ANNUAL REVIEWS

Annual Review of Phytopathology

Virulence and Ecology of Agrobacteria in the Context of Evolutionary Genomics

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Annu. Rev. Phytopathol. 2023. 61:1–23

First published as a Review in Advance on May 10, 2023

The *Annual Review of Phytopathology* is online at phyto.annualreviews.org

https://doi.org/10.1146/annurev-phyto-021622-125009

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Keywords

Agrobacterium, evolution, genomics, phytopathogens, plasmids, virulence

Abstract

Among plant-associated bacteria, agrobacteria occupy a special place. These bacteria are feared in the field as agricultural pathogens. They cause abnormal growth deformations and significant economic damage to a broad range of plant species. However, these bacteria are revered in the laboratory as models and tools. They are studied to discover and understand basic biological phenomena and used in fundamental plant research and biotechnology. Agrobacterial pathogenicity and capability for transformation are one and the same and rely on functions encoded largely on their oncogenic plasmids. Here, we synthesize a substantial body of elegant work that elucidated agrobacterial virulence mechanisms and described their ecology. We review findings in the context of the natural diversity that has been recently unveiled for agrobacteria and emphasize their genomics and plasmids. We also identify areas of research that can capitalize on recent findings to further transform our understanding of agrobacterial virulence and ecology.

1. WHAT ARE AGROBACTERIA?

Agrobacteria have the capacity or potential to cause crown gall or hairy root diseases in plants (14). These phytopathogens, capable of infecting many plant species and causing substantial economic losses, have been studied since the early twentieth century (48, 86). Knowledge of the virulence mechanism allowed for the development of agrobacteria as powerful tools for plant transformation (44). Agrobacterial phytopathogenicity depends on either tumor-inducing (pTi) or root-inducing (pRi) oncogenic plasmids, which are mobilizable and can be gained and lost. Thus, strains without either plasmid but capable of harboring them are also considered agrobacteria (14). Agrobacteria have an intricate evolutionary relationship with rhizobia (121) (**Figure 1*a***), which can induce root nodule formation on legume hosts and carry out symbiotic nitrogen fixation (14). This function is often dependent on a symbiotic plasmid (134). Together, these plant-associated bacteria are referred to as the agrobacteria–rhizobia complex (120).

Historically, agrobacteria were mostly classified into three major biovars (137). On the basis of genomic differentiation, biovar 1 (BV1) has been subdivided into multiple genomospecies with different numerical identifiers (e.g., genomospecies 1 = G1) (12, 80), and some have accepted Latin binomials (**Supplemental Table 1**). For example, G4 corresponds to *Agrobacterium radiobacter* (12), and G8, which contains the well-studied reference strain C58, corresponds to *Agrobacterium fabrum* (58). Biovar 2 (BV2) strains are genetically distinct from BV1. However, the early nomenclature is confusing because the specific epithets (e.g., *tumefaciens*, *rhizogenes*, and *radiobacter*) were used to imply phenotypes conferred by plasmids, resulting in some BV2 strains being assigned to the same Latin binomials that are associated with BV1 strains. For example, K84, a nonpathogenic BV2 strain that has biocontrol properties against other agrobacteria, is commonly known as *Agrobacterium radiobacter* (78). Nevertheless, based on recent taxonomic revisions, BV2 is now designated as *Rhizobium rhizogenes* (12, 69, 114). Biovar 3 (BV3) was referred to as *Agrobacterium vitis* (88) and later reclassified as *Allorhizobium vitis* and *Allorhizobium ampelinum* (51, 81, 82).

Importantly, these three biovars have distinct evolutionary origins within Rhizobiaceae (**Figure 1*a***). This, together with the differences in genome organization, indicates that agrobacteria are a heterogeneous group. Moreover, sister groups that share similar properties with these three biovars have been identified. For example, the sister group of BV1 (i.e., BV1-like) contains species such as *Agrobacterium larrymoorei* and *Agrobacterium rubi* (53, 82). Also, two clades of BV2-like agrobacteria were identified and contain species such as *Rhizobium leucaenae* and *Rhizobium freirei* (120, 121). The nomenclature reflects the observations that the ecological distinction between agrobacteria and rhizobia is not evolutionarily stable. For example, although most *Neorhizobium* strains are rhizobia, one *Neorhizobium* lineage naturally acquired a pTi from BV1 and is capable of inducing crown galls (36). Other rhizobia with pRi have been reported in hydroponic farms (119, 122). Owing to the heterogeneity of agrobacteria, as well as the complexity and frequent revisions of Rhizobiaceae taxonomy (137), we synthesized current knowledge of agrobacterial virulence and ecology on a phylogenetic framework. Although this framework also applies to other traits of interest, we were not able to review all of them here and thus direct readers to some excellent reviews (20, 38, 124).

2. GENOME CHARACTERISTICS OF AGROBACTERIA

All agrobacteria/rhizobia have multipartite genomes, but organization varies across the phylogeny (**Figure 1*a***). In addition to a chromosome, most lineages have one or more chromids (34). However, the delineation between chromids and plasmids is fuzzy because gene essentiality may be defined via diverse criteria. It is unclear whether the secondary replicon of BV2 strains is a

Crown gall: a plant disease caused by agrobacterial strains carrying a tumor-inducing plasmid; symptoms include abnormal tissue growth

Hairy root: a plant disease caused by agrobacterial strains carrying a root-inducing plasmid; symptoms include a massive proliferation of roots

Oncogenic plasmids: plasmids that confer upon agrobacteria the capacity to genetically transform plants and elicit abnormal growth

Plasmids: typically extrachromosomal, nonessential, autonomously replicating DNA molecules

Root nodule: structure induced by rhizobia and formed by legumes to provide an environment necessary to support activities associated with nitrogen-fixing symbioses

Symbiotic plasmid: plasmid that confers upon rhizobia the capacity to induce nodule formation and reduce atmospheric nitrogen to biologically usable forms

