AGROBACTERIUM BIOLOGY AND ITS APPLICATION TO TRANSGENIC PLANT PRODUCTION

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AGROBACTERIUM BIOLOGY AND ITS APPLICATION TO TRANSGENIC PLANT PRODUCTION

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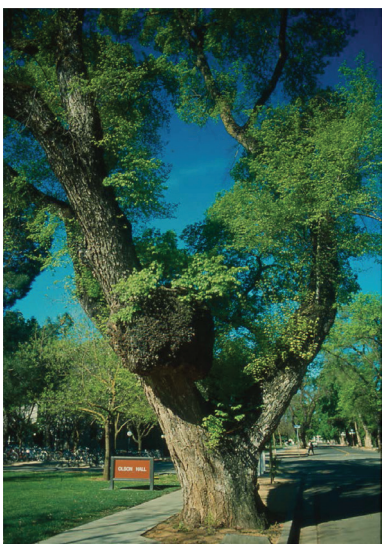

Crown gall on Elm tree (Photo by Dr. Jer-Ming Hu at UC Davis campus).

The broad host range pathogenic bacterium Agrobacterium tumefaciens has been widely studied as a model system to understand horizontal gene flow, secretion of effector proteins into host cells, and plant-pathogen interactions. Agrobacterium-mediated plant transformation also is the major method for generating transgenic plants for research and biotechnology purposes. Agrobacterium species have the natural ability to conduct interkingdom genetic transfer from bacteria to eukaryotes, including most plant species, yeast, fungi, and even animal cells. In nature, A. tumefaciens causes crown gall disease resulting from expression in plants of auxin and cytokinin biosynthesis genes encoded by the transferred (T-) DNA. Gene transfer from A. tumefaciens to host cells requires virulence (vir) genes that reside on the resident tumor-inducing (Ti) plasmid. In addition to T-DNA, several Virulence (Vir) effector proteins are also translocated to host cells through a bacterial type IV secretion system. These proteins aid in T-DNA trafficking through the host cell cytoplasm, nuclear targeting, and T-DNA integration. Genes within native T-DNAs can be replaced by any gene of interest, making Agrobacterium species important tools for plant research and genetic engineering. In this research topic, we provided updated information on several important areas of Agrobacterium biology and its use for biotechnology purposes.

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Editorial: "*Agrobacterium* biology and its application to transgenic plant production"

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Keywords: *Agrobacterium*, plant genetic transformation, T-DNA, crown gall, membrane lipid, biofilm, quorum sensing, plant defense

The extraordinary *Agrobacterium* research story started from the search for the causative agent of crown gall disease more than 100 years ago. *Agrobacterium tumefaciens* was first isolated from grapevine galls in 1897 and later isolated from Paris daisy in 1907 (Cavara, 1897a,b; Smith and Townsend, 1907). The *Agrobacterium* infection mechanism involves processing and transfer of a specific DNA fragment (the transferred-DNA, T-DNA) from a bacterial tumor-inducing (Ti) plasmid. Transfer to the plant occurs via a type IV secretion system (T4SS), after which T-DNA is integrated into the plant host genome (Gelvin, 2010; Lacroix and Citovsky, 2013). This interkingdom DNA transfer leads to overproduction of the plant hormones auxin and cytokinin, resulting in tumors. The interkingdom DNA transfer ability of *Agrobacterium* and the possibility to replace the oncogenes in the T-DNA with genes of interest has made *Agrobacterium*-mediated transformation the most popular technique to generate transgenic plants.

This Research Topic provides a collection of reviews and original research articles on *Agrobacterium* genes involved in bacterial physiology/virulence and plant genes involved in transformation and defense against *Agrobacterium*. A review by Kado (2014) provides a historical overview of how *A. tumefaciens* was first established as the cause of crown gall disease. In this review, Kado highlights key early plant pathology and milestone molecular biology studies leading to the conclusion that the expression of oncogenes in native T-DNA is the cause of tumor growth in plants. With the solid foundation of these pioneering discoveries, *A. tumefaciens* evolved from a phytopathogen to a powerful genetic transformation tool for plant biology and biotechnology research.

The first complete genome sequence of an *Agrobacterium* species (*A. tumefaciens* C58) was completed in 2001 (Goodner et al., 2001; Wood et al., 2001). The 5.67-megabase genome of this strain carries one circular chromosome, one linear chromosome, and two megaplasmids: the Ti plasmid pTiC58 and a second plasmid, pAtC58. In the review by Platt et al. (2014), the properties, ecology, evolution, and complex interactions of these two *A. tumefaciens* megaplasmids are discussed. The costs and benefits to *A. tumefaciens* strains carrying the Ti plasmid and/or the pAtC58 plasmid are discussed and presented from an ecological and evolutionary perspective. Modeling predictions are presented for the relative cost and benefits to *A. tumefaciens* strains harboring the Ti and/or the pAtC58 plasmids determined by environmental resources. Conjugation and amplification of the Ti plasmid are regulated by the TraI/TraR quorum-sensing (QS) system and conjugal opines. Lang and Faure (2014) review current knowledge of the genetic networks and molecular basis of the *A. tumefaciens* quorum sensing system. These authors also discuss the biological and ecological impact of the QS system on Ti plasmid conjugation, copy number, and interactions between *Agrobacterium* and host plants.

During the initial interaction between *Agrobacterium* and plant cells, bacteria sense various plant-derived signals in the rhizosphere with the help of Ti plasmid-encoded virulence gene (*vir* gene) and chromosomal virulence gene (*chv* gene) products. The current knowledge of how *A. tumefaciens* senses and reacts to different plant-derived signals are summarized in the review

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article by Subramoni et al. (2014), which also discusses the mechanisms of how the plant hormones auxin, salicylic acid, and ethylene, affect bacterial virulence. Finally, this review discusses the complexity and intricacy of *Agrobacterium* signaling pathways and the underlying regulatory mechanisms during the initial host cell recognition to maximize subsequent successful infection. In the original research article by Lin et al. (2014), the mechanistic regulation of the membrane sensor VirA protein is further dissected. VirA histidine kinase and the cytoplasmic response regulator VirG protein together play a central role in regulating *vir* gene expression in response to phenolics. Based on a homology model of the VirA linker region, various mutant and chimeric VirA proteins were generated and examined for their ability to induce *VirB* promoter activity. The ability of VirA to sense and respond to three separate input signals, phenolics, sugars, and environmental pH, plays a significant role in securing successful infection.

Agrobacterium attachment to plant cells is an important early step in crown gall disease progression. Motile bacteria swim toward host cells and then physically interact with host cells to form aggregates and establish a multicellular bacterial community known as a biofilm. Various genetic and environmental factors that affect *Agrobacterium* attachment and biofilm formation are reviewed in the article by Heindl et al. (2014). The functions of different types of exopolysaccharides that constitute the biofilm and underlying mechanisms involving how the second messenger cyclic-di-GMP, the ChvG/ChvI system, phosphorus levels, and oxygen tension influence bacterial attachment and virulence are also summarized. In the review article by Matthysse (2014), early studies and current knowledge of the mechanisms of polar and lateral bacterial attachment are summarized. These two mechanisms both contribute to bacterial attachment. When the environmental calcium and phosphate levels and pH values are low, polar attachment predominates. In addition, the phospholipids (PLs), phosphatidylcholine (PC), and phosphate-free lipid ornithine lipids (OLs) contribute to *Agrobacterium* virulence. In the review by Aktas et al. (2014), the biosynthetic pathways and the physiological roles of these membrane lipids are summarized. The typical eukaryotic membrane lipid PC is not frequently found in bacteria, but it constitutes almost 22% of the *Agrobacterium* membrane lipid. Interestingly, PCs and OLs may play opposite roles in *Agrobacterium* virulence. The reduction of tumor formation in a PC-deficient *Agrobacterium* mutant may result from impaired *vir* gene expressions controlled by VirA/VirG. The absence of OLs in *A. tumefaciens* may decrease host defense responses and therefore cause earlier and larger tumor formation.

Plant cells have a variety of receptors that recognize so-called microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs), and subsequently activate plant defense responses, a process known as Pattern-recognition receptor-Triggered Immunity (PTI) (Boller and Felix, 2009; Boller and He, 2009). *Agrobacteriium* may utilize effectors to hijack plant systems and evade plant defense responses. Pitzschke (2013) reviews strategies used by *Agrobacterium* to turn plant defense responses to its own advantage. Infected plant cells initiate a mitogen-activated protein kinase signaling cascade that causes VIP1 (*Agrobacterium* VirE2-interacting protein 1) phosphorylation and translocation into the plant nucleus to induce defense gene expression. On the other hand, *Agrobacterium* may hijack VIP1 to help T-DNA enter the plant nucleus. Based on the current knowledge of plant defense responses against *Agrobacterium* infection, Pitzschke (2013) discusses several biotechnological approaches to increase transformation efficiency. In another review by Gohlke and Deeken (2014), early plant responses to *Agrobacterium*, including various defense responses, hypersensitive responses, and phytohormone level alterations are discussed. The alterations in plant morphology, nutrient translocation, and metabolism caused by crown gall tumor formation are also reviewed. The authors summarize important genomic, epigenomic, transcriptomic, and metabolomic studies that reveal epigenetic changes associated with T-DNA integration and gall development. Subsequently, Hwang et al. (2015) review important pathogenic elicitors, host cell receptor molecules, and their downstream signal transduction pathways in host plants during the PAMP-triggered immune response. They highlight recent discoveries linking plant immunity to endomembrane trafficking and actin dynamic changes. Effects of both the host physiology, including hormone levels, circadian clock, developmental stages, and environmental factors, including light exposure lengths and temperature, on plant defense responses and bacterial virulence are reviewed and discussed.

In nature, evidence of ancient horizontal gene transfers (HGT) from *Agrobacterium* to plants has been observed in the genera *Nicotiana* and *Linaria*. Sequences homologous to mikimopine-type *Agrobacterium rhizogenes* pRiA4 T-DNA were first discovered in the genome of untransformed tree tobacco, *Nicotiana glauca*, and named "cellular T-DNA" (cT-DNA; White et al., 1983). Matveeva and Lutova (2014) review cT-DNA organization, distribution, expression regulation, and a possible correlation with genetic tumor formation in *Nicotiana* species. They also review recent findings of cT-DNA in the genomes of *Linaria* species and in other dicotyledonous families. The authors suggest that plants maintaining cT-DNA in their genomes may potentially benefit microorganisms in the rhizosphere by secreting opines in the root zone. They also propose that footprints of ancient pRi T-DNA insertions in the plant genome may provide selective advantage to these plants.

With this Research Topic we provide a platform for scientists to share their understanding of *Agrobacterium* biology and how *Agrobacterium* transforms plants. These contributions demonstrate how a highly active research community in plant and microbial sciences can elucidate important pathogenesis questions. Future research on *Agrobacteium* will continue to advance our understanding of plant-pathogen interactions, and provide new insights useful for plant genetic engineering.

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Historical account on gaining insights on the mechanism of crown gall tumorigenesis induced by *Agrobacterium tumefaciens*

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The plant tumor disease known as crown gall was not called by that name until more recent times. Galls on plants were described by Malpighi (1679) who believed that these extraordinary growth are spontaneously produced. *Agrobacterium* was first isolated from tumors in 1897 by Fridiano Cavara in Napoli, Italy. After this bacterium was recognized to be the cause of crown gall disease, questions were raised on the mechanism by which it caused tumors on a variety of plants. Numerous very detailed studies led to the identification of *Agrobacterium tumefaciens* as the causal bacterium that cleverly transferred a genetic principle to plant host cells and integrated it into their chromosomes. Such studies have led to a variety of sophisticated mechanisms used by this organism to aid in its survival against competing microorganisms. Knowledge gained from these fundamental discoveries has opened many avenues for researchers to examine their primary organisms of study for similar mechanisms of pathogenesis in both plants and animals. These discoveries also advanced the genetic engineering of domesticated plants for improved food and fiber.

Keywords: Ti plasmid, *Agrobacterium*, T pilus, T-DNA, type IV secretion system, type VI secretion system, opines, conjugative transfer

INTRODUCTION

Crown gall is a name given to abnormal tumor-like growths often observed at the base of the trunk and roots of trees, grapevines, and woody plants. The nature of the cause of crown gall was unknown before 1897. Not referenced by many authors who worked on this disease was the published work of Fridiano Cavara (**Figure 1**). He described in detail the galls formed at the base of grapevines that were in the Royal Botanical Gardens of Napoli (Naples), Italy. More importantly, he also described the isolation of a bacterium that he showed caused similar tumors on young grapevines. This work was published in Le Stazioni Sperimentale, Agrari Italiane (Cavara, 1897a,b; **Figure 2**). In 1904, George C. Hedgcock reported the isolation of a causal bacterium from grapevine galls that he described in a US Department of Agriculture Bureau of Plant Industry bulletin (Hedgcock, 1910, p. 21; **Figure 3**). His monograph remains not frequently cited. Most cited as allegedly the first to isolate the causal bacterium was Smith and Townsend (1907). The authors named the causal organism *Bacterium tumefaciens*. E. F. Smith had visited Cavara in Naples and learned how to isolate the causal bacterium from grapevine galls (Rodgers, 1952). He and C. O. Townsend then published the isolation of the crown gall causing bacterium from chrysanthemum. Smith worked extensively on the disease and showed that *B. tumefaciens* can induce gall formation in a number of herbaceous plants (Smith, 1911b). Subsequently, the name *B. tumefaciens* was changed briefly to *Pseudomonas tumefaciens* (Duggar, 1909) and then to *Phytomonas tumefaciens* (Bergey et al., 1923), followed by *Polymonas tumefaciens* (Lieske, 1928), and to *Agrobacterium tumefaciens* (Conn, 1942). The varying phases of the life cycle of *P. tumefaciens* were described by Stapp and Bortels (1931).

In France, Fabre and Dunal (1853) named the tumors observed on diseased grapevines as "broussin." Dornfield (1859) called the galls found on grapevines in Germany as "Grind," but the gall disease was also called "Ausschlag," "Mauche," "Krebs," "Kropf," "Raude," and "Schorf." In Italy, the gall disease on grapevines was called "rogna" (Garovaglio and Cattaneo, 1879) and "tubercoli" (Cavara, 1897a,b). In the United States, the gall disease observed on grapevines was called "black-knot" (Galloway, 1889) and likewise in Canada (Fletcher, 1890). Other names such as tubercular galls were applied to this tumorous disease that had become recognized throughout the continents wherever grapevines and woody crops were cultivated.

Eventually, nurserymen, farmers, viticulturalists, etc., became aware of the gall producing disease that occurred at the base of trees and vines near the junction of the roots to the trunk, known to these growers as the "crown," the term "crown-gall" became the common name used to recognize the tumor-forming disease.

SEARCH FOR THE AGENT THAT CAUSED CROWN GALL

Once *A. tumefaciens* was established as the cause of crown gall, the quest was initiated for the mechanism by which this pathogen induced tumors in plants. It was widely known that *A. tumefaciens* induces tumors readily by mechanical inoculation of many different plant species. Eventually, over 90 families of plants were found to be susceptible to Crown Gall disease incited by this

FIGURE 1 | Fridiano Cavara.

bacterium (Kado, 2010). In Nature, however, crown gall is found mainly on woody plants such as stone fruit trees of the genus *Prunus* and other members of the Rosaceae (rose) family, members of the Vitaceae (grape), and members of the Juglandaceae (walnut) family. There are at least 41 families of plants found to be naturally infected by *A. tumefaciens* (Kado, 2010). Experimental inoculations with *A. tumefaciens* on susceptible herbaceous plants have provided excellent opportunities to study in detail the timing of cellular transformation and the process of tumor formation.

Three schools of thought on the cause of crown gall were proposed. (1) *A. tumefaciens* caused tumors by producing one or more irritating chemicals that promoted tumor formation. (2) The phytohormone auxin was believed to play a central role in tumor formation and development. (3) Plant hosts were conditioned by *A. tumefaciens* to initiate and promote tumor formation by a tumor-inducing principle (Braun and Mandle, 1948).

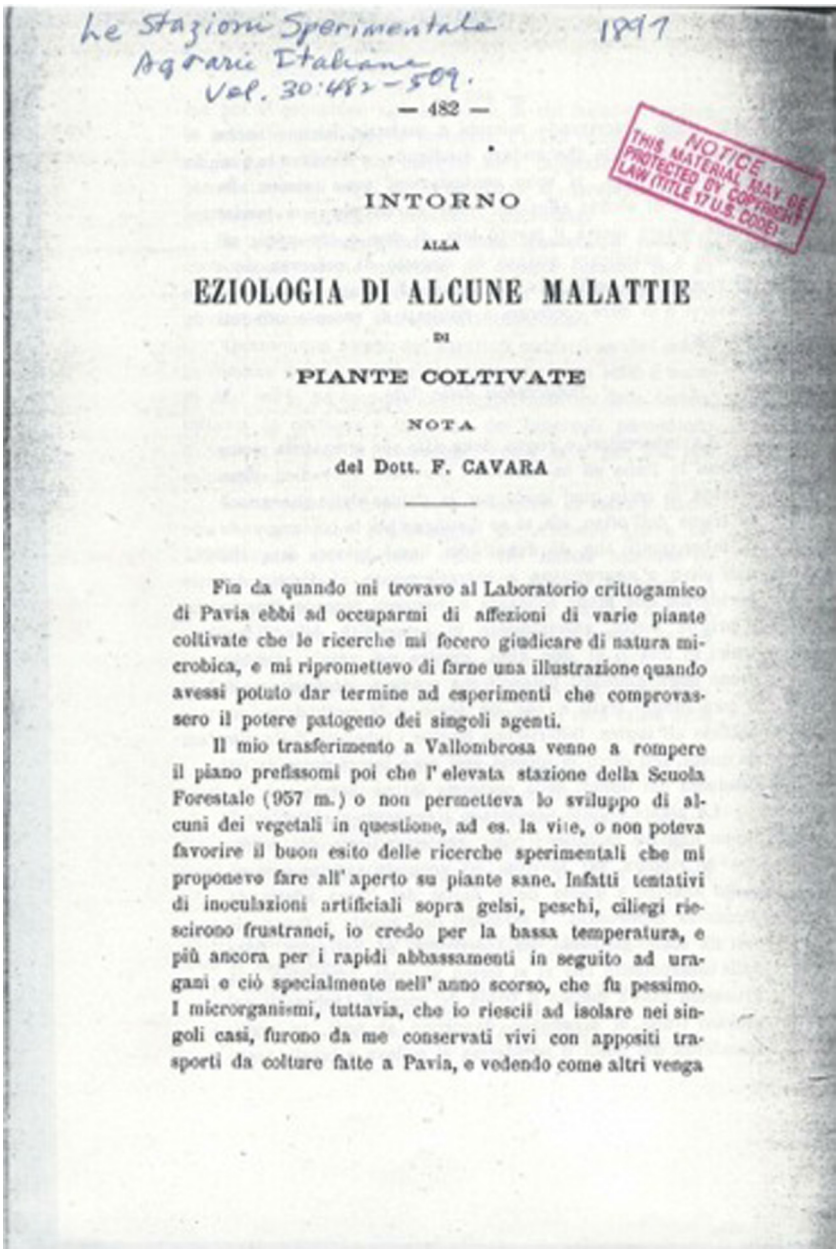
A. TUMEFACIENS PRODUCES CHEMICAL IRRITANTS THAT LED TO TUMOR FORMATION IN PLANT HOSTS

Normally, plant cells grow, develop, and multiply under stringent control. There is a mutual balance and restraint to maintain cellular order and differentiation. On the other hand, crown gall cells multiply and give rise to tissues that are not self-limiting and tax the surrounding cellular community of their energy and resources. So, the question arose among many researchers of that era, what is it that gives crown gall cells these perverse properties? In the medical field, at that early period of cancer research, it was believed that cancer was caused by some forms of external irritants. In fact, analogies between human sarcoma and crown gall were put forth by Smith (1911a; **Figure 4**).

In 1917, Smith used castor bean (*Ricinus communis* L.), a member of the Euphorbiaceae (spurge family), as the host for *A. tumefaciens* (called at that time *B. tumefaciens* Sm. and T.) to determine the mechanism of crown gall tumor growth (Smith, 1917). After a large number of tests both physical and chemical, Smith hypothesized that "dilute ammonia causes intumescences and have rendered it probable that ammonia liberated within the cell in small quantities by the imprisoned bacteria must be one of the causes of excessive and abnormal cell proliferation in crown gall." It was then thought that *A. tumefaciens* was invasive and penetrated into plant host tissues.

A. TUMEFACIENS PRODUCES PHYTOHORMONES THAT CAUSED TUMOR GROWTH

By the late 1920s, a plant growth substance named auxin (Went, 1926; **Figure 5**) was believed to play a key role in tumor growth as it was stated that "The auxin swellings bear close resemblance to the phenomena observed in some of the galls and other pathological outgrowths and there is good evidence that auxin plays an important part in such growths" (Went and Thimann, 1937). The auxin indole-3-acetic acid was found in human urine and produced by various fungi and bacteria (reviewed in Went and Thimann, 1937). Its production in plants was first confirmed in oat coleoptiles (*Avena sativa*) (Went, 1928). Subsequently, several investigators noted similarities between the reaction of plant tissues treated with indole-3-acetic acid produced by *A. tumefaciens* from tryptophan and the reaction of similar plant material inoculated with the pathogen itself (Brown and Gardner, 1936; Kraus et al., 1936; Link et al., 1937). Plant host tissue swellings and gall-like outgrowths were obtained by applying extracts from cultures of *A. tumefaciens* (then called *P. tumefaciens*) (Brown and Gardner, 1936). Using an attenuated culture of *A. tumefaciens* (then called *P. tumefaciens*), Braun and Laskaris (1942) found that the avirulent strain was capable of inducing tumors closely resembling crown gall on tomato plants when the bacteria were supplemented with either α-naphthalene acetic acid, γ-indole butyric acid, or β–indole acetic acid. These workers stated that "The discovery that synthetic growth substances were able to stimulate the development of tumors by the attenuated culture strengthened our previous belief regarding the probable role of the host growth hormones in the development of these neoplastic growths." This was somewhat contrary to the work of Locke et al. (1938) who tested an attenuated strain on decapitated tomato and *Bryophyllum* plants treated with 30 mg indole-3-acetic acid per gram of lanolin paste at the cut site and found that "… there was a slight stimulation in plants treated with the acid over untreated plants." Interestingly, these workers noticed "… the galls from virulent cultures were without chlorophyll while those from attenuated cultures were green." Based on the positive effects of phytohormones on the avirulent strain leading to tumor growth and the continued tumor growth of implanted tissue fragments from tumors initiated by the attenuated *A. tumefaciens* strain stimulated with phytohormone, Braun and Laskaris (1942) proposed that there appear to be at least two distinct phases involved in tumor formation. The first phase involves stimulation of normal cells. The second phase requires continued stimulation resulting in cellular multiplication by a growth substance, resulting in tumor formation (Braun, 1952). This premise appears to be the combination of the above two concepts, i.e., the need for a chemical irritant and the presence of phytohormones.



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NOTA

del Dott. F. CAVARA

Fin da quando mi trovavo al Laboratorio crittogamico di Pavia ebbi ad occuparmi di affezioni di varie piante coltivate che le ricerche mi fecero giudicare di natura microbica, e mi ripromettevo di farne una illustrazione quando avessi potuto dar termine ad esperimenti che comprovassero il potere patogeno dei singoli agenti.

Il mio trasferimento a Vallombrosa venne a rompere il piano prefissomi poi che l'elevata stazione della Scuola Forestale (957 m.) o non permetteva lo sviluppo di alcuni dei vegetali in questione, ad es. la vite, o non poteva favorire il buon esito delle ricerche sperimentali che mi proponevo fare all'aperto su piante sane. Infatti tentativi di inoculazioni artificiali sopra gelsi, peschi, ciliegi riescirono frustranei, io credo per la bassa temperatura, e più ancora per i rapidi abbassamenti in seguito ad uragani e ciò specialmente nell'anno scorso, che fu pessimo. I microrganismi, tuttavia, che io riescii ad isolare nei singoli casi, furono da me conservati vivi con appositi trasporti da colture fatte a Pavia, e vedendo come altri venga

FIGURE 2 | Paper by Fridiano Cavara in 1897 describing galls on grapevines from which he isolated the tumorigenic bacterium and demonstrated its gall forming activity on young grapevines.

PERMANENT AUTONOMOUS GROWTH OF CROWN GALL TISSUE *IN VITRO*: FIRST CLUES THAT A GENETIC CHANGE HAS OCCURRED

One of the most significant discoveries that have led to our current understanding of the mechanism by which *A. tumefaciens* causes crown gall was the work of White and White and Braun (1942; **Figure 6**) and Braun and White (1943). These workers showed that crown gall tumors derived from secondary tumors were bacteria-free, as determined by cultural and serological methods. This finding brought forth the idea that there was indeed some form of genetic transformation of the host plant cell that was infected by *A. tumefaciens*. Significantly, the isolated crown gall tumor tissues grew well in the absence of phytohormones (**Figure 7**). Hence, they were autonomous with respect to the need of phytohormones (auxin-autotrophic) that normal plant tissues in culture required for growth.

Further indirect evidence that a genetic transformation has taken place in crown gall is derived from the presence of rare guanidine derivatives such as octopine and nopaline in crown gall tissues. The *A. tumefaciens* strain B6 that metabolize octopine was found also to induce tumors that contained octopine (Menagé and Morel, 1964; Goldmann-Ménagé, 1971; Morel, 1972). Likewise, *A. tumefaciens* strains that metabolize

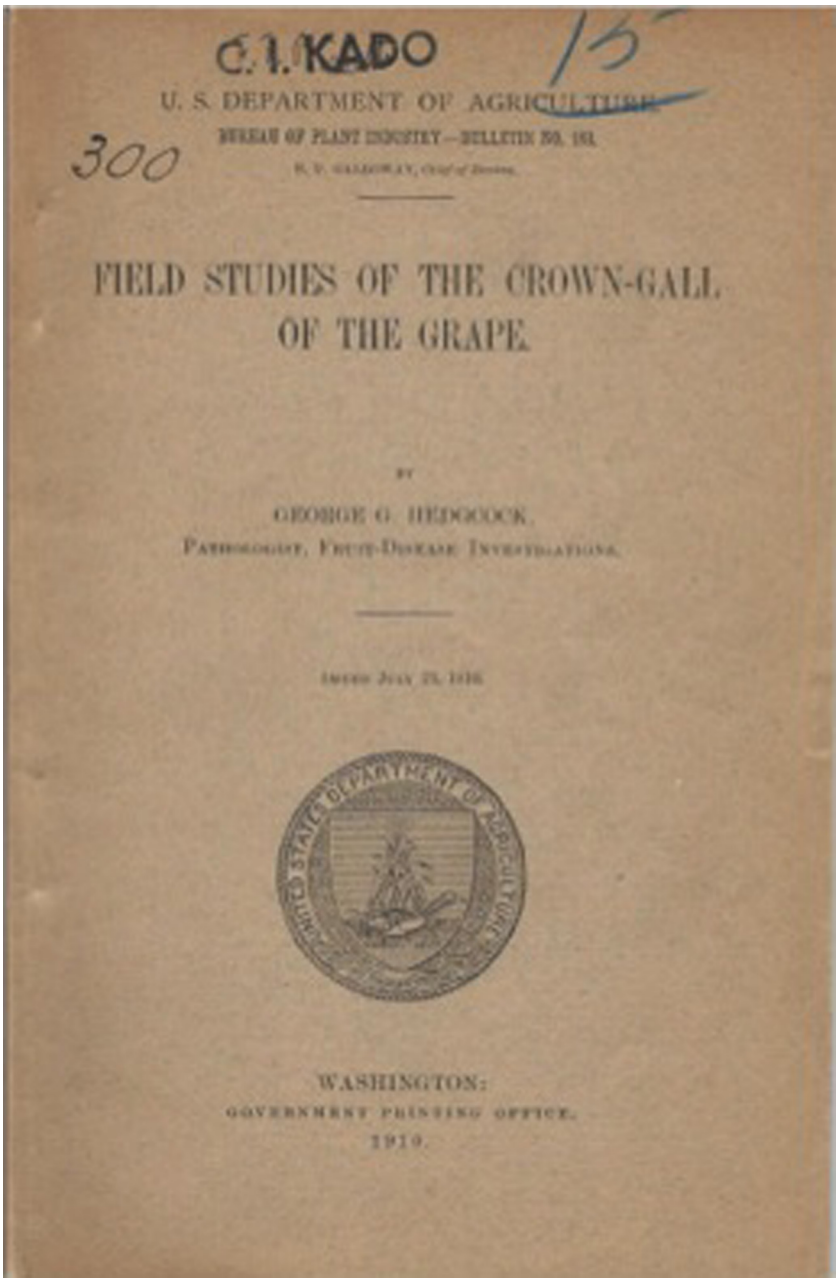
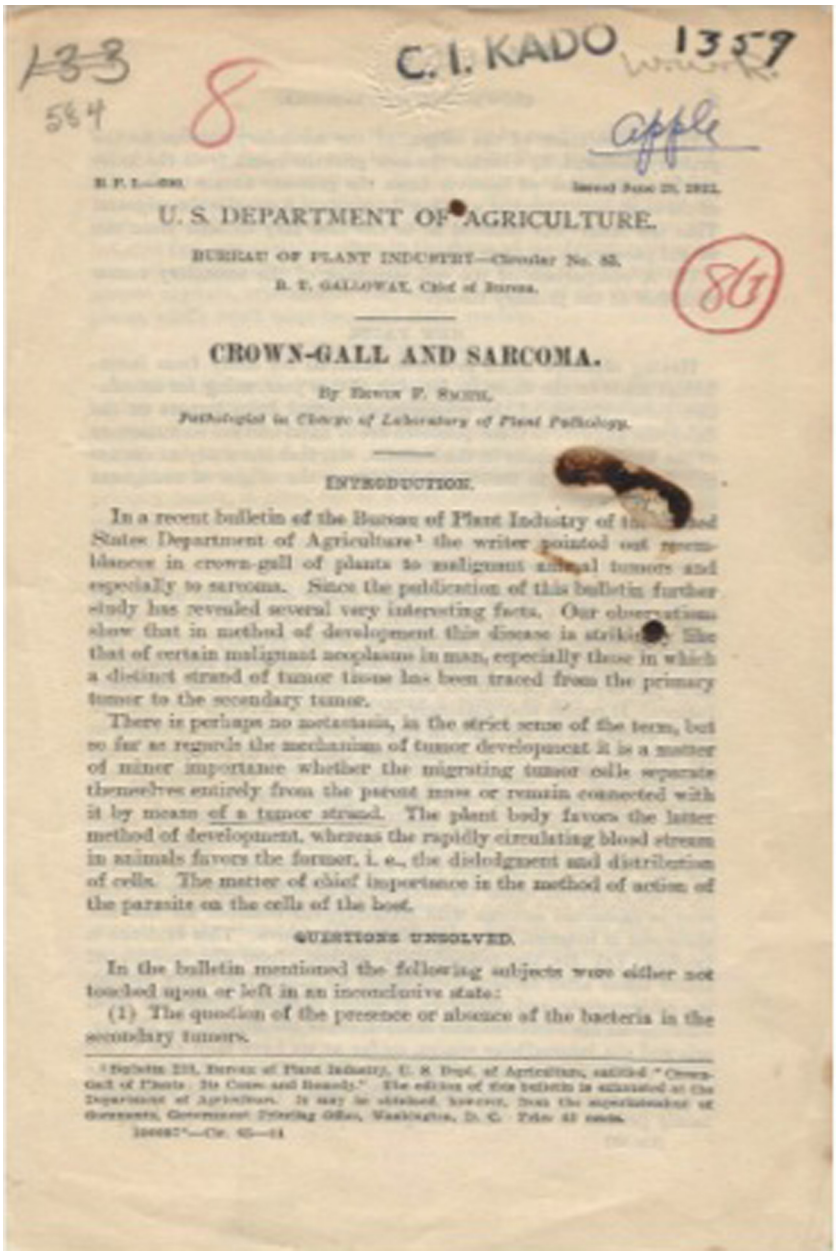


FIGURE 3 | A compendium by George Hedgcock on crown gall of grapevines published in 1910 describing his 1904 work on the isolation of crown gall producing bacterium and demonstrating tumorigenicity.

nopaline induced tumors that produced nopaline (Goldmann et al., 1969). These guanidine compounds appear to be determined exclusively by the type of *A. tumefaciens* strain used to induce crown gall and are not dependent on the plant species (Petit et al., 1970; Bomhoff, 1974). However, Wendt-Gallitelli and Dobrigkeit (1973) found octopine in habituated tobacco cells, and in the root tips of young pea and bean seedlings. These workers concluded that because of the presence of this guanidine derivative in non-transformed plant material, octopine is not exclusive to crown gall tumors. Earlier work showed that lysopine is present only in crown gall tumor tissues (Lioret, 1956). However, Seitz and Hochster (1964) found it to be produced in small amounts in normal tobacco and tomato plants. Also, Johnson et al. (1974) detected octopine in normal tobacco, sunflower, pinto bean and tobacco callus tissues. Although trace amounts of unusual guanidine compounds had been detected in the above plants, opines such as octopine and nopaline exclusively occur in crown gall tissues.

Given these suggestions that *Agrobacterium* genetically transforms plants, the idea that DNA might be transferred from



C. I. KADO 1357

apple

U. S. DEPARTMENT OF AGRICULTURE.

BUREAU OF PLANT INDUSTRY—Circular No. 83.

B. T. GALLOWAY, Chief of Bureau.

CROWN-GALL AND SARCOMA.

By ERWIN F. SMITH,

Pathologist in Charge of Laboratory of Plant Pathology.

INTRODUCTION.

In a recent bulletin of the Bureau of Plant Industry of the United States Department of Agriculture[1] the writer pointed out resemblances in crown-gall of plants to malignant animal tumors and especially to sarcoma. Since the publication of this bulletin further study has revealed several very interesting facts. Our observations show that in method of development this disease is strikingly like that of certain malignant neoplasms in man, especially those in which a distinct strand of tumor tissue has been traced from the primary tumor to the secondary tumor.

There is perhaps no metastasis, in the strict sense of the term, but so far as regards the mechanism of tumor development it is a matter of minor importance whether the migrating tumor cells separate themselves entirely from the parent mass or remain connected with it by means of a tumor strand. The plant body favors the latter method of development, whereas the rapidly circulating blood stream in animals favors the former, i. e., the dislodgment and distribution of cells. The matter of chief importance is the method of action of the parasite on the cells of the host.

QUESTIONS UNSOLVED.

In the bulletin mentioned the following subjects were either not touched upon or left in an inconclusive state:

(1) The question of the presence or absence of the bacteria in the secondary tumors.

[1] Bulletin 233, Bureau of Plant Industry, U. S. Dept. of Agriculture, entitled "Crown-Gall of Plants: Its Cause and Remedy." The edition of this bulletin is exhausted at the Department of Agriculture. It may be obtained, however, from the superintendent of Documents, Government Printing Office, Washington, D. C. Price 45 cents.

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FIGURE 4 | Paper published in 1911 by Erwin F. Smith describing the similarities and differences between crown gall and human sarcoma.

A. tumefaciens into the plant cell became a popular notion. Hence, a number of workers proposed that crown gall induction involves the transfer of bacterial DNA into plant cells (Milo and Srivastava, 1969; Quétier et al., 1969; Schilperoort, 1969; Srivastava, 1970; Srivastava and Chadha, 1970; Chadha and Srivastava, 1971; Stroun et al., 1971; Yajko and Hegeman, 1971; Heyn and Schilperoort, 1973). However, this enthusiasm was dampened when other workers failed to induce crown gall tumors by introducing purified DNA from *A. tumefaciens* into plants (Braun and Wood, 1966; Bieber and Sarfert, 1968; Stroun et al., 1971; Yajko and Hegeman, 1971).

Although bacteriophages had been found in axenically grown crown gall tissues (Tourneur and Morel, 1971), an interesting report claimed that DNA of an *A. tumefaciens* bacteriophage called PS8 was present as a plasmid in crown gall tumor cells (Schilperoort, 1969; Schilperoort et al., 1973; **Figure 8**). Also, Schilperoort (1971) found strong complementarity of *A. tumefaciens* cRNA to crown gall tissue DNA. This work could not be verified either by Eden et al. (1974), or by Farrand et al. (1975) who used DNA/RNA filter hybridization and by Chilton et al. (1974) who used renaturation kinetics in an attempt to detect bacterial and phage DNA in crown gall tumors. They stated that

they "… found no evidence for bacterial or phage DNA in the tumors examined." Drlica and Kado (1974) used DNA:DNA filter hybridization and solution enrichment techniques and found that no more than 0.02% of the crown gall tumor genome could contain *A. tumefaciens* DNA. This work left open the possibility that some traces of *A. tumefaciens* DNA might be incorporated into the plant host cell genome.

Kado and Lurquin (1976) established that exogenously added naked *A. tumefaciens* DNA to cultured tobacco cells is not stably maintained in the plant cells and nuclei. Braun and Wood (1966) found that the addition of deoxyribonuclease (DNase) at concentrations up to 5 mg/ml was completely ineffective in inhibiting tumor inception or development when the enzyme solution was applied 1–2 h prior to the time that the plants were inoculated with *A. tumefaciens* or when the bacterium and DNase were added to the wound site together. Interestingly, Braun and Wood (1966) reported that ribonuclease A (RNase) inhibited tumor formation when high concentrations (2–4 mg/ml) of the enzyme solution were applied 1–2 h prior to the time that the wound site was inoculated with *A. tumefaciens*. RNase neither affected bacterial growth, nor the virulence of the bacterium, nor the wound-healing process. These early studies suggested that the

FIGURE 5 | F. W. Went, the discoverer of auxin.

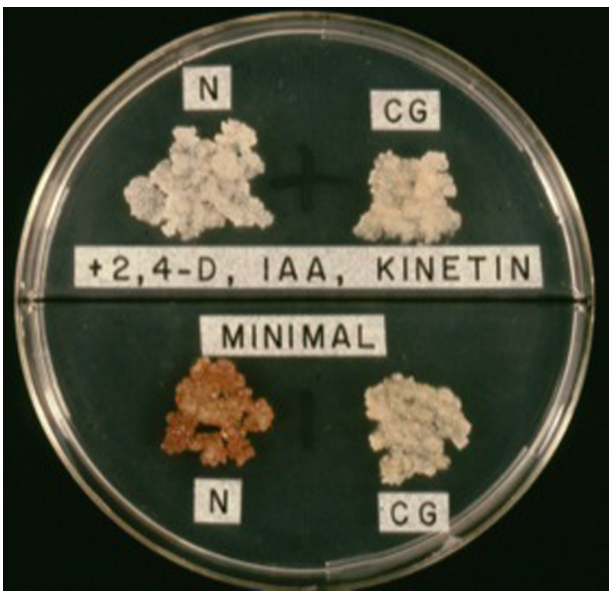


FIGURE 7 | Auxin autotrophy of crown gall tissues on hormone-free medium (lower half of bisectored petri plate). N, normal cells; CG, crown gall cells; 2,4-D, 2,4-phenoxyacetic acid; IAA, indole-3-acetic acid.

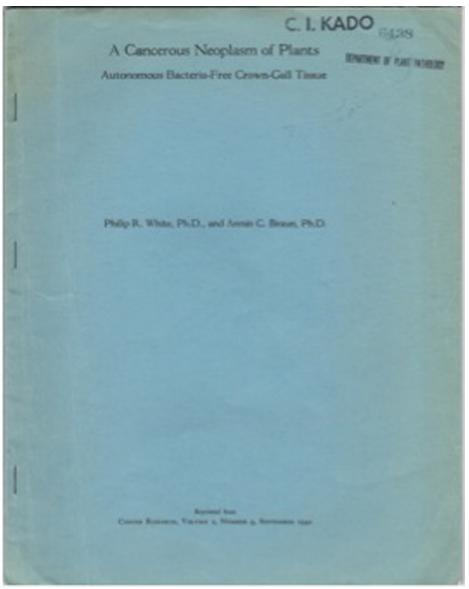


FIGURE 6 | Photo of Armin C. Braun in his greenhouse laboratory. Classic paper on auxin autonomy of crown gall tissue culture by White and Braun (1942).

FIGURE 8 | Rob Schilperoort (right), his wife (middle) with Clarence Kado (left).

bacterial DNA must gain entry into plant cells in a protective fashion. Hence, it remained possible that bacterial-specific DNA might be passed to plant cells via some form of intimate bacteria-plant cell interaction. The above studies on subjecting plant cells to naked *A. tumefaciens* DNA indicate that the release of naked DNA by *A. tumefaciens* and its uptake by plants are not the process of plant cell transformation.

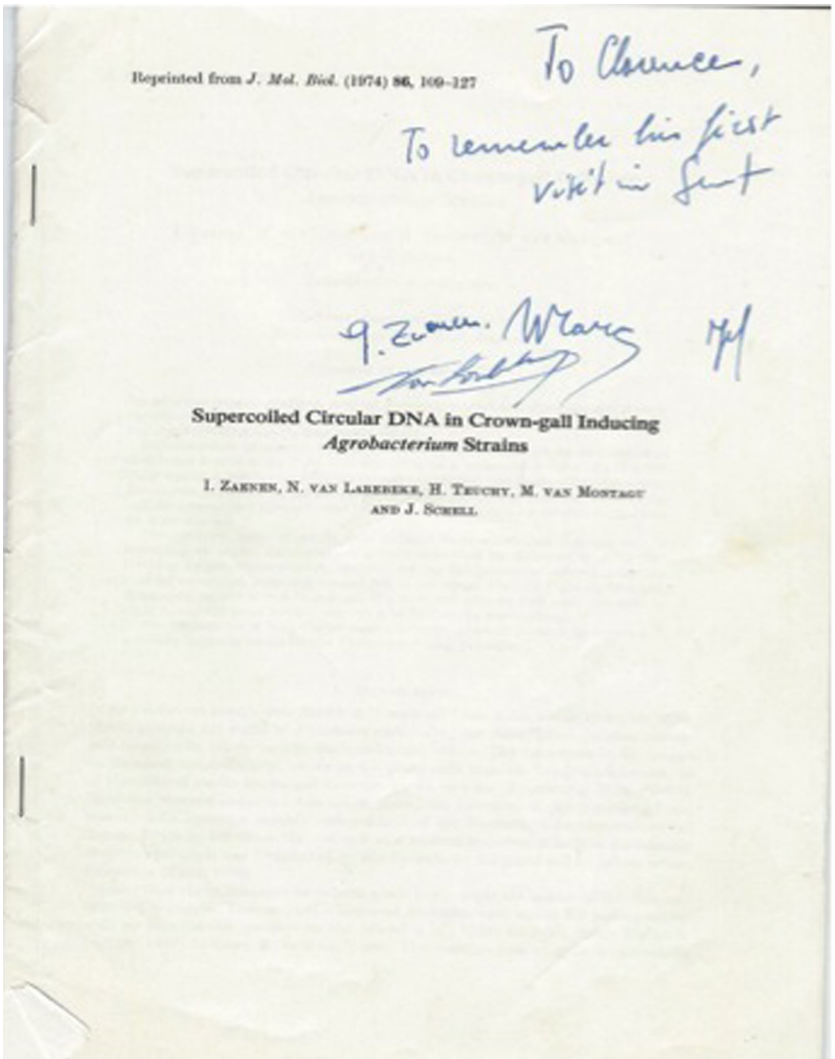
So, how is bacterial DNA transferred to plant cells? If transferred, is the DNA encapsulated or protected in some way in order to survive the transfer process? These were some of the important questions asked during that period when not much was understood about the plant-microbe interaction. Researchers began investigating how *A. tumefaciens* perceives its plant host, how it might attach to the host tissues, how it would transfer DNA and how the transferred DNA is processed in the host cells. There apparently is the absence of specific receptors on plant protoplasts onto which *A. tumefaciens* might bind and insert its DNA (Schilde-Rentschler, 1973), so binding of bacterial cells, if at all, must be at sites other than protoplastic membranes. Because wounding was required to initiate tumor formation, the plant cell wall was thought to be a barrier against effective transformation by bacteria cells. Hence, Virts and Gelvin (1985) infected *Petunia* protoplasts with *A. tumefaciens* and found bacterial DNA transferred within 2–6 h into the plant cell but most of the DNA was rapidly degraded. Earlier, Schilperoort (1969) observed attachment of bacteria to intact plant cells and later work by Krens et al. (1985) found that tobacco leaf protoplasts regenerating primary cell wall could be transformed by co-cultivation with intact *A. tumefaciens*. Apparently bacterial cellulose fibrils appear to play a role in attachment (Matthysee, 1986).

The important question arose whether or not foreign circular DNA would survive in plant cells. That question was answered by the experiments of Lurquin and Kado (1977). These workers showed that plasmids such as pBR313, a covalently closed DNA, could be taken up by plant protoplasts and remain intact in the nucleus for extended periods of time. Kerr (1969, 1971) observed that oncogenicity could be transferred from one strain of *A. tumefaciens* to another by inoculating both strains together or in succession onto the same plant. Hamilton and Chopan (1975) established that non-pathogenic strains of *A. radiobacter* or *A. tumefaciens* were converted to pathogens by surface inoculation of developing crown galls that harbored the transforming and virulent *A. tumefaciens*. The co-inoculation technique described by Kerr (1969, 1971) was called the "Kerr-cross." Although there was no definitive idea on how virulence was transferred, Roberts and Kerr (1974) elegantly stated that "… it would seem that the only other likely method of DNA transfer is through conjugation." It was well established that Hfr strains of *Escherichia coli* could transfer genetic information to *Salmonella typhimurium* (Baron et al., 1959; Miyake and Demerec, 1959). Likewise, Mitsuhashi (1977) found plasmids, called R factors, conferring antibiotic resistance that could transfer between different bacterial species via a conjugative process. Hence, the question was raised as to whether or not *A. tumefaciens* contained a conjugative plasmid.

This question was indirectly answered by an observation made by Hamilton and Fall (1971). These workers noticed *A. tumefaciens* strains C58 and Ach5 lost their virulence when sub-cultured for 5 days at 36°C. Temperatures above 31.5°C or exposure to ethidium bromide resulted in either the loss of a large plasmid or deletion of a portion of the large plasmid leading to the loss of virulence in *A. tumefaciens* (Lin and Kado, 1977). Interestingly, Braun and Mandle (1948) earlier found that 32°C was the temperature that completely stopped the transformation of normal cells to crown gall tumor cells following inoculation by *Agrobacterium*.

The importance of bacterial plasmids was confirmed by the detection and isolation of large extrachromosomal elements in virulent strains of *A. tumefaciens* but not in *A. radiobacter* strains (Zaenen et al., 1974; **Figure 9**). We had earlier explored the possibility of the existence of a plasmid in *A. tumefaciens* but failed to find any owing to the use of a plasmid isolation technique developed for *E. coli* rather than for *A. tumefaciens* (Kado et al., 1972). Interestingly, other workers showed that both large and small plasmids exist in both virulent *A. tumefaciens* and *A. radiobacter* strains (Merlo and Nester, 1977; Sheikholeslam et al., 1978). Zaenen et al. (1974) examined eight different avirulent strains and found none of them harbored large plasmids. The curious absence of large plasmids in those avirulent strains of *A. tumefaciens* or *A. radiobacter* examined by Zaenen et al. (1974; **Figure 10**) was believed to be a lucky choice of strains according to Jeff Schell (pers. commun. 1978). The conversion of virulent *A. tumefaciens* to stable avirulent strains by subculturing at elevated temperatures (32–37°) was shown to be due to the concomitant loss of a large plasmid (Watson et al., 1975). Moreover, by using the "Kerr cross" technique, an avirulent strain of *A. tumefaciens* was shown to acquire tumor-inducing ability by acquiring a 58 μm plasmid (Van Larebeke et al., 1975).

The presence of a plasmid that conferred virulence upon *A. tumefaciens* led to investigations seeking plasmid DNA in crown gall cells. Indeed, Chilton et al. (1977; **Figure 11**) detected trace amounts of a part of the plasmid in crown gall cells. The amount of foreign DNA represented 0.0011% of total DNA content of the tumor cell. This was a very significant discovery since



Reprinted from *J. Mol. Biol.* (1974) **86**, 109–127

To Clarence,
To remember his first
visit in Gent

9. Zaenen. Mary
Mpl

Supercoiled Circular DNA in Crown-gall Inducing *Agrobacterium* Strains

I. Zaenen, N. van Larebeke, H. Teuchy, M. van Montagu and J. Schell

FIGURE 9 | Classic paper first reporting the presence of an *A. tumefaciens* plasmid associated with tumorigenicity by Jeff Schell's laboratory.

no other bacterial pathogen has been shown to transfer DNA to plant cells.

AN EXTRACHROMOSOMAL ELEMENT CONFERS VIRULENCE ON *A. TUMEFACIENS*

The establishment that a large *A. tumefaciens* plasmid called the Ti plasmid (for tumor-inducing) confers virulence initiated a large number of studies on identifying plasmid genes that were transferred to the host plant cell as well as identifying the intrinsic properties of the large plasmid harbored in virulent strains of *A. tumefaciens* (Gelvin, 2000). As history of these studies show (Nester et al., 2005), emphasis shifted toward developing an understanding of the mechanism of horizontal gene transfer (HGT) by *A. tumefaciens* since this organism represent the first valid case of the inter-domain gene transfer (Bacteria to Eukarya) (Kado, 2009).

Although a number of ancillary studies on the Ti plasmid were started, the initial main efforts were on mapping the location of genes required for conferring the tumor-inducing properties on *A. tumefaciens*. The Ti plasmid of octopine strain B6-806 was physically mapped using restriction endonucleases (Chilton et al., 1978b; Koekman et al., 1979). The Ti plasmid of the nopaline strain C58 was similarly mapped by restriction endonuclease analysis (Depicker et al., 1980). Both deletion-mutational and transposon-insertional mapping were used to locate genes encoding known octopine and nopaline Ti plasmid phenotypes (Holsters et al., 1980; Degreve et al., 1981; Garfinkel et al., 1981). Southern blot analysis and heteroduplex mapping were used to identify homologous "common region" and non-homologous sequences between the octopine plasmid pTiAch5 and the nopaline plasmid pTiC58 (Engler et al., 1981). Altogether, two EcoRI fragments present in the nopaline Ti plasmid pTiC58 and homologous to a segment of the octopine plasmids pTiB6S3

FIGURE 10 | Marc van Montagu and Jeff Schell.

FIGURE 11 | Mary-Dell Chilton.

and pTiAch5 identified the region that confers oncogenicity on *A. tumefaciens* (Chilton et al., 1978a,b; Depicker et al., 1978; Schell et al., 1979). DNA reassociation kinetic analyses were used to probe four tumor lines induced by three *A. tumefaciens* strains (Merlo et al., 1980). This study revealed that a specific sector of Ti plasmid DNA, called the T-DNA (for transferred DNA) coincides with the same region of the physical map of the plasmids. The length of the T-DNA was found to vary in different tumor lines and is flanked on each end by 25 base-pair repeated sequences (Yadav et al., 1982; Wang et al., 1984). The T-DNA borders are similar to the sequences of broad and narrow host-range plasmids that are recognized by their respective nicking enzymes (Kado, 1998). The processing of the T-DNA, initiated by nicking or cleavage at the T-DNA borders, has nicely reviewed by Zambryski (1992) and Gelvin and Filichkin (1994). The right border sequence is essential for and determines the direction of DNA transfer from *Agrobacterium* to the plant genome (Wang et al., 1984). The transfer of the T-DNA, as a single-stranded molecule (Stachel et al., 1986), by a conjugal mechanism is discussed below and recently reviewed by Gelvin (2012). T-DNA is localized to the nucleus of host plant cells and covalently linked to the nuclear DNA (Chilton et al., 1980; Willmitzer et al., 1980).

The next obvious objective regarding the T-DNA was to identify its encoded functions in crown gall tumor cells. At least six discrete T-DNA-encoded mRNAs of sizes 0.73–1.75 kb were detected in octopine-producing tumor lines (Gelvin et al., 1982; Willmitzer et al., 1982) and sizes 0.67–2.7 kb were detected in nopaline tumor lines (Willmitzer et al., 1982). Polyadenylated mRNA transcribed from the T-DNA revealed a transcript 2 (designated earlier as *tms-2* revised as *iaaH*) that is directly responsible for the production of indole-3-acetic acid from indole-3-acetamide, whose formation is catalyzed by indoleacetamide hydrolase from tryptophan (Inze et al., 1984; Schröder et al., 1984). Transcript 1 (designated as *iaaM*) encodes tryptophan 2-monooxygenase (Van Onckelen et al., 1986). Several workers had reported that cytokinin biosynthesis was associated in some way with the T-DNA (Akiyoshi et al., 1983; Barry et al., 1984; Buchmann et al., 1985). The biochemical pathways for auxin and cytokinin have been reviewed (Morris, 1986). Although the T-DNA is weakly transcribed in *A. tumefaciens* (Gelvin et al., 1981), the *ipt* gene located in the T-DNA that encodes isopentenyltransferase activity is not fully expressed in *A. tumefaciens* (Heinemeyer et al., 1987). It was later shown that the *Ipt* gene was repressed by a eukaryotic-like zinc-finger protein called Ros encoded by the chromosomal *ros* gene of *A. tumefaciens* (Chou et al., 1998) and derepressed by a single amino acid substitution of Ros (Archdeacon et al., 2006). Nuclear magnetic resonance spectroscopic studies of Ros revealed a novel DNA recognition mechanism of eukaryotic promoters (Malgieri et al., 2007).

Besides phytohormone genes in the T-DNA, opine synthase genes are also located within the T-DNA. The nopaline synthase gene (*nos)* is located near the right border of the T-DNA (Depicker et al., 1982; Joos et al., 1983). The octopine synthase encoded by the *ocs* gene located in the T-DNA of octopine Ti plasmids was characterized biochemically (Schröder et al., 1981). A gene that encodes agrocinopine synthase was also located in the T-DNA of nopaline Ti plasmids, and a gene that encodes agropine synthase was identified in octopine Ti plasmids (Joos et al., 1983; Paulus and Otten, 1993). These opine synthase genes are integrated into the plant host genome. The opines produced are generally condensation products between basic amino acids and organic acids such as between arginine and pyruvate (octopine). Opines can serve as carbon and sometimes nitrogen compounds utilized by *A. tumefaciens* for nutritional and Ti-plasmid conjugational activities (reviewed in Dessaux et al., 1991; Farrand, 1993). The specificity of opine utilization by *A. tumefaciens* is not entirely tight since fluorescent *Pseudomonas* spp. associated with crown gall tumors in the field appear to catabolize opines (Moore et al., 1997).

After *Agrobacterium*-mediated transformation, these opine synthase genes are transferred to plant hosts by *A. tumefaciens*. This prompted in-depth studies on the T-DNA processing and transfer system. This historical review will not cover this aspect of the biology of crown gall. The processing and transfer of the single-stranded T-DNA covalently linked to VirD2 and then

bound with VirE2 (T-DNA complex) was nicely reviewed in Zambryski et al. (1989), Zambryski (1992), Hansen and Chilton (1999), Gelvin (2003; 2012), Citovsky et al. (2007).

Gaining detailed insights on the functions expressed by T-DNA genes directed efforts to another sector of the plasmid (designated as the *vir* region) that was required for virulence. Through initial genetic analyses (Tn5- and Tn3-lacZ induced mutagenesis) (Garfinkel and Nester, 1980; Stachel and Nester, 1986) and DNA sequencing of the *vir* region, it was initially determined that there were six operons, designated as VirA, B, G, C, D, and E, arranged in that sequential order, as a *vir* regulon (Rogowsky et al., 1990; Schrammeijer et al., 2000; Hattori et al., 2001). Each operon in the vir regulon contains the box sequence (TNCAATTGAAAPy) for both octopine and nopaline Ti plasmids (Steck et al., 1988). A *vir* gene designated *virF* was found in octopine strain A6 that confers host specificity and restricts T-DNA transfer to maize (Jarchow et al., 1991). Every *vir* operon plays an important role either in facilitating bacterial recognition of its host plant through distal and proximal interactions (Rogowsky et al., 1987; Winans, 1992), or generating a T-DNA delivery and processing system (Hooykaas and Beijersbergen, 1994). Expression of the *vir* operon is initiated by sensory detection of external chemical inducers such as acetosyringone (Stachel et al., 1985) and sinapinic acid (Rogowsky et al., 1987). Inter-communications between *Agrobacterium* and its plant host by means of chemical signals, such as precursors of lignin biosynthesis (phenols), sugars and acidic conditions in plants, leading to expression of Ti plasmid virulence genes have been extensively reviewed by Winans (1992) and Gelvin (2006). Much of the early studies focused on the encoded functional roles of *vir* genes within each operon, and those involved in T-DNA processing and its transfer to the host plant cell were key players (reviewed in Gelvin, 2012).

The early prediction by Roberts and Kerr (1974) that *A. tumefaciens* must use a conjugative process to deliver oncogenes appears to be insightfully correct. Furthermore, the prediction was made that the transfer of T-DNA from *A. tumefaciens* to plants is a conjugative system requiring a "sex" pilus (Kado, 1994). Concerted efforts by several research groups carefully analyzed the functional role of the *virB* operon (Shirasu and Kado, 1993a; Jones et al., 1996; reviewed in Zupan et al., 1998). These analyses of the VirB operon revealed striking similarities in both gene organization and sequences to genes involve in conjugative transfer of broad-host-range plasmids (Shirasu and Kado, 1993b). The *virB2* genes sequence shows similarities to *traA* of the enteric plasmid F and to *trbC* of the PilW operons of plasmid R388. The striking similarities between VirB2 and TraA in their amino acid sequences, their protein processing into a 7.2-KDa subunit, and their location in the bacterial cell brought forth the hypothesis that *virB2* encodes a VirB2 pilin subunit used in the transfer of the T-DNA (Shirasu and Kado, 1993a). Consequently, efforts were made to search for pili made by *A. tumefaciens*. A pilus of 3 nm diameter was reported by Fullner et al. (1996). Careful analyses revealed that both virulent and avirulent *A. tumefaciens* produce a common pilus of 3 nm diameter, but only the virulent induced strain, lacking the interfering flagella (Chesnokova et al., 1997), produced a long pilus of 10 nm diameter with a 2-nm lumen (Lai and Kado, 1998, 2000, 2002). This pilus was named the "T-pilus" (Lai and Kado, 1998, 2000).

