent, or a chemical agent, such as DNA, capable of initiating an alteration in the host cells. The suggestion that DNA could be the transforming agent was informed by concomitant studies of Avery, McCarty, and MacLeod, also at the Rockefeller Institute, who demonstrated that DNA was

the transforming principle responsible for transforming *Streptococcus pneumonia*.

Although the idea that DNA was involved in the transformation of plant tissues such that they could be cultured in vitro without hormones was widespread, the molecular methods of the era made acquiring evidence to support this hypothesis very difficult. Piece by piece, the weight of supporting evidence accumulated, largely through the efforts of the Eugene Nester group in Seattle (United States), the Allen Kerr group in Adelaide (Australia), the Jeff Schell group in Cologne (Germany), the Robbert Schilperoort lab in Leiden (The Netherlands), and the Marc van Montagu lab in Ghent (Belgium). In the 1970s, efforts of these groups revealed that (1) virulent *Agrobacterium* harbored a large plasmid, (2) *Agrobacterium* lost their virulence when they lost the plasmid, and (3) transfer of this plasmid into another bacterial cell carried with it the tumor-inducing principle. The definitive study, published in 1977 by Mary-Dell Chilton et al. from the Nester group in Seattle, was titled "Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis." This article showed conclusively that DNA from the bacterial plasmid was incorporated into the plant cell and demonstrated that the tumor-inducing principle was in fact DNA. The plasmid from which the transferred DNA was derived was named the Tumor-inducing (Ti) plasmid. The transferred DNA (T-DNA) was subsequently shown to be transferred to the plant nucleus, integrated into the plant genome, and stably inherited and expressed.

Even before this 1977 publication in *Cell*, many scientists had realized that *Agrobacterium* might offer a unique opportunity to transform plant cells. Recombinant DNA technology of the 1970s led to the development of methods to introduce foreign DNA into bacterial cells, but no method existed by which DNA could be introduced into higher organisms. The possibility of harnessing the Ti plasmid of *Agrobacterium* for plant transformation motivated an intensive burst of activity that very quickly led to success; in 1983, three groups reported introducing a new gene into plant cells through *Agrobacterium*-mediated transformation.

THE DEVELOPMENT OF *AGROBACTERIUM* AS A VECTOR FOR PLANT TRANSFORMATION

The Ti Plasmid T-DNA Region Is Transferred into the Host Cell

The Ti plasmid of commonly used laboratory strains is very large, more than 200 kb, and encodes more than 200 genes. Some Ti plasmids are even larger, up to 800 kb. In addition to the T-DNA region of the Ti plasmid, a second region, termed the virulence (*vir*) region is important for T-DNA transfer. The organization of Ti plasmids varies between isolates, but all carry one or more T-DNA region and one *vir* region. Much of our understanding comes from studies of pTiC58 and pTiT37 (from the nopaline-utilizing strains C58 and T37, respectively) and pTiA6 (from the octopine-utilizing strain A6).

The T-DNA region is flanked by conserved 25-bp imperfect direct repeats called the right border and left border. These borders define the region transferred into the host cell. Two classes of genes are present on T-DNA, those responsible for phytohormone production, sometimes referred to as oncogenes, and those responsible for the biosynthesis of opines. Notably, the genes within the T-DNA region are not required for DNA transfer and integration and are only highly expressed in plant cells. The genes encoded by the T-DNA have regulatory elements (promoters and terminators) that are recognized and processed by the plant cell's machinery, so that in spite of their bacterial origins they are not normally expressed in *Agrobacterium* but efficiently expressed in the eukaryotic host.