Supplemental Material >

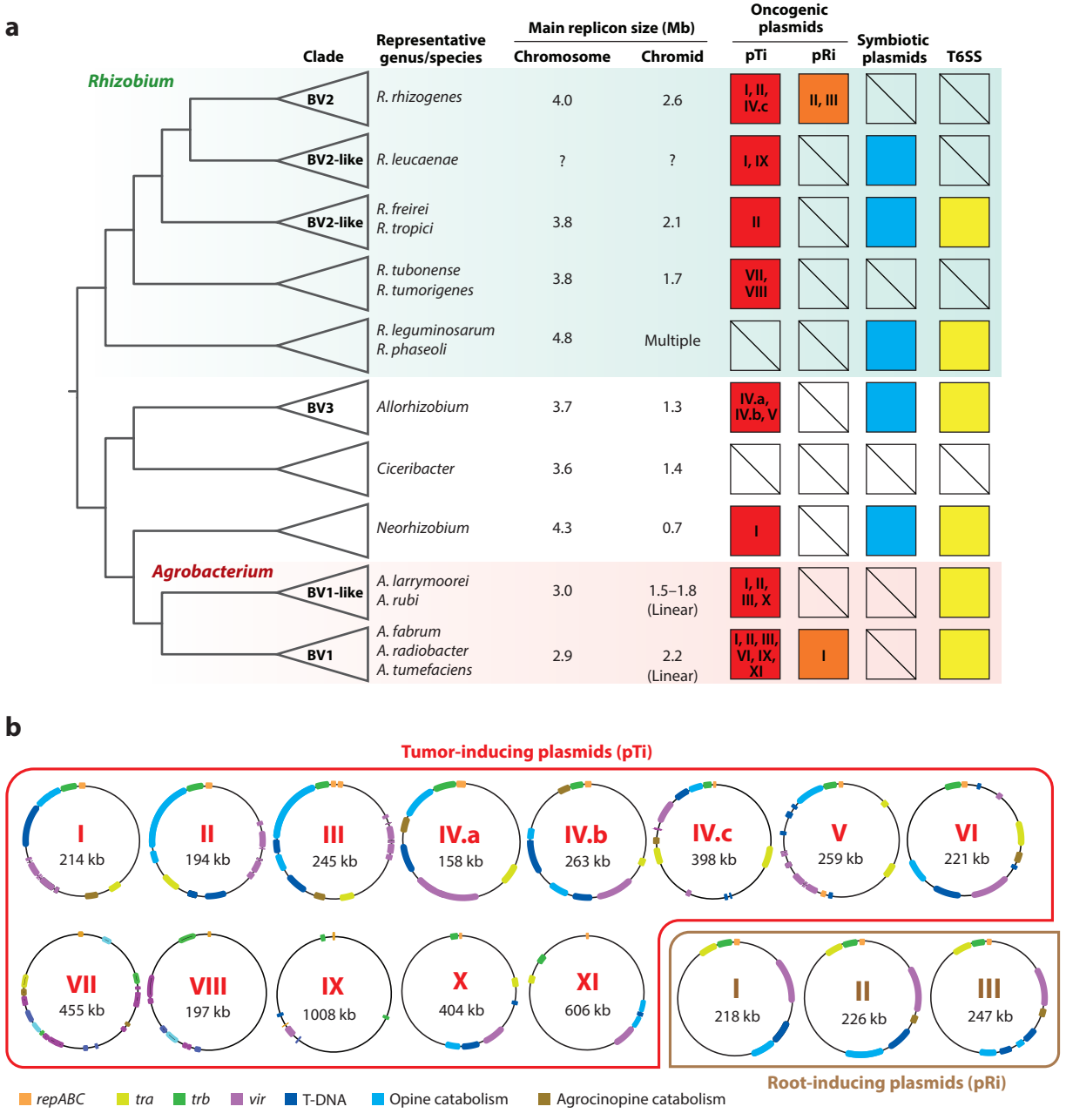


Figure 1

(*a*) Genetic diversity of agrobacteria and related lineages. The tree is redrawn from a maximum likelihood phylogeny based on 23 conserved genes; some Rhizobiaceae lineages are omitted to simplify the visualization. The distribution of oncogenic plasmids, symbiotic plasmids, and type VI secretion system (T6SS) genes are plotted. For oncogenic plasmids, the two classes [tumor-inducing plasmids (pTi) and root-inducing plasmids (pRi)] are plotted separately and the types found in each clade are labeled. Colored blocks indicate that at least one member of a clade carries the corresponding plasmid or genetic element. (*b*) Structure and organization of oncogenic plasmids by type. The presence and location of key loci are indicated by colored blocks. Plasmids are not to scale. The size of a representative plasmid for each type is indicated. Figure adapted with permissions from the American Association for the Advancement of Science and The Royal Society (United Kingdom).

chromid or a megaplasmid (106). Regardless, these secondary replicons were hypothesized to have originated from an ancestral plasmid and diversified via lineage-specific gene transfers from the chromosome (106). The partitioning into multiple replicons was hypothesized to allow for genome expansion while keeping individual replicons small for fast replication (34, 106). However, a spontaneous fusion between the chromosome and chromid was observed to have occurred in a BV1 strain, and the strain exhibited no obvious fitness defects and grew slightly faster in culture than the parental strain (65). Therefore, an alternative hypothesis is that the evolution of multipartite genomes is a consequence of stochastic processes rather than being selection driven. Although the reasons why multipartite genomes emerge remain unclear, the presence of multiple replicons requires proper coordination to ensure that they are maintained and properly segregated. Two recent studies demonstrated that direct interactions among origins of replication are crucial for maintaining genome integrity (101, 102). Additionally, plasmid transfers and recombination among replicons may have played an important role in promoting agrobacterial diversity (65, 121).

BV1 is the best-characterized lineage among agrobacteria. Based on DNA–DNA hybridization, BV1 was divided into multiple subgroups in the 1980s (96). In 2001, the first agrobacterial genome sequence was published for strain C58 (29, 127). By 2022, >200 genome assemblies were published for this lineage (121), which may be classified into at least 21 species-level taxa (**Supplemental Table 1**). Similar to other bacteria (46), a genome-wide average nucleotide identity of 95% appears to be a suitable threshold for species boundaries within BV1 (10, 120). Comparisons among BV1 strains indicated that ~85% and ~80% of the gene content is shared at within- and between-species levels, respectively (130). Furthermore, despite inferences of extensive horizontal gene transfers within BV1 (59), in most cases each species maintains a distinct gene content (10). Generally, BV1 strains have a chromosome of ~2.9 million base pairs (Mb) and a linear chromid of ~2.2 Mb. The presence of a linear chromid is a distinct trait that evolved in the most recent common ancestor of BV1 and BV1-like agrobacteria (99) and is also a defining character for the genus *Agrobacterium* in current taxonomy (89, 99).

Compared to BV1, BV2 and BV2-like (120, 121) agrobacteria have a larger chromosome that is ~4 Mb in size (**Figure 1*a***). Additionally, the BV2 chromid-like replicons are also larger than those found in other agrobacteria. Consequently, BV2 strains are predicted to have >1,000 more protein-coding genes in their genomes compared to those of BV1 or BV3 strains (106).

BV3 agrobacteria have one circular chromosome that is ~3.7 Mb in size and one circular chromid that is ~1.3 Mb. Although the partitioning of genes between chromosome and chromid is different, the overall genome size of BV3 strains is comparable to that of BV1 strains and smaller than that of BV2 strains.

Despite the progress, two key issues remain to be addressed. First, several clades (e.g., BV1-like, BV2-like, *Ciceribacter*) have limited genomic information available, which hinders analysis to better understand the genome evolution of these bacteria. Second, the majority of available genome sequences are incomplete assemblies, which do not allow for confident inference of gene content and overall organization.

3. THE ONCOGENIC PLASMIDS NECESSARY FOR AGROBACTERIAL VIRULENCE

The oncogenic plasmids are found across diverse agrobacterial lineages (**Figure 1**). The two classes, pTi and pRi, are associated with different disease phenotypes but share similar components. Each oncogenic plasmid carries virulence (*vir*) genes encoding for a type IV secretion system (T4SS) and other elements necessary for processing and transporting the transfer DNA (T-DNA) into plant cells for transformation. T-DNA regions are flanked by border sequences, which are

Biovars: a bacterial group exhibiting distinct physiological and/or biochemical characteristics, typically defined at the subspecies level

Sister groups: closest relatives in an evolutionary tree

Chromosome: in bacteria, a primary replicon with all or most of the genetic information of a cell

Chromids: secondary replicons with essential genes and those involved in plasmid-type replication and partition

Megaplasmid: very large plasmid of hundreds to more than 1,000 kilobases in size

Supplemental Material >

25-bp direct repeats required for T-DNA processing and are highly conserved in all oncogenic plasmids that have been sequenced to date. T-DNAs typically have genes for producing plant growth-promoting hormones, auxins, and cytokinins. Their expression in transformed plant cells changes plant growth patterns, leading to the formation of crown galls or hairy roots. T-DNAs also typically have genes for producing specific opines, which are diverse secondary amine derivatives or sugar-phosphodiesters (18). Because oncogenic plasmids also have genes for opine uptake and catabolism, opines produced by the transformed plant cells are hypothesized to give infecting agrobacteria a competitive advantage (i.e., opine concept) (18). Oncogenic plasmids carry other loci that are not directly involved in virulence. In addition to loci essential for plasmid replication (*repABC*) and conjugation (*tra/trb*), other genes variable in presence/absence may contribute to strain fitness within plants or in other environments (10, 120, 121).