The products of the *virB* operon are required for oncogenesis and associate with the inner and/or outer membrane of *A. tumefaciens*. The membrane association of these products was thought possibly to form some type of transport system that was distinguished as a member of the type IV secretion system (Christie, 1997; O'Callaghan et al., 1999; Christie et al., 2005). This secretion machinery is involved in the transport of the T-DNA-VirD2 complex (reviewed in Zupan et al., 1998). Comparisons between various known type IV secretion systems have revealed a high degree of conservation in their structural features (Zechner et al., 2012). Besides the type IV secretion machinery involving *virB2* genes, Ti plasmid and genomic sequence analyses have revealed two additional type IV secretion systems in *A. tumefaciens*, one of which is required for conjugative transfer of the cryptic plasmid pAtC58 (Chen et al., 2002) and the other, designated as the Trb locus is required for conjugal transfer of the Ti plasmid (Von Bodman et al., 1989; Li et al., 1999). Moreover, recent work on *Agrobacterium* secretion systems has demonstrated the presence of a type VI secretion machinery having little effect on virulence. However, it may play an ancillary role in facilitating virulence (Wu et al., 2008), as do type VI secretion systems in other pathogens equipped with this secretory system to counter-act intruding competing bacteria (Basler et al., 2013). In addition, the type VI machinery is reported to translocate a phage tail spike-like protein into target cells, cross-link with actin and serve as a tool to puncture membranes of the host cell (Pukatzki et al., 2007).

Lastly, but not the least is the insightful work accomplished on determining the fate of the transferred T-DNA complex culminating in its integration in the nuclear chromosomal DNA of the host (reviewed by Tzfira et al., 2004; Lacroix and Citovsky, 2009; **Figure 12**).

Accessory chromosomal genes assist in facilitating virulence and regulating both genes of the *vir* regulon and the T-DNA of *A. tumefaciens* (reviewed in Charles and Nester, 1994). DNA sequencing of the bacterial circular and linear chromosomes helped locate these genes of potential significance in

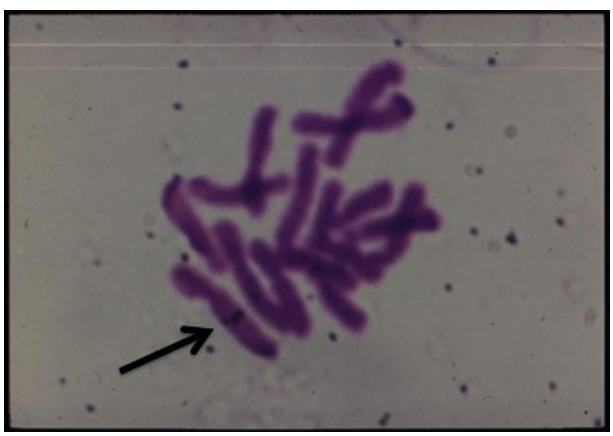


FIGURE 12 | *In situ* hybridization of labeled T-DNA integrated in the chromosome of *Happlopappus gracilis* (Quayle and Kado).

tumorigenesis (Wood et al., 2001; Goodner et al., 2011; Slater et al., 2013). Additional comparative sequence analyses between *A. tumefaciens* strains of limited host ranges may still reveal novel genes conferring host and ecological (environmental) specificity.

CONCLUSIONS AND PERSPECTIVES

The historical event of finding and isolating a tumor-inducing bacterium from grapevine galls (Cavara, 1897a,b) initiated a wonderful, long journey of scientific research that has led to our understanding and appreciation on how *A. tumefaciens* evolved to be equipped with some very sophisticated means of surviving in a hostile soil environment and on plants (Palumbo et al., 1998). This organism escaped numerous microbial competitors such as *Pseudomonas aeruginosa*, *P. fluorescens*, *Streptomyces* spp. (Hibbing et al., 2010) by swimming away from the competition (An et al., 2006) and establishing its own niche in plants in the form of overgrowths (tumors) and essentially genetically engineering the plant host to provide highly specialized organic compounds (opines) that could be specifically utilized by the tumor-inducer. An evolutionarily built-in DNA escape mechanism of purely selfish nature (Orgel and Crick, 1980) as exemplified by the conjugal chromosomal, Ti plasmid and T-DNA transfer to other microbes and to plants (Fründt et al., 1998) insured its survival. *A. tumefaciens* represents the first *living* representative of HGT, i.e., transfer between the domains Bacteria and Eukarya (Kado, 1998, 2009).

All of the pioneering research groups that contributed to these biological understanding of *A. tumefaciens* and crown gall should be applauded.

Furthermore, it is well established that applications of the HGT system between bacteria and plants (Caplan et al., 1983; Fraley et al., 1985) has led to major commercial applications that yielded many genetically engineered domesticated crop (food and fiber) plants as well as serving as a tool for investigating plant immunity responses, plant disease control through transfer of iRNA, etc. *A. tumefaciens* represents and continues to be a valuable resource for biotechnology and humanity.

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Ecological dynamics and complex interactions of *Agrobacterium* megaplasmids

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As with many pathogenic bacteria, agrobacterial plant pathogens carry most of their virulence functions on a horizontally transmissible genetic element. The tumor-inducing (Ti) plasmid encodes the majority of virulence functions for the crown gall agent *Agrobacterium tumefaciens*. This includes the *vir* genes which drive genetic transformation of host cells and the catabolic genes needed to utilize the opines produced by infected plants. The Ti plasmid also encodes, an opine-dependent quorum sensing system that tightly regulates Ti plasmid copy number and its conjugal transfer to other agrobacteria. Many natural agrobacteria are avirulent, lacking the Ti plasmid. The burden of harboring the Ti plasmid depends on the environmental context. Away from diseased hosts, plasmid costs are low but the benefit of the plasmid is also absent. Consequently, plasmidless genotypes are favored. On infected plants the costs of the Ti plasmid can be very high, but balanced by the opine benefits, locally favoring plasmid bearing cells. Cheating derivatives which do not incur virulence costs but can benefit from opines are favored on infected plants and in most other environments, and these are frequently isolated from nature. Many agrobacteria also harbor an At plasmid which can stably coexist with a Ti plasmid. At plasmid genes are less well characterized but in general facilitate metabolic activities in the rhizosphere and bulk soil, such as the ability to breakdown plant exudates. Examination of *A. tumefaciens* C58, revealed that harboring its At plasmid is much more costly than harboring it's Ti plasmid, but conversely the At plasmid is extremely difficult to cure. The interactions between these co-resident plasmids are complex, and depend on environmental context. However, the presence of a Ti plasmid appears to mitigate At plasmid costs, consistent with the high frequency with which they are found together.

Keywords: plasmids, ecology, replicon, genome, bacterial, pathogenesis, virulence

INTRODUCTION TO AGROBACTERIAL MEGAPLASMIDS

Plasmids play a key role in the ecology and evolution of bacterial populations as they frequently carry genes conferring traits such as antibiotic resistance, pathogenesis, and the ability to breakdown nutrients (Turner et al., 2002; Slater et al., 2008; Rankin et al., 2011). These independently replicating genetic elements are primarily distinguished from chromosomes by the defining characteristic of carrying only non-essential genes. In addition they tend to be smaller than bacterial chromosomes and often encode conjugative systems that allow for their horizontal transmission to other bacterial cells (Thomas and Nielsen, 2005; Harrison et al., 2010). Because they often confer phenotypes that are beneficial in particular environments, plasmids and their horizontal transfer have an important role in structuring bacterial communities and in shaping the evolution of bacterial populations (Slater et al., 2008).

Many members of the *Rhizobiaceae* have multipartite genomes that include several ecologically important plasmids (Jumas-Bilak et al., 1998). The genome of *Rhizobium etli* CFN42 provides a particularly dramatic example of this, being composed of a primary chromosome, a secondary chromosome or chromid, and five plasmids (Harrison et al., 2010; Landeta et al., 2011). Many members of the *Rhizobiaceae* family live in intimate association with plant hosts. Some, such as many rhizobia, are nitrogen fixing plant mutualists, while others, like many agrobacteria, are plant pathogens. The taxonomic status of the genus *Agrobacterium* has been debated with proposals that it be considered a species of *Rhizobium* (Young et al., 2001, 2003; Farrand et al., 2003). However, for continuity and clarity in this review we follow the convention of distinguishing between agrobacterial and rhizobial strains.

The rhizobial nitrogen fixation and agrobacterial pathogenesis functions that characterize their association with plants are largely conferred by the plasmids they carry. The conjugal Ti (tumor-inducing) and Ri (root-inducing) plasmids found in many *Agrobacterium* species carry the majority of genes underlying crown gall and hairy root disease, respectively (Escobar and Dandekar, 2003; Suzuki et al., 2009). Ti plasmids are harbored by both generalist pathogens including many *Agrobacterium tumefaciens* strains, and narrow-host range pathogens such as *A. vitis* strains that cause crown gall of grape. Ri plasmids are typically found in pathogenic *A. rhizogenes* strains that cause hairy root disease. Unless indicated otherwise, this review will focus on *A. tumefaciens*, although many of the general features of plasmid biology and plant infection are similar in *A. rhizogenes* and *A. vitis*.

Infection of a plant host involves its genetic transformation in which a large segment or segments (approximately 40 kb) of Ti plasmid-borne genes (the transferred or T-DNAs) are replicated from the plasmid via a conjugation-like mechanism, delivered into the plant cell via a type IV secretion system, and integrated into the host plant's genome (Escobar and Dandekar, 2003; Brencic and Winans, 2005). Ti plasmid virulence genes are only expressed when pathogenic *A. tumefaciens* cells encounter a specific set of environmental conditions (plant-produced phenolics, sugars, low pH, and limiting phosphate) most indicative of wounded plant tissue (Winans, 1990). Following transformation, the plant host cell machinery directs the expression of T-DNA genes, leading to T-DNA controlled synthesis of the plant hormones auxin and cytokinin, resulting in accelerated division of transformed plant cells (Drummond et al., 1977; Garfinkel et al., 1981). This gives rise to the most conspicuous symptom of crown gall disease—tumor development. Less conspicuously, but arguably of primary importance for the pathogen, the plant's expression of T-DNA genes also results in the synthesis and release of a suite of unique metabolites that are broadly termed opines (Brencic and Winans, 2005). Opine catabolic genes carried on the Ti plasmid allow the pathogen to catabolize the plant produced opines, providing a key benefit of pathogenesis to the infecting bacteria (Guyon et al., 1993; Savka and Farrand, 1997; Platt et al., 2012b). Hairy root disease caused by Ri plasmid bearing *A. rhizogenes* also involves T-DNA transfer that causes plants to produce opines, however rather than cause tumor development this disease stimulates the growth of adventitious roots. Many Ri and Ti plasmid T-DNA genes show homology, such as the Ti encoded auxin biosynthesis genes, *iaaM* and *iaaH*, and the corresponding Ri encoded *aux1* and *aux2* genes. However, several Ri plasmid T-DNAs genes show limited or no homology to genes found on Ti plasmid T-DNAs. These genes, such as *rolA*, *rolB*, and *rolC*, function in stimulating meristem formation, a key feature distinguishing hairy root and crown gall diseases (reviewed by Britton et al., 2008).

In addition to the well-studied agrobacterial virulence plasmids, agrobacteria can also harbor several less well characterized plasmids (Currier and Nester, 1976; Merlo and Nester, 1977; Albiach and Lopez, 1992). For example, some avirulent agrobacteria in nature harbor opine catabolic plasmids, which confer the ability to freeload on the benefits of pathogenesis initiated by virulent agrobacteria by catabolizing public goods in the form of opines (Merlo and Nester, 1977; Wabiko et al., 1990; Dessaux et al., 1998; Wetzel et al., 2014). The biocontrol agent *A. radiobacter* K84 is the best characterized avirulent strain harboring such a plasmid. Interestingly, K84 also produces several antimicrobials that allow it to interfere with the growth of virulent agrobacteria (Donner et al., 1993; Penyalver et al., 2001; Kim et al., 2006). For these reasons, K84 has served as a powerful commercial biocontrol agent of crown gall disease for several decades. K84's ability to catabolize opines and produce anti-agrobacterial molecules largely depends on plasmid-borne genes. Wild-type K84 harbors three plasmids. One of these, pAtK84b, confers the ability to catabolize nopaline and agrocinopine produced by crown gall infected plants and shares regions of homology with several Ti plasmids (Oger and Farrand, 2002). A second plasmid, pAgK84, carries genes underlying production and immunity to agrocin 84 (Kim et al., 2006), while a third plasmid, pAtK84a, encodes production of and resistance to agrocin 434 (Donner et al., 1993; McClure et al., 1998). Agrocin 84 specifically inhibits the growth of agrocinopine catabolic agrobacteria such as strains harboring a nopaline-type Ti plasmid (Reader et al., 2005; Kim et al., 2006). In contrast, agrocin 434 primarily inhibits the growth of biovar 2 agrobacteria, the same biovar as K84 itself (Donner et al., 1993).

Tartrate is a common nutrient present on grapevines and many *A. vitis* strains harbor a tartrate utilization plasmid, called pTr or pTar that allows them to access these nutrients (Burr and Otten, 1999). These plasmids likely provide a competitive advantage to *A. vitis* strains in colonizing their grapevine hosts (Salomone et al., 1998). Interestingly these conjugative tartrate utilization plasmids are diverse, though they harbor similar TAR regions required for the degradation of tartrate.

Several pathogenic and avirulent strains of *A. tumefaciens* carry another type of agrobacterial megaplasmid. Like the Ti plasmids, these At plasmids vary widely in their gene structure and composition, though they also share regions of homology. Non-essential for pathogenesis, the At plasmids have received considerably less attention than Ti plasmids. For this reason, they were traditionally referred to as cryptic plasmids as they were previously uncharacterized relative to the Ti plasmids. Although dispensable for virulence, the full sequence of the best characterized At plasmid, pAtC58, reveals the presence of genes involved in a range of functions including, but not limited to, chemotaxis, iron uptake, DNA damage repair, heat shock, and catabolism (Goodner et al., 2001; Wood et al., 2001; Slater et al., 2009). One set of At plasmid genes that has received particular attention are the *blcABC* genes, previously named *attKLM*, because of their initially proposed, but later refuted role in attachment (Matthysse et al., 2008). We now know, however, that attachment is largely mediated by chromosomally encoded genes (Tomlinson and Fuqua, 2009; Li et al., 2012; Xu et al., 2013), and that the products of the *blcABC* operon confer the ability to catabolize γ-butyrolactone (GBL), plant-released compounds often present at high levels in the soil and rhizosphere (Carlier et al., 2004; Khan and Farrand, 2009). In addition to GBL utilization, At plasmids confer catabolic functions that are likely to contribute to the success of *A. tumefaciens* cells inhabiting the rhizosphere. These catabolic systems include those for degradation of deoxyfructosyl-glutamine (DFG), mannopine (MOP), succinyl semialdehyde (SSA), γ-hydroxybutyrate (GHB), and γ-aminobutyric acid (GABA), and are discussed in greater detail in Sections "The Costs and Benefits Associated with the Ti and At Plasmids" and "Ecological Context of Ti and At Plasmids" of this review.

In addition to catabolic functions, there are several studies demonstrating that At plasmids can affect virulence (Nair et al., 2003; Morton et al., 2013). One such study shows that in some strains the presence of an At plasmid corresponds to an increase in the size of tumors, suggesting a positive impact on virulence (Nair et al., 2003). However, variants of pAtC58 from *A. tumefaciens* C58 have been shown to exhibit differential effects on the expression of pTiC58-encoded virulence (*vir*)

genes (Morton et al., 2013). For example, whereas a truncated form of pAtC58 (ΔAtu5207–Atu5408) has a repressive effect on *vir* gene expression, the full-length form of the plasmid had no such effect. The basis for the differences between these studies remains unclear.

In this review, we will focus on the genomic, ecological, and evolutionary significance of the two best-studied agrobacterial plasmids, the Ti and the At plasmids. We will first describe what is known about the function and regulation of replication, partitioning, and conjugation of these plasmids. Then we will discuss their diversity, ecology, and the genomic context of their evolution. Throughout, we will focus on the influence of the biotic and abiotic environmental conditions on the regulation of plasmid encoded genes and how this relates to the ecological costs and benefits associated with these plasmids. Finally, we will discuss how these genetic and ecological factors couple together to influence the evolutionary dynamics of these plasmids.

Ti PLASMIDS AND THE OPINE CONCEPT

Many agrobacterial plasmids are defined by their role in pathogenesis, as is the case for the Ti and Ri plasmids. The Ti and Ri plasmids are highly diverse and are characterized by the type of low molecular weight resources, the opines, that infectious agrobacteria cause host plants to produce. Opines are found within and around the tumors or root hairs of plant tissue that has been transformed by the T-DNA of pathogenic agrobacteria, but are not typically found in soil environments. The range of opines that are produced by the infected plant is determined by the T-DNA genes carried on the virulence plasmid, and these genes vary among strains of agrobacteria (Moore et al., 1997). The driving selective benefit for agrobacterial pathogenesis was for many years proposed to be access to the relatively exclusive opine nutrients (this was known as the "opine concept"), and subsequent experiments demonstrated that this is in fact correct (Dessaux et al., 1998).

As a family, opines are incredibly diverse with over 30 species having been characterized (Dessaux et al., 1998). Chemically, they can be separated into two structural classes: agrocinopines and secondary amine derivatives. Agrocinopines are sugar-phosphodiesters and thus represent sources of carbon and phosphorus (Oger and Farrand, 2002). The amine-derived opines are formed by the condensation of an amino acid with either a sugar or an alpha-keto acid and serve as sources of carbon and nitrogen. Amine-derived opines, such as nopaline and octopine are formed by the reductive condensation of arginine with α-ketoglutarate and pyruvate, respectively. Mannityl opines are derived from deoxyfructosyl-glutamine (DFG), the conjugation product of glutamine and a sugar (Baek et al., 2003).

Opines are usually degraded by catabolic functions that are also Ti plasmid encoded and the expression of which is inducible by the corresponding type of opine. Depending on the Ti plasmid, an *A. tumefaciens* strain can transform plants with one or more of a multiple array of opine biosynthetic genes (Dessaux et al., 1998). Corresponding opine uptake and catabolism genes are located on the non-transferred portion of the infecting Ti plasmid (Guyon et al., 1993). The transferred opine biosynthesis genes include those that function to conjugate plant-synthesized products to amino acids, creating additional substrates that can be utilized by infecting cells (Kemp et al., 1979; Hack and Kemp, 1980). Some strains of *Agrobacterium* exhibit chemotaxis toward specific opines. Chemotaxis is the directed movement of bacterial cells determined by chemical gradients (e.g., nutrients) in the environment. For *A. tumefaciens*, opine-specific chemotaxis depends upon the Ti plasmid and as such, correlates with the specific opine biosynthetic and catabolism genes encoded by the plasmid (Kim and Farrand, 1998).

Subsets of the opines, called conjugal opines, mediate horizontal transfer of the Ti plasmid from one bacterial cell to another. This occurs via activation of expression of the gene encoding the LuxR-type transcription factor TraR (described in more detail in the next section). Octopine is the conjugal opine for octopine-type plasmids, and agrocinopine A and B are the conjugal opines for nopaline-type plasmids (Farrand, 1998a). In at least one plasmid, mannityl opines activate *traR* expression and can function as conjugal opines (Wetzel et al., 2014). This process of plasmid transfer depends on the presence of specific opines produced by transformed plant cells as they enable a response to the self-produced diffusible acyl-homoserine lactone (AHL) quorum sensing signal (Zhang et al., 1993; Fuqua and Winans, 1994). Although conjugal transfer of Ti plasmids is completely dependent on the presence of the conjugal opines, the precise regulatory mechanisms vary for each Ti plasmid (Farrand, 1998b). For example, in nopaline-type plasmids, when opines are absent, the conjugation genes (*tra* and *trb*) are actively repressed by the agrocinopine-responsive transcriptional regulator, AccR (Kim et al., 2008). When opines are present, however, and cells are at a population density at which AHL molecules reach inducing levels, transcription of conjugation genes is derepressed. Similar stimulation of conjugation gene expression is mediated through the octopine-responsive transcriptional activator OccR for octopine-type plasmids. In either case, the control of Ti conjugation genes is indirect, and the opines function by elevating the expression of the *traR* gene (Piper et al., 1993; Fuqua and Winans, 1994). TraR directs the process of quorum sensing and is activated and stabilized by forming a complex with accumulating AHL molecules, resulting in the up-regulation of the *tra* and *trb* genes, as well as increased copy number of the Ti plasmid (White and Winans, 2007).

STABILITY, REPLICATION, AND PARTITIONING OF *repABC* REPLICONS

Low-copy number plasmids require efficient replication and partitioning in order to ensure their efficient transmission during the reproduction of bacterial cells. Many of the large, low-copy number plasmids and secondary chromosomes found in the genomes of agrobacteria and other alphaproteobacteria belong to the *repABC* family of replicons. The transcriptional and post-transcriptional regulation of the *repABC* operon gene products plays a central role in the replication and partitioning of this family of replicons. In this paper, we will briefly describe the regulation of *repABC* replicon replication and partitioning. We will focus on the well characterized regulation employed by the Ti plasmid and describe how this relates to the quorum sensing

dependent regulation of Ti plasmid conjugation. Several recent reviews cover these topics in detail (Mazur and Koper, 2012; Pinto et al., 2012).

The primary replication factor RepC and a nearby replication origin to which it binds are required for replication of *repABC* replicons, whereas RepA and RepB proteins coordinate replicon partitioning during multiplication of the bacterial cell. Unlike many plasmids, the partitioning (*repA* and *repB*) and replication (*repC*) genes of *repABC* replicons are typically expressed as one transcriptional unit controlled by a promoter region upstream of *repA*. RepC does not belong to a known larger protein family and the *repC* gene has only been observed in alphaproteobacteria (Pinto et al., 2012). In contrast, the RepA and RepB proteins belong to the family of ParA and ParB proteins, respectively, which includes proteins mediating the partitioning of chromosomal, prophage, and plasmid replicons. At least one agrobacterial tartrate utilization plasmid, pTar, employs a partitioning system belonging to this larger ParA-ParB family (Kalnin et al., 2000).

The multipartite genome of *A. tumefaciens* C58 is composed of a circular *oriC*-type chromosome and three *repABC* family replicons: a linear chromosome, pTiC58, and pAtC58 (Li and Farrand, 2000; Goodner et al., 2001). The replication origins of all four C58 replicons tend to generally localize to the polar region of the cell, although each site is distinct from the other replicons, suggesting that they may be targeted to distinct addresses (Kahng and Shapiro, 2003). This may contribute to the compatibility of these *repABC* replicons or reflect the mechanism(s) that allows for their stable coexistence within a bacterial cell. The location of centromere-like *par* sites composed of one or more palindromic sequences varies among *repABC* replicons. These sites play a key role in plasmid stability, partitioning, and incompatibility as they are thought to be the site where the partitioning machinery binds. In the cases of pTiC58 and pTiR10 these *par* sites are located between *repA* and *repB* within the *repABC* operon, while for other *repABC* replicons *par* sites can be found close to the *repC* stop codon or upstream of *repA* (Cervantes-Rivera et al., 2011; Pinto et al., 2012). Though not found in all *repABC* replicons, pTiC58 and pTiR10 both encode a *repD* gene located between *repA* and *repB* that overlaps the *par* sites of these plasmids (Chai and Winans, 2005a).

The transcriptional and post-transcriptional control of the replication, partitioning, and conjugation of the octopine-type Ti plasmid pTiR10 is particularly well characterized. The *repABC* operon of pTiR10 is influenced by four promoters upstream of *repA* (Pappas and Winans, 2003a,b). RepA of pTiR10 binds to an operator located downstream of the most proximal of these promoters (P4) thereby antagonizing transcription of the operon. The binding of pTiR10 RepA to this operator is thought to be enhanced by formation of a complex with RepB (Pappas and Winans, 2003b). Similarly, pTiR10 RepB binds to the *par* sites within *repD* and this binding is enhanced by the presence of RepA. Taken together, these results suggest that a RepA–RepB complex may bind both the P4 promoter upstream of *repA* and the *par* site downstream of *repA* forming a large double-stranded DNA loop structure involved in the repression of the *repABC* operon (Chai and Winans, 2005a).

The role of RepC in promoting the replication of the Ti plasmid is thought to depend on its ability to bind the replication origin (Pinto et al., 2011). As with many *repABC* replicons, pTiR10 also contains a gene between *repB* and *repC* which encodes an antisense RNA that down-regulates the expression of *repC* (Chai and Winans, 2005b). In the case of pTiR10 this gene is called *repE* and is thought to duplex with the *repABC* transcript in a way that promotes translational termination near the *repC* start codon. This post-transcriptional control, along with transcriptional autorepression of the *repABC* operon mediated by RepA–RepB complexes, likely helps maintain the low copy number state of pTiR10 under many environmental conditions.

The presence of two types of plant-produced molecules, phenolic compounds and opines, stimulates the transcriptional activity of the pTiR10 *repABC* operon leading to higher plasmid copy number when either of these plant cues are present. The opine effect is indirect, through the TraR quorum sensing mechanism, whereas the phenolic induction is mediated by the VirA-VirG two component *vir* gene regulation system. The sensor kinase VirA phosphorylates VirG in response to the presence of plant-produced phenolic compounds. Phospho-VirG binds to an upstream *vir*-box, stimulating transcription from promoter P4 of the *repABC* operon leading to the elevation of plasmid copy number to approximately four copies per cell (Cho and Winans, 2005). The VirA–VirG two component system similarly promotes *vir* gene transcription directing interkingdom gene transfer (Winans, 1991).

As described above, opine-dependent gene regulation can function through repression or activation mechanisms. For example, for octopine-type Ti plasmids such as pTiR10 the transcription of opine transport and catabolic genes is stimulated by the binding of complexes between the LysR-type transcriptional regulator OccR and octopine, one of the opines that this class of Ti plasmid engineers plants to produce (Wang et al., 1992; Fuqua and Winans, 1996). Nopaline-type Ti plasmids, typified by pTiC58, also increase expression of opine transport and catabolic genes in response to the presence of opines. Nopaline uptake and catabolism are activated by NocR, a LysR-type regulator that functions similarly to OccR (von Lintig et al., 1994). However, the best studied example of opine-responsive gene regulation for pTiC58 is derepression of agrocinopine uptake and catabolic genes by AccR, a LacI-type repressor (von Bodman et al., 1992; Kim and Farrand, 1997).

In addition to stimulating opine catabolic functions, the presence of opines in the plant tumor environment also indirectly controls Ti plasmid copy number and conjugation by inducing the expression of the quorum sensing transcriptional activator TraR, encoded on the Ti plasmid (Pappas, 2008). The inducing ligand for TraR is *N*-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL), an AHL signal molecule that is synthesized by the activity of *traI*, an AHL synthase also encoded by the Ti plasmid. TraR–AHL complexes stimulate transcription of the pTiR10 *repABC* operon from all four upstream promoters, resulting in a seven- to eightfold increase in plasmid copy number (Li and Farrand, 2000; Pappas and Winans, 2003a). Under the same conditions, TraR–AHL binds another nearby *tra* box stimulating transcription of the divergently oriented *traI*-*trb* operon that controls expression of both *traI* and

genes involved in mating pair formation (Mpf) functions required for conjugation of the Ti plasmid. The *trb* operon includes two genes, *trbJ* and *trbK*, which encode entry exclusion proteins that inhibit conjugal delivery of a Ti plasmid into the cell (Cho et al., 2009). In addition, another pair of divergent operons encoding the DNA transfer and replication functions (Dtr) elsewhere on the Ti plasmid are activated by TraR–AHL binding to an intergenic *tra* box. The Dtr functions include the conjugal nickase (TraA) and the coupling factor TraG.

While the replication, partitioning, and conjugation of the Ti plasmid are well studied, much less is known about other agrobacterial megaplasmids. In some cases, there are parallels between the conjugation of the Ti plasmid and these other plasmids. For example, AccR-dependent opine responsive regulation influences the expression of both pTiC58 and pAtC58 conjugal machinery, revealing a mechanism that promotes co-transfer of these plasmids (Lang et al., 2013). Further, the opine catabolic plasmid of K84, pAtK84b, employs opine-dependent quorum sensing to regulate its conjugation. However in contrast to most Ti plasmids, two distinct types of opines can independently induce the conjugation of this pAtK84b, with each inducing the expression of separate and functional *traR* paralogs encoded by the plasmid (Oger and Farrand, 2002). Though the frequency with which this occurs is unknown, one study has documented the transfer of pAtK84b into pathogenic agrobacteria under natural plant-tumor conditions (Vicedo et al., 1996). In this case, the K84 opine catabolic plasmid likely displaced the resident, incompatible Ti plasmid and the concurrent delivery of pAgK84 essentially converted a pathogenic agrobacterial strain into an avirulent, agrocin 84-producing competitor of the pathogen (Vicedo et al., 1996).

Within the same crown gall tumor, these researchers also observed transfer of a Ti plasmid into the K84 background, with likely subsequent recombination between the Ti plasmid and the resident pAtK84b (Vicedo et al., 1996). In this case, conjugation essentially converted the avirulent biocontrol agent into a pathogenic strain that is resistant to biocontrol by K84 and related strains, an outcome that may undermine the long-term efficacy of biocontrol by K84 (Lopez-Lopez et al., 1999). Other studies have reported the origin of pathogenic, agrocin 84 producing strains via the transfer of pAgK84 into pathogenic agrobacteria, demonstrating another threat to the utility of K84 as a biocontrol agent (Vicedo et al., 1993; Stockwell et al., 1996; Raio et al., 2009). Because of this issue, a genetically engineered derivative of K84 in which the conjugal functions of pAgK84 have been disrupted is also available for biocontrol (Jones and Kerr, 1989; Vicedo et al., 1993; Penyalver et al., 2000). These examples illustrate the recombinational modularity of the agrobacterial megaplasmids, and hint at the evolutionary histories that have led to their complex architecture and regulation.

Many plasmids encode toxin-antidote loci, which can promote their stability in growing bacterial populations and mediate within-bacterium competition among plasmids co-infecting the same bacterial cell (Gerdes et al., 2005; Van Melderen and De Bast, 2009; Cooper et al., 2010). Toxin-antidote systems are widespread among bacteria and highly diverse (Van Melderen and De Bast, 2009). Generally toxin-antidote systems involve two linked loci, one encoding a toxic factor and the other an antidote factor that prevents the toxic effects of the first factor from manifesting. Because of this, these systems can lead to the inhibition of daughter cells that do not inherit the toxin-antidote locus, thereby preventing the spread of cells lacking this locus—such as plasmid free cells—through the population (Gerdes et al., 2005). Plasmid-encoded toxin-antidote loci can similarly mediate competition between co-infecting incompatible plasmids by making it difficult to displace the resident plasmid (Cooper and Heinemann, 2000; Cooper et al., 2010). The stability of two nopaline-type Ti plasmids, pTiC58 and pTi-SAKURA, is greatly enhanced by the presence of the toxin-antidote systems they encode (Yamamoto et al., 2007, 2009). The At plasmid of C58 is highly stable despite the high selective pressure favoring lineages that lose the plasmid. This stability may reflect the effects of one or more of the putative toxin-antidote systems that this plasmid encodes (Morton et al., 2013).

Other agrobacterial plasmids encode secreted toxins that are able to mediate interference competition in addition to potentially contributing to plasmid stability. For example, the pAtK84a plasmid confers not only the ability to produce agrocin 434, which antagonizes other biovar II agrobacteria, but also resistance to the toxin (Donner et al., 1993). The same is true for the agrocin 84 plasmid, pAgK84, in that the plasmid confers the ability to produce a toxin as well as resistance to the toxin (Slota and Farrand, 1982; Ryder et al., 1987). Agrocin 84 interferes with cellular leucyl-tRNA synthetase thereby disrupting the translation of agrocin 84 sensitive strains. Importantly, pAgK84 encodes a variant leucyl-tRNA synthetase which imparts resistance to the toxic effects of agrocin 84 (Reader et al., 2005; Kim et al., 2006).

PLASMID REARRANGEMENTS AND DIVERSITY

The *repABC* family of agrobacterial plasmids is incredibly diverse (Cevallos et al., 2008). These plasmids have interspersed regions of high sequence similarity, suggesting that multiple recombination events have shaped their structure (Farrand, 1998b). These conserved blocks of sequence can be found between plasmids of different strains of the same bacterial species, but also across species, genera, and families (Farrand, 1998b; Galardini et al., 2011).

Although opine biosynthesis genes (located on the T-DNA) and opine uptake and catabolism genes are often linked together on the same plasmid, this is not always the case (Merlo and Nester, 1977). There are several examples of plasmids that lack the virulence functions all together, but still retain the genes for opine transport and catabolism. pAtK84b and pAtK112 are two examples of these plasmids, conferring the ability to catabolize nopaline and agrocinopines. Expression of a gene on the At plasmid of *A. tumefaciens* R10 is required for complete catabolism of octopine (Cho et al., 1996). Another At plasmid, pArA4, found in *A. rhizogenes* is a catabolic plasmid which provides the ability for its host bacteria to utilize MOP, mannopinic acid, and agropinic acid as sole sources of carbon (Guyon et al., 1993).

Additionally, although most Ti plasmids in *Agrobacterium* species are considered to be virulence elements, they exhibit

blocks of high sequence similarity to the symbiosis plasmids of other rhizobia (e.g., pRetCFN4d of *Rhizobium etli* and pSymA of *Sinorhizobium meliloti*). Interestingly, the conjugal pili proteins of many of these symbiotic plasmids exhibit homology to the VirB proteins encoded on the Ti plasmid (Chen et al., 2002; Ding and Hynes, 2009). However, despite the similarity between the symbiotic plasmid Type IV secretion (T4S) systems and those encoded on the Ti plasmid, the horizontal transmission of these symbiotic plasmids is regulated by the *rctAB* repression system, distinct from the quorum sensing control of Ti plasmid conjugal transfer genes and the plant-signal dependent expression of the *vir* T4S (Brencic and Winans, 2005; Perez-Mendoza et al., 2005).

Large-scale deletion events have been characterized in the pAtC58 plasmid of *A. tumefaciens* C58 (Morton et al., 2013). A series of repeat sequences (9–13 bp) was found to be distributed within the At plasmid, immediately flanking sites of large deletions (up to 0.19 Mb). These deletions were discovered in several laboratory stocks of *A. tumefaciens* C58, indicating that they occur during normal passaging. The repeated elements are not within known transposable elements and the longer repeats (11–13 bp) are more abundant on the At plasmid than on other *A. tumefaciens* C58 replicons. Strains carrying At plasmids that have incurred these deletions have increased *vir* expression and possess a higher relative fitness in lab culture compared to strains with the full length plasmids, likely due to high carriage costs associated with these deleted segments (Morton et al., 2013). A large majority of genes within the deletion intervals are represented by ABC transporters, so it could be that passage in laboratory culture favors loss of costly genes that would otherwise confer benefits specific to the natural rhizosphere environment. The variety of rearrangements in the At plasmid of *A. tumefaciens* C58 indicates that the replicon is highly adaptable and dynamic. Additionally, the repeats found flanking deletion sites are not present in the closely related *S. meliloti* (Morton et al., 2013), suggesting that they could be specific to *A. tumefaciens* and provide a mechanism for genomic plasticity.

The At plasmid of *A. tumefaciens* ANT4 has also been shown to cointegrate with the Ti plasmid (Vaudequin-Dransart et al., 1998). During matings between *A. tumefaciens* ANT4 and a plasmidless recipient, C58.00RS, some transconjugants possessed a single large replicon containing genes from both the At and Ti plasmids. It was proposed that integration of these replicons can cause gene disruption and potentially inactivate virulence functions through recombination into required regions (Vaudequin-Dransart et al., 1998). Cointegration between these replicons could have effects on the catabolic potential of *A. tumefaciens* strains, as there are numerous potential cointegration sites between the plasmids in separate isolates, in some cases disrupting opine-utilization functions. Similar cointegration events have been described previously in other members of *Rhizobiaceae* (Flores et al., 2000; Mavingui et al., 2002; Guo et al., 2003). In these cases, cointegration was shown to occur between all chromosomal replicons, but did not have strong effects on fitness or symbiotic-proficiency. The significance of cointegration between the At and Ti plasmids with respect to rhizosphere metabolism and pathogenesis remains unclear.

THE COSTS AND BENEFITS ASSOCIATED WITH THE Ti AND At PLASMIDS

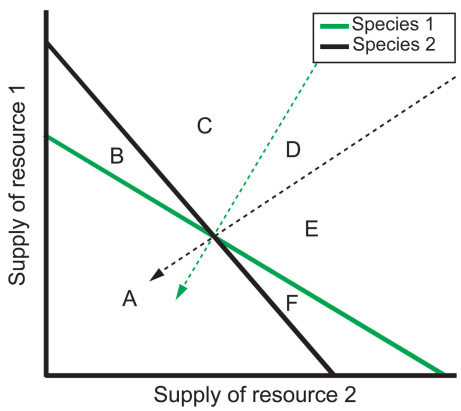
Plasmids can impose significant fitness costs on the bacterial cells that harbor them (Slater et al., 2008; Baltrus, 2013), which along with the benefits that plasmids confer play a central role in determining their ecological and evolutionary dynamics (Slater et al., 2008). The net balance of these costs and benefits determine whether a genotype with the plasmid has an advantage or disadvantage relative to a competitor genotype lacking the plasmid. Plasmid costs can result from a variety of causes, including the energetic burden of plasmid maintenance or conjugation as well as the costs associated with expressing other functions encoded by the plasmid (reviewed in Baltrus, 2013). These different forms of plasmid costs may vary in their magnitude and degree of context-dependence. The fitness cost of harboring two Ti plasmids, the octopine-type pTi15955 and the nopaline-type pTiC58, has been demonstrated to be low or even undetectable in their respective host backgrounds under laboratory conditions (Platt et al., 2012a; Morton et al., 2014). These low carriage costs likely reflect the tight gene regulation that controls expression of most genes on the Ti plasmid. Natural selection acting on plasmid genes likely favors this tight regulation, as high carriage costs antagonize the fitness of plasmid and chromosomal genes alike. Carriage of pTi15955 conferred a small competitive disadvantage against plasmidless derivatives; however, this was only measurable when the bacteria were limited for either carbon or nitrogen (Platt et al., 2012a). In contrast, when the bacteria competed under conditions that stimulated expression of *vir* genes and the *repABC* operon, cells bearing pTi15955 were at a marked competitive disadvantage (Platt et al., 2012a). This demonstrates that the expression of these genes is highly costly indicating that the infection of plant hosts comes at a significant cost to the infecting agrobacteria.

Key benefits associated with the Ti and At plasmids stem from their conferring the ability to catabolize nutrients. Consequently, resource-consumer competition models provide a useful way to describe competition among genotypes that vary in the plasmids they harbor. **Box 1** provides an overview of how the predictions of these models can be graphically represented. As articulated in the "opine concept," the primary benefit of plant pathogenesis for pathogenic agrobacteria comes in the form of the opines produced by the infected plant (Guyon et al., 1993; Oger et al., 1997; Savka and Farrand, 1997; Mansouri et al., 2002). Opines exuded by infected plants provide a nutrient source that can promote the growth of opine catabolic bacteria in the rhizosphere. For example, octopine availability can shift the outcome of resource competition between pathogenic agrobacteria harboring pTi15955 and avirulent strains lacking the plasmid (**Figure 1A**; Platt et al., 2012b). In this way, opines arise from the costly action of agrobacteria infecting host plants. Upon exudation by the plant, opines are available for any opine catabolic bacteria, which can include agrobacteria harboring opine catabolic plasmids as well as other rhizosphere bacteria (Moore et al., 1997). In addition to the benefits associated with opine catabolism following infection of a plant host, Ti plasmids also confer the ability to detoxify phenolics or even the use of these phenolics as nutrient sources (Brencic et al., 2004).

BOX 1 | The predictions of resource consumer competition models can be graphically represented and interpreted using two-dimensional plots in which the graphical axes represent concentrations of the two resources for which the competitors compete.

Each species' population grows in environments where the supply of resources is sufficiently high to support its growth. In the heuristic example shown, the solid line corresponding to each species represents environments where that species' population neither grows nor declines in size. Consequently these lines are referred to as zero-net-growth isoclines, or ZNGIs. All environments above the line have sufficient resource levels to support population growth of the species, while all environments below the line cannot support population growth of that species. In this example, the two resources are substitutable, such that each species can maintain an equilibrium population in environments with exclusively one or the other resource (e.g., the ZNGI x- and y- intercepts) or combinations of the two resources (e.g., the points on the line between the intercepts). The shape and position of ZNGIs can vary depending on the attributes of the consumer and the way in which resources influence the growth of that species. The dashed arrows are vectors representing the rate at which the associated species consumes each of the resources.

Predicting the establishment of these species in some environments is straightforward. All points in zone A are under the ZNGIs of both species 1 and species 2. Since populations of neither species can grow in zone A, then populations of neither will persist in environments with those combinations of resource levels. Similarly since populations of only species 1 can grow in zone B and only species 2 can grow in zone F, only those species can establish populations in those environments. In these models, one competitor displaces the other if growth of its population drives resource levels below the minimal needs of the other species. This occurs in zones C and E with species 1 driving resource levels below the minimal needs of species 2 in zone C, and species 2 driving resource levels below the minimal needs of species 1 in zone E. In this example, both species stably coexist in zone D since neither species drives resource levels below the minimal needs of the other species. See Tilman (1980, 1982) for a more detailed analysis of a variety of competitive scenarios.



In contrast to the Ti plasmid whose carriage cost is minimized under conditions where plasmid benefits are limited, the At plasmid carriage cost as measured in the C58 nopaline type strain, is high (Morton et al., 2014). While the reason for the observed high

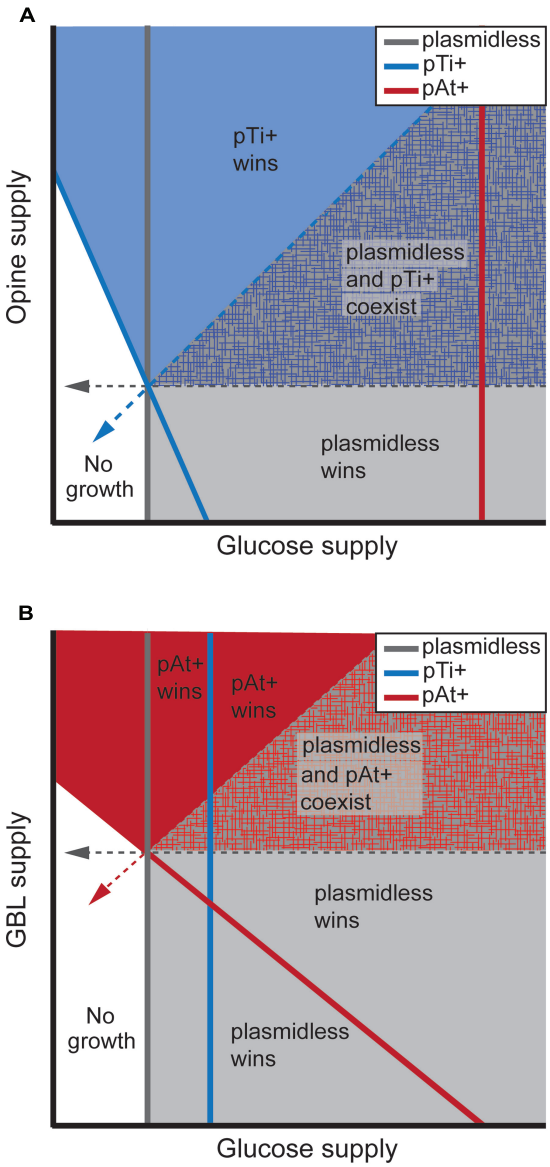


FIGURE 1 | Model predictions for the outcome of resource competition between plasmidless, Ti plasmid bearing, and At plasmid bearing agrobacteria for opines and glucose (A) and for γ-butyrolactone (GBL) and glucose (B). Solid lines represent the minimum resource levels required for growth, while dashed arrows represent the consumption vectors of the indicated strain. Plasmidless agrobacteria can catabolize glucose while pTi+ cells can catabolize both glucose and opines and pAt+ cells can catabolize both GBL and glucose. Because At plasmids are likely much more costly than Ti plasmids, the minimum amount of glucose needed to support a population of pAt+ cells is considerably greater than that of Ti+ cells (compare the x-intercepts of the zero-net-growth-isoclines of these strains). Zones of each phase plane are shaded according to which genotype or genotypes are able to persist in the corresponding environments despite resource competition with the other strains. Note that the lower left zone of each phase plane represents environments in which resource levels are too low to support growth of any of the genotypes. See Morton et al. (2014) for a more thorough model analysis that includes competition by genotypes bearing both a Ti plasmid and an At plasmid and Platt et al. (2012b) for an analysis of competition of genotypes bearing a Ti plasmid and avirulent, freeloading strains whose plasmids only contain the opine catabolism genes.

cost has yet to be determined, there are several potential explanations. For example, this At plasmid is self-conjugal, and unlike the majority of previously characterized conjugal megaplasmids, conjugation of pAtC58 is constitutive (Chen et al., 2002). That is, pAtC58 conjugates at the same frequency under a range of laboratory conditions (although it is possible that there is as yet unrecognized environmental control of this conjugation). This data correlates directly with expression analyses of the genes encoding the pAtC58 conjugal pilus, the *avhB* operon (Perez-Mendoza et al., 2005; Morton et al., in preparation). This operon encodes a Type IV secretion system responsible for pAtC58 conjugation and homologous to the VirB pilus of Ti plasmid, which mediates transfer of the T-DNA during plant infection (Chen et al., 2002). Expression of this Ti plasmid encoded system has been demonstrated to be energetically quite expensive (Platt et al., 2012a). However, given that a truncated form of the plasmid lacking the *avhB* genes still confers a significant cost to its host cells, conjugation is likely not the sole contributor to the high cost of the plasmid (Morton et al., 2013). In addition to the AvhB system, there are multiple ATP binding-cassette (ABC) transporters encoded on the plasmid. As transmembrane proteins requiring energy in the form of adenosine triphosphate (ATP) to transfer molecules across membranes, these transporters could explain a portion of At plasmid costs. Despite its high cost, pAtC58 is extremely difficult to cure and attempts to do so frequently result in genomic restructuring (Morton et al., 2014). It is perhaps the high cost of the plasmid, coupled with mechanisms that ensure its maintenance (i.e., toxin–antitoxin systems) that drives selection for the observed deletions (Morton et al., 2013).

In addition to plasmid-encoded stability functions, pAtC58 prevalence in the environment is in part explained by the catabolic benefits it provides to its host bacteria (Morton et al., 2014). The competitive advantage in the rhizosphere garnered by strains of *A. tumefaciens* harboring pAtC58 is likely attributed to the plasmid-conferred ability to catabolize such molecules as GBL and DFG as a sole carbon source (**Figure 1B**; Baek et al., 2000; Morton et al., 2014). DFG, also known as santhopine, is an Amadori compound found in decaying plant material in addition to the tumors of plants transformed by chrysopine-type strains of *A. tumefaciens*. DFG catabolism is encoded by a set of genes located adjacent to the *repABC* operon on pAtC58 called *socR* and *socABCD* (Baek et al., 2003). In addition to its prevalence in the rhizosphere, DFG is formed during catabolism of agropine (AGR) and MOP, both functions encoded by octopine/mannityl opine-type Ti and Ri plasmids (Hong and Farrand, 1994). Oxidation of MOP (taken up directly from the environment or formed by the de-lactonization of AGR) results in the formation of DFG (Kim and Farrand, 1996). The uptake and catabolism of MOP and AGR is conferred by the products of the adjacent genes, *mot, ags*, and *moc* (Hong et al., 1997; Oger et al., 1998).

Strains harboring pAtC58 are able to grow using GBLs or related compounds as a sole carbon source. This growth is dependent on a functional *blcABC* operon—previously named *attKLM*, as mentioned above (Khan and Farrand, 2009). GBLs are common plant exudates and thus pAtC58 confers the ability to catabolize resources associated with the rhizosphere environment. Many soil bacteria rely on quorum-sensing (QS) to monitor population density and regulate community behaviors accordingly (Fuqua et al., 2001). Several soil bacteria including streptomycetes produce and employ GBL derivatives as quorum sensing signaling molecules (Du et al., 2011). The *blcC* gene, encoding a lactonase, and its homologs have received considerable attention due to their potential quorum quenching effects in degrading acyl homoserine lactone (AHL) signal molecules. The BlcC lactonase can effectively degrade AHLs to form *N*-acyl-homoserines, rendering them inactive as quorum sensing signals. The transcriptional repressor *blcR* is divergently transcribed from the *blcABC* operon. Null mutants in *blcR* fail to accumulate the Ti plasmid encoded AHL, 3-oxo-C8-HSL, due to overexpression of *blcC* (Zhang et al., 2002).

Break-down products of GBLs are intermediates of the tricarboxylic acid (TCA) cycle, which can also induce *blcABC* expression by causing the dissociation of BlcR from the *blcABC* promoter, thereby inhibiting the accumulation of AHL under more natural conditions. The biological relevance of this, however, is still unclear and the subject of considerable debate. There is convincing evidence demonstrating that artificial induction of this operon during infection will cause an initial delay in tumorogenesis, but that over time these effects are negligible (Khan and Farrand, 2009). Additionally, although the *blcABC* operon confers GBL catabolism, GBL is only a minor inducer of expression of these genes. Strong expression requires the presence of the GBL breakdown products SSA, GHB, or GABA, a non-protein amino acid expressed in plant tissues in association with stress or mechanical damage (e.g., wounding; Khan et al., 2007). Please refer to Lang and Faure (2014) for a more extensive discussion on this topic.

ECOLOGICAL CONTEXT OF Ti AND At PLASMIDS

The rhizosphere is the soil at the interface of plant root tissue. Here, plant roots influence the conditions of the soil to create a dynamic environment that is rich in microbial life (Badri et al., 2009). Because of this diversity, the rhizosphere is the seat of intense resource and interference competition among the resident microbes (Raaijmakers et al., 2009). Further, the interactions that occur between plants and the rhizosphere microorganisms as well as microbe-microbe interactions have large effects on the dynamics of both plant and microbial communities (Bever et al., 2012; Philippot et al., 2013). Agrobacteria must contend with intense competition with other agrobacteria and rhizosphere microbes associated both with healthy and with crown gall-diseased plants.

The plant tumor environment is remarkably diverse and can include several different types of opine catabolic microorganisms. Though opines are relatively uncommon metabolites that provide nutrients promoting the growth of the pathogenic agrobacteria, several other soil bacteria have the ability to catabolize specific opine species (Tremblay et al., 1987; Beauchamp et al., 1990; Bergeron et al., 1990; Nautiyal and Dion, 1990; Nautiyal et al., 1991; Moore et al., 1997). Additionally, colonization by opine catabolic, avirulent agrobacteria and the *de novo* evolution of avirulent freeloaders via loss of virulence functions have both been observed (Llop et al., 2009). Pathogenic agrobacteria are likely to be at a competitive disadvantage to these avirulent, opine catabolic agrobacteria that do not pay the costs

associated with harboring the intact Ti plasmid or cooperatively infecting plants, raising the possibility that the pathogen may become displaced from the tumor environment it elicited (Platt et al., 2012a,b). While the competitive disadvantage of virulent strains against avirulent freeloaders may be sufficient to explain their exclusion from diseased environments, pathogenic agrobacteria also must contend with even more overt competitive mechanisms such as bacteriocin-mediated interference competition with other rhizosphere bacteria (e.g., Kim et al., 2006). In this way, K84 and similar opine catabolic, avirulent strains are able to highjack the crown gall environment engineered by pathogenic agrobacteria, hence K84 has utility as a biocontrol agent for certain types of crown gall disease (Farrand et al., 2007).

The non-protein amino acid GABA is involved in a wide variety of cellular responses that extends across kingdoms. In animals, GABA acts as a neurotransmitter and in plants and bacteria, the molecule is usually involved in biotic and abiotic stress responses. GABA is produced in wounded plant tissues as part of a complex defense response, where this molecule is taken up in *A. tumefaciens*. Uptake has been shown to require an ABC transporter BraDEFG (Atu2424- Atu2427) and a periplasmic binding protein (Atu2422; Chevrot et al., 2006; Haudecoeur et al., 2009b). GABA resembles break-down products of the QS signal 3-oxo-C8-HSL which is responsible for activating Ti plasmid replication and conjugation through its interaction with the regulator, TraR. Regulation of GABA uptake in A. *tumefaciens* has recently been shown to primarily occur via the sRNA AbcR1 (Wilms et al., 2011). This sRNA was found to destabilize the transcript of the proline/GABA periplasmic binding protein, Atu2422. In stationary cultures, strains deficient in AbcR1 accumulate Atu2422 and GABA to a much higher level than WT cells. It was proposed that the sRNAs serve to reduce levels of the Atu2422 transcript and minimize intracellular levels of GABA. Exclusion of GABA from the cell would prevent BlcC-mediated quorum quenching and maintain physiological AHL-regulated Ti conjugation and expression of virulence genes (Wilms et al., 2011, 2012). Additional regulation of GABA uptake has been suggested through proline as it competes with GABA for binding to the periplasmic protein, Atu2422, which is required for uptake of both molecules (Haudecoeur et al., 2009a). It is unclear, however, what role proline accumulation plays in the plant–*Agrobacterium* interaction.

While GABA stimulates expression of *blcABC* and degradation of QS signals, it has also been shown that agrocinopines stimulate production of another, Ti-encoded, lactonase, AiiB homologous to BlcC, which may be involved in the reduction in accumulation of 3-oxo-C8 HSL and a modulation of QS-mediated Ti plasmid conjugation (Liu et al., 2007). AiiB was shown to be excluded from AccR-mediated regulation, meaning that it acts independent of 3-oxo-C8 HSL levels (Haudecoeur et al., 2009b). AiiB and BlcC are thus regulated by separate pathways, suggesting they play distinct roles in the degradation of QS signals during *A. tumefaciens* plant interactions and pathogenesis.

For nopaline-type *A. tumefaciens* strains, the agrocinopines produced by the infected plant control expression of many genes, primarily through the regulator, AccR. These genes include the *arc* genes (including the *traR* gene encoding the AHL-responsive quorum sensing regulator), the *acc* genes and, more recently discovered, the *noc* genes through *nocR*. TraR is directly responsible for the expression of T4SS genes for the conjugal transfer of the Ti plasmid, meaning that agrocinopines, through AccR, control dissemination of this replicon. Similarly, it was recently shown that AccR also regulates expression of pAtC58's *rctB*, orthologs of which have been shown to be involved in control of symbiotic plasmid conjugation in related rhizobia *R. etli* and *S. meliloti* (Perez-Mendoza et al., 2005; Lang et al., 2013; Nogales et al., 2013). A recent paper demonstrated that AccR regulates the conjugation of both the Ti and At plasmids by repressing transfer of both replicons in the absence of agrocinopines (Lang et al., 2013). These results suggest that conjugation of co-resident Ti and At plasmids may be enhanced in the tumor environment. This would potentially result in co-transfer or competitive transfer of the At and Ti plasmids.

ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES OF INTERACTIONS BETWEEN THE Ti AND At PLASMIDS

Pathogenic agrobacteria pay a high cost to translocate the T-DNA into the plant's genome. However the resulting infection can benefit other individuals such as neighboring opine catabolic agrobacteria making this a cooperative behavior (Platt and Bever, 2009; Gardner and West, 2010; Platt et al., 2012a). The primary benefit of agrobacterial pathogenesis stems from the catabolism of public good nutrients, the opines that infected plants produce (Guyon et al., 1993; Oger et al., 1997; Savka and Farrand, 1997; Mansouri et al., 2002; Platt et al., 2012b). The competitive advantage of cheating genotypes threatens the evolutionary stability of any cooperative behavior (Hamilton, 1964a,b). Due to the high cost associated with infecting plants there is a strong selective pressure favoring avirulent, freeloading genotypes that retain the ability to access these benefits by catabolizing opines (Platt et al., 2012b). Non-pathogenic, opine-catabolic agrobacteria have frequently been isolated from plant crown gall tumors (Merlo and Nester, 1977; Nautiyal and Dion, 1990; Bouzar et al., 1993; Belanger et al., 1995). Growth of laboratory cultures of several strains of *A. tumefaciens* in the presence of *vir*-inducing phenolic compounds results in the origin and rapid spread of mutated strains that have generated plasmids incapable of conferring virulence (Fortin et al., 1992, 1993; Belanger et al., 1995). The evolution of avirulent agrobacteria from a pathogenic strain has also been observed in plant tumor tissues; however in this study non-pathogenic agrobacteria more often colonized the plant from the environment (Llop et al., 2009). This result is perhaps surprising given the apparent rapid rate of evolution of avirulent plasmids in the lab. Llop et al.'s (2009) observations suggest that avirulent freeloading is a successful and persistent strategy in nature and motivates future work examining the relative importance of mutation and colonization to the success of avirulent freeloaders.

Regardless of the origin, the ability of avirulent, opine-catabolic agrobacteria to invade plant tumors elicited by pathogenic agrobacteria, poses a significant challenge to the persistence of the pathogen. The costs of the Ti plasmid put the pathogen at a competitive disadvantage when opines are not present (**Figure 1**). Further, competition with avirulent, opine-catabolic agrobacteria

threatens the persistence of the pathogen in the host environment as well (Platt et al., 2012b). Thus, non-tumor soils are predicted to be a sink population for the pathogen due to competition with saprophytic agrobacteria, while freeloading avirulent bacteria can competitively displace pathogens from tumor soils. The persistence of agrobacterial pathogens, then, critically depends on the pathogen's neighbors tending to be other pathogens such that the individuals that pay the cost of infecting the plant have at least a transient exclusive access to the plant tumor environment. The conditions for pathogen persistence are relaxed if the tumor environment can support a larger population size than the healthy plant root (Platt et al., 2012b). The resolution of this tension will determine the prevalence of pathogenic strains in agrobacterial populations and therefore the disease incidence on the plant host.

Although the At and Ti plasmids are central drivers of the ecological dynamics of *A. tumefaciens* strains, there is a clear lack of knowledge regarding their frequency and distribution in nature, particularly in non-tumor environments. The spatial and temporal heterogeneity of the soil makes it difficult to define any particular microhabitat, for which the selective pressures affecting each plasmid's fitness are expected to vary (Platt et al., 2012a,b; Morton et al., 2014). Natural populations of pathogenic agrobacteria fluctuate seasonally, change across years, and can persist several years in soils that lack readily observable plants exhibiting crown gall disease (Bouzar et al., 1993; Krimi et al., 2002). In some cases the frequency of Ti plasmid bearing cells in nature has been observed to decline over time in the absence of opine-producing tumors of infected plants (Krimi et al., 2002). In contrast to this, the same study observed several instances where cells bearing a Ti plasmid appeared to have a competitive advantage over cells lacking a Ti plasmid, despite the absence of a crown gall tumor. Such observations may result from benefits conferred by chromosomal genes, the presence of cryptic tumors, or yet uncharacterized benefits conferred by Ti plasmids (Krimi et al., 2002). This highlights the importance of further work establishing the variety of factors driving the dynamics of natural agrobacterial populations.