The oncogenes (tumorigenic genes) contribute to the formation of the tumor. In the early 1980s, the characterization of these oncogenes by several groups revealed that the unrestricted proliferative growth described by Braun and his contemporaries arose as a consequence of the transfer of phytohormone biosynthetic genes from pathogen to host (see Morris [1986] and Nester et al. [1984] for reviews of these important experiments). When plant cells are subject to elevated levels of both exogenous growth regulators, they can proliferate in an uncontrolled and undifferentiated way. Gene knockout studies contributed to our understanding of oncogene functions. Plants infected with *A. tumefaciens* carrying the *tumor-morphology-rooty* (*tmr*) mutation produce rooty tumors and overaccumulate only auxin. *TMR* encodes ISOPENTENYLTRANSFERASE (IPT), a cytokinin biosynthetic enzyme, and the elevated level of auxin in the infected cells causes them to initiate root production. Conversely, the *tumor-morphology-shooty1* (*tms1*) and *tms2* mutations cause shooty tumors to form in infected tissues, as a consequence of knocking out auxin biosynthetic genes and the corresponding overaccumulation of cytokinin.

T-DNA also encodes enzymes needed for opine synthesis that, when expressed in the infected plant cells, lead to the production of opines. Genes required for opine catabolism are present on a different, nontransferred region of the Ti plasmid. The production of opines by the infected tissues activates expression of opine catabolic genes as well as genes that regulate the replication and conjugal transfer of the Ti plasmid.

In summary, when *A. tumefaciens* infects plant tissues, it transfers a few genes located in the T-DNA region of the Ti plasmid into the genome of the host plant, causing the recipient cells to proliferate and produce an *Agrobacterium*-specific nutrient and energy source, opines. Thus, Braun's suggestion that the tumor-inducing principle might be DNA was correct.

Developing *Agrobacterium* as a Plant Transformation Vector

Four major challenges had to be resolved to harness *Agrobacterium* for plant transformation: (1) the bacteria had to be made nonpathogenic (disarmed), (2) the desired genes and selectable markers had to be introduced into the T-DNA, (3) tools had to be developed with which to manipulate the very large Ti plasmid in vitro, and (4) methods had to be developed to regenerate whole plants from transformed plants cells.

The first challenge was simple because elimination of the oncogenes from T-DNA is sufficient to eliminate the pathogenicity of the bacterium and has no effect on the ability of the T-DNA to be transferred.

The second challenge, the incorporation of a foreign gene (gene of interest) into T-DNA for expression in plants, requires

optimization of gene expression in plant cells. One solution has been to affix regulatory sequences from the *nopaline synthase* gene to the target gene of interest, although a very wide range of promoters have been used to give high levels of expression or inducible or cell- or developmental-specific expression patterns. Because gene transfer is a relatively rare event, T-DNA must include one or more selectable markers to allow successful transformants to be identified; antibiotic-resistance genes flanked by plant-recognized gene regulatory sequences are commonly used.

One of the biggest challenges in working with *Agrobacterium* is the huge size of the Ti plasmid, which makes manipulating it in vitro quite challenging. Large plasmids are often low-copy number, meaning that only small amounts of plasmid DNA can be isolated. Furthermore, large plasmids have a greater tendency to break or become entangled with chromosomal DNA during in vitro manipulation; most importantly, the large size of the plasmid means that restriction endonuclease recognition sequences are usually found multiple times within the plasmid, making cloning by restriction enzymes extremely difficult. (Keep in mind that the wonderful tools derived from the PCR were not introduced into the lab until the late 1980s.) These problems were ultimately overcome by the development of a binary vector system, in which T-DNA and *vir* genes are located on separate replicons. Often this means that the T-DNA region is moved to a smaller plasmid (called binary vector) that can be manipulated easily in *Escherichia coli* but can also replicate in *Agrobacterium*. After cloning of the gene of interest, this smaller plasmid is introduced into an *Agrobacterium* strain that carries a truncated form of the Ti plasmid that contains the *vir* genes necessary for T-DNA mobilization but lacks T-DNA. This truncated *Agrobacterium* Ti plasmid is called a helper plasmid because it allows the transfer of the T-DNA region from the binary plasmid into the plant. Alternatively, T-DNA can be artificially inserted into the bacterial chromosome and launched from the *Agrobacterium* chromosome by the helper plasmid.