***k*-mer profile:** a genomic fingerprinting method based on analyses of *k*-mers, which are nucleotide sequences of length *k*

3.1. Classification of Oncogenic Plasmids

Classification is important because it establishes the foundation for inferring evolutionary relationships. Methods used, and ensuing results, can dramatically impact the resolution at which we can interpret evolutionary patterns. Historically, oncogenic plasmids were classified based on the opines produced by the transformed plants (18, 91). However, subsequent findings revealed two shortcomings of this classification scheme. First, the opine synthesis and catabolism genes are typically linked, allowing for the change of opine type by one single recombination event without loss of function. Consequently, plasmids with different backbones may be classified as the same type, or otherwise identical plasmids may be classified as different types (105, 120). Second, some oncogenic plasmids encode for multiple opine types (120), which makes them difficult to classify.

In 2020, one study expanded the number of available sequences for oncogenic plasmids to the order of hundreds (120). The large data set was essential for identifying the key genetic components among diverse oncogenic plasmids, thus revealing their commonalities and allowing for robust classification into just a few major types. In 2022, a follow-up study provided more breadth in sampling and expanded the classification scheme to include 11 types of pTi and three types of pRi (121) (**Table 1** and **Figure 1*b***). These studies showed that despite sampling hundreds of strains, most oncogenic plasmids are typically closely related within types. Also, new, and perhaps rarer, variants will be continually discovered.

Compared to the classical classification scheme based on the opine type (18, 91) or an alternative scheme that additionally considers the *vir* loci structure and T-DNA gene content (84), analyses reported in 2020 employed a more comprehensive classification scheme (120). Multiple features, including *k*-mer profile, gene content, core gene phylogeny, and plasmid structure, were all examined and found to produce consistent typing results (10, 120, 121). Therefore, we adopt this classification scheme here to review the oncogenic plasmids of agrobacteria (**Table 1** and **Figure 1*b***). Some of these newly defined types correlate to previous opine-based types; however, within-type variations in opine metabolism genes exist (**Supplemental Table 2**).

Supplemental Material >

3.2. Evolution of Oncogenic Plasmids

The ability to genetically transform plants, that is, the coordination of core *vir* genes and T-DNA borders, likely evolved once (18). The subsequent acquisition of different genes, such as those encoding for auxin and cytokinin biosynthesis, in ancestral T-DNAs led to the tumorigenic and rhizogenic phenotypes. Models suggested that following the emergence of ancestral pTi and pRi, virulence genes and elements recombined into different plasmid backbones and diversified the two classes. Each class has since diversified into distinct types, yet continues to recombine with the other and with nononcogenic plasmids.

Table 1 Classification of oncogenic plasmids

Type	Previous classification	Opine types identified
pTi I	Nopaline type	Nopaline, agropine, succinamopine, novel opine
pTi II	Octopine type	Octopine, mannopine
pTi III	Succinamopine type, agropine type, chrysopine type	Succinamopine, nopaline, agropine, mannopine, chrysopine
pTi IV.a	Nopaline type	Nopaline
pTi IV.b	Octopine/cucumopine type, octopine type	Octopine, cucumopine
pTi IV.c	None	Nopaline, octopine
pTi V	Vitopine type	Vitopine, nopaline
pTi VI	None	Nopaline, uncharacterized opine
pTi VII	None	Nopaline
pTi VIII	None	Unknown
pTi IX	Lippia type	Nopaline
pTi X	None	Unknown
pTi XI	None	Unknown, novel opine
pRi I	Cucumopine type, mikimopine type	Cucumopine, mikimopine
pRi II	Mannopine type	Mannopine, succinamopine
pRi III	Agropine type	Agropine, mannopine, succinamopine

Abbreviations: pTi, tumor-inducing plasmids; pRi, root-inducing plasmids.

The diversification of oncogenic plasmids has been promoted by homologous recombination. Highly conserved regions, such as *repABC* and *tra/trb*, appear to have the highest level of recombination, likely between oncogenic plasmids or with other rhizobial plasmids (105, 120). These regions potentially serve as common sites for cointegration between different plasmids (120). Moreover, evidence suggests that the T-DNA regions and their border sequences, as well as the *vir* loci, are intercompatible across different pTi and pRi and involved past horizontal transfers (120). Repetitive and homologous sequences, such as insertion sequence (IS) elements and other transposable elements, are also implicated in mediating recombination (92). Several of the pTi types (VII, VIII, and IX) have T-DNA and *vir* loci surrounded by IS elements (121). Additionally, two subtypes of the type VIII pTi have different plasmid backbones, yet share similar *vir* and T-DNA regions bordered by IS elements (121).

A clear example of how recombination contributes to the evolution of oncogenic plasmids was described recently and shows how even genes typically linked and functionally related can be acquired from multiple sources (121). Type VII pTi are found in *Rhizobium tumorigenes* (54, 121) and are mosaics of at least three different oncogenic plasmids. Type VII pTi have three separate *vir* loci; although each locus is partial, together these three loci provide a full complement of *vir* genes and their bacterial hosts are capable of genetically transforming plants. Type VII pTi also has homologs of both *virE* and *GALLS*, evolutionarily distinct genes that can functionally replace each other. Plasmids typically carry only one or the other (40, 100), with *virE* typically in pTi and *GALLS* typically in pRi. Their co-residence in type VII pTi suggests that they are intercompatible and do not interfere with each other. Clearly, the plasmid types vary in their alleles and virulence gene clusters, yet the genes can still interact and maintain functions. However, it remains to be seen how this variation influences host specificity and transformation efficiency.

Causing plants to produce greater quantities of opines is a potential selective pressure because it conceivably improves agrobacterial fitness. One mechanism for increasing opine production is to promote greater tissue growth of transformed plants. Unexpectedly, even the highly conserved genes, such as *tms1/tms2* involved in promoting auxin biosynthesis, were likely acquired from

different sources three separate times in the evolution of these plasmids (120). Another prediction is that competition for a common good can drive diversification to yield unique opines, which is consistent with current findings (90, 120).

Shuffling of genes between plasmids generates diversity for selection to act upon and promotes widespread dissemination of particular combinations or elements that provide a significant benefit. A common T-DNA is present in most pTi types, and phylogenies of T-DNA genes suggested it may have swept through the population (90, 120). Likewise, pRi all have at least one T-DNA region with a conserved set of genes that differs from the common pTi T-DNAs. Many variants of T-DNA can be successful in a population so long as the key factors to drive plant tissue growth and opine production are maintained. Also, the gene content of T-DNAs can vary but could be restricted if T-DNA size is a restricting factor.