Genomic characterization of natural isolates reveal that At plasmids are very commonly found in association with a Ti plasmid and strains that lack a Ti plasmid, frequently still carry an At plasmid. This is perhaps explained by the array of rhizosphere-specific catabolic functions encoded by At plasmids (DFG, GABA, and GBL). One greenhouse study shows that a strain with an At plasmid outcompetes an isogenic strain lacking the plasmid in the rhizospheres of infected plants (Morton et al., 2014). When rhizosphere-specific resources are depleted, the direction of the competitive interaction is reversed.

In addition to the direct effects of resource competition, the ecological dynamics of *A. tumefaciens* genotypes are determined by intracellular interactions. One example of this is the elevated expression of Ti plasmid virulence genes in cells that harbor a laboratory-evolved At plasmid (Morton et al., 2013). Competitions between pairs of isogenic C58 plasmid genotypes (plasmidless, carrying At plasmid, carrying Ti plasmid, and carrying both At and Ti plasmids) revealed genotype-specific interactions that differ significantly from what would be predicted based on independent plasmid costs. For example, the cost of the two plasmids together is non-additive (Morton et al., 2014). Incorporating these empirically determined plasmid-specific costs and benefits into a resource consumer model of competition shifted the predicted outcomes at equilibrium such that cells harboring an At plasmid are expected to dominate environments with a broader range of resource supply conditions (Morton et al., 2014). As a facultative pathogen *A. tumefaciens* cells occupy a range of resource environments. The environment-specific costs and benefits of the At and Ti plasmids coupled with the observed non-additive costs suggest that in a temporally dynamic and spatially structured environment, strains with both plasmids could have a competitive advantage relative to plasmidless or single plasmid genotypes.

SUMMARY AND FUTURE DIRECTIONS

Agrobacterial plasmids play a central role in the ecology and evolution of the bacteria that harbor them. These plasmids confer a wide range of phenotypes including the ability to infect plant hosts, catabolize plant-produced nutrients, and produce bacteriocins mediating interference competition with other rhizosphere bacteria. In this review we have described the wide range of ecological and molecular interactions that shape the evolution and ecological dynamics of agrobacterial plasmids.

Environmental resource levels play a key role in determining the relative costs and benefits associated with many agrobacterial megaplasmids (e.g., **Figure 1**). Consequent impacts on the competitive ability of plasmid-harboring strains thereby influence the spread and decline of these plasmids. A significant challenge remains in integrating these local scale competitive interactions into a meta-community framework that would predict population virulence levels and disease incidence. This effort will require additional information on other aspects of agrobacterial life-history, including their dispersal and dormancy. A secondary level of questions relates to the environmental benefits of the different variants of virulence plasmids. Do, for example, agrocinopine Ti plasmids confer a competitive advantage in P-limited environments while Ti plasmids conferring catabolism of secondary amine derivatives confer a competitive advantage in N-limited environments?

Conjugal transfer is a second factor determining the evolution of agrobacterial megaplasmids as it allows colonization of novel genetic backgrounds. An open question for future research is to what degree conjugation also impacts competition among variant agrobacterial plasmids. The fitness of conjugal plasmids is composed of contributions from both its vertical transmission during reproduction of the host cell and its horizontal transmission during conjugation. Because of this impact on plasmid fitness, conjugation may have a role in shaping the competitive ability of these plasmids.

Conjugation may also play a role in allowing for interactions between competing plasmids mediated by the toxin-antidote systems that they encode (Cooper and Heinemann, 2000, 2005). Several agrobacterial plasmids harbor such systems (Yamamoto et al., 2007, 2009), however their role in mediating interactions among competitor plasmids is yet uncharacterized and will be an interesting question for future research. Entry exclusion systems may play a role in mediating these affects as they provide a way

to prevent entry of the host bacterium by competitor plasmids that may evict the resident plasmid via a toxin-antidote system. An intriguing aspect of the Ti plasmid system is the hierarchical regulation of conjugation by opines and a quorum sensing system (Lang and Faure, 2014, this issue). This mechanism of gene regulation is responsive to both the levels of available resources that can be catabolized by Ti plasmid bearing cells, and the density of those cells. Why conjugation is regulated in this way, as it relates to plasmid fitness and competitive ability, is an exciting question for future work.

One of the strengths of agrobacteria as a model system for such questions is the ability to readily integrate lab, greenhouse, and field studies of this microorganism. Our understanding and ability to manipulate relevant environmental and genetic factors allows for experimental dissection of forces influencing the ecological success and evolution of these plasmids in the laboratory and more realistic greenhouse settings. Such work can be coupled with examination of the plasmid dynamics in field populations to provide a clear picture of what drives the success of agrobacterial megaplasmids in nature. These types of studies also address more general issues such as how the joint effects of dynamics in host and non-host environments influences the ecology and evolution of diseases caused by facultative pathogens. While many important diseases are caused by facultative pathogens, the dynamics of these pathogens occurring in non-host environments is poorly incorporated into most models of disease epidemiology and evolution. Consequently the experimental tractability and the established environmental context dependence to the fitness of agrobacterial pathogens makes these bacteria powerful model systems for studying the intersection of microbial ecology and disease dynamics for facultative pathogens.

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Functions and regulation of quorum-sensing in *Agrobacterium tumefaciens*

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In *Agrobacterium tumefaciens*, horizontal transfer and vegetative replication of oncogenic Ti plasmids involve a cell-to-cell communication process called quorum-sensing (QS). The determinants of the QS-system belong to the LuxR/LuxI class. The LuxI-like protein TraI synthesizes *N*-acyl-homoserine lactone molecules which act as diffusible QS-signals. Beyond a threshold concentration, these molecules bind and activate the LuxR-like transcriptional regulator TraR, thereby initiating the QS-regulatory pathway. For the last 20 years, *A. tumefaciens* has stood as a prominent model in the understanding of the LuxR/LuxI type of QS systems. A number of studies also unveiled features which are unique to *A. tumefaciens* QS, some of them being directly related to the phytopathogenic lifestyle of the bacteria. In this review, we will present the current knowledge of QS in *A. tumefaciens* at both the genetic and molecular levels. We will also describe how interactions with plant host modulate the QS pathway of *A. tumefaciens*, and discuss what could be the advantages for the agrobacteria to use such a tightly regulated QS-system to disseminate the Ti plasmids.

Keywords: quorum-sensing, opines, conjugation, genetic, plant host, quorum-quenching, gene expression regulation

INTRODUCTION

In its canonical definition, quorum-sensing (QS) refers to a process through which a bacterial population is able to monitor its cell density and accordingly to mount coordinate responses (Fuqua et al., 1994). This phenomenon relies on the synthesis, diffusion, and perception of small signal molecules (autoinducers) that allow bacteria to communicate with each other and to regulate gene expression. In the last 40 years, a number of studies have established that QS is widespread in the bacterial kingdom although the nature of the signal molecules and/or signaling networks as well as the functions regulated by QS may vary considerably depending on the species (Miller and Bassler, 2001; Frederix and Downie, 2011; Stevens et al., 2012; Pereira et al., 2013).

In Proteobacteria, the typical QS model is epitomized by the LuxI/LuxR bioluminescence system of *Vibrio fischeri* that was described as early as 1970 (Nealson et al., 1970; Eberhard, 1972). In summary, LuxI catalyzes the synthesis of an *N*-acyl-homoserine lactone, namely the 3-oxo-hexanoyl-homoserine lactone (3OC6HSL), that acts as an autoinducer and accumulates in a cell density-dependent manner. At a threshold concentration, the 3OC6HSL molecules bind to their ligands, the transcriptional factor LuxR, and the newly formed LuxR dimers induce the expression of the *lux* operon which includes the genes responsible for bioluminescence but also *luxI*. This last autoregulatory action results in an exponential increase of the production of autoinducers and accounts for the characteristic pattern of QS-dependent bioluminescence in *V. fischeri* populations which rapidly shift at the quorum concentration from an "off" state to an "on" state.

Interestingly many homologs of LuxI and LuxR proteins have been found in other bacterial species such as *Pseudomonas aeruginosa*, *Pectobacterium atrosepticum*, and *Agrobacterium tumefaciens* (Fuqua et al., 1994, 1996). The first milestone in the study of *A. tumefaciens* QS was the functional characterization of the TraR protein, the LuxR homolog (Piper et al., 1993; Zhang et al., 1993). This seminal finding opened a new area of research in horizontal transfer of virulence Ti plasmids in *A. tumefaciens* that made this phytopathogenic species a leading model for the investigation of LuxI/LuxR QS systems. In this review, we will recap the most striking results obtained in deciphering the genetic network as well as the molecular basis of *A. tumefaciens* QS. We will also present how this QS system, consistent with the phytopathogenic lifestyle of *A. tumefaciens*, is integrated into an exquisite regulatory process, including various opine-induced regulons and lactonase activities. Finally we will discuss the biological/evolutionary relevance of this complex network in terms of dissemination of Ti plasmid genes in the plant tumor environment.

OVERVIEW OF *A. tumefaciens* QS

A LuxI/LuxR TYPE QS INTEGRATING AN ANTAGONIST COMPONENT

The first insight of a QS system in *A. tumefaciens* was gained with the functional characterization of a *traR* gene, homologous to *V. fischeri luxR*, the product of which acted as a transcriptional activator in the presence of a co-inducer. Actually two versions of the *traR* gene were found almost concomitantly in nopaline- and octopine-type Ti plasmids (Piper et al., 1993; Fuqua and Winans, 1994). These genes displayed high homology between them but were located in dissimilar regions of the two Ti plasmids, the expression of each of these regions being controlled by specific opines. Along with these discoveries, the chemical structure of the co-inducer required for TraR activity was determined

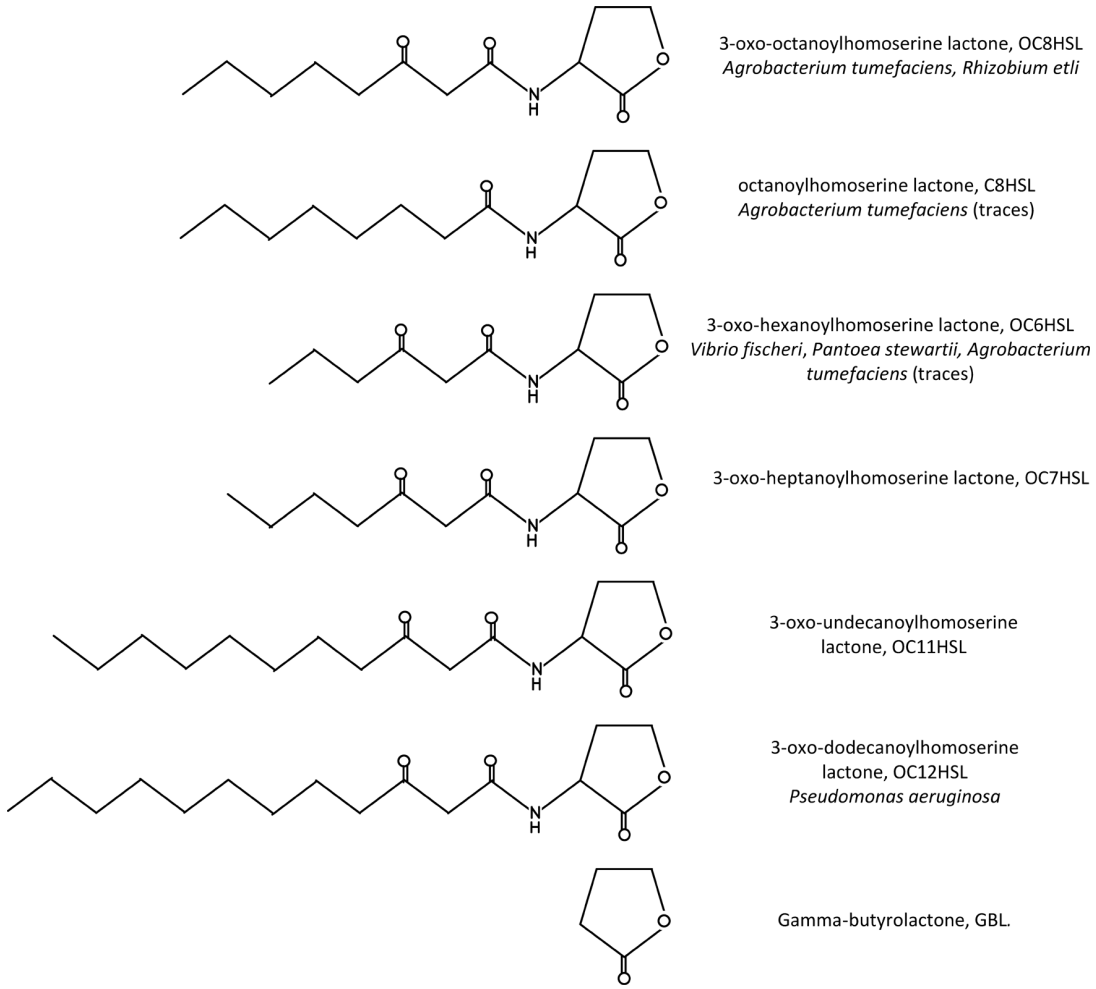

FIGURE 1 | Structures of the QS signals mentioned in this review. In parallel are indicated the full name of the molecule, its abbreviation as well as some bacterial species known to produce it.

by spectrometry analysis as 3-oxo-octanoyl-homoserine lactone (OC8HSL, see structure in **Figure 1**; Zhang et al., 1993). Soon afterward the gene *traI*, for which very closely related sequences also exist in nopaline- and octopine-type Ti plasmids, was shown to be responsible for OC8HSL synthesis (Hwang et al., 1994).

Like other LuxI/LuxR type QS systems, *A. tumefaciens* QS comprises another component that negatively modulates the activity of TraR and OC8HSL and this component is the Ti plasmid-encoded protein TraM which can suppress TraR transcriptional activity. Versions of the *traM* gene were identified in both nopaline- and octopine-type Ti-plasmids (Fuqua et al., 1995; Hwang et al., 1995). The octopine-type Ti plasmid A6 even possesses a second functional *traM* gene borne on a chromosome, surely as a result of gene duplication (Wang et al., 2006a). For long it has been thought that TraM proteins were not related to any other proteins found in the databases, but recent characterization of the *Pseudomonas aeruginosa* QslA protein contradicted this view (Seet and Zhang, 2011), suggesting that TraM-type functions might be relatively common in bacteria.

At a mechanistic level, yeast two-hybrid assays revealed that TraM and TraR could directly interact. From these data it was deduced that the association between the two proteins was responsible for the inhibition of TraR-mediated responses by preventing proper TraR binding to DNA (Hwang et al., 1999). Two subsequent findings strengthened the negative regulatory functions exerted by TraM on QS. First it was established that this protein could block TraR activity even after the transcription factor has bound to DNA (Luo et al., 2000) and second TraM was demonstrated to promote TraR proteolysis (Costa et al., 2012).

The implications of TraM action for the dynamics of the QS system will be discussed in the following section.

QS-REGULATED GENES ARE INVOLVED IN FEEDBACK CONTROL AND Ti PLASMID DISSEMINATION

Chronologically the first TraR-regulated, hence QS-regulated, genes were the OC8HSL synthesis *traI* gene and the *tra* genes involved in conjugation of the Ti plasmid (Piper et al., 1993; Fuqua and Winans, 1994; Hwang et al., 1994). Next, were the

regulatory gene *traM* (Fuqua et al., 1995; Hwang et al., 1995) and finally the *rep* genes required for vegetative replication of the Ti plasmid (Li and Farrand, 2000). Concomitantly, four 18 bp-inverted repeat operator sequences (called tra box I, II, III, and IV), the disruption of which abolished the TraR transactivation, were found in the promoter regions of the QS-regulated genes. These promoters were assigned to two distinct classes (class I-type and class II-type) according to the position of the tra boxes relatively to the transcription initiation site. In promoters of class I-type, the tra box is located approximately 65 nucleotides upstream of the transcription start site and in promoters of class II-type, the tra box is located about 45 nucleotides upstream of the transcription start site, partially overlapping with the −35 element of the promoter (**Figure 2**; Fuqua and Winans, 1996a). The *traR* gene has also been reported as being self-regulated though no tra box was detected in its promoter region (Fuqua and Winans, 1996b).

In line with the above studies, an extensive survey of QS-regulated genes has been recently carried out both in nopaline- and octopine-type Ti plasmids, using gene arrays and a TraR-overexpressing system (Cho and Winans, 2007). The results globally confirmed the previous data. Only genes located in the Ti plasmids were affected. In nopaline-type Ti plasmid, 31 genes were up-regulated in response to TraR overexpression and 25 in octopine-type Ti plasmid. Among the up-regulated genes common to the two plasmids, were the *tra*, *rep*, and *traM* genes. Moreover the operon structures, the presence of tra boxes in the promoter regions and the overall regulation of the expression of these genes were well conserved within the two plasmids.

Table 1 summarizes the identities and functions of the *A. tumefaciens* QS-regulated genes which are detailed in the following. The *traCDGyci* and *traAFBH* operons are divergently transcribed from a single class II-type promoter activated by a tra box I. These genes code for a DNA transfer and replication machinery involved in the conjugative processing of the Ti plasmid (Farrand et al., 1996; Cook et al., 1997; Cho and Winans, 2007). The proteins TraA, TraC, and TraD are notably thought to form a relaxosome at the oriT of the Ti plasmid which can also repress the expressions of both *traCDGyci* and *traAFBH* operons (Cho and Winans, 2007). The promoter of *traI-trbBCDEJKLFGHI* operon belongs to the class II-type of QS-regulated promoter but is characterized by the presence of a tra box II. The *trb* genes encode a mating pair formation system for the transfer of the Ti plasmid which is related to type IV secretion systems (Li et al., 1998). Among the proteins encoded by these genes, TrbJ and TrbK also act synergistically to implement an entry exclusion mechanism which ensures that conjugation events cannot occur between donor and recipient *A. tumefaciens* cells harboring similar Ti plasmids (Cho et al., 2009). In agreement with the gene functions, TraR-mediated up-regulation of the three *traCDGyci*, *traAFBH* and *traI-trbCDEJKLFGHI* operons results in induction of Ti plasmid conjugation. On the other hand the control of *traI* expression by TraR leads to a positive feedback loop which amplifies, through increase in OC8HSL production, the QS responses of *A. tumefaciens* (Fuqua and Winans, 1994; Hwang et al., 1994). As an illustration of this

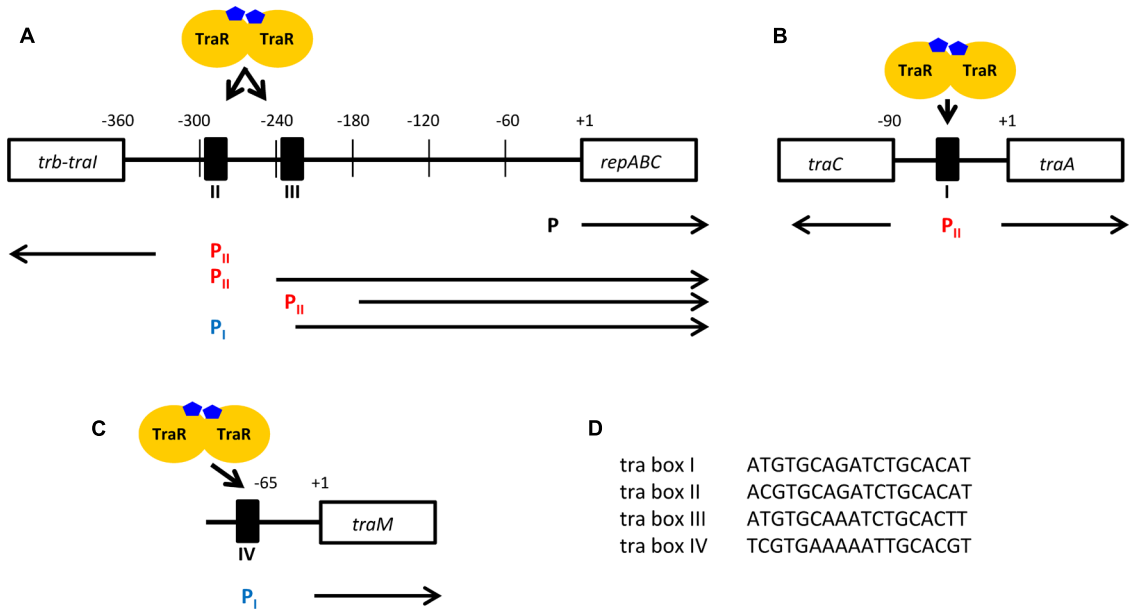

FIGURE 2 | Promoter architecture of the TraR-regulated genes in *A. tumefaciens*. **(A–C)** Representation of the regions upstream of the *traI-trbBCDEJKLFGHI* and *repABC* operons **(A)**, *traCDGyci* and *traAFBH* operons **(B)** *traM* gene **(C)**. The tra boxes (I, II, III, and IV) are indicated by black boxes. Under each tra box are presented the associated promoters, the activations of which are dependent on the binding of TraR. The promoters of class I-type are in blue while those of class II-type are in red. The fourth identified promoter controlling the expression of *repABC* in a TraR-independent way is also displayed (P). The arrows indicate the direction of transcription. **(D)** The nucleotide sequences of the four tra boxes. (Adapted from Fuqua and Winans, 1996a; Pappas and Winans, 2003a,b; White and Winans, 2005).

Table 1 | List of QS-regulated genes in nopaline- and octopine-type Ti plasmids (adapted from Cho and Winans, 2007).

Gene name	Function	atu code
traC	Conjugal transfer protein	*atu6126*
traD	Conjugal transfer protein	*atu6125*
traG	Conjugal transfer protein	*atu6124*
yci	Nuclease	*atu6122*
traA	Conjugal transfer protein	*atu6127*
traF	Conjugal transfer protein	*atu6128*
traB	Conjugal transfer protein	*atu6129*
traH	Conjugal transfer protein	*atu6130*
traI	Acyl-homoserine-lactone synthase	*atu6042*
trbB	Conjugal transfer protein	*atu6041*
trbC	Conjugal transfer protein	*atu6040*
trbD	Conjugal transfer protein	*atu6039*
trbE	Conjugal transfer protein	*atu6038*
trbJ	Conjugal transfer protein	*atu6037*
trbK	Entry-exclusion protein	*atu6036*
trbL	Conjugal transfer protein	*atu6035*
trbF	Conjugal transfer protein	*atu6034*
trbG	Conjugal transfer protein	*atu6033*
trbH	Conjugal transfer protein	*atu6032*
trbI	Conjugal transfer protein	*atu6031*
traM	Transcriptional anti-activator	*atu6131*
repA	Plasmid-partitioning protein	*atu6043*
repB	Plasmid-partitioning protein	*atu6044*
repC	Replication initiation protein	*atu6045*

effect, exogenous supply of OC8HSL to *A. tumefaciens* cells accelerated the TraR-mediated induction of Ti plasmid conjugation (Fuqua and Winans, 1996a).

Curiously the *traM* gene coding for the TraR antiactivator appears also to be up-regulated by TraR (Hwang et al., 1995). It was proposed that this regulatory mechanism allows the cells to produce TraM proteins at levels sufficient to inhibit the available TraR under conditions of basal-level expression. Later on, when the expression of *traR* is induced, the resulting increased levels of TraR protein would overcome the available TraM, thence triggering the QS response. This model actually highlights the importance of relative TraR and TraM protein levels in QS regulation and suggests that TraM significantly contributes to the quorum-dependent dimension of the system by delaying the moment when TraR is able to transactivate target genes (Su et al., 2008). Consistently, a *traM* defective strain was shown to be QS active in a cell density-independent manner (Piper and Farrand, 2000). Furthermore, a mathematical approach claimed that TraM was necessary for the existence of the *A. tumefaciens* QS "off" state (Goryachev et al., 2005). Another implication of the *traM* regulation by TraR is that the rate of TraR production must at one point exceed that of TraM production, otherwise QS activation would continuously be inhibited. Evidence that TraM is specifically transcribed from a mildly activated promoter with a tra box IV (White and Winans, 2005) is in line with this requirement. Alternatively an interesting but yet unexplored possibility to explain the induction of *traM* expression by TraR would be that this mechanism provides the cells with a mean to limit or shut off the QS process when this one is too strongly activated and becomes for instance too demanding energetically. This down-regulation loop is indeed common in other LuxI/LuxR systems (Gelencser et al., 2012). Either way a more critical examination of TraM regulation is still needed to fully clarify its role in QS. Additionally it has been shown that acetosyringone, a phenolic compound released by wounded plant cells, could also induce expression of *traM*, suggesting that during first steps of tumorigenesis TraM could efficiently inhibit QS activity (Cho and Winans, 2005).

The *A. tumefaciens* Ti-plasmids use an original system of replication and partitioning encoded in a single locus named *repABC*. While RepC is essential for replicative DNA synthesis, RepA and RepB are thought to be involved in stable partitioning of plasmids into daughter cells (Pinto et al., 2012). Initially the expression of the operon *repABC* was shown to be strongly stimulated by TraR in bacterial backgrounds with both nopaline- and octopine-type plasmids. This stimulation was also correlated with induction of vegetative replication, i.e., with a drastic increase in number of Ti plasmid copies per cell (Li and Farrand, 2000; Pappas and Winans, 2003a). However, in the array experiment mentioned previously (Cho and Winans, 2007), *repABC* up-regulation by TraR was barely detectable. The authors argued that this result was probably due to the very weak basal expression of the operon and that it did not question the role of QS in controlling the number of Ti plasmid copies because under their experimental conditions the number of Ti plasmids per cell was still higher than one. Another interpretation of this result might be that increased Ti plasmid copies culminate in a negative feedback control possibly bringing back the expression of the *repABC* genes to their basal levels, thereby avoiding continuous and anarchic replication of the replicon. The promoter architecture of *repABC* may support this hypothesis as three different TraR-dependent (repAP1, 2, and 3) and one TraR-independent (repAP4) promoters control the expression of the operon (Pappas and Winans, 2003b). Promoter repAP4 is thought to mediate the Ti plasmid replication associated with cell division but it is also autorepressed by RepA and RepB. Moreover repAP4 is located downstream of repAP1, 2, and 3. It is therefore conceivable that autorepression of repAP4 might impair activation of TraR-dependent promoters. Additionally expression of *repABC* can be induced by the virulence proteins VirA and VirG, further suggesting that the regulation of this operon is complex and might be sensitive to different physiological states (Cho and Winans, 2005; Pappas, 2008).

MECHANISTIC INSIGHTS INTO *A. tumefaciens* QS

A central aspect of the LuxI/LuxR type QS systems resides in the way autoinducers, transcriptional factors and gene promoters interact with each other. A better understanding of these mechanisms is therefore crucial to evaluate the specificity of the system.

Given the large variety of acyl-homoserine lactone derivatives which can serve as QS signals, it may also represent a privileged opportunity to get insight into possible crosstalk between different bacterial QS or to develop strategies of quorum-quenching. By combining biochemical and structural approaches with analysis of mutant strains and *in vivo* expression assays, the investigations on *A. tumefaciens* QS undoubtedly assemble one of the most elaborate sets of data in this domain.

TraI and OC8HSL SYNTHESIS

To identify the substrates of OC8HSL synthesis, the enzymatic activity of a purified *A. tumefaciens* TraI protein was tested in the presence of different molecules (More et al., 1996). It was thus determined that 3-oxo-octanoyl-acyl carrier protein (OC8-ACP) was the fatty acid donor and *S*-adenosylmethionine (SAM) the homoserine lactone precursor involved in OC8HSL synthesis. Mechanistically the synthesis reaction is proposed to occur in a "bi-ter" (two substrates, three products) way. The donation of the 3-oxo-octanoyl branch to the amine of SAM leads to the releases of first apo-ACP, then OC8HSL and finally methylthioadenosine (Parsek et al., 1999). All enzymes of the LuxI family are expected to share similar mechanisms of reaction, though variations in the acyl chain length and oxidation state at C3 of their acyl-ACP substrates exist. High-resolution crystal structures were obtained for two TraI orthologs: EsaI of *Pantoea stewartii* that synthesizes 3OC6HSLs and LasI of *Pseudomonas aeruginosa* that synthesizes 3-oxo-dodecanoyl-homoserine lactones (Watson et al., 2001; Gould et al., 2004). Analyses of these structures revealed that conserved residues in the N-terminal part of the protein were essential for SAM-binding and that selectivity of the acyl-ACP substrate was dependent on a V-shaped cleft passing through the enzyme. Other results also suggested that selectivity of LuxI-like proteins could be affected by availability of different acyl-ACP substrates. Noticeably, besides OC8HSL, *A. tumefaciens* produces traces of OC6HSL and octanoyl-homoserine lactone (C8HSL; Zhu et al., 1998).

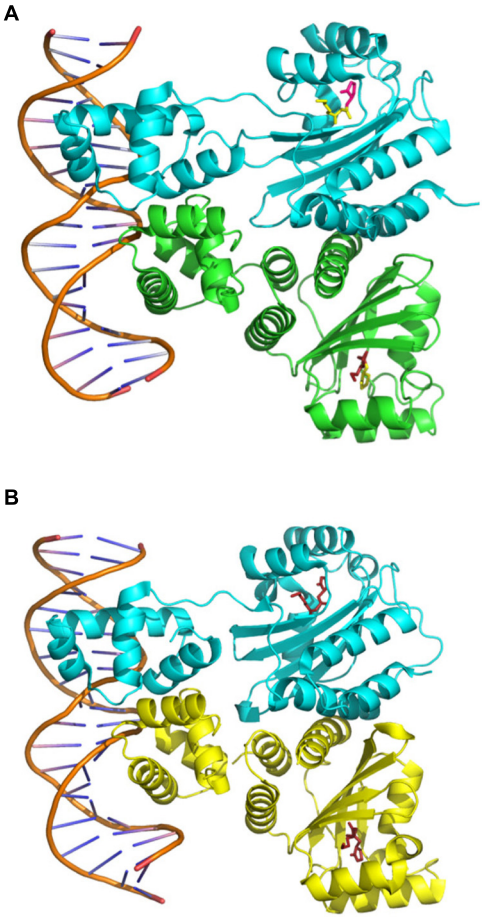

FIGURE 3 | Structures of the TraR–OC8HSL dimers in complex with DNA. The images were created using data from The Protein Data Bank (PDB; www.rcsb.org) (Berman et al., 2000) and the PyMOL Molecular Graphics System software. **(A)** PDB ID: 1H0M from Vannini et al. (2002). **(B)** PDB ID: 1L3L from Zhang et al. (2002b).

OC8HSL SPECIFICALLY INTERACTS WITH TraR

The first evidence of the interaction between TraR and OC8HSL was obtained through purified active TraR complexes which co-eluted with OC8HSLs in a ratio 1:1 (Zhu and Winans, 1999). Analysis of the protein turnover also indicated that binding of OC8HSL occurs rapidly in cells, surely during the own synthesis of TraR on polysomes (Zhu and Winans, 2001). Further crystal structures provided a mechanistic explanation for the specific interaction between TraR and OC8HSL as they revealed that the N-terminal part of TraR formed an enclosed cavity into which OC8HSL molecule could be engulfed and tightly maintained through numerous hydrophobic interactions as well as four hydrogen bounds (Vannini et al., 2002; Zhang et al., 2002b; **Figure 3**). To analyze the specificity of the interaction between OC8HSL and TraR, 31 analogs of OC8HSLs were tested for their abilities to activate TraR. Most of these compounds turned out to be potent antagonists of TraR under wild-type conditions of TraR expression and significant stimulators under conditions of TraR overexpression. These two features demonstrate that the specificity of the interaction between TraR and its ligand could be dependent on TraR concentration (Zhu et al., 1998). Moreover the 3-oxo function of the OC8HSL molecule seems to play important role in the interaction process as 3-oxo-C6-, 3-oxo-C7-, 3-oxo-C11-, and 3-oxo-C12-homoserine lactones (see structures in **Figure 1**) can also activate TraR, though with a much lower intensity than OC8HSL (Zhu et al., 1998; Luo et al., 2003b). Consistently non-conservative mutations of the threonine 129 of TraR, that was predicted to stabilize the 3-oxo group in the binding pocket, led to a strong impairment of TraR activity (Chai and Winans, 2004). In addition, alanine 49 and glutamine 58 in the N-terminal part of TraR were found to be important for the binding of the C8 acyl chain of OC8HSL since their conversion to bulkier amino acids resulted in higher affinity toward homoserine lactone derivatives with shorter acyl chain (Chai and Winans, 2004).

INTERACTION BETWEEN OC8HSL AND TraR FACILITATES FORMATION OF ACTIVE HOMODIMERS

The observation that C-terminal deletion mutants of TraR exerted strong dominant negativity over their wild-type counterparts

led to the hypothesis that TraR–OC8HSL complexes had to multimerize to be active (Luo and Farrand, 1999). Thereafter, size exclusion chromatography techniques revealed that purified active OC8HSL–TraR complexes formed homodimers, and hybrid expression reporter systems demonstrated that OC8HSL was required for this dimerization to take place (Qin et al., 2000). The existence of active OC8HSL–TraR homodimers was further supported by analysis of crystal structures which also suggested that these dimers were significantly asymmetric (Vannini et al., 2002; Zhang et al., 2002b). Two dimerization domains were identified in TraR sequence, one in the N-terminal part of the protein, partially overlapping with the OC8HSL-binding domain and another, less extensive, in the C-terminal part (Luo et al., 2003a). Several findings illustrated the role of OC8HSL binding in the maturation and dimerization process of TraR. In absence of OC8HSL, TraR proteins were intrinsically unstructured, insoluble in cells and rapidly degraded by proteases. On the opposite, presence of OC8HSL directed the release of active TraR into cytosol and enhanced the resistance of the protein against proteolysis (Qin et al., 2000; Zhu and Winans, 2001; Pinto and Winans, 2009). Additionally the proper folding of TraR and acquisition of mature ternary structure following the interaction with OC8HSL was shown to be mediated by the chaperone GroESL (Chai and Winans, 2009).

TraR–OC8HSL HOMODIMERS SPECIFICALLY RECOGNIZES tra BOXES

As mentioned above, tra boxes are 18 bp-inverted repeat operator sequences with a pronounced dyad symmetry, found in the two classes of TraR-regulated promoters (Fuqua and Winans, 1996a). The crystallization of TraR–OC8HSL complexes in presence of the tra box I sequence strongly suggested that each subunit of TraR–OC8HSL dimer binds to half of the tra box via C-terminal helix-turn-helix DNA binding motifs, thereby leading to an extensive DNA–protein interaction (Vannini et al., 2002; Zhang et al., 2002b; **Figure 3**). However, it was later demonstrated that six nucleotides at the center of the tra boxes did not interact with TraR and that yet these nucleotides contributed to proper activation of transcription, presumably by creating a flexible DNA bend (White and Winans, 2007). In parallel different screenings of TraR mutants resulted in the identification of three regions located in the N- and C-terminal part of the protein, which are critical for transactivation function but not for accumulation or DNA binding ability (Qin et al., 2004a, 2009; White and Winans, 2005). This finding suggested that these regions could cooperatively modulate the recruitment of the RNA polymerase and thereby differently control the expressions of TraR-regulated genes. Consistently some TraR mutants defective in transactivation of the *traI* promoter could still activate the *traM* promoter (Costa et al., 2009).

TraM-MEDIATED INACTIVATION OF TraR IS DUE TO OLIGOMERIC ASSOCIATION

In an effort to better understand how TraM could deactivate TraR, two crystal structures of TraM were obtained. They showed that the TraM protein can form homodimers with one unit linked to the other by an extensive hydrophobic interface (Chen et al., 2004; Vannini et al., 2004). The importance of this interface and the dimerization properties of TraM were also assessed using deletion mutants (Qin et al., 2004b). In addition, purifications of inactive TraR/TraM complexes carried out by different groups and with different biochemical techniques led to the conclusion that the inactive complexes were composed of two TraR–OC8HSL dimers and two TraM dimers both *in vitro* and *in vivo* (Chen et al., 2004; Vannini et al., 2004; Qin et al., 2007). Several domains important for this oligomerization and the resulting inhibitory effect were identified both in TraR and TraM sequences (Luo et al., 2000; Swiderska et al., 2001; Qin et al., 2007). Moreover, to explain the way TraM could inactivate DNA-bound TraR–OC8HSL dimers, a study convincingly proposed a stepwise mechanism according to which the apparition of inactive TraR–OC8HSL/TraM complexes was preceded by a nucleoprotein intermediate comprising one dimer of each protein in association with DNA (Qin et al., 2007). Interestingly the biochemical and structural properties of the TraR/TraM complexes were also investigated in the *Rhizobium* sp. strain NGR234 and led to similar conclusions regarding the mechanisms by which TraM can negatively impact TraR functions (Chen et al., 2007).

PLANT FACTORS ASSOCIATED TO *A. tumefaciens* QS

ROLE OF THE OPINES: MASTER CONTROL AND FINE-TUNING OF QS REGULATION

Opines are the small organic compounds which are produced during development of crown gall disease in transformed plant cells through the action of synthesis genes present on the T-DNA. All *A. tumefaciens* Ti plasmids harbor operons specialized in the uptake and assimilation of the opines they contribute to produce (Dessaux et al., 1992, 1998; Platt et al., 2012b). The two types most investigated in laboratories are the octopine- and the nopaline-type. Moreover, specific opines, called conjugal opines, are strictly required to enable conjugation of the *A. tumefaciens* Ti plasmid (Kerr et al., 1977; Petit et al., 1978). Therefore the finding, at the beginning of the 1990s, that this phenomenon was also dependent on the TraR/TraI QS system (Zhang and Kerr, 1991), sparked off significant interest and a number of studies aimed at understanding how these regulatory steps could be related. Successive genetic analysis, sequence determination and promoter dissections ultimately allowed the complete elucidation of the signaling pathway, clearly establishing the prominent role played by the conjugal opines for *traR* expression and QS initiation.

In the case of nopaline-type Ti plasmids, agrocinopines A and B which are a mixture of two non-nitrogenous phosphodiesters of sugars serve as conjugal opines (Ellis et al., 1982). These molecules can provoke, presumably by direct inhibitory interaction, the release of the transcriptional repression exerted by AccR, a member of the FucR family of transcriptional regulator (Beck von Bodman et al., 1992). In turn this derepression causes the expression of two divergently oriented operons: the *acc* (agrocinopines catabolism) and *arc* (agrocinopine regulation of the conjugation) operons of the Ti plasmid. The *acc* operon encodes seven proteins involved in internalization and degradation of agrocinopines plus the repressor AccR (Kim and Farrand, 1997) while the *arc* operon encodes five proteins, the fourth being TraR (Piper et al., 1999). In contrast, in octopine-type Ti plasmids, *traR* is the last

of 14 genes of the *occ* operon which codes for functions associated with octopine assimilation (Fuqua and Winans, 1996b). Octopine molecules are formed in transformed plant cells from arginine and pyruvate. Octopine is a conjugal opine as it binds to OccR, a transcriptional activator of the LysR family, thereby eliciting transcription of the *occ* operon including *traR* (Habeeb et al., 1991; Cho and Winans, 1993). Remarkably, the absence of the conjugal opines totally prevents QS-mediated conjugation of both nopaline- and octopine-type Ti plasmids. Moreover, despite the differences in *traR* location, the structures of the TraR-regulated operons are well conserved between the nopaline- and octopine-type Ti plasmids (Cho and Winans, 2007). This feature actually supports the view that *traR* and TraR-regulated genes constitute a functional unit, subjected to multiple and fortuitous recombination events in the course of *A. tumefaciens* evolution, and whose integration under the strict control of an opine regulon may have resulted in an important selective advantage for the bacteria (Piper et al., 1999; Oger and Farrand, 2001). In this sense the fact that such different molecules as agrocinopines and octopine can regulate *traR* expression in different Ti plasmids is remarkable.

Apart from the master control depicted above, opines are also involved in at least two other fine-tuning QS regulatory mechanisms. The first one was described in the *A. tumefaciens* strain R10 that harbors an octopine-type Ti plasmid. In this strain, the existence of a TraR antiactivator encoded by the Ti plasmid and different from TraM, named TrlR, was evidenced. Interestingly, TrlR expression was inducible by the opine mannopine (Oger et al., 1998). TrlR strongly resembles TraR but lacks its DNA-binding domain (Zhu and Winans, 1998). Experimental data provided evidence that TrlR could block TraR activity by forming inactive TrlR:TraR dimers (Chai et al., 2001). However, the impact of TrlR on QS implementation, especially *in vivo*, remains poorly understood. A second example of QS fine-tuning by opines is documented. In the nopaline-type *A. tumefaciens* C58 strain, expression of the Ti plasmid gene *aiiB* was shown to be induced by the agrocinopines, the same opines which are required for QS initiation (Haudecoeur et al., 2009b). Curiously *aiiB* codes for the AiiB lactonase that is highly similar to the AiiA lactonase from *Bacillus* sp. These proteins belong to a large family of Zn-hydrolases that encompasses lactonases of *Arthrobacter*, *Bacillus*, *Klebsiella*, *Mesorhizobium*, *Photorhabdus*, and *Rhizobium*. Biochemical and structural properties of AiiB were investigated. The AiiB protein is able to cleave the lactone rings of a large range of homoserine lactone derivatives, with a general preference for non-3-oxo-substituted molecules and substrates with an acyl chain longer than four carbons (Liu et al., 2007). Further conjugation experiments demonstrated the capacity of this lactonase to modulate *A. tumefaciens* QS responses both *in vitro* and *in planta* (Haudecoeur et al., 2009b). Globally the characteristics of *trlR* and *aiiB* (specific to octopine- and nopaline-type, respectively, and close homologs to *traR* and *aiiA*, respectively) suggest that these two genes could have arisen from gene duplication (for *trlR*) and horizontal gene transfer (for *aiiB*). On the other hand the conservation of an opine dependent regulation of their expression implies that there would be – somehow paradoxically – an advantage for *A. tumefaciens* cells to dampen QS communication at moments when opines, including conjugal opines, accumulate in tumors.

THE EXPRESSION OF THE OC8HSL-DEGRADING BlcC (FORMERLY AttM) LACTONASE IS INDUCED BY PLANT METABOLITES

As AiiB, the BlcC protein is a member of the AiiA lactonase family. Different studies have shown that BlcC degrades various homoserine lactone derivatives, including gamma-butyrolactone (GBL, see structure in **Figure 1**) and OC8HSLs. The *blcC* gene is part of the three-gene *blcABC* operon which codes for the catabolic pathway converting GBL to succinate, through gamma-hydroxybutyrate (GHB) and succinic semialdehyde (SSA) intermediates (Chai et al., 2007). Remarkably BlcC confers to *Agrobacterium* the ability to grow with GBL as sole source of carbon, but it does not with OC8HSLs (Carlier et al., 2004). The expression of the *blcABC* operon is tightly controlled by the transcriptional repressor BlcR. Carbon and nitrogen starvation, GBL, GHB, and SSA can all release the repression exerted by BlcR, hence allowing the expression of the *blcABC* genes (Zhang et al., 2002a; Carlier et al., 2004). The plant metabolite gamma-amino butyric acid (GABA), through conversion to SSA (Chevrot et al., 2006; Wang et al., 2006b), and the plant defense signaling hormone salicylic acid, through an unknown mechanism (Yuan et al., 2008), can also induce *blcC* expression. Based on the observations that GABA induces the expression of the *blcABC* operon and that GABA accumulates in tumors, it was proposed that the BlcC activity could coincide with QS communication during interactions between *A. tumefaciens* and plant hosts. However, in tomato tumors, the effect of BlcC on QS-dependent Ti plasmid conjugation was weak and transient (Khan and Farrand, 2009), suggesting that plant tumor tissues could exert a negative control on the expression of the BlcC expression.

The capacity of *A. tumefaciens* to take up GABA was extensively investigated in the last years. Studies revealed the involvement of two distinct transport systems. The gene *atu2422*, located on the circular chromosome is widely conserved within the *Agrobacterium* genus and codes for a periplasmic GABA-binding protein that controls GABA import through the *bra* ABC transporter (Planamente et al., 2010). Interestingly the GABA import by *atu2422* is strongly antagonized by proline, alanine, and valine, suggesting that these compounds which accumulate in tumors could also indirectly modulate the overall BlcC lactonase activity in the bacterial cells (Haudecoeur et al., 2009a). In comparison, the periplasmic binding protein encoded by the linear chromosome gene *atu4243* appears highly specific for GABA (Planamente et al., 2012). Strikingly, the expression of *atu4243* is totally repressed by *atu4232*-encoded protein and mechanisms of derepression are so far unknown (Planamente et al., 2012). Collectively these data illustrate the complexity of factors coming into play when searching to determine the impact of BlcC on *A. tumefaciens* QS. Of special interest would be the critical examination of plant metabolism to evaluate how the GABA, GBL, GHB, and SSA produced in the tumors may activate BlcC in colonizing *A. tumefaciens* cells. Such studies might reveal that the role of BlcC varies according to the metabolic status of the plant hosts.

INTERACTIONS BETWEEN THE Ti AND At PLASMIDS IN THE PLANT TUMOR

Another interesting feature of the *blcC* gene lies in its location on the companion At plasmid. This makes it the only component involved in *A. tumefaciens* QS that is not present on the Ti plasmid. Ecologically this characteristic raises interesting questions and notably that to know whether the dissociation of the At and Ti plasmids could result in a QS deregulation. To date very little is known about the maintenance of the At plasmid in *A. tumefaciens* populations. If no gene essential for the survival of *A. tumefaciens* C58 is present on the At plasmid (Goodner et al., 2001; Wood et al., 2001), the carriage of this At plasmid imposes *in vitro* high fitness costs to *A. tumefaciens* host cells (Morton et al., 2013). On the other hand, the At plasmid encodes several functions which confer or may confer a fitness advantage to agrobacteria in plant tumors (Haudecoeur et al., 2009b). Besides the degradation of butyrolactones and their derivatives mentioned above, the At plasmid is involved in the assimilation of some opines of Amadori compounds (Vaudequin-Dransart et al., 1998; Baek et al., 2005). The At plasmid also seems to have a positive impact on the virulence capacity of *A. tumefaciens* (Matthysse et al., 2008), although this point is debatable as it was recently shown that a large deletion in the At plasmid resulted in increase of the bacterial virulence (Morton et al., 2013). In conclusion, one can reasonably assume that, as for Ti plasmids, the tumor compartment is an appropriate environment for the dissemination of the At plasmid. Remarkably it was recently demonstrated that in *A. tumefaciens* C58, the conjugations of At and Ti plasmids are related events controlled by the agrocinopines-responsive regulator AccR and it was suggested that this mechanism of co-regulation could be instrumental in the conservation of the reciprocally beneficial functions carried by the two replicons (Lang et al., 2013).

OC8HSL-ASSOCIATED PLANT RESPONSES

The interactions between *A. tumefaciens* and plant hosts are mediated by several factors, from the phenolic compounds accumulated at wound sites that induce the expression of the Ti plasmid *vir* genes, to the opines produced in the tumor niche that control horizontal transfer of bacterial plasmids. It is therefore tempting to speculate on a possible implication of QS signal molecules in this generic trans-kingdom association, especially as several lines of evidence showed that *N*-acyl-homoserine lactone molecules could induce specific responses in eukaryote cells (Williams, 2007). For instance, in axenic plant systems, exogenous supply of different homoserine lactone derivatives was found to modulate plant immunity and development although the outcomes drastically differed according to the nature of the tested QS molecules (Klein et al., 2009; Hartmann and Schikora, 2012).

To our knowledge only three studies investigated the impact of OC8HSL on plants. In the first one, authors devised an inducible gene expression system based on TraR-OC8HSL activity which they introduced in *Arabidopsis thaliana* plants (You et al., 2006). To verify that induction with OC8HSL of the transferred gene did not affect the transcriptome of the transformed plants, the authors extracted RNA from 12-day-old seedlings treated or not by foliar application with 1 mM of OC8HSL for 24 h and carried out microarray experiments using Agilent technology. Processing of the data prompted them to conclude that no gene was differentially expressed by presence of the QS signal. In a second paper, a proteome analysis of *Arabidopsis thaliana* roots grown for 24 h in a hydroponic system in the presence or not of 10 μM of OC8HSL revealed that the levels of 53 proteins involved in the metabolism of carbohydrate and energy, protein biosynthesis, defense responses, and cytoskeleton remodeling, were significantly affected by the QS signal (Miao et al., 2012). The modest number of proteins differentially affected in this study suggests that plants sense *A. tumefaciens* QS signals only in a very restricted way. Noteworthy, in the two above-mentioned experiments, the used concentrations of homoserine lactone derivatives were in the micromolar and millimolar range while the concentrations at which QS molecules are active in *A. tumefaciens* are usually rather in the nanomolar range. Finally *Arabidopsis thaliana* defense responses upon exposure to OC8HSL-producing *Rhizobium etli* were recently analyzed. The results established that this condition had no impact on the plant defense (Zarkani et al., 2013), thereby strengthening the notion that plants are immune to OC8HSLs.

IMPLICATIONS AND SELECTIVE ADVANTAGES OF THE TIGHTLY REGULATED QS SYSTEM IN *A. tumefaciens*

Taken together the findings presented above described a very sophisticated system in which *A. tumefaciens* QS action is not only placed under the strict control of the conjugal opine regulon but is also modulated by various adjacent components like antiactivator or lactonases (**Figure 4**). Now we will discuss the implications of such hierarchical regulatory cascades and speculate about the selective advantages they may confer to *A. tumefaciens*.

CONJUGATION OF Ti PLASMID IN OPINE-PRODUCING TUMORS

As mentioned previously, the expression of *traR* gene requires the presence of conjugal opines. Therefore the QS system of *A. tumefaciens* functions only in host plants and only after transformed tissues have accumulated sufficient amount of conjugal opines. This restriction suggests that mature tumors are the most conducive environments for Ti plasmid dissemination and that, in these plant tumors, the selective advantages conferred to *A. tumefaciens* by a functional Ti plasmid would overcome the associated costs of maintenance. Supporting these notions, it has been demonstrated that Ti plasmid imposed a high fitness cost under conditions reminiscent of tumorigenesis but not anymore when opines were fully supplied (Platt et al., 2012a). It has also been observed that large proportion of *A. tumefaciens* cells present in mature tumors were devoid of Ti plasmids or harbored a mutated Ti plasmid (Fortin et al., 1993; Belanger et al., 1995). Thus the master control by conjugal opines could allow a large dissemination of functional Ti plasmids in an *A. tumefaciens* population characterized by a high proportion of potential recipient cells. The resulting selective advantages would be manifold. By amplifying the number of genes involved in opine assimilation, this mechanism could increase the colonizing fitness of the *A. tumefaciens* population, especially in older tumors where nutritive resources are scarcer. Multiplication of *vir* genes may also enhance aggressiveness of the bacteria. In relation, several reports already

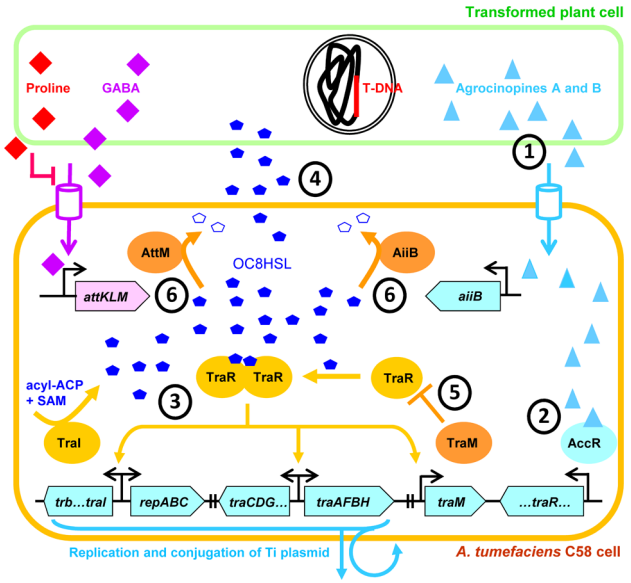

FIGURE 4 | Representation of the sophisticated hierarchical QS regulation in *A. tumefaciens* strain C58. QS-dependent conjugation and copy-number amplification of the Ti plasmid is initiated when OC8HSL and TraR reach appropriate concentration and form transcriptional active dimers. QS-signaling is positively regulated by the conjugal opines agrocinopines which are produced by the transformed plant cells **(1)** and induce production of TraR by releasing AccR repressive action **(2)**. Then, active TraR-OC8HSL dimers activate the production of TraI, thereby triggering a positive feedback in the synthesis of OC8HSLs **(3)** which are diffusible molecules **(4)**. The QS activation is delayed by the TraR-antagonist TraM **(5)**, as well as OC8HSL-cleaving lactonases AiiB and AttM **(6)** whose expressions are controlled by agrocinopines and GABA, respectively. SAM, *S*-adenosylmethionine; ACP, acyl carrier protein. The Ti plasmid genes and the At plasmid genes are in blue and pink boxes, respectively.

correlated an impairment of *A. tumefaciens* QS communication with a diminution of the crown gall symptoms (Haudecoeur et al., 2009b; Planamente et al., 2010, 2012). At last dissemination of Ti plasmids would increase the potential of migratory agrobacterial cells to initiate new infections. Interestingly Ti plasmid transfers to other bacterial species present in plant tumors may also occur, a feature that would favor genetic biodiversity. In this regard it is unfortunate that, even if the plant tumors are generally considered as privileged entry points for other bacteria, no information on plant tumor microbiomes are available at the moment.

DOES *A. tumefaciens* QS REALLY MEASURE A QUORUM OF DONOR CELLS?

Since the finding that *A. tumefaciens* QS controlled Ti plasmid conjugation, a "nagging" question remained to understand the relevance of a system in which donor cells could only monitor the density of other donors that already harbor a Ti-plasmid. Indeed as conjugation cannot happen in a cell already containing a resident Ti plasmid (Cho et al., 2009), the risk of uselessly activating, at the quorum concentration, the horizontal transfer machinery in the absence of sufficiently numerous recipient cells seems elevated. Nonetheless, as evoked previously, the master control of QS by conjugal opines might provide a way to circumvent this difficulty by allowing the conjugation of Ti plasmid only in mature tumors, i.e., in environments where the proportion of recipient cells would have extended. In such a context, the adjustment of the activation of the *tra* regulon according to a quorum of donor cells should maximize the efficiency of Ti plasmid dissemination and would be fully sensible. Under laboratory conditions, all the collected data firmly sustain the notion that *A. tumefaciens* QS functions as a cell density-dependent process. However, these conditions, using most of the time cell cultures and constant concentration of conjugal opines to initiate QS, may not reflect natural conditions. In *V. fischeri* the quorum nature of the system is defined by a production of LuxR at relatively high basal level and by a concentration of OC6HSL which increases as a function of cell density until reaching the threshold of LuxR activation (Miller and Bassler, 2001). In contrast, in *A. tumefaciens*, production of an active TraR regulator is subordinated to the presence of conjugal opines and to that of the antiactivator TraM. Taking full consideration of this characteristic implies that QS can be partly dissociated from solely functioning as a measure of population density. Another element of complexity may be brought by the non-linear accumulation of OC8HSL in tumors. Indeed plant tumors are not homogenous structures; they emerged from wound sites and underwent neoplastic expansion (Aloni et al., 1995; Veselov et al., 2003). In these complex environments colonizing *A. tumefaciens* shall form different clusters of cells more or less isolated one from the other and located in surface or intercellular spaces where diffusion rates are different as well as temporally changing. It therefore appears unlikely that the OC8HSL concentration which can be measured in a tumor or a part of the tumor does strictly mirror the cell density of the pathogen in this environment. Interestingly when they simulated the QS-induced transition in liquid cell cultures or biofilm, Goryachev et al. (2005) noticed that the first condition required a much higher threshold density than the second. They consequently came to the conclusion that *A. tumefaciens* QS served as a detector of biofilm formation rather than a sensor of cell concentration. If a growing attention has been given in the last years to mechanisms of biofilm formation in *A. tumefaciens* (Tomlinson et al., 2010; Hibbing and Fuqua, 2012), no data so far have related them to QS and very little is known about the formation of biofilms in the context of the agrobacterial interactions with plant host. However, it would definitely be relevant for the bacteria to place the coordination of Ti plasmid conjugation upon biofilm perception since the cell aggregates would constitute a very appropriate context for activation of the horizontal transfer machinery, either by minimizing the distances between donor and recipient cells or by acting as a shield against all kinds of physical or biological perturbations.

RELATIONSHIP BETWEEN QS REGULATION, Ti PLASMID CONJUGATION, AND *A. tumefaciens* HOST CELL

In the above discussion, the question of the QS-dependent dissemination of Ti plasmids was addressed only according to the selective advantages this dissemination may confer to agrobacterial cells. However, another perspective would be to consider Ti plasmids as selfish elements which somehow hijack *A. tumefaciens* cells in order to disseminate their genetic backgrounds. In this

framework Ti plasmids would take advantage of the opine and QS regulations to optimize the efficiency of their conjugations. It is furthermore important to note that the tumor conditions where the selective advantage conferred to *A. tumefaciens* cells by the Ti plasmids is the strongest coincide with the conditions where the dissemination of these Ti plasmids is the most important. The recent discovery in *A. tumefaciens* C58 that the conjugations of both Ti and At plasmids are exacerbated by conjugal opines (Lang et al., 2013) further supports the notion that Ti and At plasmids may collaborate to transform avirulent *A. tumefaciens* cells into virulent in order to perpetuate and disseminate their genetic traits.

CONCLUSION

In this review, we described the *A. tumefaciens* TraI/TraR QS system and showed how it exquisitely regulated the dissemination of Ti plasmids.

The QS systems of LuxI/LuxR type are generally thought to have originated early in evolution of Gram-negative Proteobacteria, with functional pairs of autoinducer synthases and receptors coevolving as regulatory cassettes, although in many cases these cassettes could also be inherited horizontally (Gray and Garey, 2001). In *A. tumefaciens*, the TraI/TraR system and the related QS-regulated genes are well conserved in all nopaline- and octopine-type strains studied to date, suggesting that this regulatory mechanism has been anciently selected. The target genes of *A. tumefaciens* QS are involved in the dissemination of Ti plasmids, both by replication and conjugation, but also in positive and negative feedback controls with the OC8HSL-synthesis TraI enzyme and the TraM antiactivator. Different studies demonstrated that this last protein plays a critical role in the implementation of the QS, even if it is not clear yet whether TraM is more relevant in delaying QS activation or in stabilizing and limiting QS activity.

At the molecular level, the *A. tumefaciens* QS communication has been largely deciphered. Two crystal structures have notably been obtained for TraR, in association with OC8HSL and DNA, providing a first class access to the interaction specificities of the system. Thorough biochemical investigations of active and inactive complexes also allowed to better understand multimerization processes of the QS components.