Tissue Culture Regeneration of Transformed Plants

The introduction of foreign DNA into plant cells was reported in 1980, but producing true transgenic plants required methods to regenerate whole plants from single transformed cells. Many plant cells are developmentally totipotent, meaning that they can redifferentiate into another cell type. Thus, once transformed cells are selected, they can be induced to differentiate and organize shoot and root apical meristems, usually by placing them on growth media with defined nutrients and hormones. Fertile, phenotypically normal-looking transgenic plants were first reported in 1983, and in 1985, the widely used leaf disc method was introduced, in which a hole punch is used to make leaf discs that are incubated with *Agrobacterium* and cultured to induce plantlet formation.

Early studies used tobacco (*Nicotiana tabacum*) and petunia (*Petunia hybrida*), two related species that are readily regenerated in tissue culture. Other species are less amenable to regeneration, so a regeneration method had to be developed for every plant species that is transformed. For the model species *Arabidopsis thaliana*, this task became significantly simpler in the 1990s by the development of the floral dip method of *Arabidopsis* transformation. By dipping the developing flowers into a solution of *Agrobacterium*, T-DNA is transferred to and integrated into the genome of the developing embryo. The resulting seeds can be germinated and those seedlings that have been transformed can be selected (e.g., by antibiotic resistance or other markers) and then grown into a transgenic plant; this method bypasses the need for tissue culture, but only works with a very few species and is not generally available for most plants, especially crop species.

The Development of Genetically Modified Crops

Once the technology for plant transformation had been developed, potential applications were quickly realized. Among the first transgenic plants produced were those that were resistant to herbicides (e.g., by introduction of modified plant genes encoding proteins that were no longer susceptible to the herbicide). This was followed by production of plants that were resistant to insects due to the introduction of bacterial genes encoding proteins that are specifically toxic to some insect larvae. Subsequently, genes contributing to resistance against pathogens and viruses were introduced in the mid to late 1980s, and genes conferring enhanced nutritional qualities were introduced beginning in 2000. By 2005, 67 traits had been engineered into 23 crops. Furthermore, transgenic plants represent an alternative for the production of recombinant proteins and vaccines (molecular pharming) as well as the remediation of metal-contaminated soils. Although many scientists and plant breeders see the enormous potential of *Agrobacterium*-mediated gene transfer, many consumers have expressed concerns about potential health and environmental consequences of GM crops. Although widely grown, GM crops continue to be a divisive issue. (See Teaching Tools in Plant Biology 16, *Genetic Improvements in Agriculture*, for more on GM crops).

Other Uses of *Agrobacterium*-Mediated Transformation

Agrobacterium-mediated transformation of plants has been indispensable to plant biology research. Thousands of articles have been published using this method for basic research purposes, including studies of gene expression, protein localization, and gene function. Gene functions can be assayed in stably transformed plants or using a transient assay system. T-DNA has also been useful to generate mutants. This is useful because T-DNA integrates into the genome randomly, and its insertion marks the interrupted gene with a known DNA sequence that can be used for gene identification (mapping). In the late 1990s, large-scale T-DNA insertion libraries were developed, first in *Arabidopsis* and later in rice (*Oryza sativa*). These libraries have enabled the identification of insertion mutations in most plant genes, thereby greatly facilitating reverse genetics approaches. The ability of *A. tumefaciens* strains to transfer T-DNA to yeast and other fungi has extended this technique to generate insertion mutant libraries in yeast and other fungi. For many medicinally important fungal species, *Agrobacterium*-mediated transformation remains the only genetic tool available.

What Is the Host Range of *A. tumefaciens*?

Traditionally, *A. tumefaciens* was described as having a broad host range of mostly dicotyledonous plants. Under natural conditions, crown gall tumors are not observed on monocots, which include many of the most important crop plants worldwide, the cereals. Similarly, *Agrobacterium*-mediated plant transformation is efficient for many dicotyledonous plants, but is less so for monocots.