Incompatibility groups: groups of plasmids defined based on their inability to coexist stably in the same bacterial cell line

4. NONONCOGENIC PLASMIDS OF AGROBACTERIA

Strains of the agrobacteria–rhizobia complex also harbor diverse nononcogenic *repABC* plasmids (121). These plasmids are loosely referred to as pAt regardless of gene content. Nononcogenic plasmids are not as well-studied as oncogenic plasmids, and their importance in agrobacterial fitness is unknown. Their structure and composition are difficult to determine, as available sequences are mostly derived from draft genome assemblies. No framework for classification has been established yet.

Nevertheless, nononcogenic plasmids are key to the evolution of agrobacteria. These plasmids are likely gene reservoirs and explain in part the extensive variations in oncogenic plasmids and chromids (106, 121). Moreover, *vir* loci have the potential to recombine into a nononcogenic plasmid and give birth to a new type of oncogenic plasmid. Several sequenced plasmids are cointegrates of pTi or pRi with nononcogenic plasmids (121). Type IX pTi are represented by the so-called lippia type pTi (e.g., pTiS7/73 and pTiAB2/73). These are unusually large for pTi with a size range of ~0.5–1 Mb and are associated with agrobacteria that exhibit a limited plant host range (41, 113, 121). Each of the type IX pTi has regions predicted to be acquired from nononcogenic plasmids. Emergence of new types of oncogenic plasmids can give bacterial cells opportunities to cohost oncogenic plasmids that belong to different incompatibility groups. In turn, this gives rise to more opportunities to recombine and diversify. However, the degree to which oncogenic plasmids that emerge in this manner are shared is unclear. Analyses of BV1 strains have suggested that specific pAt are largely conserved within individual genomospecies and thus may not be exchanged as broadly as pTi or pRi (121). Recombination with nononcogenic plasmids also potentially provides oncogenic plasmids with genes that confer a new fitness advantage. It is reasonable to predict that genes with no selective advantage will be lost, and eventually large cointegrates will have structures more similar to those of canonical oncogenic plasmids.

Other nononcogenic plasmids carry full or partial copies of *vir* loci but no T-DNA regions (55, 121). Their *vir* loci often diverge in sequence from those in oncogenic plasmids. Opine catabolism loci are also sometimes found on nononcogenic plasmids and can provide an advantage to strains in crown galls induced by other agrobacteria with different opine synthases (52). It is unclear whether these plasmids are relics of ancestral oncogenic plasmids that were released from selection or originated from nononcogenic plasmids acquiring some of the pTi/pRi components.

Characterizing nononcogenic plasmids will likely improve understanding of the ecology of agrobacteria. Deep and broad sampling of agrobacteria has generated a rich genomic data set. But, as described above, these are mostly draft assemblies, which hinders our ability to identify nononcogenic plasmids and carry out comprehensive comparative analysis to better understand their roles and evolution. This is a key issue to be addressed.

5. VIRULENCE GENES AND MECHANISMS

Much of what we understand about agrobacterial virulence is based on in-depth studies of a few strains. The most studied strain is C58 harboring type I pTiC58 (67), which is the wild-type progenitor of several strains used in *Agrobacterium*-mediated transformation such as EHA105 and GV3101. Another well-studied strain is Ach5 (of BV1-G1) harboring type II pTiAch5 (87), which is the progenitor of LBA4404. Two composite strains, A348 (45) and LBA1010 (83), both derived from C58 and carrying a different type II pTi, have also been studied.

Early studies used transposon mutagenesis for genome-scale identification of genes involved in pathogenicity (27, 49). In addition to *vir* loci located on oncogenic plasmids, multiple chromosomal virulence (*chv*) genes were also identified (19). Based on mutant phenotypes and molecular characterization, these genes were classified as being involved in one or more of five steps (**Figure 2**).

The degree to which our current understanding generalizes to all strain–plasmid combinations has not been systematically tested across the agrobacterial phylogeny. The *vir* and *chv* genes essential for tumorigenicity are conserved in the sense of being present in all pTi-containing strains (**Supplemental Table 3**). However, proteins encoded by these and other genes show large ranges in sequence conservation (**Figure 3**). It should be noted that many pTi-containing strains have yet to be experimentally confirmed for crown gall–inducing function. With deep sampling of strains and oncogenic plasmids, there are new opportunities to use allelic and gene presence/absence variation to gain information about virulence mechanisms.

Biofilm: an assemblage of bacteria embedded in an extracellular matrix and adhered tightly to a surface

Two-component systems: membrane-associated histidine kinase and a partner DNA-binding response regulator; often involved in sensing and responding to environmental signals