Consistent with the particular phytopathogenic lifestyle of the bacteria, *A. tumefaciens* QS system displays an original scheme including several differently acquired regulatory elements. The most important of these elements, common to all *A. tumefaciens* strains, are the conjugal opines which accumulate in tumors as a consequence of plant transformation and are strictly required for *traR* expression and hence for QS initiation. In parallel, only specific to some *A. tumefaciens* strains, lactonases such as AiiB and BlcC or supplementary anti-activator like TrlR can also modulate QS responses. This complex network of horizontal and lateral regulation suggests that there would be an advantage for *A. tumefaciens* to restrain as much as possible the window of QS activation.

Assessing reasons why a biological system has been selected is always challenging because this selection hinges on a trade-off between advantages and drawbacks which cannot be fully appreciated under laboratory conditions. By perusing different possibilities, we nonetheless hypothesized that the tight regulation of *A. tumefaciens* QS surely allowed the bacteria to disseminate the Ti plasmid in an environment where carrying the replicon would be clearly advantageous and at a moment when the energetic and physical factors would be ideal.

For the future, some important questions still remain to be answered to complete our understanding of *A. tumefaciens* QS functioning during the interactions with the host plant. For instance how do conjugal opines and TraM cooperate to produce active TraR-OC8HSL dimers? Precise dosage of conjugal opines in the course of tumor development as well as advances in knowledge of *traM* regulation might help solve this question. It would also be very interesting to better determine how the BlcC lactonase interferes with OC8HSL levels in tumors induced on different plants hosts and what are the ecological implications regarding horizontal transfers of both At and Ti plasmids. At last, analysis of bacterial populations found in natural tumors could deliver exciting results regarding abundance of potential Ti plasmid recipient cells. This kind of data might also unveil the extent of competition between the phytopathogen and other bacterial species present in plant tumors, hence leading to a novel appreciation of *A. tumefaciens* QS activity.

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Agrobacterium tumefaciens responses to plant-derived signaling molecules

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As a special phytopathogen, *Agrobacterium tumefaciens* infects a wide range of plant hosts and causes plant tumors also known as crown galls. The complexity of *Agrobacterium*–plant interaction has been studied for several decades. *Agrobacterium* pathogenicity is largely attributed to its evolved capabilities of precise recognition and response to plant-derived chemical signals. *Agrobacterium* perceives plant-derived signals to activate its virulence genes, which are responsible for transferring and integrating its Transferred DNA (T-DNA) from its Tumor-inducing (Ti) plasmid into the plant nucleus. The expression of T-DNA in plant hosts leads to the production of a large amount of indole-3-acetic acid (IAA), cytokinin (CK), and opines. IAA and CK stimulate plant growth, resulting in tumor formation. *Agrobacterium* utilizes opines as nutrient sources as well as signals in order to activate its quorum sensing (QS) to further promote virulence and opine metabolism. Intriguingly, *Agrobacterium* also recognizes plant-derived signals including γ-amino butyric acid and salicylic acid (SA) to activate quorum quenching that reduces the level of QS signals, thereby avoiding the elicitation of plant defense and preserving energy. In addition, *Agrobacterium* hijacks plant-derived signals including SA, IAA, and ethylene to down-regulate its virulence genes located on the Ti plasmid. Moreover, certain metabolites from corn (*Zea mays*) also inhibit the expression of *Agrobacterium* virulence genes. Here we outline the responses of *Agrobacterium* to major plant-derived signals that impact *Agrobacterium*–plant interactions.

Keywords: *Agrobacterium tumefaciens*, virulence, signaling mechanism, gene regulation, quorum sensing

INTRODUCTION

Agrobacterium is a genus of Gram-negative bacteria that uses horizontal gene transfer to cause tumors in many plant species with agricultural and economic importance including woody ornamental shrubs (rose), vines (grape), shade trees, fruit trees (cherry, berry, walnut), and herbaceous perennials. *Agrobacterium tumefaciens* is the most commonly studied species in this genus. *A. tumefaciens* causes typical crown-gall diseases. The disease manifests as a tumor-like growth or gall usually at the junction of the root and shoot. Infection by the species *Agrobacterium vitis* results in cane gall on grapevines while *A. rhizogenes* causes excessive formation of hairy roots or root tumors. *Agrobacterium*–plant interaction is an excellent paradigm for studying both plant and bacterial responses, as well as the role of chemical signaling in these processes. *A. tumefaciens*–plant interaction is now relatively well-understood as a result of significant findings made over the past four decades (for reviews refer to Gelvin, 2003; Brencic and Winans, 2005; McCullen and Binns, 2006; Yuan and Williams, 2012; Pitzschke, 2013). The virulence proficiency of *A. tumefaciens* is dependent on the presence of the Tumor-inducing (Ti) plasmid, which harbors a Transferred DNA (T-DNA) defined by two direct repeat sequences of approximately 25 base pairs, termed the left and right borders. Most studies have made use of nopaline metabolizing strains C58 and T37 (carrying plasmids pTiC58 and pTiT37, respectively) or the octopine utilizing strain A6 (carrying pTiA6). As a ubiquitous soil bacterium, *Agrobacterium* is capable of two lifestyles: independent free-living or acting as a pathogen in association with a plant host. When living independently, *Agrobacterium* virulence is essentially silent. Upon detection of plant-derived signals in the rhizosphere, *Agrobacterium* activates its chromosomal virulence genes (*chv* genes) and Ti plasmid encoded virulence genes (*vir* genes). *Vir* genes are directly involved in T-DNA cleavage from the Ti plasmid, T-DNA processing, transferring and integration into plant nuclei, conversely, *Chv* genes are not directly involved in the T-DNA transfer process. Instead, *chv* genes play important roles in signal transduction necessary for *Agrobacterium* pathogenicity. Since T-DNA carries genes for the synthesis of indole-3-acetic acid (IAA) and cytokinin (CK; also called oncogenic genes), their expression in plants leads to the production of a large amount of plant hormones that promote uncontrolled cell division and undifferentiated growth of plant tissues, resulting in the formation of a plant tumor and permanent plant genetic transformation.

Abbreviations: AS, acetosyringone; *chv* genes, chromosome encoded virulence genes; CK, cytokinin; DIMBOA, 2,4-dihydroxy-7-methoxy-2H-1,4-benzixazin-3(4H)-one; ET, ethylene; GABA, γ-amino butyric acid; IAA, indole acetic acid; MDIBOA, 2-hydroxy-4,7-dimethoxybenzoxazin-3-one; QS, quorum sensing; SA, salicylic acid; T3SS, type III secretion system; T4SS, type IV secretion system, T6SS, type VI secretion system; T-DNA, transferred DNA; Ti-plasmid, tumor-inducing plasmid; *vir* genes, virulence genes.

In addition to genes responsible for IAA and CK production, T-DNA also contains genes for the synthesis of opines (unusual amino acid and sugar condensates). Opines produced by transformed plant cells can be metabolized by *Agrobacterium* as a source of nutrients. In addition, opines act as signals that activate *Agrobacterium* quorum sensing (QS). QS is a special form of cell-to-cell communication by which microorganisms synthesize, release, and perceive diffusible signals such as *N*-(3-oxooctanoyl)-DL-homoserine lactone (3OC8-HSL). QS enables a single cell to sense the number of surrounding cells (cell density) and coordinates their collective behavior. In *Agrobacterium*, QS plays important roles in interaction with plant hosts, which will be discussed in later sections.

Interestingly, T-DNA encoded oncogenic genes are neither physiologically nor biologically required for the T-DNA transfer process. Therefore, T-DNA encoded genes can be deleted and replaced with genes of interest, and such genetically modified "T-DNA" can still be transferred, integrated and expressed in the plant cell. This unique ability of inter-kingdom DNA transfer makes *Agrobacterium* an important tool for genetically modifying plants, allowing for incorporation of useful traits like resistance to insects and herbicides, production of recombinant vaccines, proteins, etc. In addition, T-DNA is distally located from *vir* genes required for T-DNA transfer process. Thus, T-DNA and *vir* genes can be separated onto two plasmids without affecting T-DNA transfer into plant hosts. This feature prompted the design and construction of binary vectors that greatly facilitate DNA manipulation and plant transformation, especially considering the large size of the Ti plasmid (over 200 kb).

Agrobacterium is capable of infecting/transforming a wide variety of plant species including long-lived woody plants and cultivated plants. However, plants vary greatly in their ability to be infected/transformed by *Agrobacterium*, even among ecotypes within species, and the underlying molecular mechanisms are poorly understood (Nam et al., 1997). To mount a successful infection in nature, it is important for *Agrobacterium* to precisely and specifically recognize and respond to a combination of plant-derived signals in the rhizosphere including acidity, plant released sugars and plant-derived phenolic compounds (Stachel et al., 1985; Brencic and Winans, 2005; McCullen and Binns, 2006; Gelvin, 2012; Yuan and Williams, 2012; Pitzschke, 2013). *Agrobacterium* virulence programing and associations with plant hosts are stringently and synergistically regulated by a combination of plant-derived chemicals.

Agrobacterium RESPONSES TO ACIDIC SIGNALS CAUSED BY PLANT-DERIVED CHEMICALS IN THE RHIZOSPHERE

The rhizosphere is the narrow region (within millimeter range of roots) of soil that is directly influenced by root exudates and is densely populated by soil microorganisms. Rhizosphere is rich in not only plant-derived but microbe-derived signals as well (Winans, 1992; Phillips et al., 2004; Bais et al., 2005). Plants routinely secrete organic acids such as lactic, citric, oxalic, and malic acids as well as other secondary metabolites, resulting in acidic rhizosphere conditions (Rivoal and Hanson, 1994; Xia and Roberts, 1994; Walker et al., 2003; Phillips et al., 2004; Bais et al., 2005; Wang et al., 2006; Huckelhoven, 2007; Badri and Vivanco, 2009). Upon wounding, plants release phenolic compounds as well as neutral and acidic sugars necessary to repair damaged tissue acidifying the rhizosphere (Winans, 1992). Therefore the rhizosphere, where *Agrobacterium* primarily infects plant hosts, is typically an acidic niche driven by various plant-released chemicals.

Upon close proximity to a suitable plant host in the rhizosphere, acidic conditions and plant-derived chemicals play important roles in initiating the *Agrobacterium* virulence program, which involves various *Agrobacterium* regulatory factors and signaling pathways (Winans, 1992). A chromosomally encoded *che* cluster (chemotaxis) allows *A. tumefaciens* to be attracted to plant-derived chemicals in the rhizosphere (Wright et al., 1998). In addition, three *Agrobacterium* chromosomally encoded genes *chvA*, *chvB*, and *exoC* are involved in synthesis of extracellular oligosaccharides, such as cyclic 1,2-b-D-glucan, that allows *Agrobacterium* to attach to plant hosts (Cangelosi et al., 1989). Upon perception of acidity characteristic of the rhizosphere, *Agrobacterium* mounts both a conserved response as well as a signaling specific response to infect plant hosts. This conserved response allows *Agrobacterium* to adapt to the rhizosphere niche by modulating metabolism and cellular adaptation, such as the induction of genes coding for cell envelope synthesis, stress response, transporters of sugars and peptides (Yuan et al., 2008b).

The signaling specific response to acidity is mediated by the chromosomally encoded ChvG/ChvI two-component system, as well as other genes that allow *Agrobacterium* to initiate its early virulence program (Yuan et al., 2008a). ChvG acts as the sensor kinase while ChvI functions as the response regulator (Winans, 1990, 1992; Chen and Winans, 1991; Charles and Nester, 1993; Mantis and Winans, 1993; Li et al., 2002). The ChvG/I system is believed to recognize acidity in the rhizosphere and activates the expression of several virulence factors including *chvI*, *aopB* encoding an outer membrane protein, *katA* encoding a catalase, *pckA* encoding phosphoenol carboxykinase, and the *imp* gene cluster encoding a type VI secretion system (T6SS; Yuan et al., 2008a). A more recent study confirmed that *Agrobacterium* T6SS is indeed induced by acidity in a ChvG/ChvI dependent manner (Wu et al., 2012). Perhaps most interestingly, it was found that upon perception of acidic signals, several *vir* genes were also induced including *virG*, *virE0*, and *virH* (Yuan et al., 2008a), consistent with the observation that the ChvG/ChvI system activates the proximal promoter (P2) of *virG* (Li et al., 2002). However, to be functional, VirG requires phosphorylation signaling from another plant-derived signal, e.g., plant-derived phenolic compounds, which will be discussed in the following section. It is noteworthy that in addition to ChvG/I, another chromosomally encoded virulence gene, *chvE*, is also involved in *Agrobacterium* response to acidity and plant-derived sugars in the rhizosphere, which will also be discussed later.

Agrobacterium RESPONSES TO PLANT-DERIVED PHENOLIC COMPOUNDS

Originally it was believed that plant wounding was necessary for *Agrobacterium* infection and pathogenicity. However, recent advances have found that plant wounding is in fact not essential

for *Agrobacterium* pathogenicity since unwounded plants can also infected by *Agrobacterium* pathogens (Brencic and Winans, 2005). Besides acidic signals, plant-derived phenolic compounds are essential for the induction of *Agrobacterium* virulence (Stachel et al., 1986). Moreover, phenolics serve as chemoattractants for *Agrobacterium* (Parke et al., 1987; Melchers et al., 1989). Structural specificities of virulence inducing phenolics include the presence of a benzene ring with a hydroxyl group at position 4 and a methoxy group at position 3 (Dixon and Paiva, 1995). 3,5-dimethoxyacetophenone (acetosyringone) and hydroxyacetosyringone were the first identified inducers of *Agrobacterium* virulence (Stachel et al., 1985; Hess et al., 1991). The *Agrobacterium* VirA/VirG two-component system located on the Ti plasmid has been suggested to recognize acetosyringone as a host specific signal and activate *vir* gene expression (Winans et al., 1986; Leroux et al., 1987; Shaw et al., 1988; Winans, 1990). The membrane receptor VirA functions as a dimer with four domains; the periplasmic, cytoplasmic linker, kinase, and receiver domains. Upon phenolic signal perception, the linker domain of one VirA subunit activates the kinase domain of the opposite dimerized subunit by intermolecular phosphorylation (Chang and Winans, 1992; Turk et al., 1994; Toyoda-Yamamoto et al., 2000). However, a previous study has shown the binding of radiolabelled phenolic compounds to two small proteins other than VirA and controversy remains regarding the exact mechanism involved in phenolic detection by VirA (Lee et al., 1992). Nevertheless, the auto-phosphorylated sensor kinase VirA phosphorylates the cytosolic response regulator VirG at the conserved Asp52 (Morel et al., 1990; Lee et al., 1995). Phosphorylated VirG binds to a 12-bp *vir* box located upstream of transcription start sites of *vir* genes, thereby activating their transcription (Stachel et al., 1985; Stachel and Nester, 1986; Jin et al., 1990a,b,c; Pazour and Das, 1990; Roitsch et al., 1990). In fact, phosphorylated VirG also activates its own expression by activating *virG* transcription at the distal promoter (p1; Chang and Winans, 1992; Liu et al., 1992, 2005; Jia et al., 2002; Li et al., 2002; Yuan et al., 2008a; Wise et al., 2010).

Vir genes of *Agrobacterium* are organized in several *vir* operons. There are eight *vir* operons on the octopine-type Ti plasmid and relatively fewer *vir* genes on the nopaline-type Ti plasmid (Stachel and Nester, 1986; Rogowsky et al., 1987; Kalogeraki and Winans, 1998; Kalogeraki et al., 2000; Brencic and Winans, 2005). The *vir* operons are typically organized as *virH*, *virA*, *virB*, *virG*, *virC*, *virD*, *virE*, and *virF* transcriptional units (Brencic and Winans, 2005). *Vir* genes code for a set of proteins with different functions such as T-DNA excision and processing (*virC* and *virD*), coating and protecting T-DNA during transfer (*virE*), formation of the type IV secretion system (T4SS) responsible for the delivery of T-DNA to plant cells (*virB operon*), and T-DNA integration into plant nucleus (*virE2* and *virD4*). A study by Cho and Winans (2005) revealed that each gene on the Ti plasmid was modestly induced by plant-derived phenolic signals, while the *repABC* operon, responsible for Ti plasmid replication/partitioning, was significantly induced by phenolic signals. This suggests that the copy number of the Ti plasmid is induced by plant-derived phenolics, which is confirmed by direct binding of phosphorylated VirG to a 12-bp *vir* box upstream of the *repABC* operon (Zhu et al., 2000; Pappas and Winans, 2003; Cho and Winans, 2005). Apparently an increase in Ti plasmid copy number enhances the dosage of *vir* genes responsible for T-DNA transfer. A proteomic study corroborated VirA/VirG dependent induction of *vir* genes by identifying 11 proteins that were significantly induced in response to acetosyringone, including proteins constituting the T4SS, the single strand binding protein VirE2 that is exported to the plant nucleus, and the trans-zeatin synthesizing protein Tzs (Lai et al., 2006). Moreover, responses to phenolic inducers may be modulated by detoxification of these compounds by VirH2. VirH2 was shown to play a role in the metabolism of several phenolic compounds including ferulic acid, another inducer of *vir* genes (Brencic et al., 2004).

Agrobacterium RESPONSES TO PLANT RELEASED SUGARS IN THE RHIZOSPHERE

Agrobacterium detects and responds to plant-derived sugars through a distinct signaling pathway involving VirA and a chromosomally encoded periplasmic protein, ChvE (Cangelosi et al., 1990). Expression of *chvE* is regulated by the LysR transcriptional regulator (TraR) galactose-binding protein regulator (GbpR) in the presence of sugars (Doty et al., 1993; Peng et al., 1998). ChvE mediates *Agrobacterium* chemotaxis in response to aldose monosaccharides such as galactose, glucose, arabinose, fucose, xylose, and sugar acids. Importantly, ChvE binds plant-derived sugars and subsequently interacts with the periplasmic domain of VirA to stimulate *vir* gene expression (Cangelosi et al., 1990; He et al., 2009; Hu et al., 2013). Mutations in the periplasmic domain of VirA present the same phenotype as a ChvE mutant with both mutants unable to infect specific plant hosts (Cangelosi et al., 1990; Chang and Winans, 1992; Banta et al., 1994; Peng et al., 1998; Gao and Lynn, 2005). Recent studies also suggest that the ability of ChvE to recognize and bind different plant-derived sugars is vital in determining the host range of *Agrobacterium* (Hu et al., 2013). Interestingly, the sugar response in *Agrobacterium* has been found to be linked with the acidity responses since the absence of sugars or mutations in *chvE* disrupted acidic signaling. In addition, the affinity of ChvE for sugar acids increases with a decrease in pH (Hu et al., 2013), which reinforces an important role for acidity in modulating *Agrobacterium* virulence. It has been proposed that acidic conditions, together with the presence of sugars and a functional ChvE, promotes VirA–ChvE interactions required for efficient *vir* gene induction (Shimoda et al., 1993; Toyoda-Yamamoto et al., 2000; Gao and Lynn, 2005; Nair et al., 2011). However, mutations in *chvE* that abolish sugar sensing do not abolish *vir* gene induction by acetosyringone, although ChvE is known to interact with the periplasmic domain of VirA. This suggests ChvE and associated sugar perception play additive roles that further promote *vir* gene expression in response to sugars and phenolic compounds, while phenolics are essential *vir* gene inducing signals (Cangelosi et al., 1990; He et al., 2009; Hu et al., 2013). Apart from its signaling role, ChvE also has a role in sugar utilization as it delivers sugars to the ABC transporter MmsAB (Hu et al., 2013).

BACTERIAL AND PLANT MOLECULES INVOLVED IN T-DNA TRANSFER AND INTEGRATION

T-DNA transfer and integration into the plant nucleus is mediated by a complex set of *Agrobacterium* and host proteins. As discussed

in previous sections, *Agrobacterium* recognizes three main plant-derived signals (acidity, phenolics, and sugars) and activates *vir* genes. VirD1, a helicase, and VirD2, a site specific endonuclease, are essential for nicking the Ti plasmid and the release of T-DNA as a single stranded DNA (referred to as T-strand; Yanofsky et al., 1986; Wang et al., 1990). VirD4, the coupling protein, and VirB1–VirB11, the mating-pore-formation components, together constitute the trans-envelope channel and pilus of *Agrobacterium* T4SS apparatus. In depth studies have assigned functional roles to each protein of the T4SS complex; VirD4, VirB3, VirB4, and VirB11 constitute the ATP-dependent translocation machinery, VirB6–VirB10 form the channel and VirB1, VirB2, VirB5, and VirB7 form the pilus (Cascales and Christie, 2003). The T-strand is covalently attached by VirD2, which is subsequently bound by VirD4 and VirB11 forming the T-complex. Current findings suggest that such DNA binding by associated virulence proteins (VirD4 and VirB11) stimulates ATP hydrolysis to produce a structural transition in the membrane channel protein VirB10. This allows passage of the T-complex to the cell surface where it can be directed to the T4SS pili, followed by delivery into the plant cell by the T4SS apparatus (Cascales and Christie, 2003; Cascales et al., 2013). Notably, many Gram-negative plant and animal pathogenic bacteria employ a type III secretion system (T3SS) to inject effector proteins directly into the cytosol of eukaryotic cells and thus allow the manipulation of host cellular activities to the benefit of the pathogen (Buttner and He, 2009). However, no T4SS organelles in *Agrobacterium* reminiscent of the basal body of flagella or needle complexes of the T3SS were evident (Christie, 2004). The exact process is still unknown regarding how the T-DNA complex is delivered by T4SS into plant cells, especially how the T-DNA complex passes through cell wall and plasma membrane, subsequently moving through the cytoplasm to the plant cell nucleus (Cascales and Christie, 2003; Gelvin, 2003).

T-DNA integration into plant nuclei is thought to occur by hijacking various host systems including defense signaling, cytoskeletal networking, molecular motors, nuclear import, proteolytic degradation, chromatin targeting, and DNA repair to ensure successful plant transformation (for review see Citovsky et al., 2007). In the plant cytoplasm, the T-strand/VirD2 complex is coated along its entire length by the VirE2 ssDNA-binding protein that is transported into the plant cell independently of the T-strand complex (Vergunst et al., 2000; Citovsky et al., 2007). Both VirD2 and VirE2 carry plant nuclear localization signals and together with host protein VIP1 (VirE2 interacting protein 1), facilitate T-complex import into the plant cell for host chromatin targeting (Citovsky et al., 1992; Tzfira et al., 2001; Li et al., 2005; Djamei et al., 2007; Lacroix et al., 2008). T-DNA is thought to attach to chromatin by interacting with nucleosomal proteins and is released from the T-complex by proteolytic removal of associated proteins (Magori and Citovsky, 2011). VirF, a bacterial F-box protein, also targets both VIP1 and VirE2 for proteasome dependent degradation. The mechanism of T-DNA integration into the plant genome is thought to occur by illegitimate recombination; however, the details of many of the molecular events within the plant cell and nucleus are still unclear.

Agrobacterium METABOLIZES PLANT-DERIVED OPINES AS A SOURCE OF NUTRIENTS

Besides IAA and CK, infected plant cells produce over 20 different kinds of opines that can be classified into four families: octopine, nopaline, mannopine, and agrocinopine families (Beck von Bodman et al., 1992; Fuqua and Winans, 1996b; Piper et al., 1999). In fact, the most intensively studied Ti plasmids are the octopine and nopaline types, named after the predominant opines synthesized by transformed plant cells. Octopine is synthesized by the T-DNA-encoded enzyme octopine synthase (Ocs), which condenses pyruvate with different amino acids to produce octopine, lysopine, histopine, or octopinic acid (Dessaux et al., 1998). Nopaline is generated by nopaline synthase (Nos) in a similar condensation reaction involving αα-ketoglutaric acid and either arginine or ornithine. Opines of the mannopine and agrocinopine families are structurally more heterogeneous, which contain sugar and phosphate groups in the case of agrocinopine. Since plants cannot metabolize opines, transformed plant cells accumulate and release opines into the rhizosphere. The precise mechanism by which opines are exuded from plant cells is unknown, although the exudation of octopine and nopaline appears to depend upon the product of T-DNA gene 6a. Nevertheless, opines are present on the plant (or tumor) surface and are part of the soluble plant exudates released into the phylloplane and rhizoplane (Savka and Farrand, 1992).

Agrobacterium Ti plasmids also contain genes for opine uptake and catabolism that are located in the non-transferrable region, e.g., occ and noc regions for octopine- and nopaline-type Ti plasmids. In addition, Ti plasmids contain chemotaxic genes for their corresponding opines (Beck von Bodman et al., 1992; Kim and Farrand, 1997). *Agrobacterium* LysR-type transcriptional activator OccR (octopine catabolic regulator) and NocR (nopaline catabolic regulator) recognize and bind to opines, subsequently activating the expression of opine catabolic genes (Beck von Bodman et al., 1992; Wang et al., 1992). *Agrobacterium* metabolism of agrocinopine is much more complicated. When agrocinopines are present, the repressor agrocinopine catabolic regulator (AccR) dissociates from the promoter, allowing for expression of the *acc* operon responsible for agrocinopine metabolism. In addition, the *acc* operon is activated in response to phosphate limitation (Kim et al., 2008). Some of opine catabolic genes are also under regulation of other factors, for example the presence of certain substrates such as succinate (Hong et al., 1993). Although the rhizosphere contains species other than *Agrobacterium* that are capable of utilizing opines, they comprise of a very small minority of the bacterial population (Nautiyal and Dion, 1990). Therefore, the ability to use opines as a carbon, nitrogen, and energy source provides distinct advantages to *Agrobacterium* in the rhizosphere niche.

Agrobacterium QUORUM SENSING IS ACTIVATED BY PLANT-DERIVED OPINES

In addition to serving as a nutrition source for *Agrobacterium*, opines produced by transformed plant cells also activate *Agrobacterium* QS. In fact, the original study of *Agrobacterium* QS was relevant to Ti plasmid conjugation. In soil or cultivation at temperatures greater than 30°C, the Ti plasmid is rapidly

lost from *Agrobacterium* populations. Once infected by *Agrobacterium*, plant cells produce opines. In addition to activating genes for opine metabolism, the NocR- or OccR-opine complex also activates a LuxR-type TraR located on the Ti plasmid (Li and Farrand, 2000). When the diffusible QS signal N-(3-oxooctanoyl)-DL-homoserine lactone (3OC8-HSL) reaches a threshold level with high population density, TraR binds to 3OC8-HSL. The TraR–3OC8-HSL complex activates *traI*, a LuxI-type 3OC8-HSL synthase, and *tra*/*trb* genes coding for a second T4SS responsible for conjugal transfer of Ti plasmids (Piper et al., 1993; Zhang et al., 1993; Fuqua and Winans, 1994; Hwang et al., 1994). Since the Ti plasmid carries genes responsible for plant infection and opine metabolism, avirulent *Agrobacterium* lacking the Ti plasmid becomes infectious and capable of opine metabolism by acquiring the Ti plasmid through conjugation. Additionally, the TraR–3OC8-HSL complex activates the *repABC* operon thereby enhancing the replication and copy number of the Ti plasmid (Fuqua and Winans, 1996a; White and Winans, 2007). TraR–3OC8-HSL complex also activates the transcription of TraM, a TraR antiactivator in both octopine- and nopaline-type strains of *Agrobacterium*. TraM further modulates QS and Ti plasmid conjugation in the rhizosphere (Hwang et al., 1999). Therefore, the initial infection and T-DNA transfer leads to the synthesis of opines in plant cells. Opines activate the *Agrobacterium* TraR/TraI QS system, which activates Ti plasmid conjugation and enhances Ti plasmid copy number (up to eightfold). This typical positive feedback regulation is advantageous for maximal infection of plant hosts and opine metabolism (Zhu and Winans, 1999, 2001; Pappas and Winans, 2003; Cho and Winans, 2005; Pinto et al., 2012).

Agrobacterium QS IS FURTHER MODULATED BY OTHER PLANT-DERIVED SIGNALS

Agrobacterium QS is well regulated not only for QS signal production, but also for QS signal degradation, also known as quorum quenching. γ-amino butyric acid (GABA) significantly increases in wounded plant tissues and acidic conditions. GABA also accumulates in plants infected by *Agrobacterium* (Chevrot et al., 2006). In addition, proline significantly accumulates in plant tumors but neither in wounded nor healthy tissues (Deeken et al., 2006). The *Agrobacterium* proline/GABA receptor *atu2422* and ABC-transporter *braE* (*atu2427*) are required for GABA and proline uptake. GABA activates transcription of the *attKLM* operon located on the second plasmid of *Agrobacterium*. AttK is a NAD dependent dehydrogenase. AttL is a alcohol dehydrogenase and AttM, a γ-butyrolactonase, breaks down the *Agrobacterium* QS signal 3OC8-HSL. Proline is a competitive antagonist of GABA and is also taken up through the Atu2422-Bra ABC transporter system (Wachter et al., 2003; Haudecoeur et al., 2009). It was found that plants with relatively higher proline levels present bigger tumors and severe disease symptoms, whereas those with relatively high GABA attenuated *Agrobacterium* pathogenesis. This is likely a result of the pathogen's enhanced virulence through QS that is negatively regulated by GABA (Brugiere et al., 1999). Furthermore, it was found that a short interfering RNA, AbcR1, targets the ribosome binding site of *atu2422* and negatively affects its translation (Wilms et al., 2011, 2012).

Recent studies revealed that the plant defense signal salicylic acid (SA) also activates the *attKLM* operon thereby down-regulating *Agrobacterium* QS (Yuan et al., 2007, 2008a). It was suggested that down-regulation of QS during the initial stages of infection benefits *Agrobacterium* pathogenicity, since high levels of QS signals are known to trigger a defense response in eukaryotic hosts (Ritchie et al., 2005; Wagner et al., 2007). Therefore, *Agrobacterium* QS is under tight and complex modulation by plant-derived opine, SA, and GABA to ensure optimum infection of plant hosts and to avoid the elicitation of plant defense responses by high levels of QS signals, reflecting an evolutionary advantage. In addition, quorum quenching induced by SA and GABA might function to prevent unnecessary energy expenditure after T-DNA transfer. Moreover, since the AttM lactonase has a broad substrate range, the activation of *Agrobacterium* quorum quenching by GABA and SA likely confers *Agrobacterium* a competitive advantage by degrading QS signals from unrelated competitive bacteria occupying the rhizosphere niche (Mathesius et al., 2003; Carlier et al., 2004; Chevrot et al., 2006; Yuan et al., 2007). Furthermore, induction of *attKLM* genes allows *Agrobacterium* to metabolize other plant-released compounds such as gamma-butyrolactone to produce succinic acid for the central metabolism (tricarboxylic acid cycle; Carlier et al., 2004; Chevrot et al., 2006; Chai et al., 2007).

Agrobacterium VIRULENCE MODULATED BY PLANT HORMONES AND PLANT-DERIVED CHEMICALS

Plant hormones play important roles in plant defense and stress resistance. IAA and ethylene (ET) levels in plant tissues are elevated at the initial stages of infection by *Agrobacterium*. Following T-DNA integration, SA, IAA, and ET levels are elevated (Lee et al., 2009), while jasmonic acid (JA) levels were unchanged. However, in tumors, IAA and ET signaling pathways were activated, while JA and SA signaling pathways remained inactivated. Synthesis of IAA in crown gall occurs as a two-step process from tryptophan via indoleacetamide, mediated by-products of the T-DNA encoded *iaaM* and *iaaH* genes (Thomashow et al., 1986). T-DNA also carries an *ipt* gene responsible for CK synthesis. The *ipt* product condenses isopentenyl pyrophosphate and AMP to produce isopentenyl-AMP, which is later converted to CK by host enzymes. The elevated level of IAA and CK promote plant cell growth and tumor formation. It is now becoming evident that key phytohormones significantly influence *Agrobacterium* pathogenicity and tumor formation through both plant signaling pathways as well as direct modulation of bacterial processes (Veselov et al., 2003; Yuan et al., 2007; Zottini et al., 2007; Anand et al., 2008). The following sections are focussed on the effects of plant hormones on *Agrobacterium* pathogenicity, in particular, how *Agrobacterium* responds to these plant hormones.

Agrobacterium RESPONSES TO INDOLE-3-ACETIC ACID (IAA)

IAA is important for plant growth and development, where its functions are mediated by the asymmetric distribution of IAA both systemically and locally (Korbei and Luschnig, 2011). IAA produced by *Agrobacterium* infected cells not only contributes to tumor growth, but also affects *Agrobacterium* pathogenicity. It was found that IAA, at 25 μM concentrations, inhibits *Agrobacterium vir* gene expression while not significantly affecting *Agrobacterium*

growth. This is thought to occur by competition between IAA and phenolic inducers of *vir* genes for their interaction with the VirA/VirG two-component system (Liu and Nester, 2006). Further studies have indicated that IAA likely competes with *vir* gene inducing signals, such as acetosyringone, for association with the VirA linker domain, which is strengthened by the related molecular structures of acetosyringone and IAA. Activation of *vir* genes by acetosyringone and IAA-mediated inhibition of *vir* genes have never been genetically separated. Moreover, the inhibition of *vir* genes by IAA can be rescued by higher level of acetosyringone or incorporation of a constitutive *virA* expressing plasmid. Furthermore, IAA inhibits *Agrobacterium* growth at higher concentrations (over 50 μM) yet does not kill *Agrobacterium* (Liu and Nester, 2006). It was proposed that after successful transformation of a plant host, the synthesis of large amounts of IAA in infected plant tissues represses *vir* gene expression for energy conservation. Yet it remains unclear if the local concentration of IAA in fresh tumors reaches the inhibitory range (Liu and Nester, 2006).

Agrobacterium RESPONSES TO SALICYLIC ACID (SA)

SA is a well-known phytohormone activating plant defense responses to incompatible interactions (Zottini et al., 2007). During *Agrobacterium*–plant interactions, SA produced in infected plants modulates the *Agrobacterium* virulence program by several mechanisms (Yuan et al., 2007; Lee et al., 2009). Apart from mounting plant defense responses, SA at biologically relevant concentrations (8–10 μM) limits *Agrobacterium* growth, represses *vir* gene expression, and dampens *Agrobacterium* QS as discussed in the previous section (Yuan et al., 2007, 2008b). In fact, SA inhibits all the *vir* genes including the *repABC* operon, suggesting SA likely prevents the increase of Ti plasmid copy number. This is consistent with the observation that SA-overproducing plants display recalcitrance to *Agrobacterium* infection, whereas mutant plants impaired in SA biosynthesis and accumulation are more susceptible to tumor growth (Yuan et al., 2007; Lee et al., 2009). Similar to IAA, the inhibition of *vir* gene expression by SA can be rescued by either increasing levels of acetosyringone or incorporation of a constitutive *virA* expressing plasmid. SA likely functions as an allosteric competitive inhibitor and interferes with the interaction between the kinase domain of VirA and acetosyringone since the constitutively expressed VirA activates *vir* gene expression independent of acetosyringone (Yuan et al., 2007).

Agrobacterium RESPONSES TO ETHYLENE (ET)

ET, unlike other plant hormones, is a volatile hormone that affects many aspects of plant growth and development (Wang et al., 2013). ET also acts as a plant stress signal. ET signaling pathways are induced by various biotic and abiotic stresses including osmotic stress, salt stress, wounding, pathogen attack and flooding. These stress-induced ET signaling pathways have substantial roles in defense responses and disease resistance by accelerating senescence, abscission of infected organs and induction of specific defense proteins (Chang and Shockey, 1999). Plant tissues rich in ET, such as melons, are recalcitrant to *Agrobacterium* transformation, yet the cause for the transformation recalcitrance remains unclear. Thus, various strategies have been employed to reduce ET level to improve *Agrobacterium* transformation efficiency, including the application of an antisense ACC oxidase gene (pAP4), the final enzyme in the ET biosynthetic pathway. Recent studies have found that ET is another important factor modulating *Agrobacterium* virulence programing and determining crown gall morphogenesis (Nonaka et al., 2008). In particular, *Agrobacterium*-mediated genetic transformation was inhibited in ET-sensing melon but enhanced in ET-insensitive mutants. Further studies also revealed that *Agrobacterium* growth was not affected by ET, but the presence of ET at the beginning of *Agrobacterium* infection displays significant inhibitory activity on *vir* gene expression. Such inhibitory effects can be rescued by supplementation with acetosyringone, a *vir* gene inducer (Nonaka et al., 2008). Although the ET levels are up-regulated during *Agrobacterium* infection, plant genes for ET receptors and downstream signaling are not induced (Lee et al., 2009). These observations suggest that ET impacts *Agrobacterium*–plant interactions largely through its inhibitory effects on bacterial virulence programming.

ADDITIONAL PLANT-DERIVED *vir* GENE INHIBITORS IN NATURE

In addition to the universal phytohormones SA, ET, and IAA, monocots contain special chemicals acting as natural inhibitors of *Agrobacterium* virulence. Maize, along with other monocots, are notoriously resistant to *Agrobacterium* transformation and the cause for this has been delimited to the inhibition of *Agrobacterium vir* genes (Heath et al., 1997). In particular, metabolites derived from corn seedlings (*Zea mays*) such as 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA) inhibit the expression of *Agrobacterium vir* genes in the presence of *vir* inducing signals (Sahi et al., 1990; Zhang et al., 2000). In addition, *Agrobacterium* mutants resistant to either DIMBOA or MDIBOA were much more effective in infecting plant hosts. Moreover, *Agrobacterium* carrying constitutively active *virA* are insensitive to MDIBOA in terms of the inhibition of *vir* genes. These observations suggest that DIMBOA and MDIBOA, similar to SA, ET, and IAA, probably affect signal perception by the VirA sensor kinase prior to the VirA/G phosphorylation signal relay events.

CONCLUSION AND FUTURE PERSPECTIVES

SIGNALING INTEGRATION AND CASCADE ACTIVATION OF *Agrobacterium* VIRULENCE BY PLANT-DERIVED SIGNALS

Several lines of evidence suggests a hierarchical activation of *Agrobacterium* virulence by a combination of plant-derived signals, as illustrated in **Figure 1**. Rhizospheric acidity activates the ChvG/I system, which subsequently activates *virG* transcription at the proximal promoter (P2) to allow basal level expression of *virG*. Therefore, the ChvG/I system functions upstream of VirA/VirG system (Li et al., 2002; Yuan et al., 2008b). Upon recognizing phenolic signals such as acetosyringone, VirA becomes auto-phosphorylated and subsequently phosphorylates VirG. Phosphorylated VirG activates the expression of *vir* genes responsible for T-DNA transfer and integration. Phosphorylated VirG also activates *virG* expression at the distal promoter (p1) to further promote virulence. ChvE binds to

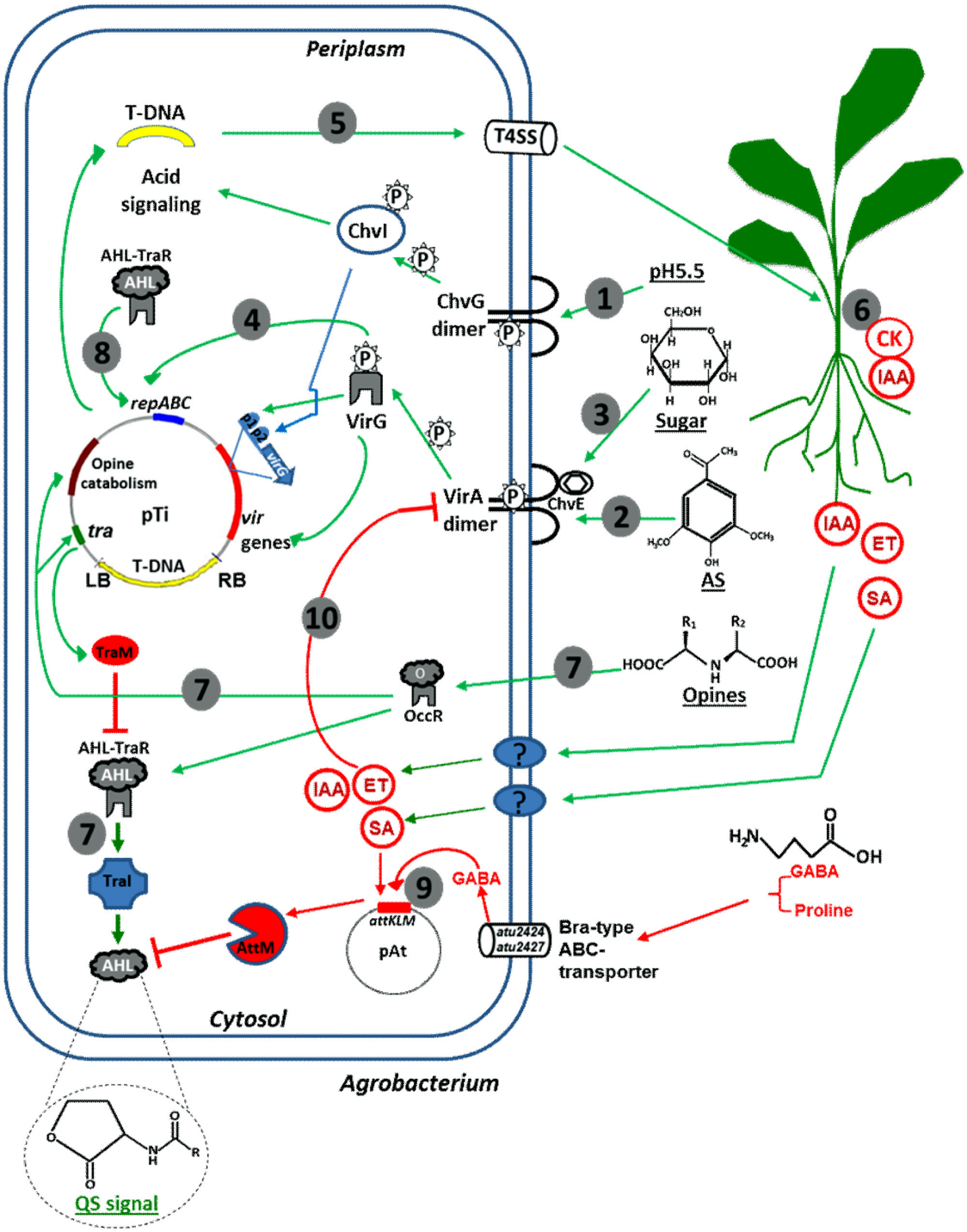

FIGURE 1 | Schematic drawing of *Agrobacterium* responses to plant-derived signals. **(1)** Upon perception of acidic conditions in the rhizosphere, the ChvG/I two-component system activates the expression of several virulence genes including *chvI* and *virG*; **(2)** Upon perception of plant-derived phenolics, the VirA/G two-component system activates all *vir* genes including *virG* to further promote *vir* gene expression; **(3)** ChvE binds plant-released sugars and interacts with the VirA to allow maximal *vir* gene expression; **(4)** *Agrobacterium* Ti plasmid copy number is up-regulated in response to phenolic compounds; **(5)** *Vir* gene products process and deliver T-DNA into plant nuclei; **(6)** Expression of T-DNA encoded genes in plants leads to the production of IAA, CK, and opines; **(7)** Opine activates *Agrobacterium* genes for opine metabolism, as well as TraR/TraI QS system that subsequently induces Ti plasmid conjugation; **(8)** QS also up-regulates Ti plasmid copy number for maximal pathogenicity; **(9)** *Agrobacterium* quorum quenching (*attKLM* operon) is activated by plant-derived GABA and SA thereby down-regulates QS; **(10)** *Agrobacterium* hijacks plant-derived SA, IAA, and ET to down-regulate *vir* gene expression.

plant-derived sugars and subsequently interacts with the periplasmic domain of VirA to allow for the maximal expression of *vir* genes. Thus, the VirA/G system couples and integrates three rhizosphere signals: acidity, sugars (monosaccharides) and phenolic compounds. Such signaling integration and cascade activation of *Agrobacterium* virulence ensures precise perception of suitable plant hosts in the rhizosphere and maximal infection, reflecting an evolutionary advantage (Li et al., 2002; Yuan et al., 2008b). Although ChvG/I regulates VirA/G, *chvG*/*chvI* are expressed independent of the VirA/G system and plant-derived phenolic signals (Charles and Nester, 1993; Peng et al., 1998).

PLANT-DERIVED SIGNALS FUNCTION ADDITIVELY AND PLAY REDUNDANT ROLES IN MODULATING *Agrobacterium* VIRULENCE, Ti PLASMID COPY NUMBER AND QS

Since Ti plasmid harbors *vir* genes as well as genes for opine metabolism, Ti plasmid copy number directly influences pathogenicity and the efficiency of opine metabolism. It was established that the *repABC* operon responsible for Ti plasmid replication and partition is induced by plant-derived phenolics through the VirA/G system. In addition, opine activated-QS further promotes the expression of the *repABC* operon. On the other hand, plant-derived SA and GABA activate *Agrobacterium* quorum quenching, which has negative impacts on Ti plasmid copy number. Furthermore, SA, IAA, and ET inhibit *vir* genes including the *repABC* operon, thereby preventing the increase of Ti plasmid copy number. The signaling complexity also applies to the modulation of *Agrobacterium vir* genes, which are activated by tripartite signals in the rhizosphere, acidity, phenolics, and plant-derived sugars, but down-regulated by SA, IAA, and ET and other natural *vir* gene inhibitors such as DIMBOA and MDIBOA (not shown in the **Figure 1**). In fact, the modulation of Ti plasmid copy number and conjugation also influences the overall *Agrobacterium* pathogenicity. It is noteworthy that *Agrobacterium* mounts distinct but overlapping cellular responses to SA, IAA, and GABA, despite the absence of any structural relation (Yuan et al., 2008a). Therefore, it is plausible that in nature, different plant-derived signals act in concert and function additively, playing redundant roles in tailoring *Agrobacterium* virulence, Ti plasmid copy number and QS (Yuan et al., 2008a).

THROUGH INFECTION OF PLANTS, *Agrobacterium* CONVERTS PLANT CELL INTO A FACTORY TO SECURE NUTRIENTS AND MAINTAIN THE GENETIC INTEGRITY IN NATURE

The evolution and survival of *Agrobacterium* as a bacterial species depends on an intricate balance of two populations of cells, those which actively maintain and those which passively lose the Ti-plasmid. Both forms are necessary for the species to sustain competitive lifestyles in either the absence or presence of a plant host. In the absence of a plant host, *Agrobacterium* harboring the Ti plasmid are at a growth disadvantage to those *Agrobacterium* lacking the plasmid, which is ascribed to the metabolic burden needed to maintain such a large Ti plasmid. In the presence of a host plant and opines, the advantage is shifted to Ti plasmid-retaining *Agrobacterium* since the Ti plasmid contains genes responsible for opine uptake and metabolism. Opines not only activates genes responsible for opine metabolism, but also activate QS-dependent functions such as induction of Ti plasmid conjugation and enhancement of Ti plasmid copy number, promoting maximal infection. In fact, the increase of Ti plasmid copy number may be advantageous for Ti plasmid conjugation. Moreover, *Agrobacterium* hijacks SA and GABA signaling to activate the *AttKLM* operon which also degrades plant-derived GABA, gamma-butyrolactone, and gammahydroxy butyrate to provide even more nutrients for the tricarboxylic acid cycle. Therefore, it is reasonable to believe that through plant transformation, *Agrobacterium* converts infected plant cells into a factory to secure nutrients, in particular opines, nutrients almost exclusive for *Agrobacterium*. In addition, QS activates Ti plasmid conjugation enabling *Agrobacterium* to maintain the Ti plasmid and genetic integrity in nature.

In summary, *Agrobacterium* pathogenicity is largely attributed to its evolved capabilities of precise recognition, response to and hijacking of plant-derived chemical signals for its own benefit. The complex inter-kingdom signaling interplay and regulatory circuits highlight elegant mechanisms of *Agrobacterium*–host co-evolution. Plant roots secrete and release a wide range of chemical cues into the rhizosphere (Bais et al., 2005), admittedly, only a limited number of plant-derived chemicals have been intensively studied for their roles in *Agrobacterium*–plant interactions. For future studies, it will be worthwhile to identify additional plant-derived chemicals that impact *Agrobacterium* pathogenicity and rhizospheric fitness. In addition, it will be very interesting to elucidate *Agrobacterium* signaling pathways and underlying regulatory mechanisms responsible for the precise perception and response to these plant-derived signals, especially at the early stage of *Agrobacterium*–plant interaction.

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Role of the VirA histidine autokinase of *Agrobacterium tumefaciens* in the initial steps of pathogenesis

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Histidine kinases serve as critical environmental sensing modules, and despite their designation as simple two-component modules, their functional roles are remarkably diverse. In *Agrobacterium tumefaciens* pathogenesis, VirA serves with VirG as the initiating sensor/transcriptional activator for inter-kingdom gene transfer and transformation of higher plants. Through responses to three separate signal inputs, low pH, sugars, and phenols, *A. tumefaciens* commits to pathogenesis in virtually all flowering plants. However, how these three signals are integrated to regulate the response and why these signals might be diagnostic for susceptible cells across such a broad host-range remains poorly understood. Using a homology model of the VirA linker region, we provide evidence for coordinated long-range transmission of inputs perceived both outside and inside the cell through the creation of targeted VirA truncations. Further, our evidence is consistent with signal inputs weakening associations between VirA domains to position the active site histidine for phosphate transfer. This mechanism requires long-range regulation of inter-domain stability and the transmission of input signals through a common integrating domain for VirA signal transduction.

Keywords: VirA, GAF domain, signal transduction, pathogenesis, two-component system, *Agrobacterium*

INTRODUCTION

Dynamic fluctuations in conformation can be essential for protein function, and large-scale adjustments are often necessary for complex cellular events ranging from allosteric enzymatic activity, regulation of overlapping signal transduction pathways, and the many intra- or inter-subunit protein-protein, protein-DNA, and protein-RNA interactions associated with information flow (Chillemi et al., 2003; Laskowski et al., 2009; Farago et al., 2010). Such protein dynamics are not typically highlighted in static structural models, but can be of critical importance to our understanding of function. The complex roles of the membrane-bound histidine kinases, which function as receptors and signal transducers to modify gene expression or protein function in response to environmental change in many prokaryotes, are critical for committing *Agrobacterium tumefaciens* to pathogenesis (Stock et al., 2000; Mitrophanov and Groisman, 2008; Cheung and Hendrickson, 2010).

The VirA histidine kinase and its response regulator VirG form a two-component stimulus-response coupling pair (Gelvin, 2000; Lin et al., 2008). This pair is the necessary first step in the regulation of transcription of the virulence (*vir*) genes on the tumor inducing (Ti) plasmid that ultimately mediate the transfer and integration of DNA into the host cell (Gelvin, 2006; Tzfira and Citovsky, 2006). The multi-domain VirA kinase (**Figure 1A**) exists as a transmembrane dimer (Pan et al., 1993; Brencic et al., 2004; Wise et al., 2005) and responds to a broad range of phenols (Melchers et al., 1989; Duban et al., 1993) and monosaccharides in low pH environments (Ankenbauer and Nester, 1990; Brencic et al., 2004; Wise et al., 2005; Hu et al., 2013). Maximal expression of the *vir* genes requires a pH sensitive monosaccharide binding to a periplasmic protein ChvE (Ankenbauer and Nester, 1990; Cangelosi et al., 1990). Both ChvE/sugar and phenols associate with VirA to regulate VirG phosphorylation (Chang and Winans, 1992). The terminal receiver domain of VirA homologous to VirG and has been shown to have both negative and positive effects on the phosphorylation cascade (Chang et al., 1996; Wise et al., 2010). Therefore, coordinated actions across the entire VirA dimer appears to be necessary for signal perception and transmission. The central position of the "linker" domain, which joins the trans-membrane helices to the kinase domain, suggests that both periplasmic and cytoplasmic inputs might be integrated here for transmission to the catalytic histidine 474, which is phosphorylated and subsequently used to phosphorylate VirG (Chang and Winans, 1992).

We have used homology models of the VirA linker to gain mechanistic insight for long-range conformational regulation of VirA activity (Wang et al., 2002; Gao and Lynn, 2007). Using mutational and chimeric protein constructs to test prediction, we now document specific interactions within and between VirA domains critical for signal transmission. These long-range structural interactions reveal additional insights into the integrator functions of the linker domain. While it is not yet clear how general these insights may be or why these specific signal inputs have been selected for broad host range evolution, it is certainly clear that sophisticated cooperative motions throughout the entire sensor kinase are exploited for the successful pathogenesis by *Agrobacterium tumefaciens*.

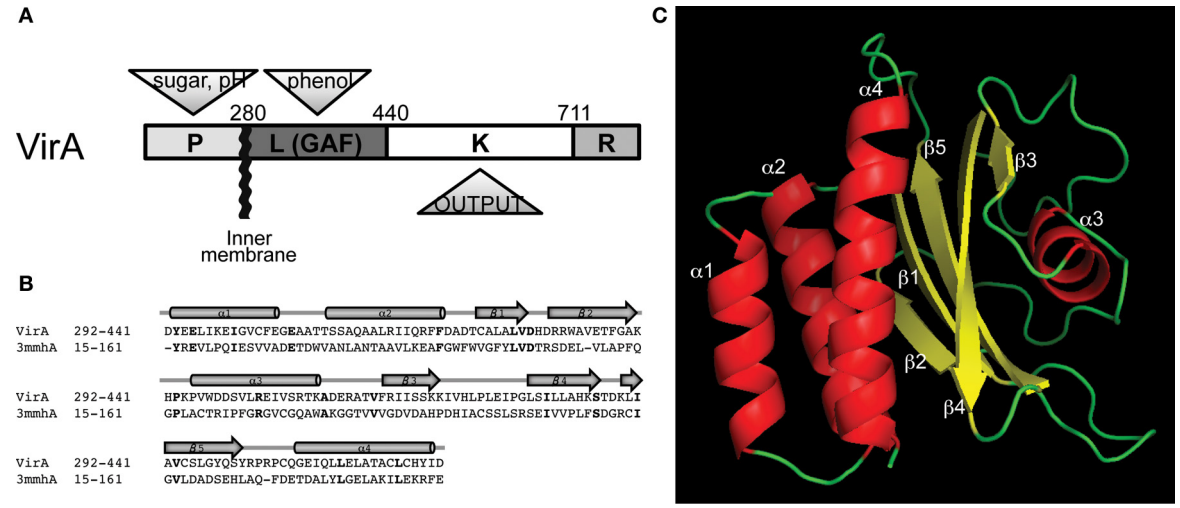

FIGURE 1 | Domain architecture of the histidine autokinase VirA. (A) Domain organization and signal inputs of VirA. Besides the conserved kinase (K), three regulatory domains exist to coordinate the signal output. The periplasmic domain (P) perceives sugar and H^+, the linker domain senses the phenol, and a receiver domain (R) locates at the C-terminus for additional regulation. **(B)** Homology between VirA (292–441) and the *Neisseria meningitidis* fRMsr protein, using Phyre2 (Kelley and Sternberg, 2009). Bold lettering indicates identical residues. **(C)** Predicted structure of the VirA linker region. The GAF-domain containing protein from *N. meningitidis* (PDB ID: 3MMH) provided a template for a predicted protein structure of the VirA linker (292–441).

MATERIALS AND METHODS

LINKER STRUCTURE MODELING

The VirA (292–441) sequence was used to perform a secondary structure homology search using Phyre2 (Kelley and Sternberg, 2009). The GAF domain was common to all but a few of the top 20 hits, and several of these protein structures were known (1VHM, 1F5M) (Gao and Lynn, 2007) (Figure S1A). The top hit was the fRMsr protein from *Neisseria meningitidis*, 12% identity with 93.4% confidence. fRMsr and other hits (e.g., 3P01 and 1F5M) were used as templates for VirA (292–441) (Figure S1B). Comparisons of the resulting GAF domains, including the previous threading of this VirA domain using Swiss Model Workspace (Gao and Lynn, 2007), provided structures that differed only slightly in the relative orientations of the secondary elements (Figure S1C).

BACTERIAL STRAINS, PLASMIDS, AND REAGENTS

The bacterial strains and plasmids used in this study are listed in **Table 1**. *E. Coli* strain XL1-Blue (Strategene) was used for routine plasmid construction. Acetosyringone (AS) used for *vir* gene induction was purchased from Sigma-Aldrich Corp. Isopropyl β-D-1-thiogalactopyranoside (IPTG) used to induce protein expression and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) used in library screening were purchased from Research Products International Corp. All cloning reagents were purchased from either New England Biolab or Promega.

PLASMID CONSTRUCTIONS

While the scheme for the design of constructs is shown in **Figure 2B**, plasmid construction procedures are described in Supplementary Materials. The plasmids are listed in **Table 1**, and the primers are listed in Table S2.

LIBRARY CONSTRUCTION AND SCREENING

The constitutively active mutants in α4 were identified by randomly mutating aa426–437 in LKR(285–829) via two-step PCR using the primers with an NNN codon replacing each residue, and the results being amplified using primers LKR285 (5′-CGGGATCCGATTGGTTAGCGCGGCGT-3′) and LKRA1 (5′-GCGGTACCGCAACTCTACGTCTTGAT-3′). The library was digested with *BamH*I and *Acc65*I and ligated into the *BamH*I and *Acc65*I digested pJZ6. These constructs were directly transformed into *A. tumefaciens* strain A136 containing pRG109 by eletroporation. To select for the constitutively "on" variants, the transformants of the mutated aa426–437 library were screened on non-inducing media plates containing X-gal. The blue colonies were extracted, sequenced, and the phenotype confirmed by site-directed mutagenesis.

β-GALACTOSIDASE ASSAYS FOR Vir GENE INDUCTION

The GCN4 leucine zipper variants, LZ(n)-426$^{K(G665D)}$, were transformed into *A. tumefaciens* strain A348-3 containing pRG150, which has *lacI*^{q} to allow chimera expression only during IPTG induction. The *A. tumefaciens* strains were grown in LB medium with appropriate antibiotics at 28°C to an OD_{600} of 0.4–0.6. The cells were pelleted by centrifugation at 4°C, 7000 × g, for 10 min. The pellet was washed with PBS, and diluted to OD_{600} ~0.1 into tubes containing a total of 1 mL induction medium (Winans et al., 1988) with 200 μM IPTG, and cultured at 28°C, 225 rpm for 15 h. β-galactosidase activity was determined as previously described (Miller, 1972), and the reading of optical densities at 600 and 415 nm was performed using a EL800 microplate reader (BIO-TEK Instruments).