More recently, using diverse *Agrobacterium* strains and isolates, recombinant Ti plasmids, and different protocols, efficient *Agrobacterium*-mediated transformation has been achieved for many plant species previously thought to be recalcitrant, and transgenic plants are routinely produced even for certain cereal crops. The reasons that *Agrobacterium* is less efficient in transferring DNA to monocots are still not fully understood, although the production of antimicrobial metabolites, a lack of *vir* gene inducers, inefficient T-DNA integration into the monocot genome, and *Agrobacterium*-induced programmed cell death have been proposed as potential mechanisms. Nevertheless, T-DNA is transferred to dicotyledonous and monocotyledonous plants by an identical molecular mechanism, which implies that any plant can potentially be transformed by *Agrobacterium* if a suitable transformation protocol is developed.

Somewhat surprisingly, *Agrobacterium*-mediated T-DNA transfer is not limited to plant cells. Under laboratory conditions, *Agrobacterium* can also transfer DNA to nonplant cells, including the Gram-positive bacterium *Streptomyces lividans*, and eukaryotic cells, including the yeast *Saccharomyces*, filamentous fungi, algae, sea urchins, and human HeLa cell lines, although there are some differences in how the DNA integrates into these more diverse host cells. These studies show that *Agrobacterium*-mediated transformation can be a useful tool for other organisms and sometimes shows a higher rate of transformation than do other methods. Studies of the transformation process on diverse species also provide new opportunities for learning about this useful pathogen.

HOW IT WORKS: INSIDE THE BLACK BOX OF TRANSFORMATION

In the time since early studies showed the potential of *Agrobacterium* as a vehicle to transfer genes into plants, we have learned a great deal more about how this interaction takes place. These findings have helped to optimize and increase the efficiency of *Agrobacterium*-mediated transformation and to expand its host range. They have also revealed the interesting dialogues that occur among bacteria and between bacteria and hosts.

Chemoattraction and Activation of *Agrobacterium* Virulence

A. tumefaciens is a soil-dwelling bacterium that is fully capable of independent living; in fact, most agrobacteria isolated from soil do not carry the Ti plasmid and are therefore incapable of pathogenesis. Those that do carry the Ti plasmid maintain most of the Ti plasmid genes in a transcriptionally inactive state, until their expression is activated by the presence of a suitable plant host.

Agrobacteria are attracted to amino acids, organic acids, and sugars extruded by plant roots and are able to move toward the source of these chemicals through a chemotactic process. Successful infection requires the production of extracellular β-1,2-glucan polysaccharides that allow attachment to the plant cell. *Agrobacterium* frequently gains entry into the plant through wounds, and compounds such as acetosyringone and monosaccharides released from wounded plant cells activate the expression of the virulence genes. Roots also affect the pH of the soil that immediately surrounds them (this is called the rhizosphere), and the full activation of *Agrobacterium*'s virulence requires the mildly acidic environment of the rhizosphere.

Although most *vir* genes are silent until induced, *virA* and *virG* are always expressed (although induced by plant signals) and encode a two-component system for the response to plant signals. The VirA protein is the sensor protein of a two-component regulator system. It forms a homodimer that spans the bacterial inner membrane and has a domain in the periplasmic space between the inner and outer membranes. The periplasmic domain binds plant phenolic compounds, such as acetosyringone, to activate the cytoplasmic protein kinase domain, leading to its autophosphorylation. The phosphoryl group from VirA is transferred to VirG, a transcriptional regulator. VirG transcriptionally activates the other *vir* genes by binding to a 12-bp DNA element called the *vir* box located upstream of the *vir* operon. Chromosomally encoded virulence genes (*Chv*s) also contribute to the induction of the *vir* regulon.