Supplemental Material >

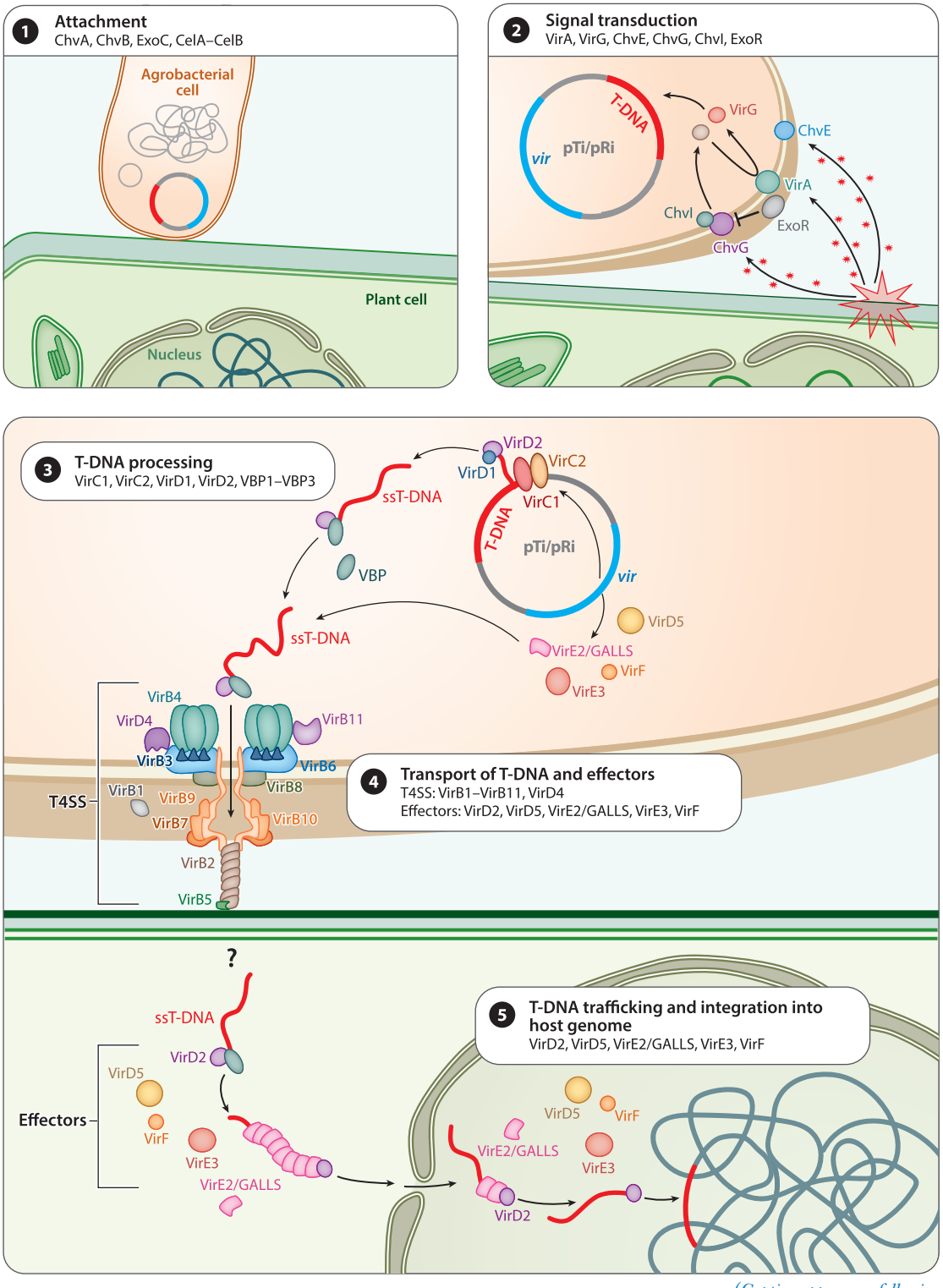
5.1. Attachment

The first step of infection likely starts with attachment of the pathogen to the host, a process that involves agrobacteria-derived exopolysaccharides (75). Experimentally generated *chvA* and *chvB* mutants are incapable of binding to the plant surface and are nonpathogenic (19, 108). ChvB is a glycosyltransferase that synthesizes cyclic β-1,2 glucan, which is transported by ChvA from the cytoplasm into the periplasm, the space between the inner and outer membranes of Gram-negative bacteria (31). Thus, it is suggested that β-1,2 glucan is responsible for attachment. However, the *Tn5::chvB* mutant also has a higher level of succinoglycan (75), an exopolysaccharide involved in biofilm formation (110); thus, the attachment deficiency of this mutant is confounded by other factors. Along the same line, although several genes involved in biofilm formation also contribute to attachment (38), biofilm formation does not appear to be directly related to pathogenicity. Interestingly, although not essential for virulence, the *celABC* and *celDE* operons encoding for the biosynthesis of cellulose fibril can promote virulence by anchoring the agrobacteria to plant cells (76). Unlike *chvA* and *chvB*, which are highly conserved in agrobacteria, *celABC* and *celDE* operons are present in only some agrobacteria and have no association with oncogenic plasmids (**Supplemental Table 3**).

5.2. Signal Transduction

Agrobacteria use two-component systems to sense chemical signals (acidity, monosaccharides, and phenolics) associated with wounded plants and activate expression of genes required for infection (126). VirA is a membrane-bound sensor histidine kinase that together with the response regulator, VirG, perceives phenolics. Although there is no evidence for binding of phenolics to VirA, genetic evidence derived by swapping *virA* alleles from different pTi indicated that VirA is responsible for discriminating between different phenolics (61).

ChvE, a sugar-binding protein, can bind to VirA for synergistic activation, especially when phenolics are at low concentrations. Acidity at a pH range of 5.5–6.0 is also critical for effective



(Caption appears on following page)

Figure 2 (*Figure appears on preceding page*)

Major steps of the agrobacteria-mediated transformation process. The key agrobacterial proteins involved in each step are labeled. Abbreviations: pRi, root-inducing plasmids; pTi, tumor-inducing plasmids; ssT-DNA, single-stranded transfer DNA; T4SS, type IV secretion system; T-DNA, transfer DNA; VBP, VirD2-binding proteins.

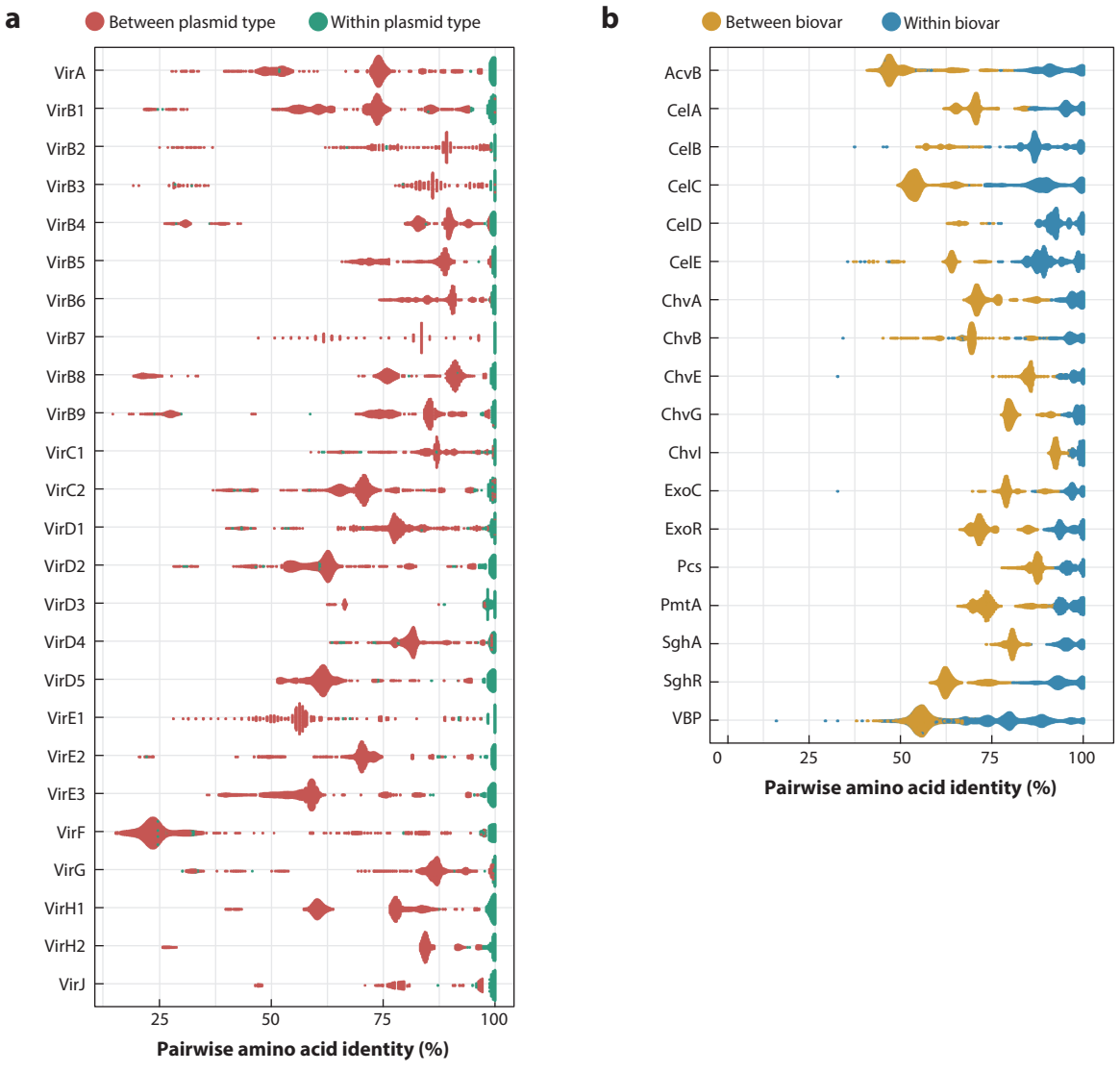


Figure 3

Sequence diversity of proteins implicated in influencing agrobacterial virulence. Comparisons were made between proteins encoded on (*a*) oncogenic plasmids or (*b*) agrobacteria chromosomes. The R package seqinR was used to calculate pairwise sequence identity. Comparisons of VirB1–VirB4/8/9 contained distant homologs of type IV secretion system–associated proteins not involved in virulence. Comparisons of VBP (VirD2-binding proteins) also included distant homologs.