Except for the LZ(n)-426$^{K(G665D)}$ variants, all of the *virA* variants and fusions were transformed into *A. tumefaciens* strain A136 containing pRG109, which carries P_{virB}-*lacZ* and P_{N25}-*virG*, for

Table 1 | Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	References
***E. coli* STRAINS**		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[*F′ proAB lacI*^{q}*Z M15* Tn*10* (Tc^r)]	Stratagene
***A. tumefaciens* STRAINS**		
A136	Strain C58 cured of pTi plasmid	Watson et al., 1975
A348-3	A136 containing pTiA6NC, Δ*PvirA* – *virA*,*virA* deletion, Km^r	Lee et al., 1992
PLASMIDS		
pYW15b	Broad-host-range expression vector, IncW, Ap^r	Wang et al., 2000
pYW33	*PN25*-6xHis-LZ-*virA(aa285–471)* in pYW15, Ap^r	Wang et al., 2002
pYW39	*PN25*-6xHis-*virA(aa285–829)(G665D)* in pYW15, Ap^r	Wang et al., 2000
pYW48	P_{virA}-*virA*(*aa1–829*) in pYW15b, Ap^r	Wang et al., 2000
pSW209Ω	*virB*::*lacZ*, IncP, Spec^r	Wang et al., 2000
pJZ4	P_{virB}-*lacZ* in pMON596, IncP Spec^r	Zhang et al., 2000
pJZ6	IncW/ColE expression vector with P_{N25}, Ap^r	Zhang, Unpublished
pRG109	P_{N25}-His$_6$-*virG* in pJZ4, Spec^r	Gao and Lynn, 2005
pRG150	*lacI*^{q} in pJZ4, Spec^r	Gao and Lynn, 2007
pRG178	P_{N25}-His$_6$-LZ(4)-*virA*(*aa426–711*)(*G665D*) in pYW15b, Ap^r	Gao and Lynn, 2007
pRG179	P_{N25}-His$_6$-LZ(3)-*virA*(*aa426–711*)(*G665D*) in pYW15b, Ap^r	Gao and Lynn, 2007
pRG180	P_{N25}-His$_6$-LZ(0)-*virA*(*aa426–711*)(*G665D*) in pYW15b, Ap^r	Gao and Lynn, 2007
pYL28	P_{N25}-His$_6$-*virA*(*aa285–829*)(*C435F*) in pJZ6, Ap^r	This study
pYL64	P_{N25}-*virA*(*aa438–711*) in pJZ6, Ap^r	This study
pYL75	P_{N25}-*virA*(*aa285–711*) in pJZ6, Ap^r	This study
pYL81	P_{N25}-*virA*(*aa446–711*) in pJZ6, Ap^r	This study
pYL99	P_{N25}-*virA*(*aa426–711*) in pJZ6, Ap^r	This study
pYL100	P_{N25}-*virA*(*aa460–711*) in pJZ6, Ap^r	This study
pYL102	P_{N25}-*virA*(*aa453–711*) in pJZ6, Ap^r	This study
pYL103	P_{N25}-*virA*(*aa467–711*) in pJZ6, Ap^r	This study
pYL108	P_{N25}-*virA*(*aa426–711*)(*C435F*) in pJZ6, Ap^r	This study
pYL136	P_{N25}-*virA*(*aa285–829*) in pJZ6, Ap^r	This study
pYL138	P_{N25}-*virA*(*aa285–829*)(*Q427F*) in pJZ6, Ap^r	This study
pYL139	P_{N25}-*virA*(*aa285–829*)(*Q427W*) in pJZ6, Ap^r	This study
pYL140	P_{N25}-*virA*(*aa285–829*)(*C435K*) in pJZ6, Ap^r	This study
pYL141	P_{N25}-*virA*(*aa285–829*)(*E430K*) in pJZ6, Ap^r	This study
pYL147	P_{N25}-*virA*(*aa426–711*)(*Q427F*) in pJZ6, Ap^r	This study
pYL148	P_{N25}-*virA*(*aa426–711*)(*Q427W*) in pJZ6, Ap^r	This study
pYL149	P_{N25}-*virA*(*aa426–711*)(*E430K*) in pJZ6, Ap^r	This study
pYL150	P_{N25}-*virA*(*aa426–711*)(*C435K*) in pJZ6, Ap^r	This study
pYL200	P_{N25}-LZ(4)-*virA*(*aa450–829*) in pJZ6, Ap^r	This study
pYL201	P_{N25}-LZ(3)-*virA*(*aa450–829*) in pJZ6, Ap^r	This study
pYL202	P_{N25}-LZ(0)-*virA*(*aa450–829*) in pJZ6, Ap^r	This study
pYL203	P_{N25}-*virA*(*aa285–829*)(*C435R*) in pJZ6, Ap^r	This study
pYL205	P_{N25}-LZ(3)-*virA*(*aa450–711*) in pJZ6, Ap^r	This study
pYL206	P_{N25}-LZ(0)-*virA*(*aa450–711*) in pJZ6, Ap^r	This study
pYL207	P_{N25}-LZ(4)-*virA*(*aa450–711*) in pJZ6, Ap^r	This study
pYL212	P_{N25}-*virA*(*aa450–711*) in pJZ6, Ap^r	This study
pYL213	P_{N25}-*virA*(*aa450–829*) in pJZ6, Ap^r	This study
pYL214	P_{N25}-LZ(-2)-*virA*(*aa450–829*) in pJZ6, Ap^r	This study
pYL215	P_{N25}-LZ(-1)-*virA*(*aa450–829*) in pJZ6, Ap^r	This study
pYL267	P_{N25}-LZ(1)-*virA*(*aa426–711*)(*G665D*) in pJZ6, Ap^r	This study
pYL268	P_{N25}-LZ(2)-*virA*(*aa426–711*)(*G665D*) in pJZ6, Ap^r	This study
pYL269	P_{N25}-LZ(-1)-*virA*(*aa426–711*)(*G665D*) in pJZ6, Ap^r	This study
pYL270	P_{N25}-LZ(-2)-*virA*(*aa426–711*)(*G665D*) in pJZ6, Ap^r	This study
pYL283	P_{N25}-*virA*(*aa285–711*)(*C449-A-D450*) in pJZ6, Ap^r	This study

(Continued)

Table 1 | Continued

Strains/plasmids	Relevant characteristics References	
pYL295	P_{N25}-*virA*(*aa285–711*)(*C449-DA-D450*) in pJZ6, Ap^r	This study
pYL296	P_{N25}-*virA*(*aa285–711*)(*C449-DALK-D450*) in pJZ6, Ap^r	This study
pYL306	P_{N25}-*virA*(*aa285–711*)(*C449-DAL-D450*) in pJZ6, Ap^r	This study
pYL307	P_{N25}-*virA*(*aa285–829*)(*K298E*) in pJZ6, Ap^r	This study
pYL308	P_{N25}-*virA*(*aa285–829*)(*K298E/E430K*) in pJZ6, Ap^r	This study

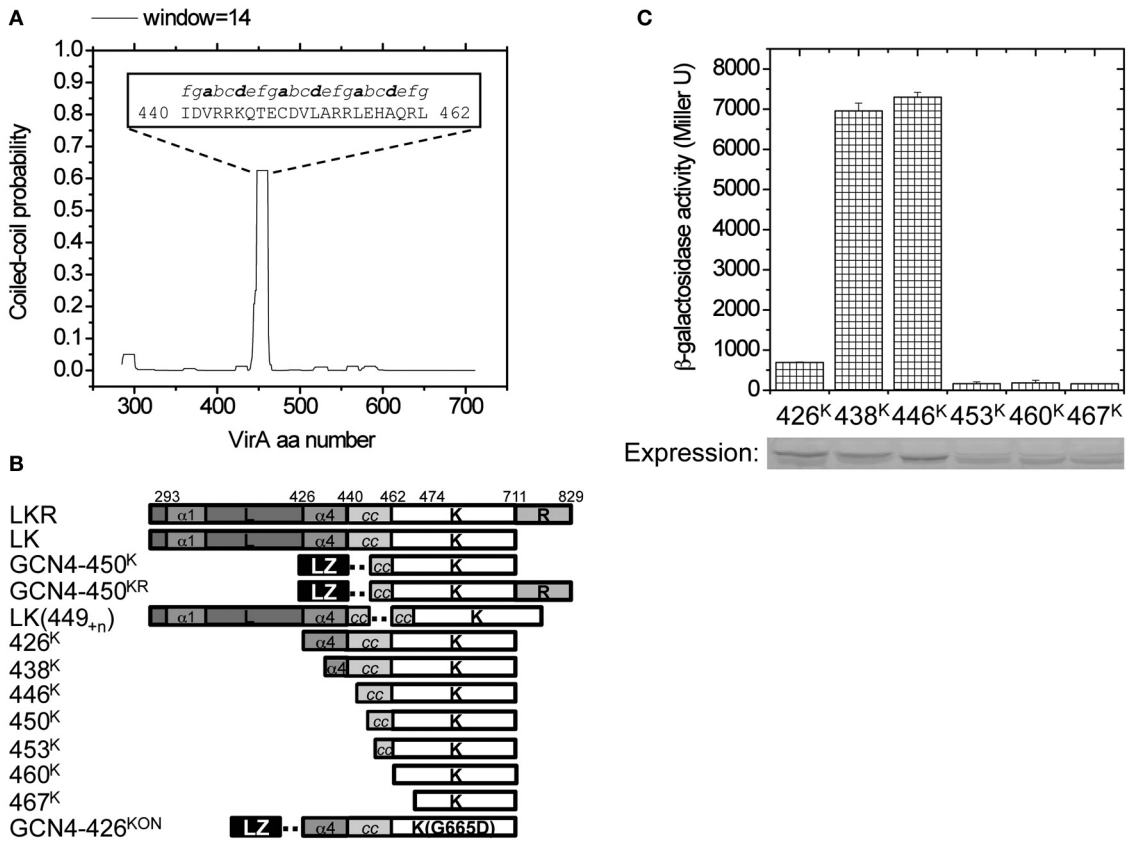

FIGURE 2 | Design of the VirA variants. (A) COILS was used to predict coiled-coil forming propensity of VirA-LK(285–711). Aa440–462 was predicted to have high coiled-coil forming probability, and the predicted heptad repeats is shown inside the figure. Heptad positions a and d are shown in bold for orientation. **(B)** The design of VirA truncations, GCN4 fusions, kinase truncations, and direct amino acid insertions. The predicted α1 and α4 of the linker domain and the coiled-coil in K are shown with the dashed line indicating the inserted adapter. **(C)** β-galactosidase activity of different kinase truncations. *A. tumefaciens* strain A136 carrying pRG109 and the kinase truncations from 426^K to 467^K were assayed for *vir* gene expression in the absence of inducers. *In vivo* protein expression of each truncation was analyzed by Western blot and shown below.

vir gene expression. The cells were grown and pelleted by the same procedure described above, and diluted to $OD_{600} \sim 0.1$ into tubes containing a total of 1 mL induction media with or without 300 μM AS, as indicated, and cultured at 28°C, 225 rpm for 15 h. The β-galactosidase activity was determined by the same method as described, from the reading of the optical densities at 600 and 415 nm.

IMMUNOBLOT ANALYSIS

A. tumefaciens strains were grown in 50 mL LB medium with appropriate antibiotics at 28°C overnight. The cells were harvested by centrifugation at 4°C, 7000 × g, for 10 min. The pelleted cells were washed with PBS and lysed on ice by sonication. The clear lysates were obtained by centrifugation at 4°C, 9000 × g, for 10 min, and analyzed by 10% SDS-PAGE followed by electro-blotting onto nitrocellulose membrane. The membrane was blocked with 3% BSA in TBS, and probed with anti-VirA polyclonal antibody (see SI methods) at 1:200 dilutions. Visualization was achieved using the goat anti-rabbit antibody conjugated with alkaline phosphatase (Amersham) at 1:1000 dilutions, followed by the 1-step NBT/BCIP development (Pierce).

RESULTS

STRUCTURAL MODEL FOR THE LINKER DOMAIN OF VirA

The linker domain, designated (L) as it connects TM2 (ending at aa279) with the kinase (K) domain of VirA

(Chang and Winans, 1992), was originally defined through mutagenesis and sequence analyses as responsible for phenol signal regulation of kinase activity (**Figure 1A**). Conservatively selecting residues 292–441 for a Phyre2 secondary structure search revealed 85% of the top 20 hits as GAF domains, so named because of their presence in c*G*MP-regulated cyclic nucleotide phosphodiesterases, *A*denylyl cyclases, and the bacterial transcription factor *F*hlA (Kelley and Sternberg, 2009). Several of these proteins have structural models, and of these, the GAF-domain containing protein fRMsr from *Neisseria meningitis* is the most similar (Gruez et al., 2010). While previous GAF domain-containing proteins are homologous to the VirA linker (Gao and Lynn, 2007), the fRMsr protein (PDB ID: 3MMH) provides a stronger template with 93.4% confidence at 98% coverage, defining the relative positioning of the α-helices and β-sheets (**Figure 1B**). Using other protein structures as templates gave similar structures with only slight changes in the orientation of the conserved secondary elements (Figure S1).

The resulting threading model of the VirA linker region (**Figure 1C**) contains a central β-sheet, arranged in a 2-1-5-4-3 strand order (**Figure 1C**), connected to a helix bundle region composed of α1, α2, and α4 that connects the linker region to the histidine kinase (see **Figure 1A**). A four-helix bundle architecture, similar to the proposed bundle in VirA, has been characterized in HAMP domains (derived from *H*istidine kinases, *A*denyl cyclases, *M*ethyl-accepting proteins, and *P*hosphatases) (Aravind and Ponting, 1999). These domains regulate signal transmission in histidine kinases (HK) (Falke and Hazelbauer, 2001; Hulko et al., 2006; Airola et al., 2010) and are thought to constitute a dimerization interface (Gao and Lynn, 2007). The α1, α2, and α4 helix region of VirA is proposed to serve as the interface in the VirA dimer based on homologies with these domains.

Initial physical analyses of this model involved over-expressing and purifying the N-terminal His_6-tagged VirA (285–471) domain (Figure S2A). The relative abundances of secondary structure determined by circular dichroism supported the threading model (∼34% α-helix, ∼20% β-sheet), but conditions were not found to sufficiently stabilize this truncated domain for further evaluation (Figure S2B). Additional sequence analysis of the full LK domains of VirA (285–711) with COILS (Lupas et al., 1991) identified strong coiled-coil propensity connecting the GAF fold to the N-terminus of the DHp domain, a region in *Thermotoga maritima* HK0853 and *Saccharomyces cerevisiae* Sln1 critical for signal transmission (Tao et al., 2002; Marina et al., 2005). Employing several scanning windows of the heptad repeats, COILS identified aa440–462 (**Figure 2A**) as having an amphipathic heptad repeat signature. Increasing the size of the scanning window lowered the probability of this region as a coiled-coil, presumably because the sequences surrounding this region do not contribute to the coiled-coil.

To directly evaluate the role of the predicted coiled-coil, we constructed a series of N-terminal truncations of the kinase domain (**Figure 2B**), starting from amino acid 426 (426^K), which includes the entire α4 of the linker (L) domain (Gao and Lynn, 2007) and extending through amino acid 467 (467^K) for complete coiled-coil removal. Most of these truncations appeared stable, but the immunoblot suggests that constructs where the coiled-coil is removed are expressed in lower amounts. To examine how well these VirA fragments are able to induce the *vir* genes, we used a well-characterized β-galactosidase assay where the VirB promoter is placed in front of a plasmid localized *lacZ* gene. As VirA receives the phenol signal, the VirB promoter is turned on and β-galactosidase is produced from the *lacZ* gene. The β-gal activity can then be assayed using its cleavage of the substrate ONPG (Miller, 1972), thereby effectively revealing the activity of the VirA protein. In the absence of inducers, VirA fragments 438^K and 446^K, which retain all or most of the coiled-coil region, have high activity, while partial (453^K) and complete coiled-coil deletion (460^K and 467^K) are expressed in lower amounts and have a lower activity (**Figure 2C**). The reduced activity of 426^K is striking and consistent with previous evidence that related HAMP-like domains can also be repressive (Gao and Lynn, 2007), suggesting that the 11 amino acids (aa426–437) in α4 contribute to that repression when inducers are absent.

FUNCTIONALLY CONNECTING THE L AND K DOMAINS

The helix bundle architecture at the dimerization interface of the GAF-fold in the L domain and the predicted coiled-coil connection to K implies a continuous helical connection being necessary for signal transmission. Previous work describing incremental fusion chimeras with the yeast GCN4 coiled-coil at aa426, just before α4, was interpreted as anchoring the relative position of the helices of the VirA dimer (Wang et al., 2002; Gao and Lynn, 2007). The aa440–462 coiled-coil, however, suggests that in-register fusions with GCN4 are possible, allowing us to define the relative registry of each VirA monomer through to the position of the active site histidine. Fusions were therefore engineered at aa450, removing the N-terminal half of the predicted coiled-coil (**Figures 2B**, **3A**), and placing the fusion just 24 residues upstream of the phosphorylated His474.

While similar results were found using the 450^K construct and GCN4 fusions (Figure S3), the effect of helix positioning was more dramatic when the receiver (R) domain is retained in the constructs (**Figure 3B**). In our experimental conditions, where the constitutive T5 promoter drives VirG expression, the R domain acts as a repressor. The protein expression appeared to be enhanced in all LZ-450^{KR} fusions compared to 450^{KR}. The 450^{KR} truncation was active, but the in-register LZ(0)-450^{KR} fusion, which is predicted to place the His474 at the same *e* heptad position, gives a 4-fold increase in activity that may be partially attributed to increased stabilization. A three amino acid insertion, LZ(3)-450^{KR}, creating a $-51°$ rotation relative to LZ(0)-450^K and moving His474 to the *a* heptad position, shows five times the activity of LZ(0)-450^{KR}. A four amino acid insertion, LZ(4)-450^{KR}, creating a $+51°$ rotation and positioning His474 at *b*, shows the same level of kinase activity as LZ(0)-450^{KR}. The "ON" and "OFF" states being regulated by the relative position of the active site His474 was further tested with LZ(-1)-450^{KR} and LZ(-2)-450^{KR} constructs, corresponding to a rotation of His474 to the *d* and *c* positions on the opposite face of the coiled-coil, and these fusions also showed little activity (**Figure 3B**).

This model was finally tested by direct insertion of amino acids at residue 449 in the center of the predicted coiled-coil, here denoted as LK(449_{+n}) where *n* is the number of amino

acids inserted (**Figure 4A**). As seen in **Figure 4B**, a +3 amino acid insertion would extend the coiled-coil by almost a single turn and create a −51° rotation, most similar to the LZ(3) fusions, and a +4 amino acid insertion would extend the coiled-coil by more than one turn and create a +51° rotation. The activity observed in LK($449_{+1,2,3,4}$) constructs follows the general pattern as the GCN4 chimeras in **Figure 3**, but the predicted registry is different; the +51° rotation LK(449_{+4}) enhanced kinase activity while the −51° rotation LK(449_{+3}) reduced activity (**Figure 4C**). We have no direct evidence that the positional variability is due to difference in expression or stability, nor do we know whether the inserts "buckle" or bend the helices in some way to transmit slightly different positional information down the helix to the histidine, and these assignments will require higher structural resolution.

MAPPING THE HELIX ASSOCIATION INTERFACES

To further investigate GCN4 fusions for controlling the dimer interface of α4–α4′, the domain was placed outside of the coiled coil region in LK to create GCN4(n)-426^{K} with the same amino acid inserts as in **Figure 3A**. Since the wild-type 426^{K} has low basal activity (**Figure 2C**), possibly due to repressive dimer association, a constitutive mutation, G665D, denoted as 426^{KON}, was used as before to increase basal activity (Chang et al., 1996; Gao and Lynn, 2007). The full range of GCN4-426^{K} fusions, LZ(0/1/2/-1/-2/3/4)-426^{KON}, mapped the possible rotations, and as shown in **Figure 5**, the activity again follows heptad orientation positioning. The highest activity was found for LZ(1)-426^{KON}, and the activity gradually diminished with rotations in either direction. By this analysis, LZ(1)-426^{ON} was assigned as the lowest energy 0 degree rotation interface, and the α4–α4′

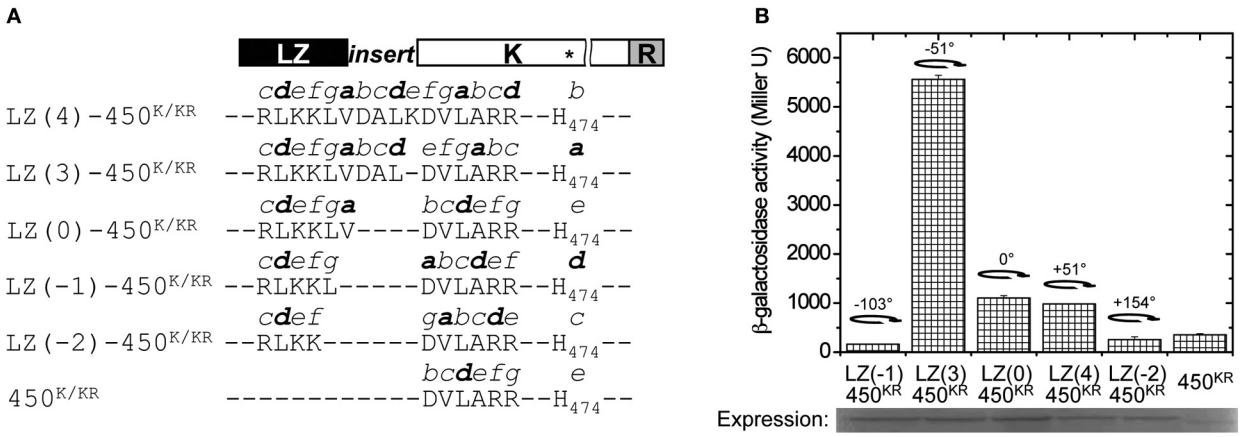

FIGURE 3 | Chimeric GCN4 fusions with 450^{K} and 450^{KR}. (A) Design of the GCN4-450 fusions. The heptad repeats from *a* to *g* were built from the registry of GCN4 and the adapters. GCN4 enforces the hydrophobic *ad* interface (shown in bold), and shifts the registry of the heptads of kinase coiled-coil according to the different adapters. The predicted position of His474 (*in the K domain) in each fusion is shown at the end of the sequence. **(B)** *A. tumefaciens* strain A136 carrying pRG109 and the indicated GCN4-450^{KR} fusions were assayed for *vir* gene expression without inducers. The degree of rotation created by each fusion is shown in the figure with the 0° rotation defined at LZ(0)-450. The protein expression of the GCN4-450^{KR} constructs was analyzed by Western blot.

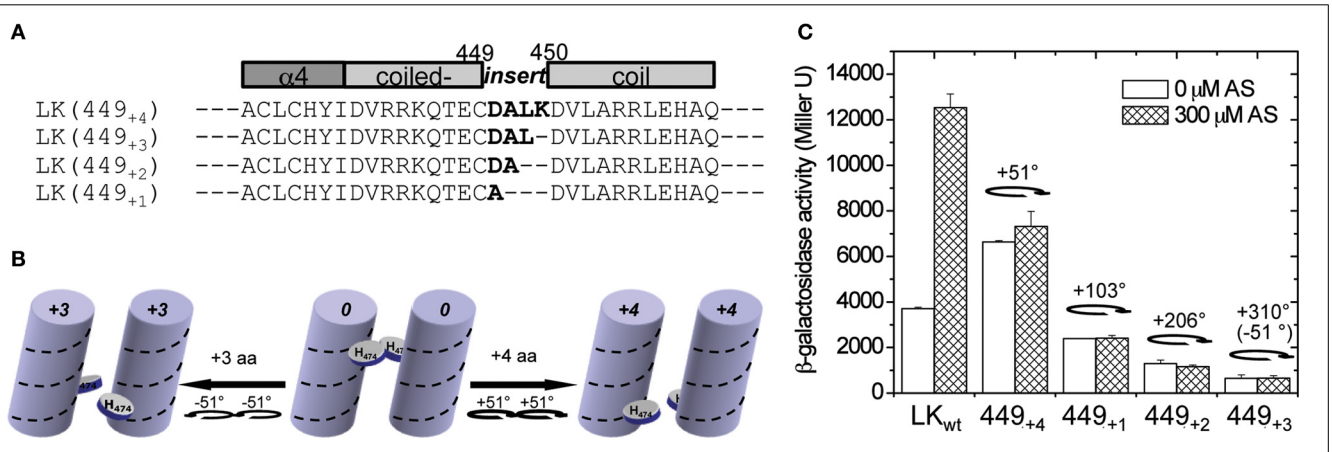

FIGURE 4 | Direct amino acid insertion within the coiled-coil. (A) The amino acids in bold were inserted between amino acids 449 and 450 in the predicted coiled-coil region. **(B)** An illustration of how His474 moves along the helix coil according to the amino acid insertion at the N′-terminus. **(C)** *A. tumefaciens* strain A136 carrying pRG109 and the LK constructs with different insertions at aa449 (449^{+n}) were assayed for *vir* gene expression with or without 300 μM AS. The degree of rotation created by the insertions is shown in the graph.

dimer interface can be designated as the "ON" conformation (**Figure 8A**).

The possibility of interactions across α4–α4′ between subunits of the dimer suggests that inputs from sugar/ChvE association might also be transmitted through α1 to the dimer interface in the GAF structure. To test this possibility, we first sought "ON" interface stabilizing mutations within α4 that could provide signal-independent activity. Residues 426–437 of LKR (aa285–829) were randomly mutagenized, and the variants were screened in A136/pRG109 on AB media plates with X-gal without phenolic inducer for active mutants. This approach yielded six constitutive mutants: three with substitutions at Cys435 (C435K, C435R, and C435F), two at Gln427 (Q427W and Q427F), and one at Glu430 (E430K).

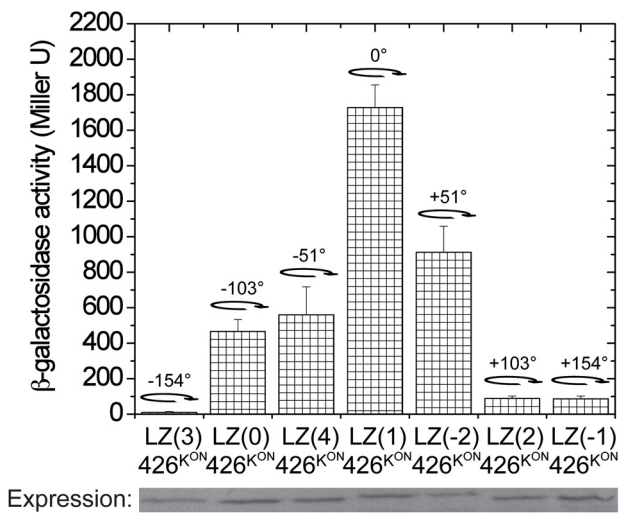

FIGURE 5 | Signal transmission through α4. β-galactosidase activity of the GCN4-426$^{K(G665D)}$ fusions. *A. tumefaciens* strain A348-3 carrying pRG150 and the indicated GCN4-426^{KON} fusions were assayed for *vir* gene expression without inducers. 200 μM IPTG was added to induce chimera expression. The degree of rotation created by each fusion is shown in the graph with the 0° rotation defined at LZ(1)-426^{KON}.

In all of these mutants, phenol induction is severely attenuated (**Figure 6A**), consistent with the "ON" interface being conformationally stabilized. These mutations were moved to 426^{K} as shown in **Figure 6B**. The hydrophobic constitutive variants (Q427F, Q427W, C435F) and the charged variant C435K enhanced 426^{K} activity, consistent with stabilization of the "ON" α4–α4′ dimer interface, but the low basal activity of 426^{K}(E430K) suggests that its constitutive phenotype in LKR is unlikely a result of α4–α4′ stabilization. The GAF models place several charged residues distributed at the helical surface of α1 (**Figure 7A**), suggesting that the constitutive phenotype of E430K might result from α4 to α1 charge interaction. While the relative positions of these helices is weakly constrained by these modeling algorithms, among the charged residues in α1, K298 is positioned close enough to form a salt-bridge with E430 in all three models with the allowance of a simple clockwise rotation. To test this possibility, a K298E mutation was constructed to complement E430K. While neither of the single E430K or K298E mutations were phenol responsive, the double mutant (K298E/E430K) restored both kinase activity and phenol inducibility (**Figure 7B**). This compensating mutation is consistent with an α1 and α4 interface impacting signal transmission, possibly connecting sugar/ChvE binding and phenol induction to conformational transmission through this helical bundle (Gao and Lynn, 2007).

DISCUSSION

Available protein structures and comparison algorithms have dramatically increased our ability to predict secondary and tertiary folds from primary sequence information. However, determining how these static structures are coupled to function, particularly in proteins not amenable to biophysical and structural analyses, remains a significant challenge. The integral membrane VirA histidine kinase of *Agrobacterium tumefaciens* is an example of remarkable signaling complexity controlling the very first commitments to pathogenesis. We have been able to predict the phenol-sensing linker domain as a GAF fold (Gao and Lynn, 2007), a structure type known to bind cyclic nucleotides, heme, simple chromophores, and branched-chain amino acids (Martinez et al., 2005; Handa et al., 2008), and to

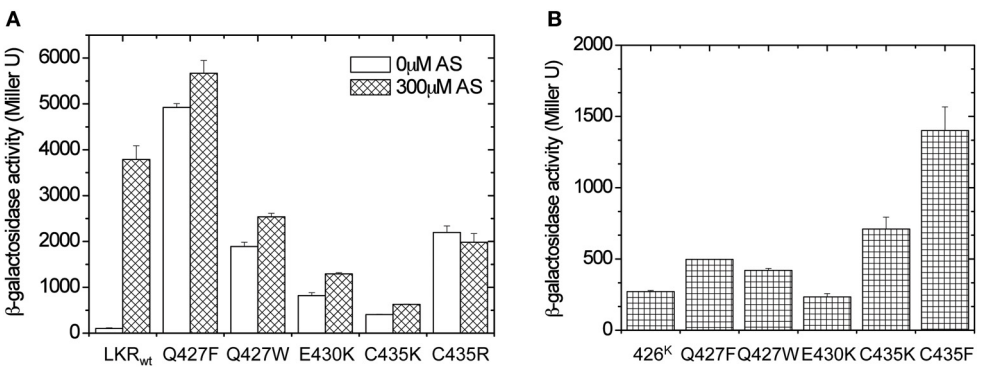

FIGURE 6 | Library screen for constitutive mutations within α4 (aa426–437). (A) β-galactosidase activity of the identified constitutively induced mutants. *A. tumefaciens* strain A136 carrying pRG109 and wild-type LKR or LKR mutants were assayed for *vir* gene expression with or without 300 μM AS. **(B)** *A. tumefaciens* strain A136 carrying pRG109 and wild-type 426^{K} or 426^{K} mutants were assayed for *vir* gene expression without inducers.

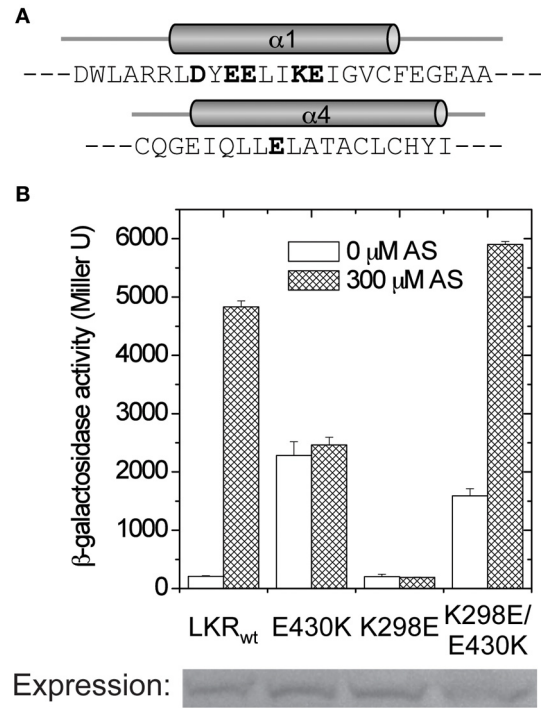

FIGURE 7 | α1–α4 salt-bridge formation. (A) The amino acids in linker α1 and α4. The charged residues are shown in bold. **(B)** *A. tumefaciens* strain A136 carrying pRG109 and LKR constructs with E430 and/or K298 mutants were assayed for β-galactosidase activity in the presence or absence of 300 μM AS.

regulate secondary messenger metabolism (Sardiwal et al., 2005; Levdikov et al., 2006; Yang et al., 2008). The GAF domain is similar to PAS domains (*P*er-*A*rnt-*S*im), an additional regulatory motif that is involved in protein functional control through interaction with a broad variety of small molecules (Ponting and Aravind, 1997; Hefti et al., 2004). Both GAF and PAS domains are observed in histidine kinases, with an estimated 9 and 33% occurrence, respectively (Gao and Stock, 2009), and successful swaps of those signal sensing domains between different HK have been described (Kumita et al., 2003; Möglich et al., 2009), possibly indicating a common signaling mechanism. The developed structural model has now been used to examine the interactions regulating signal sensing and kinase activation of VirA. Specifically, the helix bundle architecture in the VirA linker and simple rotational motion mediated by these helices was proposed, and this mechanism has been further evaluated with a diverse series of fusions and chimeric constructs.

The GCN4 leucine zipper motif was used to anchor the orientation of the continuous helix proposed to connect the linker domain and the DHp domain of the kinase. When placed in the middle of the predicted coiled-coil region (aa450), "ON" and "OFF" conformations were identified that could be proposed to arise from different relative orientations of the helices (**Figure 8A**). Amino acid insertions at the coiled-coil suggested a clockwise rotation mediates VirA activation. The VirA histidine kinase employs a *trans*-phosphorylation mechanism (Brencic et al., 2004), similar to the EnvZ histidine kinase in *E. Coli* (Cai and Inouye, 2003), meaning that the phosphorylation occurs across the subunits of the kinase dimer. A BLAST search identified VirA to have 24% identity to the *Thermotoga maritima* protein HK0853, whose entire cytoplasmic structure has been solved via x-ray crystallography (Marina et al., 2005). If VirA adopts a similar kinase fold as that of HK0853, the predicted clockwise rotation should bring the His474 in VirA closer to the ATP-binding domain of the other subunit for *trans*-phosphorylation (**Figure 8A**). This model is consistent with previous analyses (Gao and Lynn, 2007), suggesting the rotational motion controls kinase activity at the level of histidine phosphorylation rather than phosphoryl-transfer efficiency. However, HK0853 of *T. maritima* adopts a different *cis*-phosphorylation mechanism (Casino et al., 2009). The difference between VirA and HK0853 can be reconciled by the alignment of the coiled-coil region of both kinases (**Figure 8B**) and the proposed rotational mechanism. As shown in **Figure 8B**, the identified coiled-coil region of HK0853 is also located in front of the conserved H-box (Marina et al., 2005). However, when compared with VirA, an additional residue in HK0853 exists between Gly466 and Thr467 of VirA. Having this extra residue in the coiled-coil would shift the conserved histidine of HK0853 (His260) from *e* to *f* in the heptads, which involves a movement of +103° relative to the position of His474 in VirA (**Figure 8B**). Therefore, the same rotational motion in HK0853 would move His260 from an exposed surface to the ATP-binding domain of the same subunit, requiring a *cis*-phosphorylation mechanism (**Figure 8B**).

The observation of the high constitutive activities of 438^{K} and 446^{K} is consistent with the argument that the unimpeded kinase is constitutively active while regulatory domains successively repress this activity prior to signal perception release (McCullen and Binns, 2006). The kinase truncation results narrow the repressive region of the linker domain to aa426–437 (**Figure 2C**), and further lead to the hypothesis that the helical associations within the predicted helical bundle control the critical ON/OFF switch. An "OFF" interface is maintained in the un-induced state, and signal sensing switches it to the "ON" interface. Successful engineering of rotational motions at this region by similar GCN4 fusions displayed a clear rotational activation (**Figure 5**), and predicts the ON/OFF interface of α4–α4′ (**Figure 8A**). Furthermore, the control by GCN4 at both 426^{K} and 450^{K} indicates the rotational motion is coherently transmitted from the linker domain to the kinase core. Indeed, library screens for constitutive mutants identified both hydrophobic and electrostatic interactions stabilizing the dimerization interface at α4–α4′. A recent study on an engineered HK YF1 (generated by replacing the oxygen-sensing PAS domain of *Bradyrhizobium japonicum* FixL with the FMN (flavin-mononucleotide)-binding LOV (light-oxygen-voltage) domain from *Bacillus subtilis* YtvA) provided structural insight into the coiled-coil motifs mediating signal transmission between functional domains (Diensthuber et al., 2013). Furthermore, it also implies a simple motion and a fundamental mechanism that can be shared between different signal sensing domains for kinase output.

And most interestingly, this search for functional long-range interactions identified the α1 helix as a key regulator for signal

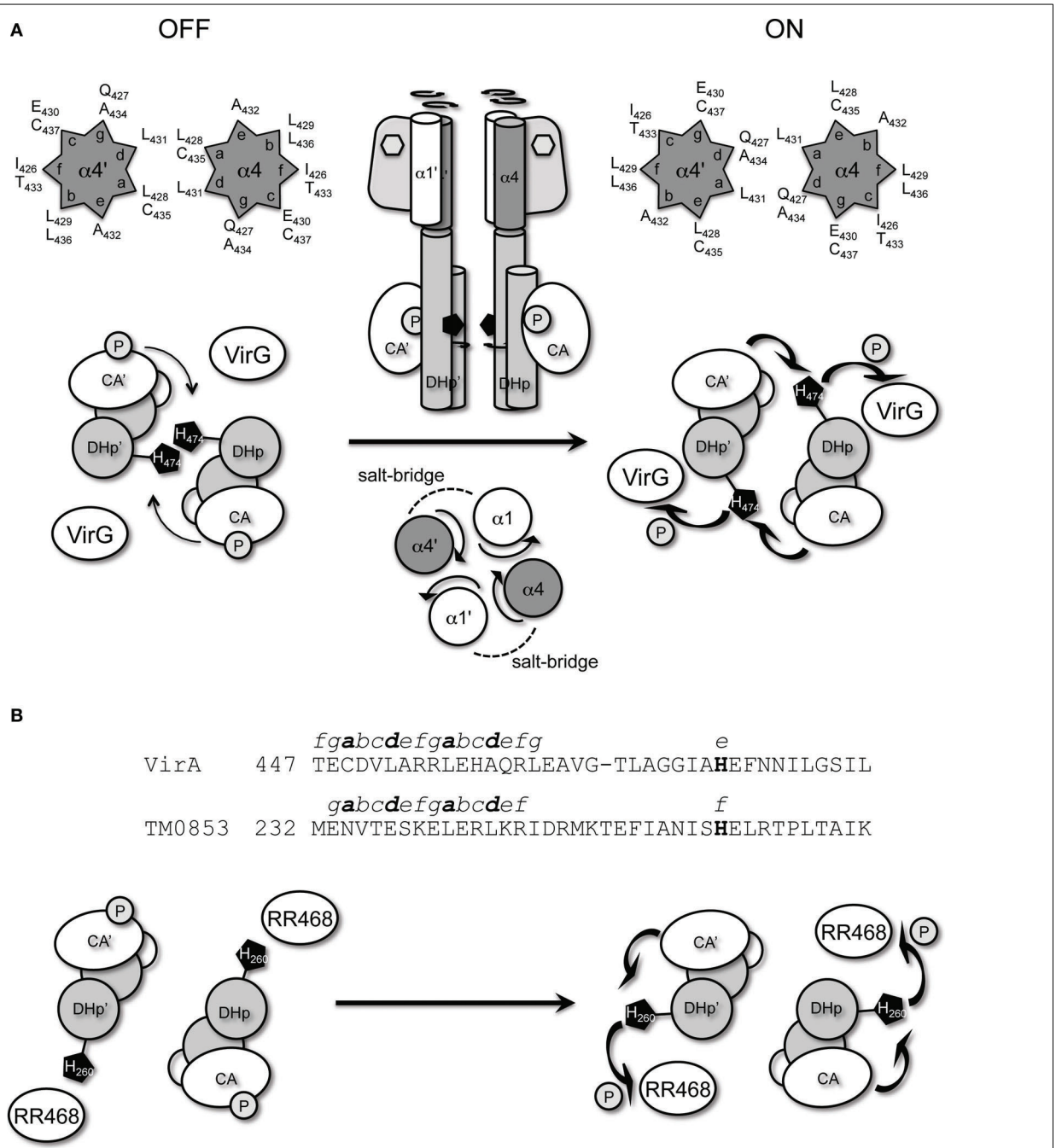

FIGURE 8 | VirA and *Thermotoga maritima* HK0853. (A) Proposed auto-phosphorylation mechanism of VirA, mediated by α4 coiled-coil. The conserved His474 of VirA, predicted to reside in the dimerization interface, is rotated clockwise upon phenolic sensing to close proximity of the ATP-binding domain at the other subunit for *trans*-phosphorylation and the subsequent VirG phosphoryl transfer. The ON and OFF α4 coiled-coil interface is represented in the helical wheel. **(B)** Sequence alignment of VirA and HK0853 at the coiled-coil region preceding the conserved histidine. The predicted heptads of the coiled-coil of both HK are shown from *a* to *g*, and the conserved histidine are shown in bold. In TM0853, the additional residue in the kinase coiled-coil shifts the registry of the conserved His260 by one residue, which creates a +103° displacement of His260 relative to VirA's His474. Therefore, the same proposed rotation upon signal sensing will move the conserved His260 in TM0853 toward the ATP-binding domain at the same subunit for *cis*-phosphorylation.

activation in this rotational mechanism. Salt-bridge associations between K298 (α1) and E430 (α4) is consistent with the computational model of the helix bundle containing α1 and α4 interfaces in the VirA dimer (Wang et al., 2002) and its regulator role in signal transmission. The other charged residues in α1 were previously found to be important in controlling a "piston-like" motion, mediated by the monosaccharide/H^+ sensing from the periplasmic domain (Gao and Lynn, 2007). Therefore, this bundle

may be the conversion point for both sugar/pH and phenol inputs to counteract the repressive region in the dimerization interface at α4–α4′. In addition, preliminary chemical cross-linking results aimed at clarifying the receiver domain's role in enhancing signal response precision indicated an association with the kinase core at this coiled-coil region (Figure S4), but the nature of this association is not yet clear.

The identified interactions point to highly cooperative long-range motions transmitting signal association within the VirA dimer to regulate the very first steps of pathogenesis. The positioning of α1, α2, and α4 vary in the three structural models for the GAF domain and indeed these kinds of structural details are the least well-defined in the structural algorithms. **Figure 8** outlines a mechanistic model that is consistent with our chimeric fusion, but the nature of the long-range transmission (Gao and Stock, 2009) has also implicated symmetry switching models (Moore and Hendrickson, 2012). A recent structural analysis identified a critical proline residue in CpxA that contributes to helix bending in that kinase (Mechaly et al., 2014), but that residue is not conserved in VirA. The range of constructs prepared here provide opportunities to identify constructs amenable to direct structural analyses and further evaluation of these models. Most importantly, the remarkably coordinated action of VirA in processing three separate input signals likely contributed significantly to the success of this pathogen. These constructs now allow the system to be simplified sufficiently to define which signal is being processed and to map the signaling landscape of the host wound site for commitment to pathogenesis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00195/abstract

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Mechanisms and regulation of surface interactions and biofilm formation in *Agrobacterium*

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For many pathogenic bacteria surface attachment is a required first step during host interactions. Attachment can proceed to invasion of host tissue or cells or to establishment of a multicellular bacterial community known as a biofilm. The transition from a unicellular, often motile, state to a sessile, multicellular, biofilm-associated state is one of the most important developmental decisions for bacteria. *Agrobacterium tumefaciens* genetically transforms plant cells by transfer and integration of a segment of plasmid-encoded transferred DNA (T-DNA) into the host genome, and has also been a valuable tool for plant geneticists. *A. tumefaciens* attaches to and forms a complex biofilm on a variety of biotic and abiotic substrates *in vitro*. Although rarely studied *in situ*, it is hypothesized that the biofilm state plays an important functional role in the ecology of this organism. Surface attachment, motility, and cell division are coordinated through a complex regulatory network that imparts an unexpected asymmetry to the *A. tumefaciens* life cycle. In this review, we describe the mechanisms by which *A. tumefaciens* associates with surfaces, and regulation of this process. We focus on the transition between flagellar-based motility and surface attachment, and on the composition, production, and secretion of multiple extracellular components that contribute to the biofilm matrix. Biofilm formation by *A. tumefaciens* is linked with virulence both mechanistically and through shared regulatory molecules. We detail our current understanding of these and other regulatory schemes, as well as the internal and external (environmental) cues mediating development of the biofilm state, including the second messenger cyclic-di-GMP, nutrient levels, and the role of the plant host in influencing attachment and biofilm formation. *A. tumefaciens* is an important model system contributing to our understanding of developmental transitions, bacterial cell biology, and biofilm formation.

Keywords: *Agrobacterium*, attachment, biofilm, cyclic-di-GMP, polarity, motility

INTRODUCTION

A biofilm is defined as a multicellular community of one or more microorganisms stably attached to a surface and frequently encased in an extracellular matrix of secreted biopolymers (Costerton et al., 1995). Biofilm formation proceeds from initial contact of an individual bacterium with a surface and reversible attachment, to stable surface association, microcolony formation, biofilm maturation, and to eventual dispersal (Dazzo et al., 1984; **Figure 1**). Biofilms can form on a wide variety of surfaces including living tissues. These multicellular structures and the processes that lead to them are of great interest as they are highly prevalent in the bacterial world, and have profound impacts on society in industrial, medical, and agricultural contexts. The physiology of bacteria within a biofilm is quite distinct from the same cells in a free-swimming, planktonic state. This is best exemplified by the observation that biofilms can manifest dramatically greater resistance to antimicrobial agents, both chemical (e.g., antibiotics, disinfectants) and biological (e.g., viruses, predatory grazing by protists). The control of biofilm growth is therefore quite challenging and a target of significant research. The initial steps of surface attachment that lead to eventual formation of a biofilm are a significant target as control of this step in the process could be used to inhibit the formation of biofilms before they are established, or to promote biofilm formation for beneficial processes. The attachment mechanisms of pathogens to host tissues overlaps with those processes that lead to biofilm formation, and for many pathogens, biofilm formation is an important or requisite component of disease progression. Additionally, the survival of facultative pathogens in environmental reservoirs, such as that for water-borne disease agents, can be dramatically enhanced within biofilms, thereby affecting disease ecology.

Agrobacterium tumefaciens is a plant pathogen which is clearly capable of surface colonization and biofilm formation on host tissues, and on abiotic surfaces. This review focuses primarily on the molecular mechanisms by which *A. tumefaciens* initially associates with surfaces and forms a biofilm, as well as the regulation of these mechanisms. Much of the data described below has been determined in the laboratory using the nopaline-type strain *A. tumefaciens* C58. More recent studies on a range of *Agrobacterium* species have revealed similar trends in biofilm formation (Abarca-Grau et al., 2011). It is acknowledged that in many cases the connection between the described attachment and biofilm formation mechanisms and ecological interactions of the

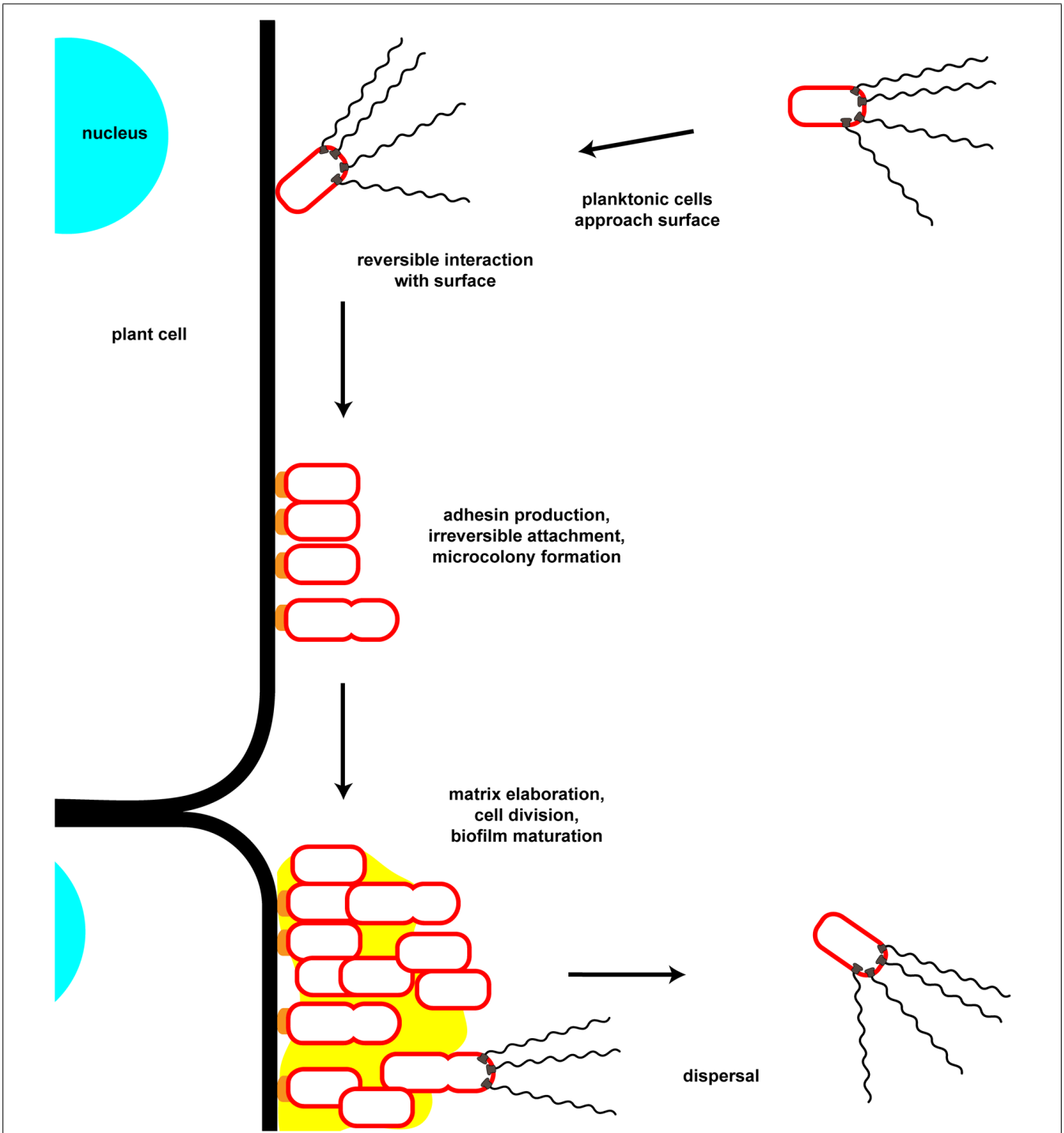

FIGURE 1 | Key steps in attachment and biofilm formation by *Agrobacterium tumefaciens*. Motile planktonic cells approach and physically interact with potential attachment substrates. Initial surface interactions are reversible and may depend on physiochemical forces at the interface of the surface with the local medium. Following these initial surface interactions the unipolar polysaccharide (UPP) is secreted by the bacterium at the point of surface contact. This irreversible surface attachment establishes a site for microcolony formation through continued growth and cell division of attached bacteria as well as aggregation of neighboring microcolonies. During and following this period cells secrete matrix components, including cellulose. As the biofilm matures cells may differentiate into various metabolic and reproductive states as the local environment within the biofilm changes. Dispersal from the biofilm may be initiated by an internal developmental cue or by an extracellular factor, as well as through release of motile daughter cells from attached mother cells. Note that in this cartoon only the outer membrane of the Gram-negative cell envelope is depicted.

bacterium within the rhizosphere remain to be experimentally validated, and much of the relevant environmental context for *A. tumefaciens*, both on and off the plant host, remains poorly understood.

PHYSICAL INTERACTIONS MEDIATING ATTACHMENT

The first step in attachment and biofilm formation is arrival at and interaction with an appropriate substrate (**Figure 1**). In the rhizosphere this step is frequently mediated by chemotaxis-directed swimming motility as bacteria are attracted toward plant exudates. For many species flagella may also serve as adhesins and there is increasing evidence that inhibition of flagellar rotation, as happens when motile bacteria abut a solid surface, stimulates adhesin production. Active motility may also be required to overcome physiochemical forces at the substrate interface. Additional motility mechanisms as well as multiple adhesin molecules, including pili and various exopolysaccharides, also participate in attachment and biofilm formation.

FLAGELLUM-DEPENDENT MOTILITY AND ATTACHMENT

There are various forms of motility observed among bacteria, all of which serve to transport bacteria, individually or collectively, through a porous or liquid environment or across a surface (Jarrell and McBride, 2008). These include flagellum-dependent swimming and swarming motility, and flagellum-independent twitching, sliding, and gliding motilities. The particular form of motility used by an individual bacterium is context-dependent and bacteria frequently possess multiple means of locomotion. *A. tumefaciens* is thought to utilize only flagellum-dependent swimming motility (Loake et al., 1988; Shaw et al., 1991; Merritt et al., 2007). Although surfactant production and swarming motility has been observed in the related species *A. vitis* this mode of motility has not yet been described for *A. tumefaciens* (Sule et al., 2009). As with many motile bacteria, in aqueous environments *A. tumefaciens* moves in a series of straight runs, with periodic redirections or tumbles. Directed movement, either toward or away from chemical and physical stimuli, functions by biasing the frequency of tumbles.

Agrobacterium tumefaciens typically has a sparse tuft of four to six flagellar filaments, sometimes described as a circumthecal arrangement (Loake et al., 1988; Shaw et al., 1991). Flagellum assembly occurs as a highly regulated process in which a master regulator(s) controls flagellar gene expression. Subsequent regulatory switches drive stepwise expression of subsets of these genes in coordination with different assembly intermediates including the basal body, the hook, and then the flagellum filament. As with several rhizobia, the master regulators of flagellar gene expression in *A. tumefaciens* are called VisN and VisR (Vital for swimming), transcription factors in the LuxR–FixJ superfamily (Sourjik et al., 2000; Tambalo et al., 2010; Xu et al., 2013). VisN and VisR are thought to function in a heterocomplex, and are required for expression of virtually all genes involved in motility. This control is, however, indirect, as VisNR primarily activate expression of another transcription factor called Rem (named in *Sinorhizobium meliloti* for Regulator of exponential growth motility), an OmpR-type two-component response regulator with no obvious partner sensor kinase. Rem is thought to directly activate transcription of the flagellar genes. As will be discussed in more detail in subsequent sections, VisNR also regulate biofilm formation, conversely with flagellar gene expression and independently of Rem, with a profound impact on the process of attachment (Xu et al., 2013).

Motility and chemotaxis play an important role in *A. tumefaciens* attachment, biofilm formation, and virulence. In the rhizosphere, *A. tumefaciens* senses and responds directly to plant exudates, chemotaxing toward plant wounds and inducing virulence gene expression (Loake et al., 1988; Shaw et al., 1988, 1991; Hawes and Smith, 1989; Shaw, 1991). Initial suggestions that flagellar-based motility may influence attachment were based on a set of transposon mutants that lost sensitivity to the flagellum-specific phage GS2 and GS6 (Douglas et al., 1982). The attachment defect in these strains, however, was later linked to pleiotropic effects caused by lesions in *chvA* or *chvB*, genes responsible for generation of β-1,2-glucans (Douglas et al., 1985). Furthermore, *chvAB* mutant strains are virulent when inoculated into plant wounds (Bradley et al., 1984). It was later shown that a putative "bald" strain of *A. tumefaciens*, engineered with disruptions in three flagellin genes (the fourth flagellin gene *flaD* was not known at that time) and microscopically devoid of flagella, was moderately reduced in virulence (Chesnokova et al., 1997). Direct experimental evidence that both chemotaxis and flagellar-based motility affect attachment and biofilm formation was provided by comparisons of defined *A. tumefaciens* mutants with either no flagella, unpowered flagella, or impaired chemotaxis. Deletion of *flgE*, encoding the flagellar hook protein FlgE, generated aflagellate, non-motile bacteria while deletion of *motA*, encoding one of the main components of the flagellar motor, resulted in non-motile cells with unpowered flagella. Aside from their lack of motility, both strains were markedly reduced in both attachment and biofilm formation on abiotic surfaces under static conditions (Merritt et al., 2007). Remarkably, under conditions of constant media flow the Δ*flgE* mutant was increased in attachment and biofilm formation relative to wild-type whereas the Δ*motA* mutant remained impaired. This result suggests that in *A. tumefaciens*, the flagellar filament is not required for attachment and is unlikely to function as an adhesin (Smit et al., 1989a). Rather, active rotation of the flagellar motor is required for both efficient attachment and biofilm formation. Increased rates of attachment and more robust biofilm generation by the Δ*flgE* mutant in a flowing environment might be explained by reduced rates of dispersal from established microcolonies and the biofilm surface.

Chemotaxis mutants, generated by deletion of either the entire chemotaxis operon or the chemotaxis sensor kinase CheA, do not tumble and are impaired for swimming as measured on motility agar plates, a standard laboratory assay for motility (Wright et al., 1998; Merritt et al., 2007). These chemotaxis mutants also manifest significant biofilm deficiencies under both static and flow conditions. By selecting for spontaneous mutants of the Δ*cheA* mutant with increased swimming motility in motility agar, Che$^-$ mutation suppressors, or *cms* mutants, were isolated. These *cms* mutants exhibited increased swimming motility on motility agar compared to their parent chemotaxis mutants and were restored for tumbling. Although they improved migration through swim agar, the *cms* mutants remained compromised in attachment and

biofilm formation (Merritt et al., 2007). Ectopic expression of a plasmid-borne wild-type *cheA* allele enhanced motility in swim agar but did not correct the attachment deficiency. The improved migration of the *cms* mutants in motility agar in the absence of true chemotaxis resembles the phenomenon known as pseudotaxis (Ames et al., 1996). Pseudotaxis has been described in several systems, including *Escherichia coli* and *Salmonella enterica*, with spontaneous suppressors of chemotaxis mutants developing mutations in flagellar switch genes that lead to increased tumbling rates (Parkinson et al., 1983; Wolfe and Berg, 1989; Magariyama et al., 1990; Sockett et al., 1992; Togashi et al., 1997). The *A. tumefaciens cms* mutants restore tumbling as well, but the basis for their attachment and biofilm deficiencies remains to be elucidated.

Ctp COMMON PILI AND REVERSIBLE ATTACHMENT

Once the bacterial cell is delivered to a surface via motility or passively by flow, it must initiate physical contact with the substratum. This is often mediated by hair-like extracellular cell surface appendages called pili (or fimbriae) that can function in cell–cell or cell–surface adhesion. Pili in Gram-negative bacteria may be divided into several categories according to their ultrastructure, protein composition, genetic determinants, and mechanism of assembly. These include the type I pili assembled by the chaperone/usher secretion system, the type IV pili assembled by dedicated machinery related to type II secretion systems, and conjugal pili assembled by type IV secretion systems (unrelated to type IV pili; Thanassi et al., 2012). The *A. tumefaciens* genome encodes at least four potential pili. These are the well-studied *virB* T -pilus associated with T-DNA transfer, conjugal pili associated with both pTi and pAt plasmids, and a locus with homology to the type IVb Tad system from *Aggregatibacter actinomycetemcomitans* (Wood et al., 2001). Of these systems, only the type IVb pilus appears to play a role in attachment and biofilm formation by *A. tumefaciens*.