Movement of T-DNA from *Agrobacterium* to the Plant Nucleus

The products of the *vir* genes located on the Ti plasmid, along with those of some chromosomally encoded virulence genes, are necessary for the transfer of T-DNA into the plant cell. The T-DNA *vir* region is ~40 kb and includes several distinct operons. The functions of the *vir* genes have been characterized in depth and reveal a surprisingly complex transformation mechanism. Some *vir* gene products operate within *Agrobacterium*, some build a macromolecular structure through which T-DNA moves, and some act within the host plant cell to ensure that T-DNA is successfully transferred to the nucleus and integrated into the host genome.

The first step in T-DNA transfer involves the action of a single-strand endonuclease that cleaves T-DNA at the border sequences. The bottom T-DNA strand is released from the plasmid by the action of VirD1 and VirD2. VirD1 is a helicase, and VirD2 is a site-specific endonuclease that recognizes the 25-bp T-DNA border sequences. VirD2 nicks only the bottom strand at each border sequence, releasing the single-stranded T-DNA molecule (referred to as the T-strand). VirD2 remains covalently attached to the 5′ end of the T-strand throughout the transfer to the host's nucleus and is thought to protect T-DNA from exonucleolytic attack at the 5′ end and guide it into and through the plant cell.

The T-strand/VirD2 complex moves into the plant cell through a large protein complex called a type IV secretion system (T4SS) that spans the bacterial inner and outer membranes and possibly the plant cell wall. The *virB* operon encodes 11 proteins that, along with VirD4, form the T4SS. The T-strand/VirD2 complex,

as well as several other proteins, such as VirE2, VirE3, VirD5, and VirF, move through the T4SS into the plant cytoplasm. Interestingly, the T4SS is found in bacterial pathogens of humans, such as *Helicobacter pylori*, which may play a role in stomach ulcers, *Neisseria gonorrhea*, which causes gonorrhea, and *Bordetella pertussis*, which causes whooping cough. Studies of the T4SS of *Agrobacterium* have contributed to our understanding of these human pathogens.

Many of the events that occur within the plant cell remain uncertain. Models have been proposed based on in vitro studies or artificial systems, but whether these models reflect the fate of T-DNA in natural interactions is not known. A working model proposes that in the plant cell cytoplasm, VirD2 and VirE2 may contribute to T-DNA movement into the nucleus. VirD2 and possibly VirE2 have nuclear localization signals that direct the complex to the plant nucleus. A plant importin-α (also known as karyopherin) also contributes to the movement of the T-complex into the nucleus.

Once the T-DNA complex enters the nucleus, it is thought to attach to the plant nuclear DNA through interactions with nucleosomal proteins. The DNA may be uncoated by the action of bacterial and host F-box proteins VirF and VBF, which target the coating proteins for proteolysis; VirD5 may protect VirF from proteolytic degradation. The exact mechanism of DNA insertion into the plant genome is not fully resolved, but it occurs via a process known as nonhomologous or illegitimate recombination. Several models for the mechanism of T-DNA insertion have been proposed, and many details remain uncertain.

COEVOLUTION, PATHOGENICITY, AND RESISTANCE

The interactions between plants and *A. tumefaciens* have been subject to millions of years of coevolutionary pressures. As with other plant–pathogen interactions, this one reveals that the pathogen has evolved methods for suppressing and evading the host's defenses and for co-opting host proteins to enhance its pathogenicity, as shown earlier for the use of host proteins during the T-DNA integration process. Here, we describe a few additional examples of coevolved interactions, some of which have only recently been discovered.

Fine-Tuning *vir* Gene Expression

Expression of the *vir* genes is strongly regulated and sensitive to environmental and host signals. Almost the entire *vir* regulon ($\sim$30 genes) is maintained in an essentially silent state, induced under very specific conditions, and silenced again once its gene products are no longer required for pathogenicity.