VirA/VirG-mediated virulence gene induction (103). The sensor histidine kinase ChvG and the response regulator ChvI constitute a chromosome-encoded two-component system for sensing acidity (62, 140). At neutral pH, ChvG is bound by ExoR, a periplasmic protein, to inhibit ChvG from activating ChvI (110, 129). Only when ExoR becomes labile in acidic environments is ChvG then released from ExoR to activate ChvI, which in turn induces the downstream genes, including *pckA* (phosphoenolpyruvate carboxykinase) (70), *katA* (catalase) (62), and *aopB* (outer membrane protein) (62). These acid-induced genes are required for full virulence, likely by helping agrobacteria cope with stresses during infection. ChvG/ChvI also upregulates the transcription of *virG* (62).

T-pilus: hairlike projections on the surface of agrobacteria that form upon perception of *vir* gene inducers

The observation that *virA/virG*, *chvG/chvI*, and *chvE* are present in all pTi-harboring strains indicates their essential role in agrobacterial virulence. A recent study synthesized past findings and coupled them to a predicted structure of VirA to infer mechanisms by which VirA coordinates perception of phenolics and sugars (107). However, there has not been a systematic study that relates the composition of phenolics and sugars from natural host species to allelic variations. It is also unknown whether these differences affect *vir* gene expression and host range. Notably, ChvE, ChvG, and ChvI show lower sequence variation across the biovars (**Figure 3**). Conversely, VirA and VirG are more variable; some of this variability is because plasmids may have homologs of these genes that encode different functions. Moreover, unlike other strains in which *virA* is located on an oncogenic plasmid, the *virA* genes of 1D1609 and strains bearing type VIII.b pTI reside in separate plasmids (35, 121). For the latter strains, evidence suggests that the *virA* gene was acquired from a source other than type VIII.b pTi. The absence of *virA* from pTi1D1609 likely explains the nonpathogenic phenotype of JP1, a composite C58-derived strain cured of pTiC58 but carrying pTi1D1609 (93).

5.3. T-DNA Processing

VirD2 is an endonuclease that binds and nicks at a specific position within T-DNA border sequences (44). The conservation of border repeats suggests that selection has also maintained the residues in proteins that process the T-DNA, which underpins the modularity of *vir* genes and T-DNAs. However, VirD2 requires other accessory factors. First, VirD1 is a helicase for generating single-stranded T-DNA, which VirD2 covalently binds to its 5′ end to form the T-complex (39). Second, the overdrive protein VirC1 has a motif required for recruiting the T-complex to the T4SS (4). Third, the C-terminal DNA binding domain of VirC2 facilitates T-DNA processing (72). In addition, three functionally redundant VirD2-binding proteins (VBP1 encoded on pAtC58 and VBP2/3 encoded on the linear chromid) are also required for recruiting the T-complex to the T4SS (32). Consistent with the expectation for essentiality, *virC1/C2/D1/D2* are highly conserved in pTi/pRi-harboring strains, whereas the presence of *vbp1/2/3* is variable (**Supplemental Table 3**). However, as previously reported, there is a high level of variation in VirD2 sequences (**Figure 3**), which can be used as markers for distinguishing types of oncogenic plasmids (23).

Supplemental Material >

5.4. Transport of T-DNA and Effectors

T-DNAs are transferred into plant cells via a T4SS (64) (**Figure 2**). VirB1–VirB11 form the translocation channel that is also required for T-pilus assembly, whereas VirD4 is an ATPase and coupling protein (56, 57). No full structure of an agrobacterial T4SS has been resolved, but that of a conjugative core T4SS complex has been determined (74). VirD4 is responsible for cargo specificity, which includes the T-DNA piloted by VirD2 as well as VirE2, VirE3, VirD5, and VirF, so-called effectors that have a C-terminal signal (115). The 12 corresponding genes of the T4SS

Supplemental Material >

Ortholog: vertically inherited gene homologs present among species related by descent

are highly conserved among all oncogenic plasmids (120, 121) (**Supplemental Table 3**). The low levels of protein sequence conservation between plasmid types are due to the inclusion of homologs from other plasmid-borne T4SS-associated genes (**Figure 3*a***).

The role of the T-pilus is unclear, and it does not appear to function as a conduit for T-DNA transfer but may enhance transformation efficiency or virulence. The T-pilus is composed of VirB2 arranged in a five-start helical assembly with a positively charged lumen in association with the phosphatidylcholine lipid (2). Indeed, the biogenesis of phosphatidylcholine is critical for the stability of T4SS proteins (1). A mutant deficient in both *pmtA* and *pcs*, encoding phospholipid N-methyltransferase and phosphatidylcholine synthase, respectively, required for phosphatidylcholine biosynthesis is nonpathogenic (123). Conversely, amino acid substitution variants of several VirB proteins resulted in a phenotype in which their uncoupling mutants remain tumorigenic but without detectable T-pilus formation, suggesting that the T-pilus is not required for virulence (28, 132). However, uncoupling mutants were attenuated in transient transformation of *Arabidopsis* seedlings (132).

VirJ is another protein involved in T-DNA transfer. Its corresponding gene is in some but not all oncogenic plasmids (**Supplemental Table 3**). VirJ is functionally redundant with and presumably evolved from the chromosomally encoded AcvB (94), which is highly conserved. The translated sequence of *virJ* is largely conserved among oncogenic plasmids that carry it, and ortholog clustering analysis grouped it with AcvB sequences (**Figure 3**). A recent functional characterization of AcvB indicated its role in membrane lipid homeostasis (30).

5.5. T-DNA Trafficking and Integration into Host Genome

VirD2 harbors a nuclear localization signal that is recognized by importin α implicated in transporting T-DNA molecules across plant nuclear pores for integration into plant chromosomes (6). VirE2 is a single-stranded DNA binding protein required for virulence (44). Although an obvious function of VirE2 is to protect single-stranded T-DNA from degradation, VirE2 is not associated with T-DNA molecules inside agrobacterial cells (9). Instead, biochemical and genetic evidence demonstrated that VirE2 acts only inside plant cells (112). But whether VirE2 binds to T-DNA molecules in plant cells remains unknown. Recent data showed that VirE2 interacts with a plant nucleoporin protein CG1, and the ability of VirE2 to localize in nuclei requires the presence of a T-DNA and VirD2 (63). CG1-deficient plants are more resistant to transient transformation and crown gall formation. In all, data suggested that VirE2 associates with T-DNA molecules piloted by VirD2 to mediate translocation through nuclear pores (63). Compared across plasmid types, VirE2 is diverse in sequence (**Figure 3**).

VirF, VirE3, and VirD5 are accessory proteins that influence host range or the transformation process (44). Interestingly, *virF* is absent from types V, IX, and XI pTi and present in all others; types I and VI pTi have two homologs, whereas type III pRi has three homologs (120). This pattern is a consequence of there being at least two different genes annotated as virF (**Supplemental Table S3**). Relative to the typical VirF of strain A6, the alternative variant present in strain C58 has a large unrelated central region flanked by homologous amino and carboxy-termini. The *virE3* and *virD5* genes are more prevalent among characterized pTi (**Supplemental Table 3**). Consistent with these patterns, VirF sequences are highly divergent and can have <25% amino acid identity between plasmid types (**Figure 3**). VirE3 and VirD5 also differ in sequence between plasmid types.