Type IV pili are widespread among diverse bacteria. They are common among Gram-negative species or proteobacteria such as enteropathogenic and enterohemorrhagic *E. coli*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, and *Vibrio cholerae* (Strom and Lory, 1993; Craig et al., 2004; Craig and Li, 2008). Type IV pili are generally 6–9 nm wide, composed primarily of one major pilin subunit, and often aggregate laterally to form bundles. In many species cycles of extension and retraction of type IV pili generate a significant mechanical force, enabling a variety of non-adhesive functions including twitching motility, DNA uptake during transformation, and phage infection (Mattick, 2002). Type IV major pilin subunits are usually synthesized as a prepilin monomer with an N-terminal hydrophilic leader peptide. Type IV pili are grouped into two categories: type IVa pili, whose pilin subunits have short leader peptides (<10 residues) and are 150–160 residues long, and type IVb pili, whose pilin subunits have longer leader peptides (15–30 residues) and are either long (180–200 residues) or are very short (40–50 residues; Mattick, 2002; Thanassi et al., 2012).

The Tad (tight adhesion) system was originally discovered in the periodontal pathogen *Aggregatibacter actinomycetemcomitans* where it mediates attachment and biofilm formation in the oral cavity and may contribute to infective carditis caused by this organism (Scannapieco et al., 1983, 1987; Rosan et al., 1988; Tomich et al., 2007). More recently homologous systems have been identified in many bacterial and archaeal species, including *Yersinia pestis*, *V. cholerae*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa* (Kachlany et al., 2000; Tomich et al., 2007). The *tad* locus is responsible for biogenesis of adhesive Flp (fimbrial low-molecular-weight protein) pili, within the type IVb pilus subclass, which are often involved in biofilm formation and pathogenesis. Several Alphaproteobacteria closely related to *A. tumefaciens*, including *Caulobacter crescentus* and *S. meliloti*, also encode genes homologous to the *Aggregatibacter actinomycetemcomitans tad* locus (Skerker and Shapiro, 2000; Fields et al., 2012). In *C. crescentus* this locus, the *Caulobacter* pilus assembly locus (*Cpa*), is responsible for generating developmentally regulated polar pili that are required for surface interactions and attachment (Skerker and Shapiro, 2000; Bodenmiller et al., 2004; Li et al., 2012). The *A. tumefaciens* genome sequence revealed the *ctpABCDEFGHI* (cluster of type IV pili) locus homologous to the *Aggregatibacter actinomycetemcomitans tad* locus (Wood et al., 2001; Tomich et al., 2007). For the *A. tumefaciens* locus, *ctpA* is predicted to encode the major pilin subunit and *ctpB* the prepilin peptidase that cleaves the leader peptide for pilin maturation. The remaining *ctp* genes encode components of the biosynthetic machinery and related secretion apparatus. Transmission electron microscopy (TEM) of *A. tumefaciens* reveals the presence of thin filaments, significantly thinner than flagella, arranged around the cell surface and frequently shed into the external milieu. These filaments are absent in TEM images of mutant strains deleted for *ctp* genes suggesting that these genes encode Flp-type pili (Wang et al., 2014). As in *C. crescentus*, the Ctp pilus, or a component thereof, may be involved in attachment and subsequent biofilm formation. Mutations in *ctpA*, *ctpB*, or *ctpG* (a predicted ATPase responsible for energizing pilus biogenesis) result in partial but significant decreases in attachment and biofilm formation, and a notable decrease in reversible surface interaction compared to the wild-type strain. Taken together, these results indicate that the *ctp* locus is involved both in pilus assembly, attachment and biofilm formation. Unexpectedly, mature pilin subunits themselves appear to contribute to attachment and biofilm formation, even in mutants for which the Ctp pilus does not assemble (Wang et al., 2014). Modulation of surface interactions by pilin proteins independent of pili has been reported in other bacteria. For example, the minor pilin subunits of *P. aeruginosa*, PilX and PilW, modulate intracellular levels of the second messenger cyclic diguanylate monophosphate (cyclic-di-GMP, or c-di-GMP) and consequently inhibit swarming motility in this pathogen (Kuchma et al., 2012).

POLAR ATTACHMENT TO SURFACES

At some point weak, reversible surface interactions can transition to more stable associations (**Figure 1**). Several well-studied biofilm-forming bacteria such as *P. aeruginosa* transition from transient interactions in which single cell poles engage the surface, to a longitudinal position (Petrova and Sauer, 2012). This is thought to represent the switch to highly stable, irreversible attachment. Polar surface binding is evident in many micrographs of *A. tumefaciens* associated with plant tissues (Pueppke and Hawes, 1985; Brown et al., 2012), and is consistent on abiotic

surfaces in flowing and non-flowing environments, and within complex biofilms (Li et al., 2012; Xu et al., 2012, 2013). It is not clear that polar surface interaction is the only way in which *A. tumefaciens* engages with surfaces, but it is certainly a common mode of interaction. In contrast to the switch from polar to non-polar interactions observed for *P. aeruginosa* and other bacteria, many stably attached *A. tumefaciens* remain associated by a single pole. Recent studies have suggested a model in which during T-DNA transfer to plants *A. tumefaciens* transitions to lengthwise interactions and transfers the DNA via type IV secretion complexes interspersed in an arrayed pattern along the length of the cell (Aguilar et al., 2010, 2011; Cameron et al., 2012). This work uses very high resolution deconvolution microscopy and contradicts previous studies indicating that the type IV secretion complexes localize predominantly to poles (Lai et al., 2000; Atmakuri et al., 2003, 2007; Judd et al., 2005a,b). It is certainly possible that although *A. tumefaciens* might establish stable polar interactions with surfaces, upon induction of the Vir system and initiation of T-DNA transfer to plant cells, it switches to a lengthwise association. Although we observe consistent polar association with both living plants and abiotic surfaces, the two models are not mutually exclusive. Polar attachment is also consistent with the asymmetric budding division of *A. tumefaciens* (described below) where newly born daughter cells are released from the attached mother cell (Brown et al., 2012). The relationship between polar surface binding and the orientation of the *A. tumefaciens* cell during T-DNA transfer has yet to be explained, and new insights may require time lapse analysis of surface binding and T-DNA transfer.

EXTRUSION OF A UNIPOLAR POLYSACCHARIDE ADHESIN

The stable polar attachment of individual cells to surfaces and to other cells seemed likely to be mediated by adhesin molecules in some manner localized to the cell pole. Unipolar attachment mediated by a polarly localized polysaccharide-containing adhesin is particularly common among Alphaproteobacteria, and is best studied in the Caulobacteraceae and Rhizobiaceae families. Among the stalked members of the Caulobacteraceae this adhesin is called the holdfast and has been extensively studied in *C. crescentus*, in which it is produced at the end of the polar stalk (Poindexter and Cohenbazire, 1964; Poindexter, 1981). In the related *Asticcacaulis biprosthecum*, and *Asticcacaulis excentricus* with non-polar stalks, the holdfast is not localized to the stalk ends, but rather the holdfast localizes to the cell pole (Poindexter and Cohenbazire, 1964; Umbreit and Pate, 1978; Merker and Smit, 1988). In these bacteria, holdfast synthesis and export occurs via a Wzy-type mechanism related to capsule biosynthesis in *E. coli* (Smith et al., 2003; Toh et al., 2008; Cuthbertson et al., 2009). The holdfast of *C. crescentus* is well-characterized in terms of synthesis, export, and physical properties, yet little is known regarding its composition (Tsang et al., 2006; Berne et al., 2013). Based on lectin binding the holdfast is thought to contain *N*-acetylglucosamine residues and is anchored to the cell surface via a functional amyloid protein (Merker and Smit, 1988; Hardy et al., 2010). The strength of this adhesive is remarkable and it has been described as "nature's strongest glue" (Tsang et al., 2006). Several Rhizobiaceae also attach to surfaces via a polysaccharide adhesin localized to a single cell pole (Dazzo et al., 1984). *Rhizobium leguminosarum*, for example, has a unipolar glucomannan adhesin (Laus et al., 2006). This polysaccharide contains largely glucose and mannose sugar residues, plus detectable amounts of galactose and rhamnose, and is required for specific binding to pea roots, recognized by a lectin produced by peas. Current data show that this unipolar glucomannan interacts directly with a plant lectin rather than acting as a general adhesin. An additional acidic polysaccharide has also been shown to participate in attachment to plastic surfaces and biofilm formation in *R. leguminosarum*, although there is no indication that this polysaccharide is polarly localized (Russo et al., 2006; Williams et al., 2008). More recently a glucomannan-independent acidic polysaccharide-dependent polar attachment has been observed for *R. leguminosarum*, a mode of attachment that is also dependent on the presence of plant arabinogalactan-like glycoproteins (Xie et al., 2012).

The unipolar polysaccharide (UPP) of *A. tumefaciens* is an extracellular polysaccharide with facile similarity to both the *C. crescentus* holdfast and the glucomannan exopolysaccharide of *R. leguminosarum* (Tomlinson and Fuqua, 2009; Xu et al., 2012). Like the holdfast of *C. crescentus* and *Asticcacaulis biprosthecum*, the UPP is produced at a single cell pole upon surface contact (Li et al., 2012; Xu et al., 2012). Wild-type *A. tumefaciens* rarely produces the UPP during planktonic or colony growth (Xu et al., 2013). The *C. crescentus* holdfast is also developmentally regulated and this may be the case as well for the *A. tumefaciens* UPP (Janakiraman and Brun, 1999; Kim et al., 2013). The UPP is known to play an essential role in attachment and biofilm formation on abiotic surfaces, and may also be required for efficient binding to host plants (Xu et al., 2012, 2013). Although it is not yet known how its adhesive strength compares to the *C. crescentus* holdfast, it is clearly an effective cellular adhesin.

Visualization of the UPP was achieved by staining surface-adhered cells with fluorescently labeled wheat germ agglutinin (WGA), an *N*-acetylglucosamine-specific lectin known to label the holdfast of *C. crescentus* (Tomlinson and Fuqua, 2009). Later it was shown that the *N*-acetylgalactosamine-specific lectin *Dolichos bifloris* agglutinin (DBA) similarly labeled a polarly localized structure (Xu et al., 2012). Thus, the UPP is likely to contain at least two sugars, *N*-acetylglucosamine and *N*-acetylgalactosamine. The first gene verified to be required for UPP biosynthesis was *uppE*, a homolog of *C. crescentus hfsE*, the initiating glycosyltransferase for holdfast synthesis. The *uppE* locus was identified in a screen for *A. tumefaciens* mutants that were deficient in attachment and biofilm formation (Xu et al., 2012). It is clear that *uppE* and the surrounding genes comprise an incomplete Wzy-type polysaccharide biosynthesis cluster, *uppABCDEF* (Atu1235–1240), and are orthologous to the genes required for unipolar glucomannan in *R. leguminosarum* (Williams et al., 2008). This suggests that both adhesins may share structural or functional similarities. Nonetheless, the unipolar glucomannan of *R. leguminosarum* and the UPP of *A. tumefaciens* are clearly not identical, perhaps reflecting different host preferences and lifestyles. It is hypothesized that additional genes are involved in UPP biosynthesis as several key functions including a flippase (Wzx) and a polysaccharide polymerase (Wzy) homolog have not yet been identified.

Interestingly, the requirement for *uppE* is conditional. Phosphate limitation abrogates the requirement for *uppE*. Genetic analysis revealed a conditional redundancy for *uppE* and a paralogous initiating glycosyltransferase, Atu0102 (Xu et al., 2012). The *uppE* gene is required for UPP biosynthesis under phosphate-replete conditions whereas *uppE* and Atu0102 function redundantly under conditions of limiting phosphate. The underlying basis for this conditional functional redundancy remains unclear, but may involve the intracellular signal c-di-GMP.

CONTACT-DEPENDENT ATTACHMENT AND JUST-IN-TIME ADHESIN DEPLOYMENT

Thus far only the general requirement for the UPP in *A. tumefaciens* attachment and biofilm formation has been described. It was also noted that the UPP is not produced by planktonic cells, or cells in colonies. How is such temporal control over UPP synthesis and export achieved? In several genera of Alphaproteobacteria, including *C. crescentus*, *A. tumefaciens*, and *A. biprosthecum*, contact with a solid surface stimulates production of a polar polysaccharide-containing adhesin such as the holdfast and UPP. Biosynthesis and export of this adhesin enables the transition from reversible to irreversible attachment (Li et al., 2012). Surface sensing, and subsequent adhesin production, was demonstrated to be pili- and flagellum-dependent in *C. crescentus*, requiring inhibition of the flagellar motor. The exact molecular mechanism by which inhibition of flagellar rotation regulates adhesin production is not clear. This would not, however, be the first example of the flagellum being used as an environmental sensor. In the pathogenic marine bacterium *V. parahaemolyticus*, it has been shown that the polar flagellum senses surface contact, enabling differentiation of this organism into a swarming motility-competent cell type (McCarter and Silverman, 1990; Gode-Potratz et al., 2011). In *V. cholerae* flagellum interaction with a surface results in a transient loss in membrane potential that ultimately effects the transition to the attached state (Van Dellen et al., 2008). More recently, inhibition of the MotA/MotB stator in *Bacillus subtilis* was demonstrated to effect poly-γ-glutamate (PGA) production, an extracellular capsular polymer (Chan et al., 2014). It is intriguing to imagine that a similar mechanism might extend into the Alphaproteobacteria.

The mechanism of surface sensing and consequent adhesin production in *A. tumefaciens* and *Asticcacaulis biprosthecum* is not known. It is hypothesized that flagellar rotation and pili may participate, as in *C. crescentus*. Of note, and described earlier, polar adhesin production and just-in-time deployment functions normally in non-piliated *A. tumefaciens* mutants (Wang et al., 2014). Importantly, contact-dependent polar adhesin production in *A. tumefaciens* was also shown to efficiently occur on the plant root surface (Li et al., 2012). It is likely that the regulatory signals that direct just-in-time deployment of the *A. tumefaciens* UPP adhesin control additional aspects related to attachment and biofilm formation. Indeed it has been suggested that the elaboration of cellulose fibrils occurs only after the initial attachment process in both *A. tumefaciens* and *R. leguminosarum* (Matthysse et al., 1981; Smit et al., 1987). Just-in-time deployment of the UPP is hypothesized to prevent occlusion of the adhesive by soluble ligands and unproductive autoaggregation of planktonic bacteria, also allowing conservation of resources until the bacterial cell is proximal to a solid surface. As mentioned above and described below, planktonic *A. tumefaciens* cells generally do not generate the polar adhesin unless key regulatory components and signaling circuits are disrupted.

BIOFILM COMPOSITION

Over time surfaces colonized by irreversibly attached individual *A. tumefaciens* cells may undergo a profound transition to a multicellular state, the biofilm (**Figure 1**). Biofilms comprise a community of bacterial cells attached to a surface and surrounded by a hydrated macromolecular matrix (Costerton et al., 1995). Matrix components may include one or more extracellular polymeric substances, including exopolysaccharides, extracellular DNA (eDNA), and protein components (Flemming and Wingender, 2010). The *A. tumefaciens* genome encodes for production of at least six polysaccharide species, several of which play roles in attachment and biofilm formation. These include the UPP adhesin (described above), cellulose, succinoglycan, cyclic β-1,2-glucans, β-1,3-glucan (curdlan), and outer membrane lipopolysaccharide (LPS). Thus far there are no data suggesting that either eDNA or proteinaceous components are found as structural elements in the matrix of the mature *A. tumefaciens* biofilm. A possible role for a protein adhesin, the so-called rhicadhesin (Rhizobiaceae calcium-binding adhesin) protein has been shown for attachment. The matrix of many bacterial species contains one or more functional amyloid proteins as a structural element, with perhaps the most well-known examples being CsgA (curlin) of *E. coli* and TasA of *B. subtilis* (DePas and Chapman, 2012). Several strains of *A. tumefaciens* and related strains from *R. etli* encode a cluster of genes with homology to the functional amyloid curlin, but these have yet to be assigned any physiological role in these bacteria.

CELLULOSE

Cellulose is frequently found as a component of the biofilm matrix in many organisms including several members of the Rhizobiaceae (Karatan and Watnick, 2009; Flemming and Wingender, 2010; Bogino et al., 2013). Cellulose, perhaps the most abundant organic polymer on Earth, is produced by nearly all plants and many bacteria, as well as within the animal and fungal kingdoms (Delmer, 1987; Römling, 2002; Matthysse et al., 2004; Sagane et al., 2010). Cellulose is a homopolymer of β-1, 4-linked glucose monomers with individual cellulose fibers consisting of thousands of individual subunits. The mechanism of prokaryotic cellulose biosynthesis has been well-studied in the Alphaproteobacterium *Gluconacetobacter xylinus* (Ross et al., 1987). Homologous systems for cellulose biosynthesis were later found in *A. tumefaciens*, *E. coli*, and *Salmonella enterica*, among others (Amikam and Benziman, 1989; Matthysse et al., 1995b; Zogaj et al., 2001). Prior to identification of synthetic and regulatory genes involved in cellulose production in *A. tumefaciens*, a role for cellulose in attachment to plant surfaces was reported (Matthysse et al., 1981). The production of cellulose by *A. tumefaciens* results in loose aggregation of planktonic cells (flocculation), pellicle formation in static cultures, and loose attachment to surfaces. Although not absolutely required for virulence, cellulose mutants do show a slightly reduced ability

to colonize plants and induce tumor formation (Matthysse, 1983). Overproduction of cellulose enhances attachment to plant roots in *A. tumefaciens* (Matthysse et al., 2005). Cellulose synthesis in *A. tumefaciens* requires genes in two operons, *celABCG* and *celDE* (Matthysse, 1995; Matthysse et al., 1995b, 2005). The *celA* gene encodes a protein homologous to the catalytic subunit of cellulose synthase (BcsA) from *G. xylinus*, and contains a PilZ domain at the C-terminus, allowing for potential allosteric regulation via c-di-GMP. CelB homologs are also known to bind c-di-GMP and likely function as regulatory subunits of cellulose synthase. CelC has homology to secreted endoglucanases while *celD* and *celE* are soluble, cytoplasmic components involved in early steps of cellulose polymerization. Several negative and positive regulators of cellulose synthesis have been identified, including CelG and CelI (Matthysse et al., 1995a). Mutations in either *celG* or *celI* results in increased cellulose production, indicating that these gene products encode negative regulators of synthesis. Mutations in the *A. tumefaciens* homologs of *divK* or *pleD* (*celR*) also affect cellulose production (see Coordination of Division and Development; Barnhart et al., 2013, 2014). Similar results have been observed in *R. leguminosarum* (Ausmees et al., 1999). As described below, many regulatory aspects of cellulose synthesis parallel that of UPP regulation, with c-di-GMP being one of the primary regulators.

ROLE OF OTHER POLYSACCHARIDES IN ATTACHMENT AND BIOFILM FORMATION

As mentioned earlier, aside from the UPP and cellulose, *A. tumefaciens* produces at least three additional exopolysaccharides: succinoglycan, cyclic β-1,2-glucans, and curdlan (Nakanishi et al., 1976; Hisamatsu et al., 1978; Zevenhuizen and Vanneerven, 1983; Karnezis et al., 2003). The major acidic EPS produced by *A. tumefaciens* is succinoglycan, the product of the *exo* genes (Cangelosi et al., 1987). The role of succinoglycan in the biology of *A. tumefaciens* is unclear. Mutants unable to synthesize succinoglycan are fully virulent, efficiently attach to plant surfaces, and are not diminished in biofilm formation (Tomlinson et al., 2010). In contrast, in *S. meliloti* succinoglycan (also called EPS I) is required for biofilm formation and productive interaction with the plant host (Cheng and Walker, 1998; Fujishige et al., 2006). It was recently proposed that the physiochemical properties of succinoglycan contribute to aggregation in *S. meliloti*, and that this may eventually lead to productive biofilm formation (Dorken et al., 2012). It is possible that succinoglycan may play a similar role in some environments for *A. tumefaciens*, although at present there are no supporting data to this effect. In both *A. tumefaciens* and *S. meliloti* succinoglycan synthesis is negatively regulated by a periplasmic protein, ExoR (Chen et al., 2008; Tomlinson et al., 2010). ExoR is itself sensitive to pH and thus it is possible that one function of *A. tumefaciens* succinoglycan is related to acid tolerance (Lu et al., 2012; Wu et al., 2012).

β-1,2-Glucans may be generated in linear or cyclic forms and are synthesized by many rhizobia (Breedveld and Miller, 1994). In *A. tumefaciens* β-1,2-glucans are cyclic, the product of the ChvB synthase (Puvanesarajah et al., 1985; Zorreguieta et al., 1988; Castro et al., 1996). The *chvB* (*ch*romosomal *v*irulence) gene was originally isolated in a transposon screen for mutants unable to attach to plant cells and required for virulence (Douglas et al., 1982). A second locus adjacent to *chvB* also identified in this screen is *chvA*, the product of which is required for export of β-1,2-glucans into the periplasm where they are believed to play a role in osmoadaptation (de Iannino and Ugalde, 1989; O'Connell and Handelsman, 1989). While the genes directing synthesis of cyclic β-1,2-glucans were isolated due to their attachment and virulence phenotypes, a direct role for this polysaccharide species in attachment has not been demonstrated. Rather, impaired osmoregulation within the periplasmic space results in pleiotropic effects on the cell surface, several of which likely contribute to the attachment deficiency (Breedveld and Miller, 1998). As well as being deficient in attachment to plant surfaces, mutants in *chvA* or *chvB* also show a modest decrease in biofilm formation (Xu et al., 2012).

Curdlan is a neutral β-1,3-glucan produced by many bacteria and utilized as a gelling agent in the food industry (McIntosh et al., 2005). While most work on curdlan biosynthesis has been performed in the curdlan-overproducing strain *Agrobacterium* sp. ATCC 31749, genome analysis of *A. tumefaciens* indicates that the curdlan synthesis genes are conserved. Although the regulation of curdlan synthesis in *Agrobacterium* sp. ATCC 31749 shares many features with regulation of other exopolysaccharides, no biological function has been described for this polysaccharide species in *A. tumefaciens* (Ruffing and Chen, 2012). Deletion of *crdS*, encoding the curdlan synthase homolog in *A. tumefaciens* has no effect on attachment and biofilm formation (Xu et al., 2012).

Early work suggested that *A. tumefaciens* LPS was required for attachment to plant surfaces (Lippincott and Lippincott, 1969; Whatley et al., 1976). This work demonstrated inhibition of attachment to wound sites with crude preparations of LPS. It is unclear what other inhibitors may have been present in this preparation. Other than these findings, there are no other data supporting a role for LPS in attachment and biofilm formation, although many of the genes encoding LPS synthesis would be essential, and genetic studies might therefore not reveal a role for this surface polysaccharide. The localization of LPS on the outer leaflet of the outer membrane certainly might impart an influence on surface interactions, and in other bacteria LPS has been demonstrated to impact attachment to surfaces.

RHICADHESIN AND RAPS

Although the UPP and cellulose are important adhesins mediating attachment and biofilm formation in *A. tumefaciens*, it is possible that additional adhesins may contribute to either process. The activity of these putative adhesins may be discernible only under particular circumstances, indicative of temporal or developmental regulation or a specific plant host interaction. One possible adhesin present in the rhizobia is the calcium-dependent protein rhicadhesin, originally identified in *R. leguminosarum* strain 248 (Smit et al., 1987). Under calcium-limiting conditions *R. leguminosarum* was reduced both in its ability to agglutinate to glass and to attach to pea root hair tips. This same activity was described for *A. tumefaciens* strains 1251 and LBA1010 (Smit et al., 1987, 1989b). Rhicadhesin was further characterized as a small (14 kDa), soluble, extracellular component inactivated by heat and protease treatment (Smit et al., 1989a,b). The gene or genes encoding

rhicadhesin have yet to be identified and therefore it is unclear that the rhicadhesin activity isolated from each strain is due to homologous proteins.

An additional set of calcium-binding adhesins were identified in *R. leguminosarum* and *R. etli* in an elegant experiment designed to identify the rhicadhesin coding sequence (Ausmees et al., 2001). Using a phage-display cloning approach the genes for four Rap (*Rhizobium*-adhering proteins) proteins were isolated. The phylogenetic distribution of these proteins is limited compared to rhicadhesin and it is unlikely that they represent the same activity. The Rap proteins were originally proposed to be agglutinins secreted by the PrsD–PrsE type I secretion system. These proteins recognize a polar cell-surface receptor on the bacterium and are capable of mediating autoagglutination and possibly attachment to plant roots, glass, and polystyrene (Russo et al., 2006). Recent work has demonstrated that RapA2 of *R. leguminosarum* specifically binds the acidic exopolysaccharide in a calcium-dependent manner and may contribute to development of the biofilm matrix in this organism (Abdian et al., 2013). No Rap proteins have been identified in *A. tumefaciens* and thus, as for rhicadhesin, any role for these proteins in attachment or biofilm formation by *A. tumefaciens* is speculative.

THE ROLE OF THE At PLASMID

Initial attempts at isolation and characterization of *A. tumefaciens* mutants that were impaired in early stages of attachment were extensively reported but ultimately raised several questions that have yet to be fully resolved. Tn5 transposon mutagenesis and microscopic observation of mutants unable to attach to carrot suspension culture cells led to the identification of a 29-kb region of genomic DNA that was hypothesized to harbor multiple *att* genes involved in attachment (Matthysse, 1987; Matthysse et al., 2000). At the time of the initial isolation and characterization of the *att* genes the complete genome sequence of *A. tumefaciens* had not been published. The *A. tumefaciens* C58 genome sequence revealed that the *att* genes were located on the accessory plasmid, pAtC58 (Goodner et al., 2001; Wood et al., 2001). This result conflicted with earlier reports that the pAt plasmid was not required for virulence (Hooykaas et al., 1977; Rosenberg and Huguet, 1984; Hynes et al., 1985). It was later confirmed that although the pAt plasmid can mildly influence virulence and ecological fitness of the organism, pAtC58-cured derivatives remain fully virulent with no obvious attachment or virulence deficiency (Nair et al., 2003; Morton et al., 2013). It was further reported that several of the original *att* transposon insertions generated dominant negative alleles and thus the effect of the intact genes was questioned (Matthysse et al., 2008). It seems likely that the pAt plasmid may influence *A. tumefaciens* ecology by broadening the scope of nutritional resources in the rhizosphere via genes that impart catabolism of several common soil compounds (Baek et al., 2005; Chai et al., 2007). Nonetheless, the role of this plasmid and the *att* genes in attachment and biofilm formation, if any, is unclear.

IMPACT OF THE PLANT HOST ON ATTACHMENT AND BIOFILMS

It is clear that association of bacteria with plant tissues is profoundly, in some cases, dominantly, influenced by the host plant. Nutrient exudation, surface chemistry and defense responses all combine to influence which bacteria efficiently colonize the plant, establishing beneficial, neutral, or pathogenic interactions. In several cases, specific receptors have been identified, such as plant lectins that recognize specific polysaccharides produced by colonizing rhizobia (van Rhijn et al., 2001). There are several candidates for plant surface receptors for *A. tumefaciens*, as well as other plant functions that are required for *A. tumefaciens* infection and T-DNA transfer. Using a collection of T-DNA disruption libraries in the host plant *Arabidopsis thaliana* several candidate plant receptors for *A. tumefaciens* were identified (Gelvin, 2010). These include mutants for an arabinogalactan protein, AtAGP17, a cellulose synthase-like protein, CslA-09, and β-expansin, so-called *rat* mutants (resistant to *Agrobacterium* transformation; Nam et al., 1999; Zhu et al., 2003; Gaspar et al., 2004). Using an analogous screen for *Arabidopsis* mutants that were hypersusceptible to *Agrobacterium* transformation (*hat* mutants) the putative plant receptor protein AT14A was identified as required for efficient attachment (Sardesai et al., 2013). Direct screens for proteins that interact with the Vir machinery also identified potential targets (Hwang and Gelvin, 2004). It remains unclear which of these candidate functions plays a major role in initial attachment, and it is certainly plausible that attachment processes which lead to T-DNA transfer are not identical to those that result in benign associations. There remains much to learn about the bacterial population dynamics on plant tissue surfaces, the impact of plant structures and its response to the colonizing bacteria, and how these influence the outcome of interactions of plants with *A. tumefaciens* in the natural environment.

REGULATION OF ATTACHMENT AND BIOFILM FORMATION

The transition of bacteria from the motile to the sessile lifestyle, and then to the biofilm mode of growth involves several phenotypic changes mediated at both transcriptional and post-translational levels. Following initial surface contact, flagellar motility is often repressed post-translationally utilizing mechanisms ranging from rotational slow-down to complete flagellar ejection (Shapiro and Maizel, 1973; Aldridge and Jenal, 1999; Blair et al., 2008). Repression of motility allows for stabilization of surface interactions and irreversible attachment mediated by one or more adhesins (Foster and Hook, 1998; Hinsa et al., 2003; Tsang et al., 2006; Berne et al., 2013; Xu et al., 2013). Once irreversibly attached to a surface individual cells can aggregate, forming microcolonies that become enmeshed by the biofilm matrix (Flemming and Wingender, 2010). Within the biofilm cells may communicate, grow, divide, and die, resulting in a metabolically and developmentally heterogeneous population (Stewart and Franklin, 2008). Although establishment of a biofilm is often considered an irreversible process for an individual bacterium there are occasions when the biofilm matrix is actively degraded resulting in dispersal of embedded cells. While dispersal has been observed for attached and biofilm-associated *A. tumefaciens* the mechanism by which this occurs, and how it is regulated, has not been described (Hibbing and Fuqua, 2012). Surface contact, environmental conditions such as oxygen and phosphate levels and pH, and intracellular signaling molecules, often integrated through

transcriptional regulatory pathways or posttranscriptional controls have been shown to directly influence attachment and biofilm formation in *A. tumefaciens* (**Figure 2**).

CYCLIC-DI-GMP

One of the primary signaling molecules that controls the motile-to-sessile transition in diverse bacteria is now recognized to be c-di-GMP (**Figure 3**; Hengge, 2009; Römling et al., 2013). Cyclic nucleotides are widespread in both prokaryotes and eukaryotes, with phenotypic effects ranging from nutrient utilization and cell division (cAMP), to cyst formation and pathogenesis (cGMP), to cell cycle control (c-di-AMP; Botsford and Harman, 1992; Beavo and Brunton, 2002; Witte et al., 2008; Gomelsky, 2011; Marden et al., 2011; An et al., 2013). C-di-GMP was first described as a

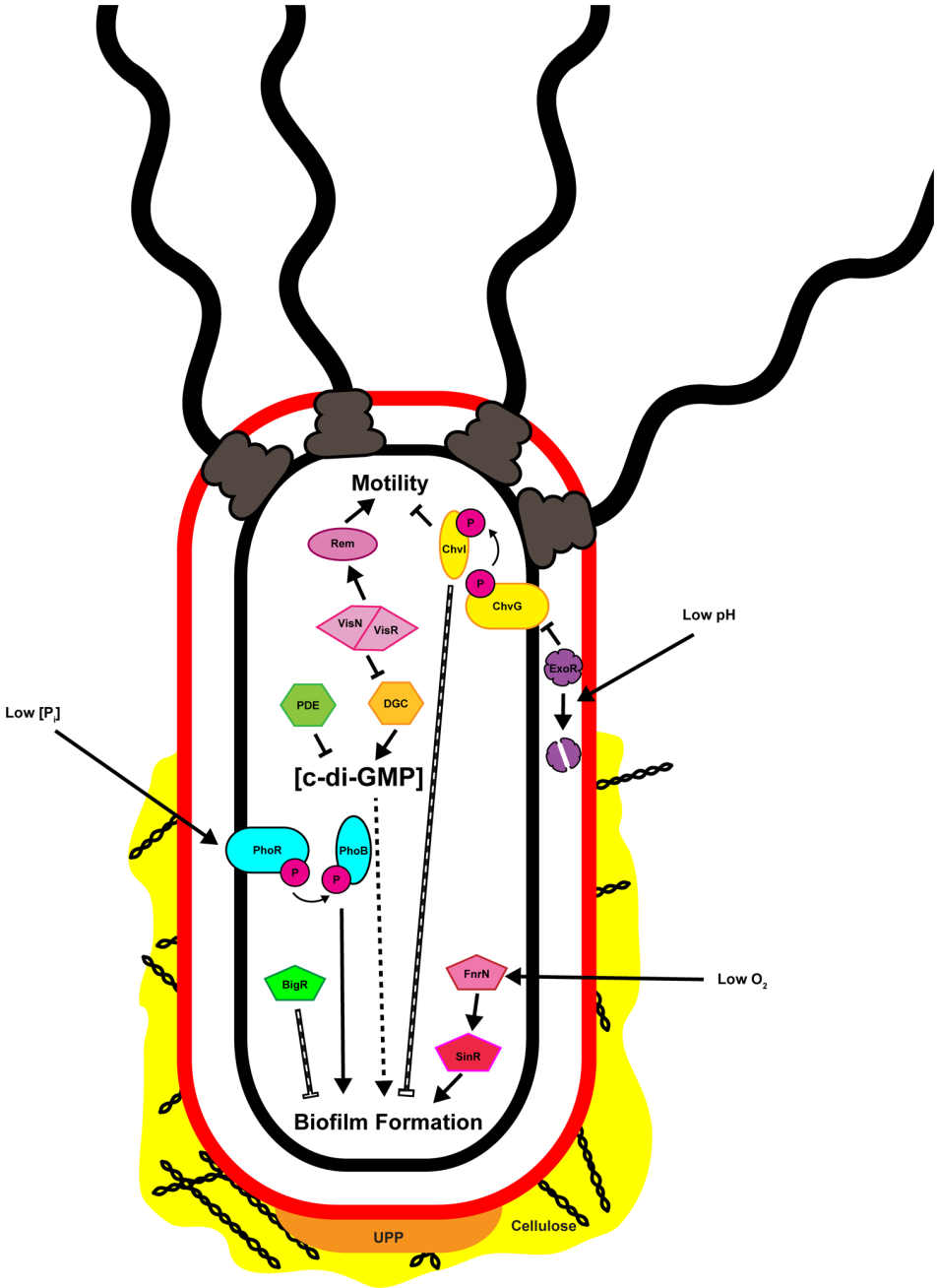

FIGURE 2 | Multiple inputs regulate attachment and biofilm formation by *Agrobacterium tumefaciens*. Depicted in the image are the known factors regulating attachment and biofilm formation and discussed in the text. Solid black arrows and bars indicate direct positive or negative regulation, respectively. Hashed arrows and bars indicate regulation that is indirect or where the molecular mechanism has not been defined. Note that the cell envelope is represented only by the outer (red) and inner (black) membranes, and the periplasmic peptidoglycan is not shown.

molecule that could activate cellulose synthase in *G. xylinus* and *A. tumefaciens* (Ross et al., 1987; Amikam and Benziman, 1989). Over two decades of research has discovered a variety of bacterial phenotypes regulated by c-di-GMP, including biofilm formation, cell cycle progression, and motility, among others (Römling et al., 2013).

The intracellular concentration of c-di-GMP is controlled by the opposing action of two enzymatic functions: diguanylate cyclases (DGCs), that synthesize c-di-GMP from two molecules of the common nucleotide GTP, and phosphodiesterases (PDEs), that degrade it (**Figure 3**; Schirmer and Jenal, 2009). DGC proteins are characterized by a GGDEF catalytic motif (Paul et al., 2004). Many DGCs also contain an allosteric inhibitory region known as the I-site (Chan et al., 2004). C-di-GMP-specific PDEs are characterized by the presence of either an EAL or HD-GYP catalytic motif (Schmidt et al., 2005; Ryan et al., 2006; Rao et al., 2008). The ubiquity of c-di-GMP signaling was evident early on, with GGDEF- and EAL-containing domains recognized as conserved domains of unknown function (DUF1 and DUF2, respectively) prior to demonstration of their enzymatic activity. Many bacteria have multiple proteins with GGDEF and EAL domains, often associated with other regulatory domains. Many proteins also have both DGC and EAL domains, and the same protein may catalyze c-di-GMP synthesis and degradation. Each domain can be individually regulated, hinting at the complexity and diversity of c-di-GMP-specific signaling. C-di-GMP generally functions allosterically by binding to regulatory domains in proteins or RNA molecules. There are several common c-di-GMP-binding domains found in bacteria including the PilZ domain, at least one two-component response regulator, degenerate (non-functional) EAL domains, and I-sites proximal to inactive GGDEF domains. Binding of c-di-GMP to these domains may be transduced to *cis* regulatory domains within the same protein or to *trans* signal transduction partners that ultimately effect a c-di-GMP-dependent phenotype (Pratt et al., 2007; Römling et al., 2013). Several transcription factors are c-di-GMP responsive, transducing the signal to changes in gene expression (Hickman and Harwood, 2008; Leduc and Roberts, 2009; Krasteva et al., 2010). In addition, riboswitches that specifically sense c-di-GMP with extremely high affinity ($K_D \sim 1$ nM) have been shown to modulate transcriptional activity and RNA splicing (Sudarsan et al., 2008; Lee et al., 2010).

Although c-di-GMP can control a wide range of phenotypes, a common regulatory pattern of c-di-GMP signaling entails altered levels reciprocally affecting two primary phenotypes: motility and attachment. Increasing c-di-GMP levels generally leads to reduced motility and concomitant enhanced attachment. Examples of c-di-GMP-dependent motility phenotypes include the complete flagellar ejection seen in *C. crescentus*, and the reduction of swimming velocity by interaction of a c-di-GMP binding protein with the flagellar motor, observed for *E. coli* (Aldridge and Jenal, 1999; Boehm et al., 2010). C-di-GMP levels may affect both adhesin production and maintenance of these adhesins on the cell surface. This is demonstrated by control of secretion of MRP adhesin in *Pectobacterium atrosepticum* and preservation of the LapA adhesin on the *Pseudomonas fluorescens* cell surface (Newell et al., 2011; Perez-Mendoza et al., 2011). In addition, production of biofilm matrix components is often influenced by c-di-GMP. A recent example is the allosteric control of poly-β-1-6-*N*-acetylglucosamine (poly-GlcNAc) synthesis and secretion in *E. coli* by direct allosteric control of the biosynthetic enzyme complex by c-di-GMP (Steiner et al., 2013). Finally, virulence can be modulated by c-di-GMP signaling, as seen in *Y. pestis* and *V. cholerae* (Pratt et al., 2007; Bobrov et al., 2011).

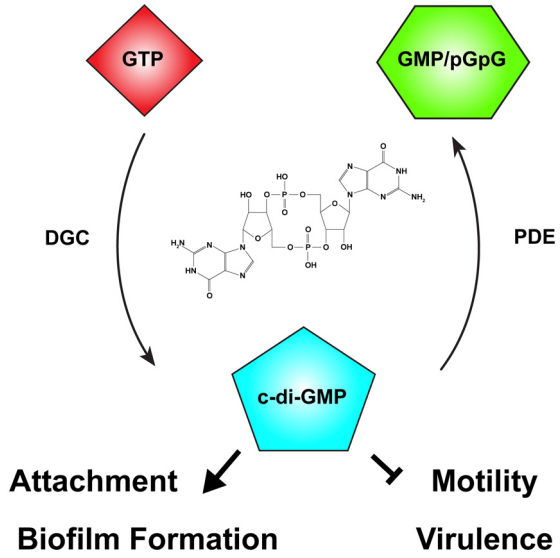

FIGURE 3 | The second messenger cyclic-di-GMP. Cyclic diguanylate monophosphate, or cyclic-di-GMP (c-di-GMP) is a common second messenger in prokaryotic systems. C-di-GMP is generated from two molecules of guanosine triphosphate (GTP) by diguanylate cyclases (DGC) and degraded by phosphodiesterases (PDE) to the linear form, 5′-phosphoguanylyl-guanosine (pGpG), and ultimately to two molecules of guanosine monophosphate (GMP). In many bacteria, including *Agrobacterium tumefaciens*, c-di-GMP levels reciprocally regulate the transition between motility and attachment. In *A. tumefaciens* globally or locally increased c-di-GMP levels positively regulate attachment and biofilm formation while negatively regulating motility. The effect of c-di-GMP on virulence in *A. tumefaciens* has not been described, although in many bacteria virulence is negatively regulated by elevated c-di-GMP levels. The chemical structure of c-di-GMP is included in the center of the figure.

Agrobacterium tumefaciens possesses 33 proteins predicted to be involved in modulating intracellular levels of c-di-GMP (16 GGDEF, 1 EAL, 1 HD-GYP, 13 GGDEF-EAL). This large number of proteins likely reflects the importance of c-di-GMP signaling in the control of *A. tumefaciens* phenotypes. One *A. tumefaciens* phenotype influenced by c-di-GMP was recognized early on with the observation that cellulose synthase activity in crude extracts increased upon the addition of micromolar levels of c-di-GMP (Amikam and Benziman, 1989). It followed from this observation that cellulose-dependent attachment to plant surfaces was also likely influenced by c-di-GMP levels. Ectopic expression of the wild-type *A. tumefaciens* PleD (homologous to *C. crescentus* PleD, the first characterized GGDEF DGC protein) artificially elevated the intracellular levels of c-di-GMP, resulting in a drastic increase in both cellulose and UPP production (Paul et al., 2004; Xu et al., 2013). Increased production of cellulose and UPP coincided with enhanced cellulose-dependent aggregation, UPP-dependent rosette formation, attachment to glass and PVC

coverslips, and biofilm formation. C-di-GMP signaling in *A. tumefaciens* appears to follow the paradigm of inverse regulation of motility and attachment as reduced motility was also observed upon c-di-GMP elevation (Xu et al., 2013).

Several activities are regulated by c-di-GMP in *A. tumefaciens*, but it remains unclear how the activity of the various DGCs and PDEs is controlled and how this control is integrated with the motile-to-sessile switch and the production of adhesive polysaccharides. Recently, it was suggested that increased attachment under conditions of limiting phosphate was mediated, at least in part, by a PhoB-dependent increase in c-di-GMP levels (Xu et al., 2012). Thus, environmental conditions seem likely to contribute to regulation of DGC and PDE activity. Transposon mutagenesis of a strain engineered to lack all known exopolysaccharides except UPP identified several mutants with increased UPP production. Of particular interest are four genetic loci in which multiple transposon mutants were isolated (Xu et al., 2013). These loci include two LuxR-type transcription factors (*visN* and *visR*), a CheY-type single domain response regulator (*rrpX*), a putative short-chain dehydrogenase/pteridine reductase (*pruA*), and a dual GGDEF-EAL protein. Further analysis of the role of VisN and VisR identified three DGC homologs that are regulated through VisNR.

VisN/VisR

VisN and VisR are members of the LuxR–FixJ family of transcriptional regulators that play a critical role in regulating motility in several members of the Rhizobiaceae, including *A. tumefaciens* (Sourjik et al., 2000; Xu et al., 2013). VisN and VisR were first identified as global regulators of motility in *S. meliloti* (Sourjik et al., 2000). The C-termini of both VisN and VisR show strong homology to the DNA-binding domain of LuxR. The N-termini, however, share little homology either with one another or with other known LuxR-family transcriptional regulators, although these N-terminal domains are conserved among orthologs within the Rhizobiaceae. VisN and VisR are believed to function together to regulate transcription of chemotaxis and flagellar motility genes in *S. meliloti*, presumably forming heteromultimers (Sourjik et al., 2000; Rotter et al., 2006; Xu et al., 2013).

As mentioned above, VisN and VisR were originally identified as negative regulators of UPP synthesis and, consequently, attachment and biofilm formation (Xu et al., 2013). Mutations in either *visN* or *visR* also result in a loss of motility in *A. tumefaciens*, consistent with their role as positive regulators of motility in *S. meliloti* and *R. leguminosarum* (Sourjik et al., 2000; Tambalo et al., 2010; Xu et al., 2013). Inverse regulation of motility and biofilm formation by VisNR resembles c-di-GMP-dependent regulation of these same phenotypes in *A. tumefaciens*. Phenotypic and transcriptomic analysis identified three DGCs, *dgcA*, *dgcB*, and *dgcC*, as components of the VisNR regulatory network (Xu et al., 2013). Curiously, deletion of *dgcA*, *dgcB*, or *dgcC*, alone or in any combination does not affect average cytoplasmic levels of c-di-GMP in *A. tumefaciens* cells. This observation supports models where local pools of c-di-GMP and c-di-GMP-dependent effectors play a more defined role in regulating developmental phenotypes, over and above mean cytosolic concentration.

Microarray analysis of the VisNR regulon identified *dgcB* and *dgcC* as transcriptionally regulated by VisNR. DgcA, which plays the dominant role in VisNR-dependent regulation of biofilm formation, was not recognized to be transcriptionally regulated by VisNR. Similarly, microarray analysis of a positive regulator of attachment, ExoR (described below), does not reveal any obvious candidates for transcriptionally controlled regulators of biofilm formation, with the exception of a number of uncharacterized DGC genes (Heckel et al., in review). These observations suggest that control of biofilm formation through the VisNR and ExoR regulons proceeds primarily through post-transcriptional mechanisms. Two other classes of genes are commonly regulated by VisNR and ExoR: the *exo* genes controlling succinoglycan biosynthesis and the *imp* genes controlling type VI secretion (Wu et al., 2012; Xu et al., 2013; Heckel et al., in review). Both of these gene groups, however, are oppositely regulated by VisNR and ExoR. The *exo* and *imp* genes display reduced expression in a Δ*visR* mutant and enhanced expression in Δ*exoR* strains, suggesting positive regulation by VisNR and repression by ExoR (Heckel et al., in review).

ExoR-ChvG/ChvI

The periplasmic regulator ExoR is a positive regulator of attachment and biofilm formation in *A. tumefaciens* (Tomlinson et al., 2010). ExoR was originally described as a repressor of exopolysaccharide synthesis in *S. meliloti* (Doherty et al., 1988). Additional phenotypes affected in *S. meliloti exoR* mutants include increased biofilm formation, reduced motility, loss of prototrophy, and reduced symbiotic efficiency (Yao et al., 2004; Wells et al., 2007). Several of these phenotypes are consistent with *A. tumefaciens exoR* mutants, including enhanced production of succinoglycan and reduced motility, although in contrast to *S. meliloti* these mutants exhibit attachment and biofilm defects (Tomlinson et al., 2010; Heckel et al., in review).

ExoR exerts its effects primarily through direct inhibition of the two-component system ChvG/ChvI (**Figure 2**; Wells et al., 2007; Chen et al., 2008; Wu et al., 2012; Heckel et al., in review). The ChvG/ChvI two-component system, homologous to ExoS/ChvI of *S. meliloti*, is an acid-responsive signaling system required for virulence (Charles and Nester, 1993; Mantis and Winans, 1993; Li et al., 2002). A genetic interaction between ExoR and ExoS (ChvG) was originally identified in *S. meliloti* (Doherty et al., 1988; Fujishige et al., 2006; Wells et al., 2007). Direct interaction between periplasmic ExoR and the periplasmic portion of the ExoS (ChvG) histidine kinase was eventually demonstrated for both *S. meliloti* and *A. tumefaciens* (Chen et al., 2008; Wu et al., 2012). Under neutral conditions ExoR represses activity of ExoS (ChvG), and through this interaction also negatively regulates the DNA-binding activity of the ChvI response regulator. Upon acidification of the periplasm ExoR is degraded by an unidentified protease, derepressing ExoS (ChvG) activity, resulting in phosphorylation of ChvI and transcriptional activation of several ChvI-regulated genes (Chen et al., 2008; Lu et al., 2012; Wu et al., 2012). The ExoR-ChvG/ChvI signaling trio is well-conserved among the Rhizobiales, and is responsive to environmental signals relevant to the ecology of the individual organism. For example, in the intracellular mammalian pathogen *Bartonella*

henselae BatS/BatR, homologous to ChvG/ChvI, is activated at a pH of 7.4, the physiological pH of mammalian blood (Quebatte et al., 2010). For *A. tumefaciens*, low pH is a virulence-inducing signal that is common to the rhizosphere, allowing the ExoR-ChvG/ChvI system to play a distinct role in the ability of the bacteria to sense and respond to potential host plants (Winans, 2008).

Although ExoR-ChvG/ChvI activity and regulation in *A. tumefaciens* is quite similar to that in *S. meliloti* there are two important differences. First, in *A. tumefaciens* mutations in this pathway dramatically diminish attachment and biofilm formation, whereas in *S. meliloti* these mutations enhanced biofilm formation (Fujishige et al., 2006; Tomlinson et al., 2010). Second, while *exoR* is readily deleted from the genome of *A. tumefaciens*, it has been historically difficult to obtain such a mutant in *S. meliloti*. This suggests that control of this important regulatory circuit has diverged in these lineages, perhaps to support the commensal lifestyle of *S. meliloti* and pathogenicity in *A. tumefaciens*, respectively.

ENVIRONMENTAL AND NUTRITIONAL INPUTS

As with other bacteria, *A. tumefaciens* is responsive to local environmental conditions. As discussed below, efficient induction of the virulence genes of the tumor-inducing (Ti) plasmid occurs under conditions that mimic those found in the plant host rhizosphere. These conditions include low pH and limiting phosphate concentrations. Full virulence induction also requires the presence of plant phenolics such as acetosyringone. The integration of the virulence response with environmental conditions allows for expression of the full suite of virulence genes to occur at a location most likely to result in a productive host–pathogen interaction. Attachment and biofilm formation are also responsive to local environmental conditions. Within and around a microbial biofilm there are expected to be differing environmental conditions such as gradients of oxygen tension, redox potential, and metabolites (Stewart and Franklin, 2008; Koley et al., 2011). Multiple environmental and nutritional inputs have been shown to regulate attachment and biofilm formation by *A. tumefaciens*, including oxygen levels and phosphate concentrations. The pH impacts attachment and biofilm formation through the ExoR-ChvG/ChvI regulatory pathway described above (**Figure 2**). Oxygen tension is proposed to affect biofilm maturation through two independent regulatory pathways, SinR/FnrN and BigR (biofilm growth-associated repressor), both of which are described further below (**Figure 2**).

Phosphorus levels and biofilm formation

In *S. meliloti* the production of two exopolysaccharides, EPS I (succinoglycan) and EPS II (galactoglucan), is differentially regulated by phosphate concentration (Rinaudi et al., 2006; Rinaudi and Giordano, 2010). Both of these exopolysaccharides participate in productive biofilm formation in *S. meliloti*, with increased biofilm levels under P_i limitation (Rinaudi and Gonzalez, 2009). In *A. tumefaciens* limiting P_i levels increase attachment and biofilm formation, an effect that is not succinoglycan-dependent (Danhorn et al., 2004; Tomlinson et al., 2010; Xu et al., 2012). This effect was regulated by the canonical PhoR/PhoB phosphate-sensing two-component system (**Figure 2**). *A. tumefaciens* is unusual in that both the *phoR* and *phoB* genes are essential, under phosphate-replete and phosphate-limiting conditions (Danhorn et al., 2004; Xu et al., 2012). Increased attachment under limiting P_i is directly mediated by the UPP adhesive polysaccharide. Interestingly, experimental analysis of the *upp* biosynthetic genes in low phosphate revealed a conditional redundancy for the *uppE* gene, described above (Xu et al., 2012). The effects of P_i levels on attachment and biofilm formation have been observed in other Rhizobiaceae, including *R. leguminosarum*, indicating that it may be a conserved response among these bacteria (Janczarek and Skorupska, 2011). However, an inverse relationship between phosphate concentration and biofilm formation is not universal. For example, with *Pseudomonas fluorescens* elevated phosphate levels increased adherence in a PhoR/PhoB-dependent manner and ultimately through c-di-GMP (Monds et al., 2001, 2007).

Redox regulation of biofilm formation

As biofilm growth and maturation proceed the local within-biofilm environment experiences several changes, including a reduction in available oxygen, particularly for actively aerobic bacteria (Stewart and Franklin, 2008). In order to survive microaerobic conditions, many bacteria, including *A. tumefaciens*, undertake a respiratory shift from oxic to anoxic conditions, utilizing nitrate rather than oxygen as a terminal electron acceptor (Bueno et al., 2012). In many Alphaproteobacteria, including *A. tumefaciens*, this process, denitrification, is regulated by one or more members of the FNR (fumarate and nitrate reductase) family of transcriptional regulators. *A. tumefaciens* has four such regulators: FixK, FnrN, NnrR, and SinR. Three of these, FnrN, NnrR, and SinR, clearly play a role in regulating denitrification genes in low-oxygen environments, including at the plant interface (Baek et al., 2008). In addition, both SinR and FnrN have been shown to affect biofilm maturation (Ramey et al., 2004b).

The *sinR* locus was initially identified in *A. tumefaciens* during a screen to isolate mutants deficient in biofilm formation (Ramey et al., 2004b). SinR mutants attach and initiate biofilm formation but are deficient in biofilm maturation, never reaching the same structure and cell density achieved by wild-type *A. tumefaciens*. Directly upstream of *sinR* is a canonical FNR-type binding site, and both FnrN and SinR regulate expression of *sinR*. While mutations in FnrN do not display a decrease in biofilm formation Δ*sinR* Δ*fnrN* double mutants approximate the Δ*sinR* phenotype. Ectopic expression of *sinR* in wild-type, Δ*sinR*, Δ*fnrN*, and Δ*sinR* Δ*fnrN* backgrounds accelerates biofilm maturation and leads to the formation of denser biofilms on both abiotic and plant surfaces (Ramey et al., 2004b).

Oxygen-sensing FNR homologs frequently acquire an oxygen-labile $[4Fe–4S]^{2+}$ cluster under low-oxygen conditions, leading to dimerization, DNA binding, and regulation of target genes (Lazazzera et al., 1996). In *A. tumefaciens*, only FnrN is predicted to function in this manner and FnrN upregulates both *sinR* and denitrification genes under low-oxygen conditions (Ramey et al., 2004b; Baek et al., 2008). Together these data suggest that FnrN allows for coordinate regulation of biofilm maturation and respiration under microaerobic or anoxic conditions, allowing *A. tumefaciens* to adjust to local environmental conditions. Although FnrN and SinR both ultimately affect biofilm maturation their

regulatory networks are poorly defined, and it is unclear which target genes play a role in biofilm maturation.

BigR is a member of the ArsR/SmtB subfamily of metal-sensing winged-helix transcription factors. In contrast with most members of this family, BigR and its homologs act as redox switches that, upon oxidation, form an intramolecular Cys–Cys disulfide bond. The resulting conformational change reduces the affinity of BigR for its DNA binding site and allows for derepression of the *bigR* operon (Guimaraes et al., 2011). Thus far BigR has been shown to regulate the activity of a single operon, found in both *Xylella fastidiosa* and *A. tumefaciens*, encoding a putative sulfur dioxygenase Blh, BigR itself, and at least three additional putative membrane proteins, one of which likely acts as a sulfite exporter. The *bigR* operon is induced when either *X. fastidiosa* or *A. tumefaciens* is grown as a biofilm on glass coverslips, and a *bigR* mutant generated thicker biofilms on both glass coverslips as well as *Nicotiana tabacum* roots (Barbosa and Benedetti, 2007). It was proposed that detoxification of metabolically generated hydrogen sulfide by Blh would be particularly important under conditions of low oxygen tension such as those found within a biofilm. The mechanism by which BigR would be oxidized in these conditions, thus derepressing transcription of the necessary detoxification genes including *blh*, is not understood, although the authors speculate that hydrogen sulfide-induced reactive oxygen species may play a role (Guimaraes et al., 2011).

MULTICELLULARITY AND DEVELOPMENT

During its lifetime a single *A. tumefaciens* bacterium must precisely coordinate cell growth and division with current environmental conditions, including whether or not it is entering or exiting the multicellular biofilm mode of growth. It is now recognized that many, if not most, rhizosphere bacteria exist primarily as residents of a single-species or polymicrobial biofilm. Within the rhizosphere *A. tumefaciens* may attach to and form a biofilm on soil particles or at interfaces on the plant host. Participation as a member of a multicellular community, therefore, is a normal and regulated aspect of *A. tumefaciens* biology with important consequences for its ecology.

A NOVEL FORM OF CELL DIVISION AMONG DIVERSE ALPHAPROTEOBACTERIA

Many members of the Rhizobiaceae, including *A. tumefaciens*, are morphological rods and it was presumed that cell division proceeded in much the same way as in the well-studied *E. coli*, *B. subtilis*, and the more closely related Alphaproteobacterium *C. crescentus*. In these model systems division occurs via binary fission. In these systems, individual cells elongate longitudinally by the insertion of new cell wall peptidoglycan and membrane material throughout the length of the cell, followed by septation and cytokinesis. The processes of elongation and septation in these bacteria are directed by conserved protein complexes including the MreB-containing elongase and FtsZ-containing divisome (Margolin, 2009). Other bacteria, such as the Actinobacteria, are known to elongate at the cell poles. In these bacteria pole-directed growth is dependent upon the conserved protein DivIVA and its homologs. Cell growth and division in *A. tumefaciens* and several other Rhizobiales contrasts with both of these known mechanisms for rod-shaped growth. These bacteria lack elongase component homologs as well as DivIVA, but retain one or more copies of FtsZ plus additional divisome components. Time-lapse microscopy coupled with fluorescent protein tracking and selective labeling of outer membrane components detailed a novel budding growth pattern common among *A. tumefaciens*, *S. meliloti*, *Brucella abortus*, *Ochrobactrum anthropi*, and *Hyphomicrobium denitrificans* (Fujiwara and Fukui, 1974; Latch and Margolin, 1997; Brown et al., 2012; Zupan et al., 2013). Budding occurs by insertion of new cell wall and membrane material at a single pole only, followed by septation and cytokinesis (**Figure 4**). Cell division results in two morphologically similar but distinct cell types. One cell, the mother cell, retains old cell wall material while the newly budded daughter cell contains *de novo* synthesized material. Importantly, polar growth was observed in bacteria attached to plant roots with the mother cell attached to the root surface by the UPP and the daughter cell budding into the medium (Brown et al., 2012).