Certain phenolic compounds derived from wounded plant tissues are potent inducers of *vir* gene expression and act through the VirA/VirG two-component system, but *vir* gene expression is also regulated by other signals found in the rhizosphere, particularly root-exuded sugars and root-induced acidification. The *Agrobacterium* chromosomal virulence gene *chvE* encodes a periplasmically localized sugar binding protein. When ChvE binds certain plant-derived sugars, it promotes autophosphorylation of VirA and enhances its signaling. The *chvG* and *chvI* genes encode a two-component system activated under acidic conditions. ChvI is a periplasmically localized pH sensor that activates ChvG, which induces expression of a set of acid-inducible genes, including *virG*. Thus, the chromosomally encoded virulence genes act synergistically with those on the Ti plasmid to ensure strong induction of the *vir* regulon in the presence of an appropriate host.

After T-DNA has been successfully transferred, *vir* gene expression is no longer necessary. Infected plant cells produce elevated levels of auxin and ethylene hormones, which can suppress *vir* gene expression, as does the defense signal salicylic acid (SA). Specifically, SA interferes with the activation of the VirA protein by acetosyringone.

Quorum Sensing and Quorum Quenching

As with many other bacteria such as the light-emitting bacterium *Vibrio fischeri*, *A. tumefaciens* regulates the expression of genes in a population-density dependent manner, based on the accumulation of an acyl homoserine lactone (AHL). This mode of regulation has been called quorum sensing, and the signals are referred to as autoinducers, quorum signals, or quoromones. Quorum sensing in *Agrobacterium* is induced after the initial plant infection takes place, and the precise details are slightly different in different strains; here, we describe the response of the octopine-utilizing strains.

The successful transfer of T-DNA leads to octopine production by the plant. The secreted octopine is recognized by *A. tumefaciens* by a transcriptional regulator called the octopine catabolism regulator (OccR). When bound to octopine, OccR activates transcription of the *occ* operon, which includes genes involved in octopine uptake and catabolism; also in this operon is the *traR* gene that encodes a transcription factor that is activated by octopine. TraR bound to AHL activates an operon that includes *traI*, which encodes an enzyme for the synthesis of AHL, and the AHL produced binds more avidly to TraR, thus forming a positive feedback regulatory loop. Other TraR-regulated genes are involved in Ti plasmid replication and conjugal transfer (horizontal DNA transfer between bacterial cells through a specialized conjugal transfer apparatus). As most *Agrobacterium* isolated from the soil do not carry a Ti plasmid, conjugal transfer can spread the plasmid into these strains. The net result is that after the initial infection event, the octopine produced as a result of plant transformation spreads the plasmid into other *Agrobacterium* in the soil, conferring on them the ability to utilize opines and increasing the number of pathogens. It can be considered that the Ti plasmid promotes its own replication and dispersal and thereby also confers a fitness advantage to the bacteria.

Recent studies have shown that plants are able to perceive bacterial quormone signals and respond to them through developmental changes, defense responses, and initiating quormone degradation processes known as quorum quenching. Quorum quenching involves elimination of the quorum signal from the environment. The *Agrobacterium* gene *attM* encodes an AHL lactonase, which is believed to degrade and therefore quench the quormone signal. The plant-derived compounds SA (produced as part of the defense response) and γ-amino butyric acid, which accumulates in crown gall tumors, induce expression of AttM. Interestingly, quorum quenching can benefit the

bacterium because it occurs fairly late in the infection process, beyond the time when the quorum sensing responses are needed. More studies of the fitness advantages conferred by quorum quenching to the plant and bacterium will help to resolve which partner has the most to gain from it.

EVADING THE HOST'S IMMUNE RESPONSES

Agrobacterium is recognized by the host plant as a pathogen and elicits plant defense responses. Plants have receptors through which they can recognize conserved pathogen-associated molecular patterns (PAMPs). One of the best characterized PAMPs is flagellin, the protein that makes up bacterial flagella. The *Agrobacterium* flagellin protein, like that of several related symbiotic rhizobia, is not recognized by the plant and does not trigger an immune response. However, another conserved PAMP, EF-Tu, does; *Brassica* plants have an EF-Tu receptor called EFR, mutation of which makes *Arabidopsis* plants more susceptible to infection by *Agrobacterium*; conversely, transfer of the *Arabidopsis* EFR receptor into other plants (such as tomato) makes them more resistant to *Agrobacterium* and other bacterial pathogens.