6. TRANSCRIPTOMICS

In the context of evolutionary genomics, analysis of gene content and sequences provides a first look at diversity. However, to link such genetic variation to functions, examination of

gene expression is critical. Earlier transcriptomic studies have informed on the virulence of agrobacteria, such as how C58 responds to salicylic acid, a plant-derived hormone associated with immunity (139). Findings also highlighted the role of an acidic environment, hypothesized to mimic rhizosphere conditions, in reprogramming C58 gene expression and demonstrated a key role of ExoR, a regulator whose repression is alleviated upon degradation in acidic conditions (37, 129, 140). Multiple surveys in the mid-2010s have also associated virulence with small noncoding RNAs, a subset of which regulate gene expression (17, 60, 77, 97, 125).

However, few studies have capitalized on the diverse agrobacterial strains to identify conserved transcriptome changes to inform on core traits essential for host infection. Also, examination of differential expression regulation could provide clues into functions that contribute to differences in host specificity and variations in virulence. Such an approach requires transcriptome changes to be framed relative to genome differences to draw conclusions. A recent study compared transcriptome changes of strains C58 and 1D1609 (BV1-G7) grown in culture with and without acetosyringone, a virulence gene inducer (35). These two strains were selected because they exhibit different infection efficiencies in a host species-dependent manner. Intriguingly, although most of the genes that were induced by acetosyringone are located on pTi, nearly all the repressed genes are located on the chromosome and chromid. Moreover, homologs with the same expression pattern account for <50% of those differentially expressed genes. These findings suggest that phenotypic variation may involve divergence in both gene content and expression.

7. HOST RANGE

Pathogen host range is a frequently invoked but loosely defined concept influenced by biological and technical factors (79). A host can be defined based on showing disease in natural or field settings or following artificial inoculations. For agrobacteria, a host can be defined based on transformability in the laboratory. If properly contextualized, any criterion is reasonable. But selection has shaped host specificity only in natural/field settings and approaches that use genomic data to study host specificity will be more effective if this criterion is used. The taxonomic rank of the pathogen must also be properly contextualized. Agrobacteria, collectively, as well as BV1, have a broad host range. Members have been isolated from diseased tissues of diverse plant species, and many strains tested in laboratory settings can cause disease or transform several plant species (11). However, some lineages of agrobacteria may have a limited host range. BV3 strains have been repeatedly cultured from grapevine only (88). But some strains, when inoculated, can induce gall formation on tobacco and tomato and are thus confusingly referred to as having a wide host range (50). It is important to note that grapevine itself is not restricting interactions to BV3 strains only, as pathogenic BV2 strains have also been cultured from diseased grapevines (120). Other lineages such as those in BV1-like are also considered more restricted in host range, although conclusions may be impacted by the limited breadth of sampling (54). Lastly, even for a particular host plant species, cultivar specificity has also been observed (43, 50).

Notwithstanding the vagueness of its definition, the host range of agrobacteria is predicted to be influenced by chromosomal and plasmid-encoded genes necessary for host perception (61, 86). The mobilization of oncogenic plasmids confounds the ability to strongly draw conclusions, but there are patterns hinting at the role of plasmids in microbial and plant specificity (120, 121). Some plasmid types are widespread among agrobacteria, such as type I pTi, which is found in both BV1 and BV2 (**Figure 1**). Others are more limited. Types II and III pTi and type I pRi have been found almost exclusively in BV1, whereas types II and III pRi have been found only in BV2. Types IV.a, IV.b, and V pTi are so far limited exclusively to BV3. However, subtype IV.c pTi has been

found in BV2. Similarly, oncogenic plasmids show patterns for kinds of plant host. Type I pTi and types II and III pRi were found primarily in strains isolated from woody plants, whereas type III pTi and type I pRi were found primarily in strains isolated from herbaceous plants. Early work also showed the host range of a grapevine-limited BV1 strain could be extended by replacing its oncogenic plasmid with a type II pTi from another BV1 strain, suggesting its original pTi was the limiting factor (71). In other studies, transfer of a pTi from limited host range BV3 into a BV1 strain cured of its native pTi led to restricted virulence in grapevine (109, 136). The limited host range of this pTi is likely explained by the lack of a functional cytokinin biosynthesis gene from the T-DNA and *virA*/*virC*. Similarly, cytokinins synthesized in agrobacterial cells demonstrably modulate tumorigenesis efficiency in a host-dependent manner (42).

Microbe-associated molecular patterns (MAMPS): molecules, typically derived from conserved genes of microbes, with potential to be perceived by pattern recognition receptors of the host

FLS2: a plant-encoded leucine-rich receptor–like kinase involved in pattern recognition of bacterial flagellin

Recent results from genome analyses of strains seemingly limited to *Lippia* hosts also support oncogenic plasmids impacting host range (3, 41, 120, 121). The three strains examined are members of BV1 and BV2-like, but they each have unique and unusually large oncogenic plasmids. Why these plasmids influence host range is unclear, as they and several others like them are simply mosaics. Plausible explanations are that mosaics have unique compositions of virulence genes and/or expression of their genes is misregulated. Massive scrambling of genomes has been implicated in causing misregulated gene expression, increasing virulence, and narrowing host range (95). Expression differences in *vir* genes have been implicated in affecting the host range of agrobacteria (8).

Plant immunity may influence the host range of agrobacteria (135). Pattern-triggered immunity (PTI) perceives microbe-associated molecular patterns (MAMPs) and elicits responses that suppress pathogen growth (47). One of the better-known MAMPs is flg22, which is derived from bacterial flagellin and elicits FLS2-dependent PTI (22). The flg22 sequence of C58 differs from that of other phytopathogens and evades detection by several plant species (22). However, some plant species, such as wild grape, encode a variant of FLS2 that can bind C58 flg22 and dramatically reduce transient transformation rates and prevent significant visible disease symptoms (24). It would be valuable to examine allelic variation of MAMP-encoding genes and test whether the FLS2 variant of wild grape confers immunity to different agrobacteria, especially members of BV3. With the knowledge that PTI limits transformation, approaches have been developed to use hosts lacking functional MAMP receptor-encoding genes or conditions that compromise PTI to increase transformation efficiency (118, 142).

Microbes deploy toxins and proteins to blunt PTI and give populations opportunities to establish in plants. BV1 strains are hypothesized to secrete effectors, such as VirF, via the T4SS (26). Many other Gram-negative bacteria use a type III secretion system (T3SS) to deploy effectors (7). Because BV1 strains lack a T3SS-encoding locus, researchers ingeniously engineered one strain to express and deliver T3SS-associated effectors derived from *Pseudomonas syringae* (98). The engineered strain elicited lower relative expression of defense-associated genes in *Arabidopsis* and had higher rates of transformation in multiple plant species. Unlike BV1, BV2 strains have a T3SS-encoding locus (15, 106). Whether the T3SS of BV2 strains is used during infection remains unknown, as do the identities of its effector genes.