COORDINATION OF DIVISION AND DEVELOPMENT

Although at first glance it may not be readily apparent, the *A. tumefaciens* life cycle resembles that of the more overtly asymmetric *C. crescentus* (**Figure 4**). *C. crescentus* exhibits a complex, biphasic life cycle that results in the generation of two non-identical cell types: a sessile, non-motile mother cell that often remains attached to a surface, and a motile daughter cell called the swarmer cell (Brown et al., 2009; Curtis and Brun, 2010). The regulatory components underlying this growth, division, and differentiation are well conserved among the Alphaproteobacteria (Brilli et al., 2010). The core architecture of this coordination of division and development (CDD) pathway includes two multicomponent His-Asp phosphorelays converging on multiple response regulators affecting diverse physiological outputs, including c-di-GMP production, motility, biofilm formation, and DNA replication (**Figure 4**). In *C. crescentus*, the master regulator of cell cycle progression is the response regulator CtrA. CtrA directly binds DNA and both blocks replication initiation and affects transcription of multiple target genes. CtrA activity is modulated by phosphorylation and proteolysis via the CckA/ChpT phosphorelay. Activity of the CckA hybrid histidine kinase is, in turn, modulated by the single-domain response regulator DivK. Phosphorylation and dephosphorylation of DivK is mediated by the PdhS family of histidine kinases. These kinases include PleC and DivJ in *C. crescentus* plus additional PleC/DivJ homolog sensor kinases in other bacteria (Hallez et al., 2004, 2007; Pini et al., 2013). *A. tumefaciens* encodes four PdhS proteins: PleC, DivJ, PdhS1, and PdhS2. While several CDD components are essential in *A. tumefaciens*, deletion of many of the non-essential components (PleC, PdhS1, PdhS2, and DivK) affected biofilm formation. Loss of PleC, PdhS1, or DivK disrupted biofilm formation. In contrast mutation of *pdhS2* increased attachment and biofilm formation. These data indicate that the ability to attach to a surface and form a biofilm is integrated into the overall cell cycle program of *A. tumefaciens* (Kim et al., 2013). One mechanism by which this may be achieved is through the response regulator PleD. As described above, PleD is one of several DGCs in *A. tumefaciens* responsible for biosynthesis of the second messenger c-di-GMP (see Cyclic-di-GMP). The activity of PleD is

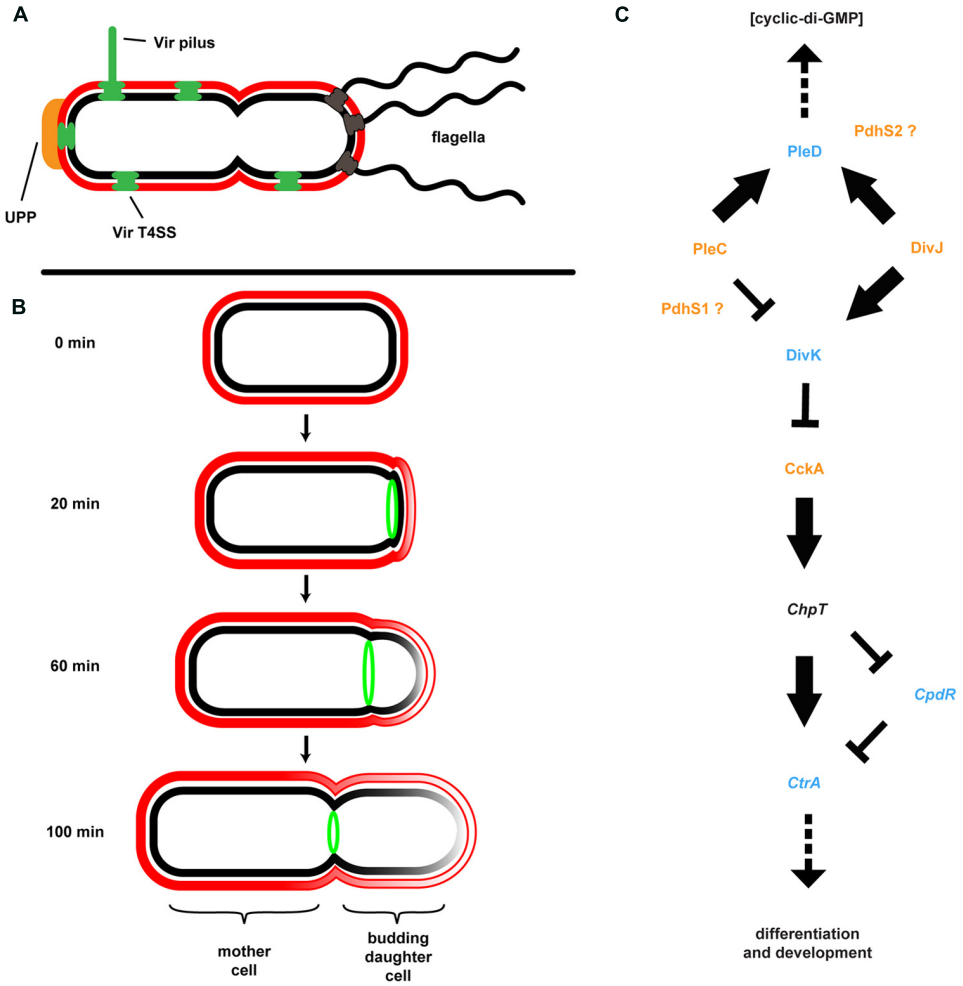

FIGURE 4 | *Agrobacterium tumefaciens* generates and maintains multiple developmental asymmetries. (A) Shown are morphological features of *A. tumefaciens* known to localize primarily to one pole of the bacterium, including multiple flagella, the unipolar polysaccharide (UPP), Vir pilus and type IV secretion system (T4SS). **(B)** Cell division in *A. tumefaciens* occurs by a polar budding mechanism. The cell division protein FtsZ (green) appears at the site of early constriction, from which the daughter cell buds and at which cytokinesis occurs. The rate of budding indicated is for growth in defined medium (ATGN) on agar pads. **(C)** The coordination of division and development (CDD) regulatory pathway. Proteins for which genetic and/or phenotypic data confirm the suggested pathway architecture are in bold typeface. Italicized proteins are present in the *A. tumefaciens* genome but do not have experimental support; placement of these proteins in the pathway is based on data from other model systems. Histidine kinases are colored orange, response regulators are colored blue, and a single Hpt phosphotransferase (ChpT) is in black text. The location of PdhS1 and PdhS2 is suggested by current data, but not confirmed. Note that the bacterial cell envelope in all panels is depicted as described in **Figure 3**.

regulated by phosphorylation. In *C. crescentus* and *S. meliloti*, and likely in *A. tumefaciens*, the histidine kinases interacting with PleD are the PdhS family members (Curtis and Brun, 2010; Pini et al., 2013; Sadowski et al., 2013). Deletion of PleD results in a moderate increase in biofilm formation and attachment, although there are other DGCs that appear to have more profound effects (Xu et al., 2013). A complete understanding of CDD regulation of these processes, the effectors, and the molecular mechanisms involved awaits full elucidation (Barnhart et al., 2013, 2014; Kim et al., 2013).

VIRULENCE

Though studying the motile-to-sessile transition is illuminating in and of itself for understanding bacterial development, it is critical to keep in mind the role that this transition may play as part of the pathogenic lifestyle of *A. tumefaciens*. Virulence of *A. tumefaciens* is mediated by the Ti plasmid, a part of which, called the T-DNA, is translocated into plant host cells and integrated into the host genome to cause tumor formation (Watson et al., 1975; Chilton et al., 1977; Leemans et al., 1981). A critical part of the *Agrobacterium*–plant interaction is attachment of the bacterial cell to a host plant cell, followed by translocation of the T-DNA via a type IV secretion apparatus that spans the bacterial cell wall and somehow provides access to plant cell cytoplasm (Lippincott and Lippincott, 1969; Beijersbergen et al., 1992, 1994). Although attachment to plant tissue frequently leads to biofilm formation, it is clear that in laboratory conditions, biofilm formation is not required for T-DNA transfer (Escudero and Hohn, 1997; Ramey et al., 2004a;

Brencic et al., 2005). However, T-DNA transfer is notably inefficient, and attached *A. tumefaciens* cells may be subject to the plant defense response (Veena et al., 2003; Zipfel and Felix, 2005; Zipfel et al., 2006). In natural infections, the large, concentrated population of *A. tumefaciens* cells within a biofilm that forms at a potential infection site may help to overcome these barriers and promote the overall likelihood of a successful T-DNA transfer. Though dense bacterial populations may not be required for virulence *per se*, they are required for pTi maintenance and conjugal dissemination within populations of *A. tumefaciens* associated with infected plants (Fuqua and Winans, 1994).

Biofilms in plant tumors would provide an optimal environment for pTi conjugation, assuring maintenance of the plasmid – and the capacity for infection – among populations of *A. tumefaciens*. There exists an additional relationship between biofilm formation and virulence. In low-phosphate environments, such as in the rhizosphere, both biofilm formation and virulence gene expression are enhanced in *A. tumefaciens* (Winans, 1990; Danhorn et al., 2004). As described above, the phosphate-sensing two-component system PhoR/PhoB mediates an enhanced adherence phenotype, while the pTi-encoded two-component system VirA/VirG mediates the virulence response (Winans, 1990; Danhorn et al., 2004). These regulatory systems potentially work in parallel to allow *A. tumefaciens* cells to attach to plant cells and express virulence genes in a timely manner.

DISPERSAL

The final "step" in the life of a biofilm is dispersal of members of the microbial community away from the site of attachment and into the environment (**Figure 1**). The ability to inhibit biofilm formation, dissociate the biofilm matrix, or induce active dispersal of the biofilm community is economically, ecologically, and medically relevant. There are multiple known activators of biofilm dispersal in diverse bacteria, including quorum sensing, production of small molecules such as nitric oxide, and secretion of matrix-degrading exoenzymes such as the glycoside hydrolase dispersin or nucleases (McDougald et al., 2012). The D enantiomers of amino acids have also been implicated in biofilm dispersal, although this may be due to indirect effects on protein synthesis (Cava et al., 2011; Leiman et al., 2013). Departure of motile daughter cells away from the attached mother cell upon septation may also serve as a coordinated aspect of biofilm development.

Although dispersal of individual cells from a mature biofilm is proposed to occur at some point in the lifetime of most, if not all, of these multicellular communities, there are few experimental details for this activity in the Rhizobiaceae, including *A. tumefaciens*. Dispersal of *R. leguminosarum* biofilms on abiotic surfaces has been observed but the regulation and mechanism of dispersal, and relevance to surface association with the plant host, have not been defined (Russo et al., 2006). In *A. tumefaciens* the addition of cell-free *P. aeruginosa* culture supernatant stimulated dispersal, although the identity of the active compound secreted by *P. aeruginosa* was not identified (An et al., 2006; Hibbing and Fuqua, 2012). These data suggest that regulated dispersal may be a component of the normal developmental program in *A. tumefaciens*.

CONCLUSIONS, FUTURE DIRECTIONS, OUTSTANDING QUESTIONS

It is clear that *A. tumefaciens* actively associates with a variety of surfaces in the environment, including but not restricted to those associated with plant hosts. As a metabolically plastic heterotrophic bacterial species, *A. tumefaciens* and its avirulent, plasmidless relatives can occupy a wide variety of environmental niches, and the ability to productively attach to surfaces and form multicellular biofilms is an important and well developed process under complex regulatory control. The asymmetric polar division process exhibited by *A. tumefaciens* is well suited for cells attached via their poles to surfaces in which the mother cell remains sessile and the newly budded daughter cell is released into the environment. Parallels with the well-studied biphasic life cycle of *C. crescentus* are instructive and have led to numerous insights into *A. tumefaciens* cell biology. The molecular targeting mechanisms that lead to polar localization and attachment, along with their coordination, are areas under active study. The orchestration of cell division with the assignment of specific functions to the old pole of the cell or the newer pole created with each round of cell division is a natural extension of such studies. How cytoplasmic c-di-GMP pools are modulated during the transition of motile cells to a sessile state, and the mechanisms by which this is linked to surface contact remain to be discovered. These processes are relevant to *A. tumefaciens* whether or not it is associated with host plants. In the context of plants, *A. tumefaciens* has evolved remarkable mechanisms for colonizing and manipulating its host, most notably culminating in interkingdom gene transfer, neoplastic growth and opine production. It remains unknown how the attachment and biofilm formation mechanisms that are the primary focus of this review are integrated with the events leading to T-DNA transfer. Mutants that are severely hampered in attachment remain virulent as measured using *in vitro* plant inoculation assays. It is unclear whether this is a limitation of these assays, or whether the events and processes leading to T-DNA transfer are truly distinct from those which mediate general surface attachment and subsequent biofilm formation. One plausible explanation is that in the natural environment, there is a temporal progression from general surface attachment, to the induction of *vir* genes and elaboration of the type IV secretion system, plus whatever additional intimate interactions with the plant cells are driven by these functions (including the potential shift to lateral association), and eventual T-DNA transfer. What is required to evaluate this hypothesis is the ability to follow the process from tissue colonization through T-DNA transfer in real time. As yet the tools and approaches for such dynamic monitoring have not been applied to this process, but such a high resolution view of *A. tumefaciens* interactions with plant hosts is a goal for future research.

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Attachment of *Agrobacterium* to plant surfaces

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Agrobacterium tumefaciens binds to the surfaces of inanimate objects, plants, and fungi. These bacteria are excellent colonizers of root surfaces. In addition, they also bind to soil particles and to the surface of artificial or man-made substances, such as polyesters and plastics. The mechanisms of attachment to these different surfaces have not been completely elucidated. At least two types of binding have been described unipolarpolysaccharide-dependent polar attachment and unipolar polysaccharide-independent attachment (both polar and lateral). The genes encoding the enzymes for the production of the former are located on the circular chromosome, while the genes involved in the latter have not been identified. The expression of both of these types of attachment is regulated in response to environmental signals. However, the signals to which they respond differ so that the two types of attachment are not necessarily expressed coordinately.

Keywords: *Agrobacterium*, attachment, adhesion, exopolysaccharides, bacterial binding

INTRODUCTION

Most terrestrial bacteria are found living on surfaces. *Agrobacterium tumefaciens* lives in the upper layers of the soil and in the rhizosphere. These bacteria can bind to a variety of inanimate surfaces including quartz sand, glass, plastic, polyester, and cellulose (Tomlinson and Fuqua, 2009). Considering the range of substrates to which the bacteria are able to bind, the bacteria presumably can also bind to particles in the soil. In addition, *A. tumefaciens* binds to the surface of plants, particularly to roots and root hairs, and to the surface of fungi (Matthysse et al., 1978; Bundock et al., 1995; Matthysse, 1996; Piers et al., 1996). Roots release a number of organic compounds into the soil including dicarboxylic acids, amino acids, and sugars (Lugtenberg et al., 1999). Thus, the colonization of the root surface may be advantageous for *A. tumefaciens*. That binding to roots promotes bacterial growth is illustrated by the interaction of two isogenic strains of *E. coli* differing only in adhesin genes which can and cannot bind to alfalfa sprouts (Jeter and Matthysse, 2005). When the strains are inoculated individually with the sprouts only the strain which can bind grows. In addition, when the strains are inoculated together, once again only the strain which can bind grows. Thus, the binding of one strain did not promote the binding or growth of the other strain. The experiment suggests that binding to the root would confer a considerable advantage over simple presence in the rhizosphere. Binding to the root also results in the formation of a biofilm (Ramey et al., 2004). Many studies have shown that bacteria in biofilms, such as those on the root epidermis, are protected from toxic compounds including antibiotics and from predation by protists (Ramirez and Alexander, 1980; Stewart and Costerton, 2001; Danhorn and Fuqua, 2007).

EARLY STUDIES OF THE ATTACHMENT OF *A. tumefaciens* TO PLANT CELLS

The importance of bacterial attachment to the plant surface was first recognized by Lippincott and Lippincott (1969). They showed that prior exposure of the plant wound site to avirulent *A. tumefaciens* resulted in inhibition of tumor formation by virulent bacteria and that the mathematics of the inhibition fit a one-particle dose–response curve suggesting that the avirulent bacteria were occupying sites and making them unavailable to the virulent bacteria. Additional studies of attachment of *A. tumefaciens* to plant cells and wound sites were carried out in the next 20 years. The techniques generally used in these early studies of attachment rely on indirect measurements of bacterial adhesion: competition between various bacterial strains as seen in the experiment described above, removal of bacteria from sites by washing (Lippincott and Lippincott, 1967), and inhibition of tumor formation by treatment of the wound site or the bacteria with surface extracts of the bacteria or plant cells prior to inoculation of the bacteria into the wound site (Whatley et al., 1976; Lippincott et al., 1977; Neff et al., 1987; Wagner and Matthysse, 1992). The first method requires that there be a limited number of discrete attachment sites where bacterial binding can initiate tumors so that the avirulent strain can occupy these sites and block binding of virulent bacteria. It has the advantage that only binding to sites which result in tumor formation is measured. The second method only produces results if the bacteria are bound reversibly. The third method depends on the extracts being tested having no other effects on the plant or bacterium in addition to their effects on the binding site. These experiments were carried out when there was little information on plant defense responses to bacteria and many of them are difficult to interpret due to possible stimulation of plant defense responses by the extracts which could then inhibit tumor formation without having any significant effect on bacterial binding. Extracts which were shown to inhibit tumor formation include pectin (Lippincott et al., 1977; Neff et al., 1987), bacterial lipopolysaccharides (LPS) (Whatley et al., 1976), and plant cell wall proteins (Gurlitz et al., 1987; Wagner and Matthysse, 1992). Reviews of experiments prior to 1986 concerning attachment of *A. tumefaciens* to plant cells have been published by Lippincott and Lippincott (1975) and Matthysse (1986).

Direct observations of bacterial binding to plant cells have been made using plant tissue culture cells and seedling roots of a variety of plants including *Arabidopsis thaliana*, tomato, tobacco, and carrot. Microscopic studies have the advantage that the site and orientation of bacterial attachment can be observed. Their major disadvantage is that large numbers of bacteria are usually required. Bacterial attachment can also be measured using radioactive bacteria or by washing the tissue and determining the number of bacteria bound (retained) using viable cell counts. Washing the tissue has the advantage that reversible and irreversible binding can be distinguished (Neff and Binns, 1985). These methods allow detection of small numbers of bacteria but they may remove (and thus fail to detect) bacteria which are loosely bound to the plant tissue.

POLAR ATTACHMENT MEDIATED BY THE UNIPOLAR POLYSACCHARIDE (UPP)

Visually, the most prominent type of attachment of *A. tumefaciens* to surfaces under a variety of conditions is polar binding of the bacteria (for example, see **Figure 1**). On root hairs or polyester threads, polar attachment of bacteria gives the appearance of a bottlebrush. This binding occurs early in the interaction of the bacteria with both biological (plant and fungal) and non-biological surfaces (Li et al., 2012). Polar attachment of *A. tumefaciens* is mediated by the unipolar polysaccharide (UPP; Tomlinson and Fuqua, 2009). This extracellular polysaccharide was first described in *Rhizobium leguminosarum* where it mediates polar attachment to root hairs (Laus et al., 2006). The *R. leguminosarum* UPP has been shown to be composed largely of mannose and glucose (Laus et al., 2006; Williams et al., 2008). Lectins from the plants nodulated by this bacterium, pea and vetch, bind the polysaccharide. *R. leguminosarum* mutants which are unable to make the UPP are deficient in binding to root hairs under acidic conditions (pH 5.6) but not under more alkaline conditions (pH 7.2) in the presence of calcium ions (Laus et al., 2006; Downie, 2010). *A. tumefaciens* makes a similar polysaccharide localized to one pole of the cell (Tomlinson and Fuqua, 2009). The genes required for its synthesis are located in two adjacent operons (*Atu1235–Atu1239*) in *A. tumefaciens* strain C58. Deletion of these genes results in mutant bacteria which fail to show prominent polar binding to inanimate surfaces, fungi, and plants (**Figure 1**). The formation of the UPP is required for biofilm formation on a wide variety of surfaces (Danhorn and Fuqua, 2007).

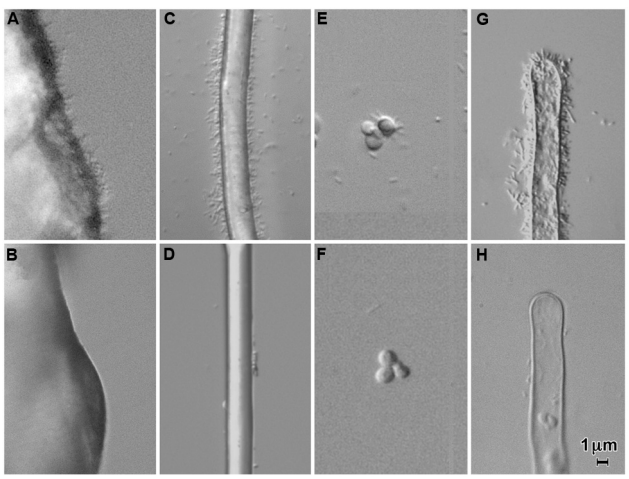

FIGURE 1 | Attachment of *Agrobacterium tumefaciens* strain C58 (A,C,E,G) and a UPP-deletion mutant of C58 (B,D,F,H) to quartz sand (A,B), polyester thread (C,D), yeast (*Saccharomyces cerevisiae*; C,F), and tomato root hairs (G,H) in a 1/10 dilution of MS medium containing a 1/20 dilution of AB minimal medium. Note the copious attachment of wild-type cells and the large decrease in attachment in a UPP deletion mutant. Approximately 10^6 bacteria per ml were incubated with the substrate for 24 h.

The UPP reacts with wheat germ agglutinin (WGA), a lectin which binds to *N*-acetyl-glucosamine (Tomlinson and Fuqua, 2009; Xu et al., 2013). Fluorescent WGA has been used to visualize the presence of the UPP in bacteria growing under various circumstances. Studies using fluorescent WGA have shown that the UPP is rarely made by planktonic bacteria (Li et al., 2012). Shortly after the bacteria come into contact with a surface, UPP is visible at the attached pole (Tomlinson and Fuqua, 2009; Barnhart et al., 2013; Xu et al., 2013). How the bacteria detect the presence of a surface and how this triggers the elaboration of the UPP is not known.

Attachment of bacteria to surfaces mediated by the UPP appears to be irreversible. Bound bacteria are retained after washing of the substrate to which the bacteria are bound (Tomlinson and Fuqua, 2009; Barnhart et al., 2013). In particular, the washing required for the detection of the UPP by fluorescent WGA does not appear to remove the bacteria.

Several genes and environmental conditions involved in the regulation of the production of UPP have been identified. These include concentrations of phosphate (Xu et al., 2012) and calcium (Matthysse, manuscript in preparation) in the environment and regulation via the intracellular, signal molecule cyclic-di-guanylic acid (c-di-GMP) in response to unidentified signals (Xu et al., 2013). The increased binding and biofilm formation seen with phosphorus limitation is dependent on the presence of functional UPP genes in the bacteria. Overexpression of the regulator involved in the uptake of phosphorous, *phoB*, increases the amount of UPP present and thus bacterial surface binding (Xu et al., 2012). Increased calcium ion concentrations (3 mM or greater) cause a reduction in UPP and a consequent decrease in polar bacteria binding (Matthysse, manuscript in preparation). The mechanism of this effect is unknown. The *exoR* gene involved in the regulation of succinoglycan synthesis and flagellar gene expression is also involved in the regulation of biofilm formation (Tomlinson et al., 2010). A deletion of *exoR* results in decreased biofilm formation on roots but individually bound bacteria are still seen. ExoR mutants retain virulence. c-di-GMP also plays a role in the regulation of the production of the UPP (Xu et al., 2013). Constitutive expression of *pleD*, a diguanylate cyclase also called *celR*, results in the synthesis of UPP not just at the pole of the cell but distributed all over the bacterial surface. Deletions of a gene *visR* required for motility result in increased biofilm formation and increased the production of the UPP. VisR was shown to inhibit the expression of the diguanylate cyclase genes *dcgA* and

dcgB and thus a deletion of *visR* should increase their activity. Mutations in a guanylate phosphodiesterase (*Atu3495*) resulted in higher levels of c-di-GMP and of the UPP (Xu et al., 2013). When VisR is expressed, the cells are motile and the synthesis of UPP is inhibited due to the lack of synthesis of c-di-GMP by DcgA and DcgB. Thus the regulation of the elaboration of the UPP is complex and is integrated with pathways in the bacterium controlling motility (*visR* and *exoR*), regulation of other exopolysaccharides (*exoR* and *pleD* aka *celR*), and phosphate uptake (*phoB*).

Binding to surfaces involving the UPP does not require the presence of the Ti plasmid and strains lacking pTi show binding indistinguishable from that of virulent strains (Tomlinson and Fuqua, 2009). None of the regulatory pathways involved in the control of UPP synthesis are known to be influenced by genes located on pTi. A UPP deletion mutant retains virulence on all plants tested including *Kalanchoe daigremontiana,* potato, and tomato (Tomlinson and Fuqua, 2009). Thus it seems likely that there is a second mechanism of attachment of the bacteria to the plant surface which is involved in the transfer of the T DNA.

UPP-INDEPENDENT ATTACHMENT

Although the UPP mediates the visually and numerically prominent polar binding of *A. tumefaciens* to surfaces, it is not required for virulence (Tomlinson and Fuqua, 2009). In a UPP deletion mutant or under conditions in which the UPP is not made, bacterial binding to the surface of plants can still be observed (**Figure 2**). This binding involves very few bacteria compared to that mediated by the UPP. It may require the presence of the Ti plasmid. Attachment of *A. tumefaciens* strain C58 to carrot suspension cells incubated in Murashige and Skoog medium (MS) was observed to be dependent on the presence of the Ti plasmid (Matthysse et al., 1978) as was bacterial attachment to protoplasts in a medium containing 60 mM $CaCl_2$, 7 mM sodium acetate, and 247 mM mannitol pH 5.8 (Aguilar et al., 2011). The number of bacteria observed to be attached was low in both of these experiments.

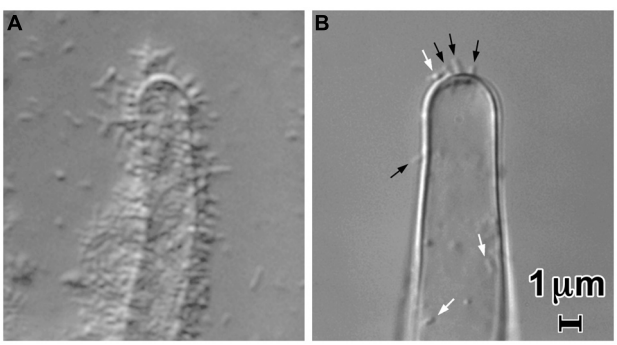

FIGURE 2 | Attachment of *A. tumefaciens* strain C58 to tomato root hairs in a 1/10 dilution of MS medium containing a 1/20 dilution of AB minimal medium (A) and in 60 mM $CaCl_2$, 7 mM sodium acetate, and 247 mM mannitol pH 5.8 (B). Note the large decrease in attachment in the presence of the $CaCl_2$-containing medium Laterally attached bacteria are visible at the white arrows in **(B)**; polarly attached bacteria are indicated by the black arrows. Approximately 10^6 bacteria per ml were incubated with cut tomato roots for 24 h.

In MS medium, bacterial binding to tissue culture cells and root hairs was both polar and lateral. In 60 mM $CaCl_2$, 7 mM sodium acetate, and 247 mM mannitol binding to protoplasts was exclusively lateral. No UPP could be detected on bound or planktonic bacteria in either medium suggesting that it was not made under these conditions (Matthysse, manuscript in preparation). The factor determining whether UPP was produced appeared to be the calcium ion concentration. MS medium contains 3 mM $CaCl_2$ at a pH of 5.6. Addition of calcium to media in which UPP is ordinarily synthesized resulted in reduced or undetectable UPP production by the bacteria and reduced bacterial binding (**Figure 2**).

In the absence of the production of UPP or cellulose bacterial binding appears to be reversible and the bacteria can be removed from the plant surface by water washing (Lippincott and Lippincott, 1967; Sykes and Matthysse, 1986). Cellulose production and irreversible bacterial binding appear to occur about 2–4 h after the inoculation of the bacteria into wound sites or plant cell suspension cultures (Lippincott and Lippincott, 1967; Matthysse, 1983; Neff and Binns, 1985). These experiments were all carried out in media which contained more than 3 mM calcium and thus there was probably little UPP produced by the bacteria.

In bacteria incubated with plant protoplasts in 60 mM $CaCl_2$, 7 mM sodium acetate, and 247 mM mannitol pH 5.8 bacterial binding to the plant cells was observed to be lateral. Under these conditions, the T pilus was also localized laterally in the bacteria (Aguilar et al., 2011). However, when bacteria were grown under inducing conditions with low calcium ions, the T pilus was reported to be exclusively localized at the end of the bacteria (polar localization; Lai et al., 2000). Polar localization of the VirB proteins (except VirB2) which assemble the T-pilus in cells incubated under inducing conditions in low calcium concentrations was shown by Judd et al. (2005). The observations showing polar and lateral localization of the Ti pilus differ in the medium used which may affect the position of the pilus. Low calcium would favor the elaboration of the UPP which could conceivably help to direct the T pilus to the cell pole. Lateral vs polar attachment of the bacteria may also be affected by the plant surface to which the bacteria are attached. The experiment showing lateral orientation of the bacteria involved bacterial attachment to tobacco protoplasts. The receptors to which the bacteria bind are likely to differ in nature and/or orientation between intact plant cells and protoplasts. Thus, the lateral bacterial attachment observed by Aguilar et al. (2011) could be a result of using tobacco protoplasts. However, bacteria bound to glutaraldehyde-fixed carrot protoplasts were observed in both lateral and polar orientations (Matthysse et al., 1982).

The role of pTi in bacterial attachment is unclear. Genes on pTi which may be involved in binding have not been identified. It is possible that binding is mediated by the T pilus itself, in which case VirB2 which makes up the shaft of the pilus or VirB5 which is found at the tip of the pilus are the obvious candidates for the proteins involved (Aly and Baron, 2007; Christie et al., 2014). Some mutations in *virB5* which alter or delete the carboxy-terminal amino acids of the protein result in bacteria which can transfer pTi to other bacteria but when inoculated onto plants (*K. daigremontiana*) the bacteria were avirulent (Aly and Baron, 2007). It is not known which steps in DNA transfer are blocked in

these mutants. Exogenous VirB5 enhanced DNA transfer from the bacteria to the plant as measured by a transient gene expression assay. The exogenous VirB5 had no effect on bacterial binding to roots under conditions where the majority of the binding is mediated by UPP (Lacroix and Citovsky, 2011). Whether VirB5 affects binding under conditions where UPP-mediated attachment is not seen is unknown. Binding of VirB2 to host proteins found on the surface of *Arabidopsis thaliana* roots has been described suggesting that VirB2 may play a role in bacterial attachment to host plants (Hwang and Gelvin, 2004). Thus VirB2 and VirB5 pilus proteins may play a role in bacterial attachment. Other genes which play a role in bacterial binding may also be located on pTi; these could potentially include both genes for adhesins or regulatory genes which control the expression of adhesin genes located elsewhere in the genome.

CELLULOSE-MEDIATED ATTACHMENT

Agrobacterium tumefaciens like many other bacteria is capable of making cellulose (Matthysse et al., 1981). The genes required are located in two adjacent operons on the linear chromosome (Matthysse et al., 1995). The cellulose synthase CelA of agrobacteria shares a high degree of homology with the cellulose synthases of other proteobacteria including rhizobia, *Gluconacetobacter xylinus*, and *Escherichia coli*. *A. tumefaciens* and the rhizobia which produce cellulose synthesize the exopolysaccharide in microfibrils emerging from many points scattered over the cell surface. In contrast, in bacteria such as *G. xylinus* and *P. fluorescens* cellulose fibrils emerge from a linear array of sites on one side of the cell and the cellulose produced forms a sheet (Brown et al., 1976; Cannon and Anderson, 1991; Spiers et al., 2003). This difference in the geometry of cellulose production influences the type of aggregates the bacteria form in solution and on surfaces and correlates with sequence differences in the *celB* gene. Cellulose fibrils bind tightly to other cellulose fibrils and thus cellulose synthesis results in the formation of bacterial aggregates which may be free in solution or bound to the cellulose on the plant surface. Bacteria in aggregates of *A. tumefaciens* produced by cellulose tend to be tangled in the cellulose in random orientations. Cellulose-producing *A. tumefaciens* will also bind to non-living materials containing cellulose such as Whatman filter paper (Matthysse, 1983). The production of cellulose by attached bacteria results in the formation of large clumps of attached bacteria on filter paper as well as on plant surfaces.

Cellulose synthesis is known to be regulated by a number of genes. Mutations in *celG* (*Atu8186*, the last gene in the operon containing *celABCG*) result in overproduction of cellulose (Matthysse et al., 2005). An RNA or protein product of the gene must be involved as the cellulose overproduction in a *celG* mutant can be reduced to wild-type levels by the provision of the gene on a plasmid. Mutations in *celI* (*Atu3105*) which has homology to transcriptional regulators also cause overproduction of cellulose (Matthysse et al., 2005). No additional information is available about the function of this gene.

In many bacteria including *A. tumefaciens* cellulose synthase (the product of the *celA* gene) can be directly regulated by c-di-GMP which binds to a *pilZ* site in the carboxy-terminal end of the protein (Amikam and Benziman, 1989; Ross et al., 1991). The active site where UDP-glucose is bound is located in the amino-terminal end. Regulation by c-di-GMP acts directly on the enzymatic activity of the protein and can be observed in cell-free extracts of the bacteria by measuring rate of incorporation of UDP-glucose into cellulose. Overexpression of either of two genes encoding a diguanylate cyclase, *Atu1297* or *Atu1060*, causes increased cellulose synthesis. A deletion of *Atu1297* (also known as *celR* or *pleD*) reduces the synthesis of cellulose and as well as (an)other undefined exopolysaccharide(s). This deletion also increased polar attachment of *A. tumefaciens* to the plant surface and biofilm formation on glass due to an increase in the amount of UPP present (Barnhart et al., 2013, 2014). Thus, regulation by c-di-GMP serves to integrate the synthesis of cellulose and UPP. However, *Atu1297* and *Atu1060* have other effects on virulence in addition to their effects on cellulose and UPP synthesis. To examine the effects of these genes on processes other than cellulose synthesis, the effects of overexpressing either *Atu1297* or *Atu1060* were examined in a cellulose synthase (*celA*) deletion mutant. Overexpression of either gene resulted in reduced virulence (Barnhart et al., 2013). Deletion of cellulose synthase by itself has little effect on virulence but does render bacterial binding more fragile so that the bacteria can be removed by water washing (Matthysse, 1983). Overproduction of cellulose causes the formation of large aggregates of bacteria on surfaces but has little effect on virulence (Matthysse et al., 2005).

THE ROLE OF OTHER EXOPOLYSACCHARIDES: CYCLIC-β-1,2-D-GLUCAN, SUCCINOGLYCAN, LIPOPOLYSACCHARIDE, AND CURDLAN

Bacterial mutants (*chvA* and *chvB*) which fail to synthesize the periplasmic polysaccharide cyclic-β-1,2-D-glucan were the first mutants shown to be defective in binding to plant cells (Douglas et al., 1982). Inability to synthesize this polysaccharide has pleiotropic effects including increased sensitivity to osmotic stress, overproduction of succinoglycan, and reduced motility (Douglas et al., 1985; Puvanesarajah et al., 1985). The effects of *chvB* mutations are temperature sensitive. The ability to bind to plants, motility, and virulence are all restored in c*hvB* mutants when incubation of the bacteria with the plants is carried out at temperatures below 16°C (Bash and Matthysse, 2002). Addition of cyclic-β-1,2-D-glucan to the solution has no effect on the attachment of wild-type *A. tumefaciens* to plant cell surfaces (Puvanesarajah et al., 1985). It seems likely that the effect of *chvA* and *chvB* mutations is indirect, resulting from multiple defects caused by the absence of the glucan polysaccharide from the periplasmic space rather than from the absence of a molecule which plays a direct role in attachment.

Succinoglycan is the most abundant of the exopolysaccharides produced by *A. tumefaciens* growing on agar plates in the laboratory. However, its role in the life of the bacteria in nature remains obscure. Bacterial mutants unable to synthesize succinoglycan retain virulence and show no obvious defects in binding to plant surfaces (Tomlinson et al., 2010). Overproduction of succinoglycan is seen in *chvA, chvB*, and *exoR* mutants (Puvanesarajah et al., 1985; Tomlinson et al., 2010). All of these mutants show reduced binding to roots and reduced motility. However, unlike *chvA* and *chvB* mutants, *exoR* mutants retain virulence on potato

disks. An *exoAexoR* double mutant that cannot make succinoglycan recovered the ability to bind to roots, but did not recover wild-type motility suggesting that the overproduction of succinoglycan was responsible for the lack of binding of the *exoR* mutants (Tomlinson et al., 2010). The role, if any, played by excess succinoglycan in the phenotype of *chvA* and *chvB* mutants is unknown.

There is little information about a possible role for LPS in the attachment of *A. tumefaciens* to plant cells. The addition of purified LPS from *A. tumefaciens* strain C58 inhibited bacterial binding to carrot suspension cells in MS medium (Whatley et al., 1976; Matthysse, 1987b). However, the effects of added bacterial substances on binding may be due to their ability to elicit plant defense reactions rather than a direct effect on binding. A study using an inhibitor of LPS biosynthesis found no effect on the initial attachment although the drug did inhibit the formation of cellulose fibrils (Goldman et al., 1992).

Agrobacterium tumefaciens strain C58 has intact genes for the biosynthesis of curdlan; however, this strain has not been observed to make curdlan. Curdlan synthase (*crdS*) mutants retain virulence and are able to colonize roots (Matthysse, unpublished observation). Other strains of *Agrobacterium* such as LTU50 and ATCC1379 are used in industry to produce large amounts of curdlan (McIntosh et al., 2005). These strains lack pTi and thus are not virulent. LTU50 is able to colonize plant roots and has been observed to bind to root hairs (Aracic et al., unpublished observations). Curdlan production in LTU50 is negatively regulated by the presence of combined nitrogen (McIntosh et al., 2005). When bacterial growth is limited by the absence of available nitrogen and an abundant carbon source such as glucose is present, the bacteria produce large amounts of curdlan. Bacteria growing in 4% glucose can convert 95% of this glucose into curdlan (McIntosh et al., 2005). LTU50 incubated with tomato roots in MS medium rapidly run out of combined nitrogen and begin to make curdlan. The bacteria embedded in a curdlan matrix form a blanket-like structure covering the roots. This structure is fragile and easily removed by water washing (Matthysse, unpublished observation). Bacteria embedded in curdlan are protected from phagocytosis by protists such as *Dictyostelium discoideum* (Aracic et al., unpublished observations). Thus, curdlan production is likely to increase bacterial survival in soil.

PROTEIN ADHESINS

A 14-kDa calcium-binding protein named rhicadhesin has been reported to be involved in the binding of rhizobia and *A. tumefaciens* to root hairs (Smit et al., 1989a,b). Rhicadhesin is reported to be released from the surface of the bacterial cell when the cells are placed in medium with low concentrations of calcium. Addition of the purified protein inhibited the binding of rhizobia and *A. tumefaciens* to pea roots (Smit et al., 1992). The purified protein was also able to restore the binding of an *A. tumefaciens chvB* mutant to pea roots and virulence on *K. daigremontiana* (Swart et al., 1994). The gene encoding this protein has not been identified. However, the protein is made by rhizobia lacking the sym plasmid and by *A. tumefaciens* lacking pTi suggesting that the relevant gene(s) are chromosomal (Smit et al., 1987). There are many possible reasons why the rhicadhesin gene has not been identified. Among the likeliest is the existence of multiple copies of the gene so that a mutation in one copy has no evident phenotype or the possibility that mutations in the gene are lethal. The role of rhicadhesin in attachment remains uncertain. It was defined by its ability to inhibit bacterial attachment. The major case in which it promotes attachment involves its addition to *chvB* mutants. However, as discussed above the phenotype of these mutants probably result from indirect effects of the lack of cyclic-β-1,2-D-glucan. Thus, the mechanism of the restoration of the wild-type phenotype may be indirect. The experimental data do support a role for rhicadhesin in the structure and stability of the bacterial surface. The definition of its role in attachment will have to await the identification of the gene(s) encoding this protein.

Other protein adhesins which play a role in the binding of *R. leguminosarum* to roots have been identified. These include the Rap proteins which are secreted bacterial proteins that bind to the surface of the bacteria. RapA1 is a calcium-binding protein with two binding sites which agglutinates the bacteria by binding at the pole. These genes for these proteins are restricted to only a few members of the *Rhizobiaceae* (Ausmees et al., 2001). The overexpression of RapA1 from the gene cloned into a plasmid resulted in increased bacterial binding to roots but had no effect on binding to abiotic surfaces (Mongiardini et al., 2008). The gene is not required for nodulation. The suggested role for this protein is in root colonization by the bacteria. RapA2 is also a calcium-binding protein. It interacts with the acidic exopolysaccharide of the bacteria and is apparently a calcium-dependent lectin (Abdian et al., 2013). No genes homologous to the *rap* genes have been identified in *A. tumefaciens*.

Several genes on the cryptic plasmid pAT (*att* genes) have been identified as being involved in attachment. Transposon insertions in these genes block attachment in calcium-containing medium in which the UPP is not made (Matthysse, 1987a; Matthysse et al., 2000). The mutations have no effect on attachment in medium in which the UPP mediates the majority of bacterial attachment. Their effect on the synthesis of the T pilus is unknown. The genes cannot be required for virulence as bacterial strains lacking pAT are virulent (Nair et al., 2003). The transposon insertions in some of the *att* genes (*attC* and *attG*) resulted in dominant-negative mutations (Matthysse et al., 2008) suggesting that they act by causing the synthesis of partial proteins (affected gene translated to the site of the insertion) or partial protein complexes (only some of the genes in an operon expressed) perturbing the bacterial surface so as to block the ability of the bacteria to bind to plants in medium containing moderate levels of calcium ions. Whatever the mechanism of action of the transposon insertion mutations in genes found on pAT, it appears certain that the effects of these mutations, similar to those of the *chvA* and *chvB* mutations, on bacterial attachment are indirect and that the genes do not encode molecules directly involved in bacterial attachment.

GENERAL CONCLUSIONS

It appears that *A. tumefaciens* has at least two mechanisms by which it can bind to plant surfaces (**Figure 3**). One, the UPP,

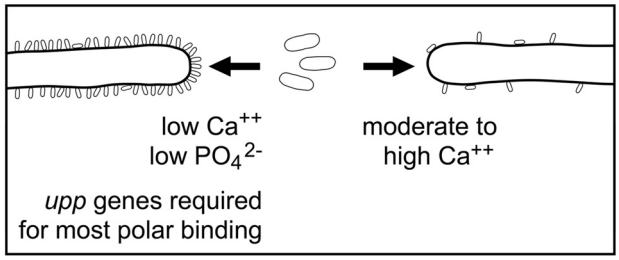

FIGURE 3 | A model of the initial binding of *A. tumefaciens* to root hairs. In the presence of low levels of calcium UPP is formed and mediates polar bacterial attachment to the root hair surface. This results in binding of large numbers of bacteria to the root hair. The genes for synthesizing the UPP are required for this binding. In the presence of moderate or high levels of calcium UPP is not formed and bacterial binding is sparse.

is quite non-specific and aids the bacteria in binding to a wide variety of both animate and inanimate surfaces. This binding is visually striking because it is a polar attachment and results in the binding of large numbers of bacteria to the surface. The UPP is produced optimally under conditions of low calcium, low phosphate, and acidic pH. UPP-mediated binding to surfaces is likely to play a prominent role both in attachment to soil particles and in colonization of plant surfaces. The genes for the production of this exopolysaccharide are located on the chromosome and appear to be widely distributed in the agrobacteria and rhizobia.

The second mechanism of attachment is mediated by unknown molecule(s). It can be detected when the interactions between the bacteria and surfaces are carried out in media containing moderate to high concentrations of calcium where the UPP is not produced or by the examination of the binding of UPP mutants. The numbers of bacteria bound are very small when compared with bacterial binding mediated by the UPP. This UPP-independent attachment may result in both polar and lateral attachment to plant surfaces. It is not known what conditions control the polar vs lateral orientation of the bacterium or whether bacteria bound in these two orientations use different mechanisms of attachment. No mutants unable to show UPP-independent attachment have been identified. Thus it is not known whether more than one type of UPP-independent attachment exists nor is there any information on the genes or adhesins involved in this binding. It seems clear that the major mechanism of attachment of *A. tumefaciens* to surfaces both biological and inanimate has been identified as the binding of the UPP but there clearly remains more to be discovered about the surface interactions of this bacterium with its plant hosts particularly those which result in T-DNA transfer.

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Membrane lipids in *Agrobacterium tumefaciens*: biosynthetic pathways and importance for pathogenesis

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Many cellular processes critically depend on the membrane composition. In this review, we focus on the biosynthesis and physiological roles of membrane lipids in the plant pathogen *Agrobacterium tumefaciens*. The major components of *A. tumefaciens* membranes are the phospholipids (PLs), phosphatidylethanolamine (PE), phosphatidylglycerol, phosphatidylcholine (PC) and cardiolipin, and ornithine lipids (OLs). Under phosphate-limited conditions, the membrane composition shifts to phosphate-free lipids like glycolipids, OLs and a betaine lipid. Remarkably, PC and OLs have opposing effects on virulence of *A. tumefaciens*. OL-lacking *A. tumefaciens* mutants form tumors on the host plant earlier than the wild type suggesting a reduced host defense response in the absence of OLs. In contrast, *A. tumefaciens* is compromised in tumor formation in the absence of PC. In general, PC is a rare component of bacterial membranes but amount to ~22% of all PLs in *A. tumefaciens*. PC biosynthesis occurs via two pathways. The phospholipid *N*-methyltransferase PmtA methylates PE via the intermediates monomethyl-PE and dimethyl-PE to PC. In the second pathway, the membrane-integral enzyme PC synthase (Pcs) condenses choline with CDP-diacylglycerol to PC. Apart from the virulence defect, PC-deficient *A. tumefaciens pmtA* and *pcs* double mutants show reduced motility, enhanced biofilm formation and increased sensitivity towards detergent and thermal stress. In summary, there is cumulative evidence that the membrane lipid composition of *A. tumefaciens* is critical for agrobacterial physiology and tumor formation.

Keywords: membrane lipids, phospholipid biosynthesis, phosphatidylcholine, phosphorus-free lipids, ornithine lipids, glycolipids, betaine lipids, *Agrobacterium tumefaciens*

INTRODUCTION

The structure of biological membranes is mainly defined by heterogeneous amphipathic phospholipids (PLs) forming the phospholipid bilayer. PLs contain a diacylglycerol (DAG) as hydrophobic component with saturated or unsaturated fatty acyl chains of variable length and a polar head group attached to the phosphate group (Korn, 1966; van Meer et al., 2008; Wolf and Quinn, 2008). The general structure of PLs and common head groups are shown in **Figure 1**. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are zwitterionic lipids whereas phosphatidic acid (PA), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylserine (PS), and phosphatidylinositol (PI) represent the anionic lipid class. Contrary to previous assumptions based on the fluid mosaic model (Singer and Nicolson, 1972), the lipid distribution in pro- and eukaryotic membranes is dynamic and asymmetric (Fadeel and Xue, 2009; Clifton et al., 2013). Specialized lipid micro domains (in eukaryotes referred to as lipid rafts) serve as platform for various cellular processes such as signal transduction and transport (Edidin, 2003; Zhang et al., 2005; Pike, 2006; Donovan and Bramkamp, 2009; Lingwood and Simons, 2010; LaRocca et al., 2013).

All biological membranes share the same basic membrane structure but the lipid composition differs tremendously between the domains of life and even within a domain. The lipid repertoire of eukaryotic cells is very complex. Combination of different head groups and variations in fatty acid tails results in more than a thousand different lipids. The major lipids in eukaryotes are PLs with PC as the most abundant, followed by PE, PS, PI, and PA (van Meer et al., 2008). PG is also present in eukaryotes and is used as precursor for CL synthesis, exclusively found in mitochondria (Bligny and Douce, 1980). Further important constituents of eukaryotic membranes are sphingolipids (SLs) and cholesterol, which are enriched in lipid rafts (Lingwood and Simons, 2010; Sonnino and Prinetti, 2013).

Bacterial membrane lipids are more diverse than previously thought (Parsons and Rock, 2013). Most bacteria, like the Gram-negative model organism *Escherichia coli* have a simple membrane lipid composition with the major PLs PE, PG, and CL (Ames, 1968; Cronan, 2003; López-Lara et al., 2003; Dowhan, 2009). However, many other bacteria are known to produce additional and uncommon lipids. PS is abundant in eukaryotic membranes but most prokaryotes contain only minor PS amounts as it serves as precursor for PE biosynthesis (Bunn and Elkan, 1971; López-Lara et al., 2003). Although PI is a rare component of bacterial membranes it is a major lipid in *Mycobacterium tuberculosis* where it is essential for viability (Jackson et al., 2000). SLs have been described in *Sphingobacterium, Sphingomonas,* and *Bacteroides* species (Heung et al., 2006). *Sphingomonas paucomobilis* contains two glyco-SLs in its outer membrane important for pathogenesis (Kinjo et al.,

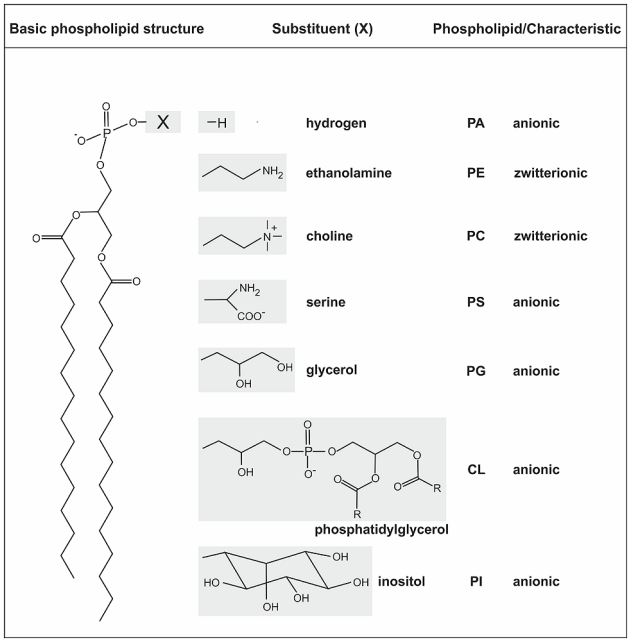


FIGURE 1 | General structure of phospholipids and common head groups. PLs contain two fatty acids ester-linked to glycerol at C-1 and C-2, and a polar head group attached at C-3 via a phosphodiester bond. The fatty acids in PLs can vary in carbon group length and saturation degree. The different common polar head groups and charges are indicated. PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin; PI, phosphatidylinositol.

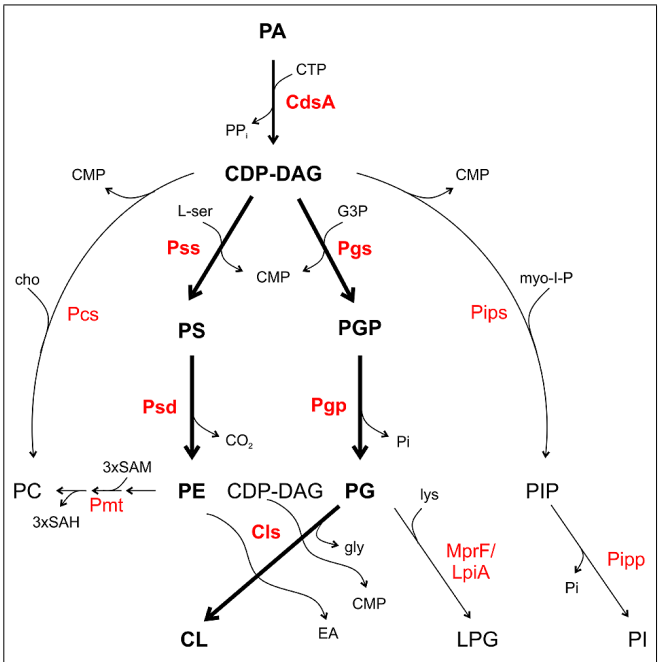


FIGURE 2 | Phospholipid pathways and enzymes in bacteria. CDP-DAG is the central precursor for synthesis of the PLs. Thick arrows and boldface letters indicate the most common pathways and enzymes in bacteria. For details see text. CMP, cytidine monophosphate; CTP, cytidine triphosphate; EA, ethanolamine; cho, choline; G3P, glycerol 3-phosphate; gly, glycerol; lys, lysine; L-ser, L-serine; myo-I-P, myo-inositol 1-phosphate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

2005; Mattner et al., 2005). Some bacteria such as *Methylococcus capsulatus* or *Rhodopseudomonas palustris* TIE-1 can also synthesize steroid lipids and/or sterol homologues (hopanoid lipids; Tippelt et al., 1998; Bode et al., 2003; Doughty et al., 2011). The membrane of the Gram-positive model organism *Bacillus subtilis* comprises lysyl-PG (LPG) and up to 40% neutral glycolipids (GLs; Salzberg and Helmann, 2008). In some bacteria such as *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, and *Rhodobacter spaeroides* phosphate limitation stimulates the production of phosphate-free lipids including ornithine lipids (OLs), sulfolipids, betaine lipids, and GLs (López-Lara et al., 2003, 2005; Vences-Guzmán et al., 2012; Geske et al., 2013; Parsons and Rock, 2013). The major eukaryotic membrane lipid PC is not widespread in bacteria. It has been estimated that ∼15% of all bacterial species produce PC (Sohlenkamp et al., 2003; Aktas et al., 2010; Geiger et al., 2013). It is frequently found in symbionts or pathogens and in bacteria with extensive intracytoplasmic membranes (Hagen et al., 1966; Goldfine, 1984; Geiger et al., 2013). Often, PC is critical for bacteria–host interactions.

COMMON METABOLIC PATHWAYS FOR PHOSPHOLIPIDS IN BACTERIA

All major PLs in bacteria are formed from a common precursor, namely cytidine diphosphate diacylglycerol (CDP-DAG) generated by a CDP-DAG synthase (CdsA) using PA and cytidine triphosphate (CTP; **Figure 2**; Zhang and Rock, 2008; Parsons and Rock, 2013). CDP-DAG can be directly converted to PS, PG phosphate (PGP) or in some bacteria to PI phosphate (PIP) and PC. These reactions are catalyzed by specific CDP-alcohol phosphatidyltransferases releasing a CMP molecule from CDP-DAG and transferring the phosphatidyl moiety to different polar head groups (Sohlenkamp et al., 2003; Parsons and Rock, 2013). PS synthases (Pss) use L-serine as the phosphatidyl acceptor to generate the anionic lipid PS, which serves as precursor for PE synthesis via PS decarboxylases (Psd). In *mycobacteria*, a PIP synthase (Pips) converts CDP-DAG and myo-inositol 1-phosphate to PIP which is dephosphorylated via a PIP phosphatase (Pipp) to PI (Morii et al., 2010; Morii et al., 2014). PG synthases (Pgs) transfer the phosphatidyl group from CDP-DAG to a glycerol-3-phosphate (G3P) resulting in PGP, which serves as precursor for PG synthesis by PGP phosphatases (Pgp). Two PG molecules are condensed via a cardiolipin synthase (Cls) to CL. Most bacteria possess more than one Cls. *E. coli* encodes three Cls with distinct specificities. ClsA uses two PG molecules for CL formation whereas ClsC condenses a PE and PG molecule to CL. Like the other Cls enzymes, ClsB utilizes PG but the second substrate is unknown (Pluschke et al., 1978; Nishijima et al., 1988; Guo and Tropp, 2000; Tan et al., 2012). In *Streptomyces coelicolor* a eukaryotic-type Cls using CDP-DAG and PG for CL synthesis was identified. This enzyme belongs to the CDP-alcohol phosphatidyltransferase family and seems to be common in actinobacteria (Sandoval-Calderón et al., 2009).

In several Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, PG is converted to the positively charged lipid

LPG by aminoacylation using lysyl-tRNA as the lysine donor by the Mprf (multiple peptide resistant factor) enzyme (Ernst and Peschel, 2011). In *Staphylococcus aureus*, LPG confers resistance towards cationic antimicrobial peptides (CAMPs) by perturbation of the electrostatic attraction of CAMPs (Kilelee et al., 2010; Andrä et al., 2011). MprF homologs namely LpiA (low pH inducible A) are also present in some Gram-negative bacteria such as *Rhizobium tropici* and *Sinorhizobium medicae* and confer tolerance to acid stress and selected cationic peptides (Reeve et al., 2006; Sohlenkamp et al., 2007).

Two common PC synthesis pathways operate in bacteria: the PE-methylation pathway and the PC synthase (Pcs) route. Several bacteria contain both PC synthesis pathways such as *A. tumefaciens* and *S. meliloti*. However, some species like *Rhodobacter sphaeroides* or *Zymomonas mobilis* only have the methylation pathway for PC synthesis. Some important pathogens including *Borrelia burgdorferi*, *Brucella abortus,* or *Pseudomonas aeruginosa* only possess the Pcs pathway (Martínez-Morales et al., 2003; Sohlenkamp et al., 2003; Aktas et al., 2010; Geiger et al., 2013). In the methylation pathway, one or several phospholipid *N*-methyltransferase (Pmt) enzymes transfer a methyl group from *S*-adenosylmethionine (SAM) to the amino group of PE generating the intermediates monomethyl-PE (MMPE) and dimethyl-PE (DMPE) and finally PC (**Figure 2**). The methyldonor SAM is converted to *S*-adenosylhomocysteine (SAH) during this reaction. In the bacteria-specific Pcs pathway, choline is condensed with CDP-DAG to PC releasing a CMP molecule (Sohlenkamp et al., 2000; Aktas et al., 2010; Solís-Oviedo et al., 2012; Geiger et al., 2013).

A eukaryotic-like CDP-choline pathway has been postulated in *Treponema denticola* (Kent et al., 2004) and might be also present in other *Treponema* species. This pathway involves a choline kinase (LicA) generating choline phosphate which serves as substrate for a CTP: phosphocholine cytidylyl transferase (LicC) to produce CDP-choline. In the final step, PC is formed by transferring the phosphocholine moiety to DAG by a CDP-choline transferase (CPT; Kent et al., 2004; Geiger et al., 2013).

Recently, a new PC biosynthesis route was discovered in *Xanthomonas campestris*, which produces PC via a yeast-like two-step acylation of the precursor glycerophosphocholine (Moser et al., 2014) demonstrating that quite different strategies acting on the head or tail group have evolved for PC synthesis in bacteria.

Following this general information, the remainder of this review will present an overview of biosynthetic pathways and enzymes for membrane lipids in the plant pathogen *A. tumefaciens* and discuss the physiological relevance of those lipids in this organism.