Agrobacterium has evolved several strategies by which to evade the host's immune responses. For example, upon initial contact, it transfers T-DNA into the host cell rapidly. Additionally, *Agrobacterium* can suppress the plant's defensive hypersensitive response and detoxify reactive oxygen species. Some of the host's defenses themselves have been co-opted to enhance the bacterium's success. As described previously, SA shuts off the quorum sensing and virulence programs after they are no longer needed, thereby reducing the metabolic load demanded by Ti plasmid replication. One of the more fascinating models of the co-option of the host's defenses has been described as a Trojan horse strategy and involves the VIP1 protein that interacts with VirE2 that may facilitate entry of the T-complex into the nucleus. VIP1 is a transcription factor that is a target of phosphorylation by defense-induced protein kinases. When phosphorylated, VIP1 is translocated into the nucleus. When the nuclear import machinery brings the VIP1 complex into the nucleus, it may bring along its dangerous hidden T-DNA cargo.

Plant Genes Involved in Susceptibility or Resistance

Studies have identified proteins that conferred resistance or hypersensitivity to *Agrobacterium* transformation, such as *rat* (resistant to *Agrobacterium* transformation) and *hat* (hypersusceptible to *Agrobacterium* transformation) genes. *RAT* genes encode a protein that may contribute to T-DNA integration and a VirB-interacting protein that may facilitate contact between the T4SS and the host cell. Other identified genes include those involved in bacterial attachment, cytoplasmic trafficking, and transgene expression (see Gelvin, 2010). A better understanding of the plant's contributions to the infection process can help to identify methods by which to protect plants, such as almond (*Prunus dulcis*) or walnut (*Juglans regia*) trees or grape vines that are susceptible to crown gall, and also may lead to more efficient plant transformation methods, particularly for the important monocotyledonous crop plants.

BEYOND *A. TUMEFACIENS*

Agrobacterium is a genus in the family *Rhizobiacea*, which includes the symbiotic nitrogen-fixing bacteria of the genera *Rhizobium* and *Sinorhizobium*. Members of this family have complex genomes that include large plasmids on which pathogenicity and symbiosis genes can be found and, because the plasmids are mobile, make species definitions complex. Traditionally, bacteria of the *Agrobacterium* genus have been defined based on the disease symptoms they elicit. An alternative approach has been to classify these bacteria based on nonpathogenic metabolic properties that are more likely to be encoded in the major chromosome and has led to a description of three biovars. More recently, genomic sequencing projects have provided more insights and led to one species being reassigned to the *Rhizobium* genus. The nomenclature assigned to this group continues to be refined.

A. vitis (Biovar III) and *Agrobacterium rubi*

A. vitis causes crown gall disease, but its host range is limited to grape and a few other species. The causal agent was initially considered to be a variant of *A. tumefaciens*, but extensive testing led to its being assigned as a new species in 1990. The genome of the sequenced *A. vitis* strain comprises two chromosomes and five plasmids, including a large Ti plasmid that contains four distinct T-DNA regions. *A. vitis* is unusual among *Agrobacterium* in that it can induce necrosis in its host and induce a hypersensitive response in nonhost plants, such as tobacco. *A. rubi* is a pathogen of caneberries of the genus *Rubus*, including blackberry and raspberry. As with *A. tumefaciens* and *A. vitis*, *A. rubi* transfers T-DNA into the host plant cell and induces gall formation.