Theory predicts that if agrobacteria deploy effectors to dampen PTI, they may elicit another form of inducible immunity. Effector-triggered immunity (ETI) occurs in plants that encode resistance proteins that perceive cognate microbial effectors (47). ETI is often associated with a programmed cell death called the hypersensitive response. Agrobacterial inoculations have been associated with necrosis in some plants (128), but whether these are indicative of ETI is unclear. At least for nonpathogenic BV3 strain F2/5, genes associated with eliciting necrosis were not predicted to encode an effector (141).

8. ECOLOGICAL INTERACTIONS: THE TYPE VI SECRETION SYSTEM

Agrobacteria likely compete with other bacteria throughout their natural life cycle. During attachment, cells may compete for access to plant wounds. After infection, transformed tissues are a rich source of opines and other nutrients (16) and are inhabited by diverse bacteria species (21, 25), including nonagrobacterial species that can catabolize opines (85). Various mechanisms may be deployed by agrobacteria, and the type VI secretion system (T6SS) likely provides a powerful weapon to gain an advantage against competitors.

The T6SS delivers effectors into the extracellular milieu or adjacent cells (13, 116) and T6SS-encoding loci are broadly distributed among Proteobacteria (104). Depending on the biochemical functions and destination of secreted effectors, T6SS can function in metal acquisition, antihost, or antibacterial activities (33, 66). In the case of interbacterial competition, effector genes are necessarily paired with cognate immunity genes to prevent self-intoxication.

Molecular studies have been reported for diverse agrobacteria. The primary T6SS gene cluster is present in many BV1 and BV3 strains but absent in BV2 (131) (**Figure 1*a***). This cluster is conserved in sequence and composition. It consists of an *imp* operon encoding the main T6SS components and an *hcp* operon encoding other components such as effectors and the cognate immunity proteins (10, 130, 131, 133). T6SSs of BV1 strains have been shown to give cells a growth advantage in culture and in planta when competing against other agrobacteria or other species of bacteria (73). In most tested strains, the T6SS is regulated in part by ChvG/ChvI (73, 110, 129). However, some strains show different patterns of regulations, suggesting that the T6SS may be activated by different environmental signals (131).

Susceptibility of agrobacteria to T6SS attack has also been analyzed in a phylogenetic and ecological framework. Outcomes differ in intraspecies or interspecies contexts, suggesting the importance of genetic relatedness in interbacterial competition (130). Although the strain-pairs competing at intergenomospecies levels exert antagonism, those competing at intragenomospecies levels tend to exhibit no or minor fitness costs regardless of effector-immunity incompatibility. It is possible that different agrobacteria vary in T6SS susceptibility. Indeed, a screen using mutants in the *Escherichia coli* Keio collection (5) as prey cells identified several genes that affect susceptibility to T6SS attacks by C58 (68). It is also notable that environmental factors influence T6SS-mediated interactions. Prey cells tend to be more susceptible when competing in a nutrient-poor environment (138).

Notably, activation of the T6SS and T4SS may be coordinated. In C58, T6SS is activated by acidity, whereas T4SS is activated upon sensing phenolics, sugar, and/or acidity, with the signals enriched in plant wounding sites and the apoplast. However, the acid activation of T6SS is not universal in all T6SS-harboring agrobacteria (131). Interestingly, although T6SS secretion is activated at pH 5.5, a T4SS inducer represses T6SS activity (129). The significance and conservation of this phenolics-repressed T6SS secretion remain unknown. Nevertheless, this observation suggests that the activities of agrobacterial T6SS and T4SS may be coordinated spatially and temporally during infection.

The role of the T6SS in pathogenesis requires further investigations to understand disease ecology. Strains lacking a functional T6SS can induce crown gall formation at wild-type levels when inoculated directly on wounded tomato stems and potato tuber discs (133). When inoculated into soil, disease incidences on tomato stems/roots caused by two T6SS mutants were significantly lower than wild-type C58 (117). Nevertheless, the composition of the bacterial communities in crown galls was similar regardless of whether their development was triggered by a T6SS mutant or wild-type strain (117). Thus, T6SS-mediated competition may have an early role in antagonizing competing bacteria prior to transformation. Once crown galls have formed, T6SS activity does not appear to shape their microbiota. This is consistent with findings from a Tn-Seq screen

Tn-Seq: transposon sequencing; a method that couples high-throughput sequencing with transposon mutagenesis to infer genes with fitness effects

in crown galls, in which T6SS genes were not among those identified as compromising the fitness of agrobacteria (111).

9. CONCLUSIONS

Agrobacteria are rare among living organisms in having the capacity to directly transfer DNA across biological domains. These bacteria reprogram plant growth to construct a new ecosystem, provide new and greater sources of nutrients, and shape microbial communities. This ability is driven by cooperation between genes in agrobacteria chromosomes and oncogenic plasmids. Recent efforts in evolutionary genomics have documented tremendous diversity among agrobacteria and their plasmids, inferred processes that have shaped them, and placed findings in a framework. This helps to contextualize current understanding and provide new directions of research on bacteria empowered by plasmids to have the remarkable capacity to genetically modify plants.

SUMMARY POINTS

1. It is crucial to study agrobacteria based on a phylogenetic framework.
2. More than 300 agrobacterial strains and oncogenic plasmids have been sequenced, classified, and placed in an evolutionary context.
3. Agrobacteria are a polyphyletic group and some have been more intensively studied than others.
4. Diverse plasmids have influenced the independent emergence of multipartite genome structure and virulence.
5. Oncogenic plasmids are genetic reservoirs that have the capacity to broadly mobilize traits as well as shuffle and generate new gene combinations.
6. Despite their potential to rapidly diversify, most extant oncogenic plasmids exhibit similar gene organization and composition and can be classified into a few types.
7. A large proportion of knowledge regarding mechanisms of agrobacterial virulence is based on the study of a few strains and oncogenic plasmids.
8. The type VI secretion system (T6SS) influences the ecology of agrobacteria by mediating interbacterial competition.

FUTURE ISSUES

1. The need to effectively communicate about these bacteria regarding taxonomy and historical records while maintaining flexibility to keep pace with an evolving understanding of their biology remains to be resolved.
2. To better understand variation in genome content and structure, the genome assemblies of strains representing each lineage need to be completed.
3. The roles of chromosomal and plasmid-associated genes in determining host range remain to be better characterized.
4. The natural variation in gene content and sequences can be leveraged to study mechanisms of virulence.

5. Improvement of transformation in different plant species and cultivars may be achieved by exploring the diversity of agrobacterial strains and plasmids.
6. The roles of nononcogenic plasmids in agrobacterial fitness remain to be investigated.
7. The ecological roles of the type VI secretion system (T6SS) in agrobacteria–plant interactions remain to be inferred.
8. The evolution and divergence of BV3 agrobacteria remain to be better studied.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank the members of our laboratories for their discussions and insights that contributed to this review. The A.J.W. laboratory is supported in part by start-up funds from the Department of Botany and Plant Pathology, Oregon State University. The J.H.C. laboratory is supported in part by the National Institute of Food and Agriculture, US Department of Agriculture (2022-67013-36883). The E.-M.L. and the C.-H.K. laboratories are supported in part by the National Science and Technology Council of Taiwan and Academia Sinica (AS-GC-111-L02). Y.W. is supported by the Taiwan International Graduate Program. The funders had no role in manuscript preparation or the decision to publish.

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Annual Review of Phytopathology

Volume 61, 2023

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Errata

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