Agrobacterium radiobacter (aka *Rhizobium radiobacter*)

A. radiobacter is avirulent, does not carry a functional Ti plasmid, and does not cause disease symptoms. Strain K84 (aka biovar II) has recently been reassigned to the genus *Rhizobium* (see Velázquez et al., 2010). Whatever you call it, strain K84 is particularly important and widely used as a biocontrol agent that can protect plants against some pathogenic strains of *Agrobacterium*. Its effectiveness in biocontrol is linked to its production of anti-*Agrobacterium* compounds, such as agrocin 84, which is taken up through the opine uptake mechanism. Inside the cell, agrocin 84 inhibits a tRNA synthetase; the enzyme found in *R. radiobacter* itself is insensitive to the antibiotic. Biocontrol may also occur due to the production of a rhizobial iron siderophore, which allows *R. radiobacter* to compete very effectively with other bacteria for iron and limit their growth.

A. rhizogenes (Biovar II)

A. rhizogenes is somewhat different from the other *Agrobacterium* species in that it induces root formation rather than crown gall formation on its host and carries a root-inducing (Ri) plasmid rather than a Ti plasmid. Under natural conditions, its host range is limited to a few perennial dicotyledonous plants, but in the lab it can be induced to infect many more species. In spite of much

effort, the functions of the oncogenic genes carried on the T-DNA of the Ri plasmid, known as *root oncogenic loci*, or *rol*, genes, remain unclear. Rather than inducing hormone synthesis in the host plant, they may alter the host plant's sensitivity to hormones.

A. rhizogenes has been exploited as a tool for research and industrial purposes. Studies of gene expression in roots can be performed in *A. rhizogenes*–induced roots by introducing the gene of interest into the T-DNA region of the Ri plasmid. In addition, gene silencing (by RNA interference) can be performed in roots of chimeric plants that have normal leaf and shoot tissue but transformed roots (so-called composite plants); this RNA interference approach in roots is particularly useful if silencing of the targeted gene prevents the regeneration of fertile plants or prevents seed production by transformed plants. Industrially, the root masses induced by *A. rhizogenes* can be cultured for long periods of time and have been exploited as a rich source of plant-produced compounds. The benefits of these bioreactor-cultured roots are that wild plants are thus protected from overharvesting (which is a particular problem for many plants that produce medicinally important compounds) and that an efficient culture system can lower the cost of the compounds. Examples of compounds harvested from *A. rhizogenes*–induced root masses include ginsenosides from root cultures of *Panax ginseng* and ginkgolides from *Ginkgo biloba*. In addition, metabolic engineering can be applied to these plant tissues to enhance or modify the compounds produced, including the production of novel recombinant proteins such as antibodies.

SUMMARY AND ONGOING RESEARCH

Almost every molecular biologist works with *E. coli* at some point in their career, and *A. tumefaciens* is as ubiquitous in the labs of plant molecular biologists. This unusual and useful pathogen, which was once thought to hold the secrets of human cancers, has proven to be the key through which scientists can transform plants readily. *Agrobacterium*-mediated transformation of plants underlies many of the most important scientific breakthroughs of the past 30 years, including the discovery of transcriptional gene silencing by small RNAs. The release of an annotated T-DNA insertion library of *Arabidopsis* opened the door fully to the powerful reverse genetic approaches that are enabling its genome to be functionally annotated, and insertional mutagenesis is contributing to the characterizations of other, even nonplant genomes. The introduction of genes into crop plants, through *Agrobacterium* or other methods, can have far-ranging benefits from pathogen resistance (e.g., rainbow papaya, *Carica papaya*) to nutritional enhancement (e.g., golden rice).

Nevertheless, we still don't fully understand how this process works, in the lab or in the wild. Many questions about the contributions of plant genes in resisting or facilitating the infection process remain, and there are clearly opportunities to manipulate the system to favor infection in the lab, for transformation purposes, and to discourage infection in the field, to protect plants. Without question, *Agrobacterium* is a very useful, and interesting, pathogen.

This review is dedicated to Eugene W. Nester in honor of his retirement in 2009 at the age of 79. We thank Allan Downie (John Innes Centre), Stanton Gelvin (Purdue University) and Vitaly Citovsky (Stony Brook University) for critical reading of this article.

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RECOMMENDED READING

(This is a representative list of sources to help the reader access a huge body of literature. We apologize in advance to those whose work is not included.)

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