MEMBRANE LIPID REPERTOIRE AND PHOSPHOLIPID BIOSYNTHESIS ENZYMES IN *A. tumefaciens*

Agrobacterium membranes contain a rich setup of polar lipids (Randle et al., 1969; Das et al., 1979; Thompson et al., 1983; Vences-Guzmán et al., 2013; Moser et al., 2014). The lipid repertoire of several *Agrobacterium* strains has been quantified. Under full nutrition, *A. tumefaciens* membranes are mainly composed of the PLs PE and PG (account together ∼45%), PC (∼22%), CL (∼15%), MMPE (∼15%) and traces of DMPE (∼4%; Moser et al., 2014). Two-dimensional thin layer chromatography and mass spectrometry analysis revealed that *A. tumefaciens* membranes also contain two OLs (Geske et al., 2013; Vences-Guzmán et al., 2013). A broad variety of membrane lipids in this organism is reflected by a lysine-containing lipid with a backbone structure similar to OLs (Tahara et al., 1976). Most of the PL synthesis pathways and enzymes in *Agrobacterium,* except for PC synthesis, are still uncharacterised. However, with the exception of a *pgp* gene, homologs for all common PL biosynthesis genes described above are encoded in the *A. tumefaciens* genome (**Figure 3**; Wood et al., 2001).

The putative *A. tumefaciens pss* (*atu1062*) gene is homologous to the *pss* gene from the non-pathogenic, high beta-1,3-glucan (curdlan) producing *Agrobacterium* sp. ATCC31749 (Karnezis et al., 2002; Ruffing et al., 2011). Functional analysis of its recombinant Pss protein in *E. coli* demonstrated a Mn^{2+}-dependent [^{3}H]serine incorporation into a chloroform-soluble product, most likely PS. Localisation studies in *E. coli* and topology predictions suggest that Pss is an integral membrane protein of ∼30 kDa with eight transmembrane domains (TM). A cytosolic loop connecting the second and third TM contains a conserved motif ($DX_2DGX_2ARX_5S/TX_2GX_3DSX_2D$) characteristic for amino alcohol phosphatidyltransferases and thought to be involved in catalysis. A *pss* mutant is unable to produce PE suggesting that PE synthesis exclusively occurs via decarboxylation of PS. Loss of PE seems to be compensated by increased PG and CL levels in the *pss* mutant. Interestingly, the PE-deficient mutant is dramatically reduced in curdlan production and grows poorly in minimal medium. This growth defect can be compensated by Mg^{2+} ions, which presumably stabilize the membrane. However, curdlan production of the mutant strain cannot be cured by Mg^{2+}.

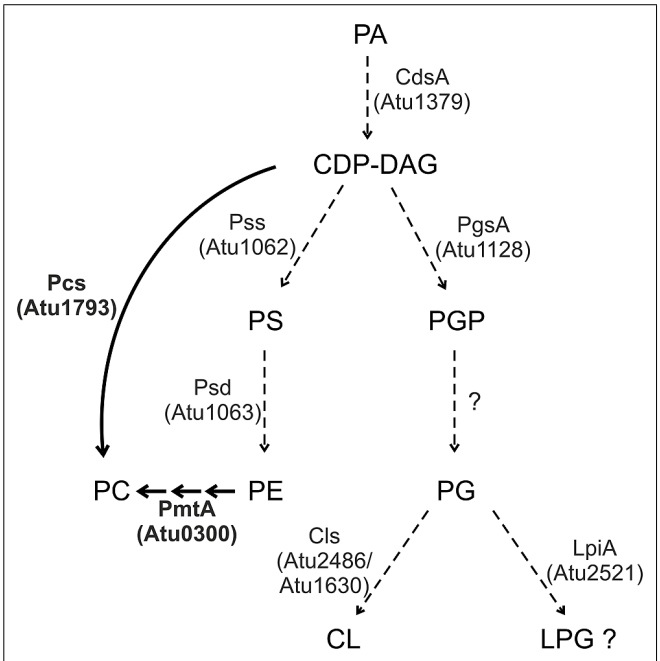


FIGURE 3 | Phospholipid synthesis in *A. tumefaciens*. Characterized pathways are indicated by thick arrows and enzymes in boldface letters. Dashed arrows indicate the putative pathways. For details see text.

PE seems to be required for proper assembly and function of the integral inner membrane protein curdlan synthase as shown for several other membrane proteins (Karnezis et al., 2002, 2003; Raja, 2011; Bogdanov and Dowhan, 2012).

A. tumefaciens codes for a putative LPG synthase (Atu2521, LpiA) but LPG has not yet been identified in this organism. LPG is a major lipid in some Gram-positive bacteria but only low levels are formed in Gram-negatives. Transcription of the related *lpiA* gene in *S. medicae* is activated at low pH and is required for survival during acid stress. However, LPG was not detected even under acidic conditions in this organism suggesting production of very small amounts or rapid turnover of LPG (Reeve et al., 2006). Small amounts of LpiA-produced LPG were detected in *R. tropici* CIAT899 (~1% of the total lipids) in low pH minimal medium. Here, LPG confers resistance against the cationic peptide polymyxin B under acidic growth conditions (Sohlenkamp et al., 2007). Interestingly, *lpiA*/*mprF* homologs are present in many bacteria interacting with eukaryotes such as symbionts, pathogens and commensals suggesting that LPG might be important for bacteria–host interactions (Vinuesa et al., 2003; Sohlenkamp et al., 2007). Since low pH is one of the signals inducing virulence factors in *A. tumefaciens*, it will be of great interest to determine whether *lpiA* contributes to *Agrobacterium* pathogenesis.

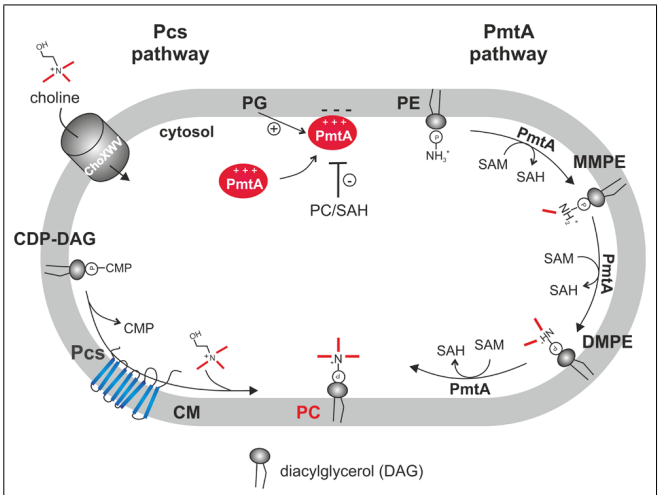


FIGURE 4 | Phosphatidylcholine biosynthesis in *A. tumefaciens*. In the PC synthase pathway, the integral membrane protein Pcs condenses CDP-DAG and choline to PC. Choline is taken up via the ChoXWV transporter. In the PmtA pathway, a single peripheral phospholipid *N*-methyltransferase (PmtA) converts PE via three successive methylations to PC. PmtA is stimulated by the anionic lipid PG and inhibited by PC and SAH. CM, cytoplasmic membrane.

THE METHYLATION PATHWAY IN *A. tumefaciens*

The two PC biosynthesis pathways and corresponding enzymes (Pcs and PmtA) in *A. tumefaciens* have been well characterized (**Figure 3**). Initial work on PC synthesis in *Agrobacterium* demonstrated incorporation of the ^{14}C-methyl moiety of SAM into MMPE, DMPE, and PC and ^{14}C-choline uptake and incorporation into PC (Kaneshiro and Law, 1964; Sherr and Law, 1965; Kaneshiro, 1968). In earlier studies, two distinct Pmts were postulated in *A. tumefaciens*. A soluble Pmt catalyzing MMPE formation only and a Pmt associated with the particulate cell fraction producing all methylated PE-derivatives (Kaneshiro and Law, 1964). The *A. tumefaciens* genome, however, contains only a single constitutively expressed *pmt* gene (*pmtA*, *atu0300*) on the circular chromosome (Wessel et al., 2006; Klüsener et al., 2009). The lack of MMPE, DMPE, and PC in a *pmtA* mutant grown without choline demonstrated that PmtA is the only enzyme responsible for MMPE, DMPE, and PC synthesis via the methylation pathway (Wessel et al., 2006). Purification of recombinant PmtA from the soluble cell fraction suggests that it is a peripheral membrane protein reversibly attaching to its site of action, the membrane (Aktas and Narberhaus, 2009; Aktas et al., 2011a). PmtA is a monomeric small enzyme (~22 kDa) catalyzing the methylation of PE to MMPE, DMPE, and PC. *In vitro* lipid binding experiments with PmtA revealed strong binding to the anionic lipids PI and PG. Interestingly, overall PmtA activity is stimulated by PG. Association of peripheral proteins with membranes is often mediated via electrostatic interactions with negatively charged PLs such as PG and a similar mechanism is proposed for the *A. tumefaciens* PmtA enzyme (**Figure 4**). SAM binding by PmtA occurs only in the presence of its substrates PE, MMPE, DMPE or the end product PC. PG alone does not influence SAM binding suggesting that two distinct binding sites for its substrates or products and for PG exist (Aktas and Narberhaus, 2009).

In vitro PmtA activity is negatively regulated by the end products SAH (via interfering with SAM binding) and by PC. End product-mediated inhibition might also be relevant *in vivo* to balance proper lipid composition (Aktas and Narberhaus, 2009). Like all Pmt enzymes, PmtA contains a highly conserved N-terminal SAM binding motif [VL(E/D)XGXGXG] (Sohlenkamp et al., 2003). Within this motif, the amino acids E58, G60, G62, and E84 were found to be essential for activity and SAM binding (Aktas et al., 2011a). *A. tumefaciens* PmtA seems to follow an ordered Bi–Bi reaction mechanism with initial substrate binding followed by a conformational change allowing SAM binding. Subsequently, the methyl group might be transferred to the lipid substrate releasing the first product SAH followed by the release of the methylated lipid product (Aktas et al., 2010).

Bacterial Pmts are classified into *Sinorhizobium* and *Rhodobacter* type enzymes. Enzymes belonging to the *Sinorhizobium* family including *A. tumefaciens* PmtA, are homologous to rRNA methylases, whereas *Rhodobacter*-like Pmt enzymes are similar to UbiE, ubiquinone/menaquinone biosynthesis methyltransferases. Similarities between these two Pmt families are restricted to the conserved SAM-binding motif (Sohlenkamp et al., 2003; Aktas et al., 2010; Geiger et al., 2013). The product spectrum of Pmt enzymes varies in different organisms. While *A. tumefaciens* and *S. meliloti pmtA* release small amounts of the intermediates MMPE and DMPE, expression of *R. sphaeroides pmtA* in *E. coli* exclusively resulted in PC formation (Arondel et al., 1993; de Rudder et al., 2000; Klüsener et al., 2009). The *Sinorhizobium* type PmtA from *X. campestris* produces MMPE exclusively and is unable to further methylate it to DMPE and PC (Moser et al., 2014).

Most bacteria contain one Pmt enzyme for all three methylation steps but in some cases several Pmts with different specificities are required (Sohlenkamp et al., 2003). In the soybean

symbiont *Bradyrhizobium japonicum*, PmtA methylates PE to MMPE, which serves as substrate for PmtX1-catalyzed methylation to DMPE and PC. *B. japonicum* encodes three further Pmt enzymes with distinct specificities (PmtX2-4), which are not expressed under standard laboratory conditions. PmtX1 and PmtX2 are similar to *R. sphaeroides* PmtA, whereas PmtA, PmtX3 and PmtX4 are homologous to *S. meliloti* PmtA (Minder et al., 2001; Hacker et al., 2008a,b). Like *B. japonicum*, *Rhizobium leguminosarum*, *Rhodopseudomonas palustris,* and *Rhizobium etli* seem to encode more than one *pmt* homolog (López-Lara et al., 2003; Martínez-Morales et al., 2003).

THE PC SYNTHASE PATHWAY: A MEMBRANE-INTEGRATED ENZYME USES EXOGENOUS CHOLINE FOR PC SYNTHESIS

The second PC synthesis pathway in *A. tumefaciens* is catalyzed by the Pcs enzyme (**Figure 4**). Like *pmtA*, the *pcs* gene (*atu1793*) is located on the circular chromosome and is constitutively expressed (Wessel et al., 2006; Klüsener et al., 2009; Wilms et al., 2012). Pcs uses exogenous choline, which is transported via the high-affinity choline transport system ChoXWV. A *choXWV*-deficient strain is largely impaired in choline transport but can still produce PC when choline is present suggesting alternative choline uptake systems in *A. tumefaciens* (Aktas et al., 2011b). Similar to *A. tumefaciens*, the Pcs pathway in *S. meliloti* and *B. abortus* rely on exogenous choline delivered by a homologous Cho transport system (de Rudder et al., 1999; Dupont et al., 2004; Herrmann et al., 2013). Choline is a major component of eukaryotic membranes liberated by phospholipases from PC. Large amounts of free choline is found in homogenized plant tissues (Zeisel et al., 2003) and a recent study showed that considerable choline pools are also present on leaf surfaces. *Pseudomonas syringae* produces PC exclusively via the Pcs pathway and contains three choline transport systems with different specificities (Chen and Beattie, 2008). *P. syringae* exhibits chemotaxis towards choline and other quaternary amines. Extracellular choline is scavenged by *P. syringae* and enhances fitness during leaf colonization (Chen et al., 2013).

An *A. tumefaciens pcs* mutant produces PC via the remaining PmtA pathway and conversely PC production in a *pmtA* mutant depends on extracellular choline which might be delivered by the host plant. Only a *pmtA*/*pcs* double mutant lacks PC excluding alternative PC synthesis pathways in this organism (Wessel et al., 2006). Both *A. tumefaciens* PC biosynthesis pathways can be functionally reconstituted in *E. coli* demonstrating that PmtA and Pcs do not require *A. tumefaciens* specific cofactors or substrates (Klüsener et al., 2009).

The best-characterized Pcs enzyme derives from *S. meliloti* (de Rudder et al., 1999; Sohlenkamp et al., 2000; Solís-Oviedo et al., 2012). It catalyses the transfer of a phosphatidyl group from CDP-DAG to choline releasing a CMP molecule and PC. Enzyme activity depends on divalent cations like Mn^{2+} or Mg^{2+} and on detergents such as triton X100 (de Rudder et al., 1999). A topological study suggested that sinorhizobial Pcs is an integral membrane protein containing eight TM with N- and C- termini located in the cytosol. Pcs is a member of the CDP-alcohol phosphotransferase (CDP-OH-PT) protein superfamily containing a modified version of a conserved CDP-OH-PT motif ($DX_2DGX_2ARX_{12}GX_3GX_3D$) characteristic for this enzyme family. Most of the conserved amino acids are located within a cytosolic loop connecting the TM domains II and III and are critical for enzyme activity as shown via mutagenesis (Solís-Oviedo et al., 2012; Geiger et al., 2013). Since the membrane-bound nature of Pcs enzymes has precluded their purification and biochemical characterisation, the precise reaction mechanism of Pcs enzymes is presently unknown but most likely proceeds via a sequential Bi–Bi reaction as in other CDP-OH-PT enzymes (Geiger et al., 2013).

It is not clear why two PC biosynthesis pathways operate simultaneously in *Agrobacterium* and some other bacteria. Although the Pcs pathway is energetically more favorable than the PE-methylation route, under conditions of choline limitation during competition with other bacteria, the Pmt pathway might be beneficial. In *Agrobacterium* both pathways seem to be constitutively present. PmtA activity is detected even in the presence of choline, when Pcs is active (Wessel et al., 2006; Klüsener et al., 2009). When two alternative PC synthesis pathways are present in eukaryotes, PC production via PE-methylation is repressed in the presence of choline used by the CDP-choline dependent pathway (Vance and Ridgway, 1988; Vance et al., 1997). It remains to be examined whether PmtA and Pcs pathways produce distinct PC pools with different fatty acyl chains as it is the case in eukaryotes (DeLong et al., 1999). Clearly, PC biosynthesis in *Agrobacterium* deserves further studies.

NON-PHOSPHORUS LIPIDS AND BIOSYNTHETIC PATHWAYS

Since inorganic phosphate is limiting in most soils, bacteria have evolved exquisite strategies to deal with phosphate deficiency. One strategy is to partially replace membrane PLs by phosphorus-free lipids as shown for *S. meliloti*, *Pseudomonas fluorescens*, *R. sphaeroides,* and *A. tumefaciens*. Various phosphorus-free lipids appear in these organisms upon phosphate limitation such as sulfolipids, GLs, betaine lipids, or OLs (Benning et al., 1995; López-Lara et al., 2003; Geiger et al., 2010; Zavaleta-Pastor et al., 2010; Geske et al., 2013).

The *A. tumefaciens*-related α-proteobacterium *S. meliloti* has served as model system in this context. Its membranes are composed of the PLs PG, PE, MMPE, and PC when grown under phosphate-rich conditions. Phosphate limitation triggers the degradation of PE, MMPE, and PC and accumulation of the phosphate-lacking lipids DGTS-(*N*,*N*,*N*,-trimethyl)homoserine (DGTS), sulfoquinovosyl-DAG (SQD), and OLs. Phosphate-dependent membrane remodeling is regulated by the PhoR-PhoB system: under phosphate-limitation, the response regulator PhoB activates expression of genes responsible for OL and DGTS synthesis and for synthesis of an intracellular phospholipase C (PlcP). PlcP degrades the PLs PE, MMPE, and PC to the corresponding phosphoalcohols and DAG. Inorganic phosphate is released from the phosphoalcohols by yet unknown phosphatases and is used as a source for essential phosphate-dependent biological processes. The released DAG serves as substrate for the formation of the non-phosphorus lipids DGTS and SQD (Geiger et al., 1999; Zavaleta-Pastor et al., 2010).

Ornithine lipids are fatty-acylated amino acids free of phosphate and glycerol. The non-proteinogenic amino acid ornithine is connected via its α-amino group to a 3-hydroxy fatty acid and

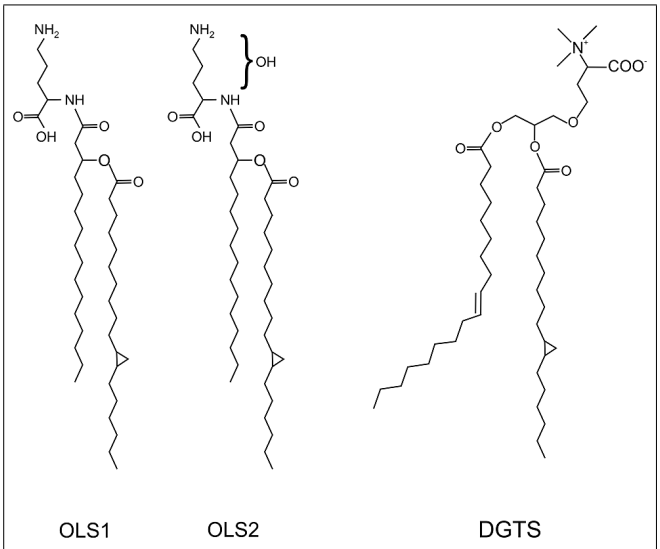
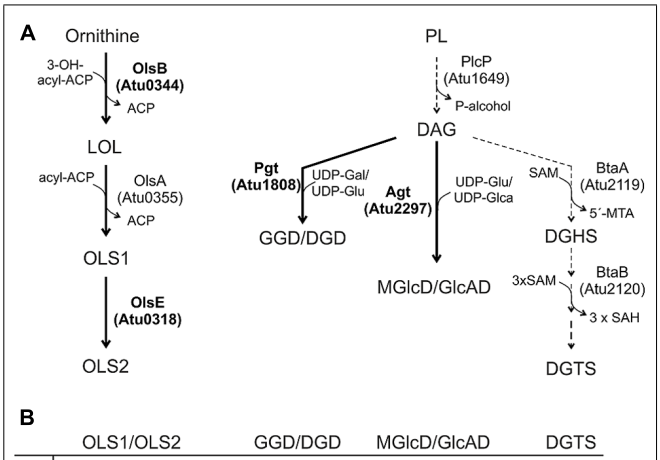


FIGURE 5 | Structure of the ornithine lipids OLS1/OLS2 and diacylglycerol trimethylhomoserine (DGTS) in *A. tumefaciens*. The OLs contain C16 3OH and C19:0 cyclo fatty acids. OLS2 is hydroxylated within the ornithine moiety. DGTS contains 18:1 and 19:0 cyclo fatty acids (Geske et al., 2013; Vences-Guzmán et al., 2013).



B

	OLS1/OLS2	GGD/DGD	MGlcD/GlcAD	DGTS
+P	~24/>1 %	-	-	-
- P	~11/~9%	~5 %	~2/8 %	~20%

FIGURE 6 | Phosphorus-free lipid synthesis pathways in *A. tumefaciens*. (A) Characterized pathways are indicated by thick arrows and enzymes in boldface letters. Putative biosynthetic pathways/enzymes are indicated with dashed arrows (Geske et al., 2013; Vences-Guzmán et al., 2013; Semeniuk et al., 2014). For details see text. **(B)** Relative proportion of phosphorus-free lipids in *A. tumefaciens* C58C1 under phosphate-replete (+P) and phosphate depleted (−P) conditions (Geske et al., 2013). Gal, galactose; Glu, glucose; Glca, glucoronic acid.

a second fatty acid chain is esterified to the 3-hydroxy group of the first fatty acid (**Figure 5**). OLs are widely distributed among eubacteria but absent from archaea and eukaryotes. Biosynthesis of OLs occurs via an acyl-ACP dependent two-step acylation of ornithine by two different acyltransferases. The first OL acyltransferases were discovered in *S. meliloti* (Weissenmayer et al., 2002; Gao et al., 2004). Acylation of ornithine occurs here via OlsB at the α-amino group to form lyso-ornithine (LOL), which in turn is acylated by OlsA at the 3-hydroxyl group to form OL (Gao et al., 2004; Geiger et al., 2013). Some bacteria modify their OLs by hydroxylation of the ornithine moiety or the ester- or amide-linked fatty acid. Three different OL hydroxylases are known in bacteria so far. OlsE homologs hydroxylate the ornithine moiety and the fatty acid portion is hydroxylated by OlsD (amide-linked) or OlsC (ester-linked) hydroxylases (Geiger et al., 2010; González-Silva et al., 2011; Vences-Guzmán et al., 2012). Several studies showed a contribution of hydroxylated OLs in microbe–host interactions and pH or thermal stress resistance (Rojas-Jiménez et al., 2005; González-Silva et al., 2011; Vences-Guzmán et al., 2011, 2012). It has been suggested that the additional hydroxyl groups increase the interaction between lipids via hydrogen bonds and thus, decrease the membrane fluidity and permeability, which might be advantageous under different stress conditions (Geiger et al., 2013). A recent study revealed a modification of OLs via methylation of the ornithine head group to mono-, di- and trimethyl-OL in planctomycetes isolated from an acidic and nutrient-poor ecosystem (Moore et al., 2013). Methylation of OLs increases their polarity and confers a more cylindrical shape, which possibly increases membrane stability similar to the bilayer forming lipid PC. Therefore, producing methyl-OLs might be an adaptation strategy to cope with acidity and nutrient scarcity in these organisms (Moore et al., 2013).

TWO ORNITHINE LIPIDS ARE SYNTHESIZED IN *A. tumefaciens*

In contrast to *S. meliloti* and *R. sphaeroides*, which produce only minor amounts of OLs under phosphate-replete conditions, *A. tumefaciens* accumulates significant amounts of two different OLs namely OLS1 and OLS2 even under full nutrient supply (Geske et al., 2013; Vences-Guzmán et al., 2013). In *A. tumefaciens* the OLs are composed of the fatty acids C16 3OH and C19:0 cyclo as shown by mass spectrometry analyses. OLS2 is the hydroxylated form of OLS1 containing the hydroxyl group within the ornithine moiety (Geske et al., 2013; Vences-Guzmán et al., 2013; **Figure 5**). *Agrobacterium* encodes the three *ols* homologs *olsA* (*atu0355*), *olsB* (*atu0344*), and *olsE* (*atu0318*) on the circular chromosome (Vences-Guzmán et al., 2012). *olsE* and *olsB* mutants in the *A. tumefaciens* A208 strain revealed that *olsB* is essential for formation of both OLS1 and OLS2 whereas *olsE* is only required for OLS2 synthesis (**Figure 6**). Heterologous expression of *olsE* resulted in OLS2 formation providing further evidence that OlsE is the hydroxylase responsible for OLS2 formation. Thus, the first step in OL synthesis in *Agrobacterium* is mediated by the acyltransferase OlsB forming ornithine to the lyso-ornithine lipid (LOL). Subsequently, LOL might be acylated via the putative OlsA to form OLS1. OLS2 formation is completed by hydroxylation of the ornithine moiety by OlsE (Vences-Guzmán et al., 2013).

Under low phosphate conditions, both OLs accumulate to a total amount of 45–50% whereas the total PL content decreases in *A. tumefaciens* A208. A putative Pho box is located in the promoter region of *olsB* suggesting PhoB-induced expression under phosphate starvation (Geske et al., 2013; Vences-Guzmán et al., 2013). In the *olsB* mutant, lack of OLs seems to be compensated by an

increase in DGTS and GL accumulation under phosphate reduced conditions (Vences-Guzmán et al., 2013).

Ornithine lipids production under phosphate starvation conditions seems to vary in different *Agrobacterium* strains. In contrast to *A. tumefaciens* A208, the total amount of OLs does not change under phosphate-limiting conditions in *A. tumefaciens* C58C1 but the degree of hydroxylation is ninefold increased (Geske et al., 2013; Vences-Guzmán et al., 2013). Deviations in the experimental setups such as the growth media and the precise phosphate concentrations might account for these differences.

A. tumefaciens PRODUCES FOUR DIFFERENT GLYCOLIPIDS AND A BETAINE LIPID UNDER PHOSPHATE DEPRIVATION

Glycolipids contain carbohydrate residues, which are glycosidically bound to the 3-position of a *sn*-1,2-DAG (Shaw, 1970). Different GLs are produced in bacteria under phosphate starvation. The photosynthetic bacterium *R. sphaeroides* produces the unique GL glucosylgalactosyl-DAG (GGD) with α-glucose (1⟶4)-linked to β-galactose (Benning et al., 1995). A series of GLs found in the nitrogen-fixing symbiont *Mesorhizobium loti* differs from the rhodobacterial GL. *M. loti* produces the GLs GGD, digalactosyl-DAG (DGD), and different molecular species of triglycosyl-DAG with various combinations of galactose and glucose in the head. All of the sugars are in β-configuration and (1⟶6)-linked to each other. Additionally, *M. loti* contains two further GLs with yet unknown head groups (Devers et al., 2011).

A. tumefaciens produces under phosphate deprivation four different GLs and DGTS accounting to 35% of the total lipids (Geske et al., 2013). The GLs have been identified recently as GGD and DGD with a β-configuration and monoglucosyl-DAG (MGlcD) and glucuronosyl-DAG (GlcAD) with a α-configuration (Geske et al., 2013; Semeniuk et al., 2014). The relative amount of these lipids in *A. tumefaciens* C58C1 is given in **Figure 6B**. Similar to *M. loti*, GGD and DGD are synthesized in *A. tumefaciens* via a processive glycosyltransferase namely Pgt (**Figure 6**) by a successive transfer of glucosyl and/or galactosyl residues to DAG. Functional characterisation of Pgt in *E. coli* and *Pichia pastoris* and overexpression in *Agrobacterium* revealed a broad substrate specificity concerning the glycosyl acceptor (DAG or ceramides) and sugar residues (uridine diphospho, UDP-galactose or UDP-glucose). However, Pgt favors DAG over ceramide and UDP-galactose over UDP-glucose (Hölzl et al., 2005). The promoter region of *pgt* contains a predicted Pho box suggesting an induced Pgt synthesis upon phosphate limitation mediated via the PhoR-PhoB system. A *pgt* mutant lacks GGD and DGD but the remaining lipids accumulate wild type-like (Geske et al., 2013).

Synthesis of MGlcD and the acidic GlcAD in *A. tumefaciens* is catalyzed by a single promiscuous glycosyltransferase namely Agt encoded by *atu2297* (**Figure 6**). Enzyme assays with recombinant Agt in *E. coli* protein extracts provided evidence that Agt uses UDP-glucose and UDP-glucuronic acid as sugar donors for MGlcD and GlcAD synthesis, repectively (**Figure 6**; Semeniuk et al., 2014). *A. tumefaciens* Agt is the first described glycosyltransferase using sugars with different chemistry. An *A. tumefaciens agt* mutant is deficient in MGlcD and GlcAD formation and loss of these GLs is compensated by a twofold increase in GGD and DGD. Remarkably, while DGTS and all other PLs are not influenced in the *agt* mutant, PC amount is strongly reduced. Deletion of both *pgt* and *agt* genes results in the loss of all GLs, which is compensated by a strong DGTS accumulation. Similar to the single *agt* mutant, the PC content of the double mutant is strongly reduced. One reason might be that PC is degraded to provide DAG for GGD/DGD synthesis in case of the *agt* mutant or for DGTS synthesis in the double mutant. It is unclear, however, why specifically PC and no other PL is turned over to supply DAG for the synthesis of phosphate-free lipids. Another reason might be that reduction of the bilayer-stabilizing PC in membranes missing the acidic GlcAD is necessary to sustain membrane structure and fluidity (Semeniuk et al., 2014). In *S. meliloti*, loss of the acidic glycolipid SQD is compensated by an increase of the anionic and bilayer-forming lipid PG (Weissenmayer et al., 2000).

Since loss of all GLs has no impact on growth and virulence even under phosphate-limited conditions, *A. tumefaciens* seems to compensate the lack of all GLs by DGTS (Geske et al., 2013; Semeniuk et al., 2014). A *S. meliloti* mutant deficient in all phosphate-free lipids shows decreased growth under phosphate starvation but is not influenced in nodule formation on its host alfalfa (López-Lara et al., 2005) suggesting that these lipids function as bulk membrane lipids. Whether lack of all GLs and DGTS impacts *A. tumefaciens* physiology and virulence remains to be seen.

The acidic GlcAD in *A. tumefaciens* might be the counterpart of the glycolipid SQD which is absent in *Agrobacterium* but widespread in photosynthetic organisms and present in a few non-photosynthetic bacteria such as some rhizobia (López-Lara et al., 2003). The role of SQD in these organisms is still unclear. It has been speculated that SQD might have a special role in photosynthesis or is required for nodule formation and nitrogen fixation. However, SQD-free mutants of the photosynthetic purple bacterium *R. sphaeroides* and the nitrogen fixing *S. meliloti* are not compromised in photosynthesis and symbiosis, respectively suggesting no general function of bacterial SQD in these processes (Benning et al., 1993; Weissenmayer et al., 2002; López-Lara et al., 2003).

DGTS-(*N*,*N*,*N*,-trimethyl)homoserine is a betaine-ether linked glycerolipid abundant in membranes of plants, algae, and fungi and is found in a few bacteria (Dembitsky, 1996; López-Lara et al., 2003). In *Agrobacterium* membranes DGTS is a major non-phosphorus lipid (∼20 mol%) during phosphate starvation (**Figure 6B**). Similar to PC, DGTS is a zwitterionic lipid containing a quaternary amino head group (**Figure 5**). It has been observed that the content of PC and DGTS within a cell is reciprocal. Organisms containing major amounts of PC produce only traces of DGTS and vice versa (Geiger et al., 2010). The structural similarity and the inverse relationship between DGTS and PC concentrations led to the speculation that these two lipids are functionally interchangeable (López-Lara et al., 2003; Geiger et al., 2010; Devers et al., 2011).

DGTS-(*N*,*N*,*N*,-trimethyl)homoserine synthesis in *R. sphaeroides* and *S. meliloti* occurs via the BtaA/B system (Klug and Benning, 2001; López-Lara et al., 2005). BtaA is a SAM/DAG 3-amino-3-carboxypropyl transferase that converts DAG to DAG-homoserine (DGHS) using SAM as homoseryl donor. Subsequently, DGHS is threefold methylated via BtaB, a SAM:DAG-homoserine-*N*-methyltransferase, to DGTS.

Expression of the sinorhizobial *btaA* and *btaB* genes is PhoB regulated. BtaA (*atu2119*) and BtaB (*atu2120*) homologs which have not been characterized yet are encoded in the *A. tumefaciens* genome suggesting a similar DGTS biosynthesis and regulation (Yuan et al., 2006; **Figure 6**). In *A. tumefaciens*, DGTS and GL accumulation under phosphate limitation also seems to be controlled not only on transcriptional level of the responsible biosynthesis genes but also via DAG substrate availability. A PlcP homolog, encoded by *atu1649* in the *A. tumefaciens* genome (Geske et al., 2013) suggests a similar membrane remodeling mechanism as described in *S. meliloti* (Zavaleta-Pastor et al., 2010; Geiger et al., 2013). Interestingly, phosphate starvation results not only in the replacement of PLs by non-phosphorus lipids in *A. tumefaciens* but also in changes in fatty acid composition of DAG and PLs with a shift from 18:1 to 19:0 cyclo fatty acids (Geske et al., 2013). Whereas under full nutrition PLs are mainly composed of 18:1 (50–60%) fatty acids and contain low proportions of 19:0 cyclo (20 and 40% in PC) fatty acid, phosphate limitation results in a decrease in 18:1 (10%) and a strong increase in 19:0 cyclo (60%) fatty acids (Geske et al., 2013). *A. tumefaciens* codes for a putative cyclopropane fatty acid (CFA) synthase presumably responsible for this modification (Geske et al., 2013). Cyclopropanation of pre-existing unsaturated fatty acids is widespread in bacteria and maximal activity is observed during stationary phase. The biological role of CFA containing lipids in bacteria is not fully understood. Accumulation of CFAs in *E. coli* is correlated with acid tolerance and seems to be important for pathogenic bacteria–host interactions as shown for *Mycobacterium tuberculosis* (Chang and Cronan, 1999; Glickman et al., 2000; Zhang and Rock, 2009). A twofold increase in CFA content under phosphate starvation and acid conditions is also observed in *S. meliloti*. Here, two CFA synthases have been described, with Cfa1 essential for cyclopropanation of fatty acids under tested conditions. Both *cfa* genes are not required for symbiotic nitrogen fixation in *S. meliloti* (Saborido Basconcillo et al., 2009). Whether cyclopropanated lipids are required for *A. tumefaciens* virulence remains to be determined.

IMPORTANCE OF MEMBRANE LIPIDS FOR *A. tumefaciens* PHYSIOLOGY AND PATHOGENESIS

PHOSPHATIDYLCHOLINE IS CRUCIAL FOR AGROBACTERIUM VIRULENCE

Although the typical eukaryotic membrane lipid PC is rarely found in bacteria it is a main constituent of *A. tumefaciens* inner and outer membranes suggesting an important role for this organism (Klüsener et al., 2009). Indeed, loss of PC causes different physiological defects. A PC-deficient mutant is impaired in growth on solid medium at elevated temperatures and is unable to grow in the presence of the anionic detergent SDS. Furthermore, it is less motile and produces larger amounts of surface-attached biomass (Klüsener et al., 2009). The motility defect is explained by reduced flagellar proteins (FlaA and FlaB) in minimal medium (Klüsener et al., 2009, 2010). The most striking phenotype of a PC-deficient mutant is its defect in tumor formation due to loss of the VirB/D4 Type 4 SS (T4SS) essential for T-DNA transfer (Wessel et al., 2006). In response to plant stimuli the two component system VirA/G controls the expression of 11 transcriptional units, among them the *virB* and *virD* operons encoding the T4SS. The homodimeric histidine kinase VirA is anchored in the inner membrane. Plant-released signals, e.g., phenolic compounds are recognized by a cytoplasmic linker domain whereas acidic pH and monosaccharides are perceived by the periplasmic domain (Nair et al., 2011). The global response to PC-deficiency in *A. tumefaciens* as determined by proteomics and transcriptomics shows that the VirA/G-controlled *vir* gene expression under virulence-induced conditions is drastically reduced thus explaining the absence of the T4SS (Klüsener et al., 2010). Only a limited set of other genes coding for membrane-related proteins were changed in the absence of PC. Expression of *virG* in the PC-deficient mutant was also dramatically reduced suggesting that lack of virulence gene induction is due to low *virG* expression. Since the loss of *vir* gene expression in a PC-deficient mutant cannot be complemented by expression of a plasmid-encoded wild type *virG* but by a constitutively active VirG, it seems that a non-functional VirA sensor kinase is responsible for the loss of virulence gene expression in the PC-lacking *Agrobacterium* mutant. These observations suggest that signal transduction between VirA and VirG is impaired in the absence of PC, possibly due to limitations in membrane insertion or folding of VirA (**Figure 7**). It remains to be seen whether the observed phenotypic defects in the PC-deficient mutant are PC-specific or a consequence of altered bulk physico-chemical properties of the membrane in the absence of PC. The structural organization of membranes is defined by the physical properties and shape of membrane lipids. Cylindrical-shaped lipids such as PG or PC are bilayer-forming lipids whereas cone-shaped lipids such as PE are considered non-bilayer forming lipids (van Meer et al., 2008). However, non-bilayer lipids can form bilayer-structures depending on solvent conditions, alkyl chain composition, and temperature.

We do not know yet whether loss of other PLs such as PE, PG, or CL *in A. tumefaciens* causes similar effects on physiology and VirA/G-mediated signal transduction. It has been shown that PE can act as molecular chaperone for proper folding and function of membrane proteins such as the lactose permease LacY in *E. coli* (Bogdanov et al., 1999; Bogdanov and Dowhan, 2012). Replacement of PE by PC during reconstitution of the ABC multidrug exporter HorA from *Lactobacillus brevis* into membrane vesicles altered the orientation of TM helices and abolished transport function (Gustot et al., 2010). The effect of PC depletion on membrane proteins (Klüsener et al., 2010) suggests that PC and probably other PLs play a role in membrane protein homeostasis in *A. tumefaciens*.

It is important to note that the requirement of PC for productive host–microbe interactions is not restricted to *A. tumefaciens*. PC-deficient *S. meliloti* mutants are unable to establish nitrogen-fixing symbiosis with their host plant alfalfa (Sohlenkamp et al., 2003). Reduced PC levels in *B. japonicum*, the symbiont of the soybean *Glycine max* cause formation of nodules with impaired nitrogen fixation activity (Minder et al., 2001). A PC-deficient mutant of the intracellular human pathogen *Legionella pneumophila* shows lowered cytotoxicity and adhesion to the host cell. Loss of PC affects the Dot/Icm T4SS, system which delivers virulence factors into the cytosol of infected cells and is required for intracellular growth (Conover et al., 2008). In *P. syringae* PC is essential for secretion of the HrpZ harpin effector protein possibly

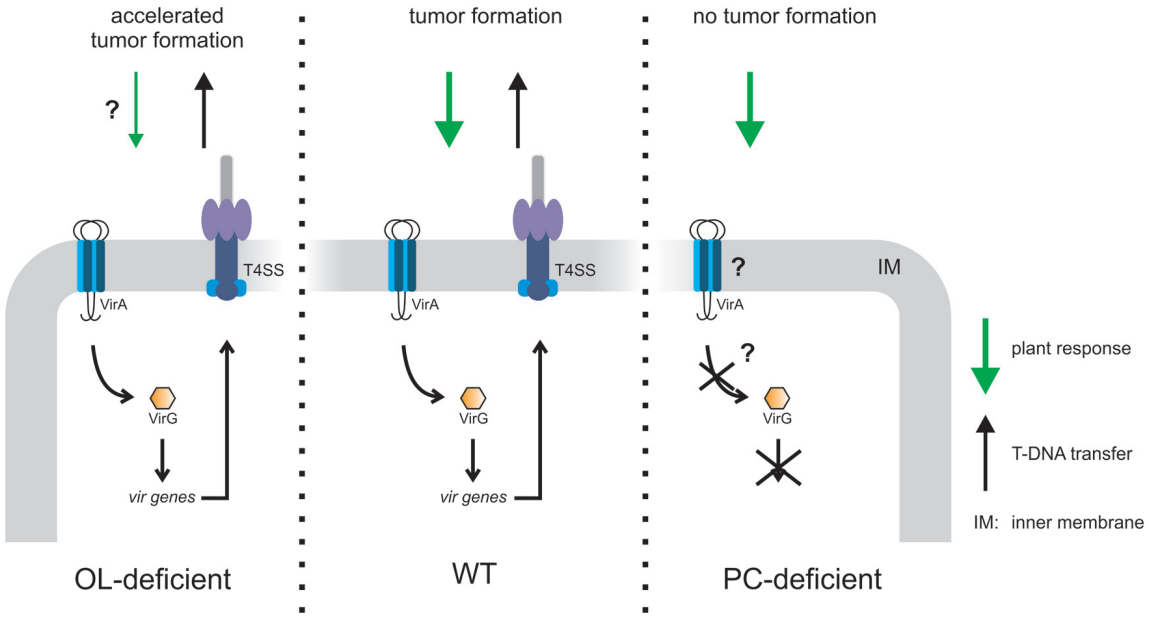


FIGURE 7 | Model of PC- or OL-dependent effects on *A. tumefaciens* infection efficiency. OL-lacking *A. tumefaciens* induces accelerated tumor formation on potato disks compared to wild type (WT) probably due to reduced plant defense (Vences-Guzmán et al., 2013). A PC-deficient mutant is unable to elicit tumors on *Kalanchoë* leaves since VirA/G controlled *vir* gene expression is impaired. As a consequence, the type4 secretion system (T4SS) is not produced which is essential for tumor formation (Wessel et al., 2006; Klüsener et al., 2010).

due to a non-functional T3SS (Xiong et al., 2014). *B. abortus*, the causative agent of brucellosis produces PC via the Pcs pathway. A *pcs* mutant is defective in PC formation and attenuated in virulence when assayed in the mouse model (Comerci et al., 2006). PC is not generally critical for physiology or microbe–host interactions. Loss of PC in the opportunistic pathogen *P. aeruginosa* did not affect physiology and virulence (Malek et al., 2012). It is important to note here that PC is only a minor (∼4%) component of *P. aeruginosa* membranes (Geiger et al., 2013).

LACK OF THE HYDROXYLATED ORNITHINE LIPID OLS2 IN *A. tumefaciens* CAUSES ACCELERATED TUMOR FORMATION

Although various bacteria deficient in OL biosynthesis have been characterized, the function of OLs still is largely unclear. OLs have been implicated in high-temperature tolerance in *Burkholderia cepacia* (Taylor et al., 1998). In *Bordetella pertussis* and *Flavobacterium meningosepticum* OLs are involved in hemagglutination and stimulation of macrophages (Kawai and Yano, 1983; Kawai and Akagawa, 1989; Kawai et al., 1999). In *Rhodobacter capsulatus* OL is critical for optimal yields of cytochrome c (Aygun-Sunar et al., 2006). In Gram-negative bacteria OLs are enriched in the outer membrane. It has been postulated that the zwitterionic OLs increase outer membrane stability via stabilization of the negative charges of LPS. Hydroxylation of OLs often correlates with bacterial stress response (Vences-Guzmán et al., 2012). It is speculated that the additional OH group increases hydrogen bonding between the lipid molecules as shown for the 2-hydroxylated lipid A in *Salmonella typhimurium*. This would decrease the membrane fluidity and make it less permeable (Gibbons et al., 2000; Vences-Guzmán et al., 2012).

In *A. tumefaciens* A208 grown at low temperatures (15°C) unmodified OLS1 is completely hydroxylated to OLS2 suggesting a role of this modified OL in temperature stress. However, lack of both OLs has no impact on growth even under high osmolarity or at low temperature. Interestingly, *A. tumefaciens* A208 mutants devoid of OLS2 induce about 1 week earlier tumors and consequently, the tumor size is increased compared to wild type induced tumors (Vences-Guzmán et al., 2013). OLs share a 3-acyloxyacylamide structure with lipid A of Gram-negative bacteria, which is an elicitor in plant–microbe interactions (Scheidle et al., 2005; Silipo et al., 2008; Madala et al., 2011). It has been speculated that hydroxylated OLs cause a plant defense response, which might be lowered in the absence of OLs thus explaining accelerated tumor formation (**Figure 7**).

The role of OLs in plant interaction cannot be generalized and it seems that OLs have different functions in different bacteria. In contrast to *A. tumefaciens*, the two modified OLs P1 and P2 are necessary for a successful symbiotic interaction in the nitrogen-fixing symbiont *R. tropici* CIAT899, which is highly tolerant to different environmental stresses (Rojas-Jiménez et al., 2005). The OL in *S. meliloti* is required for normal growth under phosphate-limiting conditions but not necessary for symbiotic performance (López-Lara et al., 2005).

CONCLUSION

Recent progress in lipid analysis technologies has revealed a surprising diversity in bacterial membrane lipid biosynthesis. The membrane composition is very dynamic and substantially remodeled in response to environmental changes. A future challenge will be to define the physiological role of specific lipids at the molecular

level. The phenotypic characterisation of lipid biosynthesis mutants has already provided interesting insights into the *in vivo* function of various lipids but has considerable limitations. Sometimes it is difficult to interpret whether the observed phenotypes are direct or indirect because most bacteria are able to compensate the loss of one lipid by changing the overall lipid composition. One interesting model organism in this context is *A. tumefaciens*, the natural genetic engineer of plants. Two specific membrane lipids, the PL PC and a phosphate-free lipid OL affect virulence with opposing outcomes. PC-deficiency causes a loss of virulence gene expression and tumor formation whereas lack of OLS2 accelerates tumorigenesis. Biophysical and biochemical studies combined with genetic manipulation are needed to understand the precise molecular mechanisms, by which these lipids influence membrane properties and *Agrobacterium*-mediated tumor formation.

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Agrobacterium infection and plant defense—transformation success hangs by a thread

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The value of *Agrobacterium tumefaciens* for plant molecular biologists cannot be appreciated enough. This soil-borne pathogen has the unique capability to transfer DNA (T-DNA) into plant systems. Gene transfer involves both bacterial and host factors, and it is the orchestration of these factors that determines the success of transformation. Some plant species readily accept integration of foreign DNA, while others are recalcitrant. The timing and intensity of the microbially activated host defense repertoire sets the switch to "yes" or "no." This repertoire is comprised of the specific induction of mitogen-activated protein kinases (MAPKs), defense gene expression, production of reactive oxygen species (ROS) and hormonal adjustments. *Agrobacterium tumefaciens* abuses components of the host immunity system it mimics plant protein functions and manipulates hormone levels to bypass or override plant defenses. A better understanding of the ongoing molecular battle between agrobacteria and attacked hosts paves the way toward developing transformation protocols for recalcitrant plant species. This review highlights recent findings in agrobacterial transformation research conducted in diverse plant species. Efficiency-limiting factors, both of plant and bacterial origin, are summarized and discussed in a thought-provoking manner.

Keywords: *Agrobacterium tumefaciens*, transformation, plant defense, reactive oxygen species, VIP1

INTRODUCTION

In their natural habitats, plants live in close contact with a myriad microorganisms. Plant-microbe associations can be mutually beneficial, such as the root nodule symbiosis with nitrogen-fixing bacteria or the more wide-spread association of plant roots with arbuscular mycorrhizal fungi (reviewed in Parniske, 2008; Markmann and Parniske, 2009). In contrast, pathogenic fungi or bacteria impair plant development and cause various disease symptoms in their hosts. The gram-negative *Agrobacterium tumefaciens* of the family Rhizobeaceae is a "special case." It is a biotroph pathogen, which markedly alters the physiology and morphology of infected host plants. What makes *Agrobacterium* so special is its capability for interkingdom gene transfer. In nature, wild type *A. tumefaciens* (as well as *A. rhizogenes* and *A. vitis*) causes "crown gall disease," characterized by the growth of tumor-like structures (calli) on host species. The genetic information for this anatomical reprogramming is encoded on the tumor-inducing (Ti) plasmid. The transfer DNA (T-DNA) derived from the Ti plasmid is imported into the host cell's cytoplasm and subsequently into the nucleus (Gelvin, 2003, 2005; Dafny-Yelin et al., 2008; Pitzschke and Hirt, 2010b). T-DNA transport is mediated by agrobacterial virulence factors, and—involuntarily—supported by proteins of the attacked host. Over the last decade, microbiologists and plant scientists have disclosed an impressive portfolio of agrobacterial infection strategies, some of which resemble those in other pathogen-host interactions. Plant defense mechanisms counteracting these strategies are equally diverse and impressive.

PRINCIPAL STEPS

The principal steps and factors involved in *Agrobacterium*-mediated plant transformation are comparatively well-understood, and reviews can be found in e.g., (Gelvin, 2009, 2010a,b; Pitzschke and Hirt, 2010b). Briefly, agrobacteria sense phenolic substances that are secreted by wounded plant tissue. Reception of these signals drives the expression of bacterial virulence (*vir*) genes. Subsequently, Vir proteins are produced, and single-stranded T-DNA molecules are synthesized from the Ti plasmid. The T-complex, i.e., T-DNA associated with certain Vir proteins, is injected into the host cytoplasm. A sophisticated network of bacterial and plant factors mediates translocation of the T-DNA to its final destination, the host cell's nucleus.

Agrobacterium inserts substrates (T-DNA and virulence proteins including VirD2, VirE2, VirE3, VirD5, and VirF) into the host cell by a type IV secretion system (Cascales and Christie, 2003). This strategy is also employed for the delivery of microbial factors by other plant pathogens, including *Xanthomonas campestris* (Thieme et al., 2005) and *Burkholderia* (Engledow et al., 2004). Likewise, mammalian pathogens including *Bordetella pertussis*, *Legionella pneumophila*, *Brucella* spp., and *Helicobacter pylori*, use type IV machineries to export effector proteins to the extracellular milieu or the cell cytosol (Christie and Vogel, 2000). Remarkably, under laboratory conditions, agrobacteria can genetically transform virtually any type of eukaryote, ranging from yeast (Bundock et al., 1995) to human cells (Kunik et al., 2001) (reviewed in Michielse et al., 2005; Lacroix et al., 2006). The T-complex, consisting of T-DNA, bacterial virulence proteins (VirE2, VirD2) and the host factor

VIP1 (VirE2-interacting protein 1) is imported into the nucleus. Subsequently, the proteinaceous components are stripped off, releasing the T-DNA from the T-complex. This step relies on degradation of VirE2, VirD2, and VIP1 by the plant SCF proteasomal machinery (see below). The bacterial F-box protein VirF, which is contained in and confers substrate specificity to the SCF complex, participates in this degradation. If the T-complex disintegrates *before* it is in contact with the host's chromatin, the delivered transgenes are expressed for only a few days. The loss of transgene activity at later stages likely results from the T-DNA being degraded by host nucleases (Gelvin, 2003). In contrast, if the T-DNA is shielded *until* the T-complex is in contact with chromatin, stable transformants can be obtained. Due to its affinity for histones, VIP1 most probably guides the T-DNA to its target destination, the chromatin (Lacroix et al., 2008).

Since the discovery of the gene transfer mechanism (Schell and Van Montagu, 1977; Holsters et al., 1978), *Agrobacterium* strains have been converted ("disarmed") into efficient delivery systems for the genetic manipulation of plants. While transient expression approaches can provide rapid answers on e.g., subcellular localization, protein-protein interaction and promoter/effector relationships (Andrews and Curtis, 2005; Li et al., 2009; Pitzschke, 2013b), genetic engineering requires the transgene(s) to be stably integrated in the host genome.

The so-called disarmed/non-oncogenic *A. tumefaciens* strains employed are deprived of their Ti properties, and the T-DNA region is used as a vehicle for the introduction of tailor-made DNA sequences. Any DNA sequence placed between T-DNA "border sequences" (Ti-plasmid-derived 25-bp direct repeats) can be transferred (Gelvin, 2012). Disarmed strains, therefore, facilitate transformation, but do not provoke callus growth or other abnormalities caused by oncogenic strains. Consequently, phenotypic abnormalities that may be exhibited by transformed plants are primarily due to the particular transgene being expressed. Furthermore, by using armed and disarmed strains side-by-side, host responses that are independent of or dependent on Ti sequences can be distinguished.

TRANSCRIPTIONAL RE-PROGRAMMING OF HOST CELLS

The advent of full genome sequencing and microarray technologies has created the opportunity to draw a complete picture on *Agrobacterium*-induced changes at the transcript level. Gene expression profiling data have been generated for various plant species, and comprehensive databases (e.g., http://www.plexdb.org) and bioinformatics resources even allow comparison of transcriptional responses across multiple plant species (Dash et al., 2012). One major finding from diverse microarray studies was that agrobacteria largely modify host gene expression, particularly that of defense-related genes.

This fact had already been recognized in the "pre-microarray era." cDNA-AFLP analysis of *Ageratum conyzoides* plant cell cultures enabled the identification of (non-oncogenic) *Agrobacterium*-induced transcripts, many of which encoded putative defense factors (Ditt et al., 2001). In a subsequent study the same research group observed an anti-correlation between *Agrobacterium*-mediated transformation efficiency and defense gene expression levels (Ditt et al., 2005). By the approach of suppression subtractive hybridization and DNA macroarrays, Veena Jiang et al. (2003) provided the first insight into the molecular kinetics of *Agrobacterium* -plant interactions. Transcriptional responses of tobacco BY-2 cell cultures to a subset of agrobacterial strains, impaired in T-DNA and/or Vir protein transfer, were monitored over a 36-h-period. All strains elicited a general defense response during early stages of infection. However, expression of defense-related genes was repressed at later stages—exclusively by the transfer-competent strains. More detailed expression profiling of selected genes furthermore disclosed the "unintentional" participation of the host cellular machinery in the transformation process (Veena Jiang et al., 2003).

MICROBIAL ATTACK AND PLANT DEFENSE

Microbes attempting to invade their hosts betray themselves by the presence of so-called microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). These molecules, which are recognized as "non-self" initiate the first line of defense, known as PAMP-triggered immunity (PTI) (Nurnberger et al., 2004; Sanabria et al., 2008; Boller and He, 2009) (see below). Pathogens, in turn, aim to overcome PTI activation by injecting certain effector proteins into the host cytoplasm. Perception of these pathogen-encoded effectors by cognate intracellular plant proteins raises the second line of defense, effector-triggered immunity (ETI) (Bonardi and Dangl, 2012; Gassmann and Bhattacharjee, 2012). This response is characterized by the induction of localized apoptosis (hypersensitive response, HR) and systemic defense signaling. Plants capable of activating ETI can thus not only restrict pathogen spread, but they can also fortify themselves against subsequent attacks (Shah and Zeier, 2013).

MAMPs AND THEIR PERCEPTION

MAMPs are best described as molecular "signatures" typical of whole classes of microbes (Boller and Felix, 2009). MAMP perception through specific cell-surface-located proteins ("pattern recognition receptors") is a conserved strategy of eukaryotic innate immune systems. Because MAMPs initiate defense responses in many plant species, they are also referred to as "general elicitors" (Nurnberger et al., 2004). Prominent examples of MAMPs include oligopeptide elicitors such as those derived from EF-Tu (elongation factor thermo unstable), flagellin, and cryptogein (a fungal sterol-scavenging protein), as well as glycol-conjugates, including bacterial lipopolysaccharides and peptidoglycan, and the fungal MAMPs beta-glucan, chitin and chitosan oligosaccharides (reviewed in Silipo et al., 2010).

The two undoubtedly best-characterized MAMP receptors in plants, FLS2 and EFR, recognize the oligopeptides flagellin and EF-Tu, respectively. Owing to their composite structure, these membrane-located leucine-rich repeat-receptor-like kinases (LRR-RLK) convert and transmit perceived "attack signals" into the interior of cells to initiate appropriate defense responses. On the contrary, the primary "aims" of pathogens are to claim nutrients from and multiply to high levels in their hosts. To avoid or block defense responses during early stages of infection, pathogens have two options: (1) evade recognition and "sneak in" or (2) "step in self-consciously" and counteract the

elicited warfare attack. Biotrophs, such *as Pseudomonas syringae*, *A. tumefaciens*, *Xanthomonas campestris,* and *Botrytis cinerea*, have developed sophisticated strategies to block defense signaling in their hosts at several steps (Pitzschke et al., 2009c).

A total of 292 and 165 *LRR-RLK* genes were retrieved from the rice and *Arabidopsis* genomes, respectively (Hwang et al., 2011). These large numbers provide an idea of the versatility of LRR-RLK applications. Specific roles have been ascribed to individual family members. Studies in individual LRR-RLK mutants have contributed to our understanding of pathogen perception in general. They also demonstrate the similarity of early plant responses to agrobacteria and other microbial pathogens.

For instance, *fls2* mutants fail to recognize flagellin and are more susceptible to infection by the pathogen *Pseudomonas syringae* (Zipfel et al., 2004). Similarly, mutants deficient in EFR, the receptor for the agrobacterial MAMP EF-Tu, are hypersensitive to *Agrobacterium*-mediated transformation (Zipfel et al., 2006). These examples demonstrate that "ignoring" the invader is not advisable. Instead, perception is the first and mandatory step to restrict bacterial invasion. *FLS2* gene induction upon pathogen exposure or flagellin treatment (Boutrot et al., 2010), as well as *EFR1* induction by EF-Tu-derived peptides (Zipfel et al., 2006) reflect additional host mechanisms to better target the suspected invaders.

MAPK SIGNALING

One of the early intracellular events following pathogen perception is signal transduction and amplification through mitogen-activated protein kinases (MAPKs) (Nakagami et al., 2005; Pitzschke et al., 2009c; Huang et al., 2012; Rasmussen et al., 2012). MAPK cascades are conserved eukaryotic signaling modules. Their minimal components, a MAPK kinase kinase (MAPKKK), a MAPKK and a MAPK, represent multigene families. Exogenous or developmental signals are perceived by a receptor which subsequently (directly or indirectly) initiates the MAPK cascade. Once activated, a MAPKKK phosphorylates its downstream MAPKK which in turn phosphorylates and thereby activates its downstream MAPK (Nakagami et al., 2005). MAPK-mediated phosphorylation of target proteins can alter their properties, such as subcellular location, DNA-binding specificity, enzymatic activity or stability. There is ample evidence for disturbed MAPK signaling markedly affecting biotic and abiotic stress tolerance (Rohila and Yang, 2007; Pitzschke and Hirt, 2009; Pitzschke et al., 2009a; Rodriguez et al., 2010; Sinha et al., 2011; Persak and Pitzschke, 2013; Zhang et al., 2013b). It is very likely that such a scenario will hold true in many plant species.

MAPK SIGNALING AND THE MULTIFUNCTIONAL PROTEIN VIP1

In the context of agrobacteria and pathogen defense, one member of the *Arabidopsis* MAPK family has merited special attention: MPK3. This protein is activated within few minutes upon treatment with pathogens or bacterial elicitor-derived peptides such as flg22 and elf18 (Djamei et al., 2007; Lu et al., 2009). MPK3 is an important positive regulator in defense signaling (Nakagami et al., 2005; Pitzschke et al., 2009c). From a pathogen's point of view, activation of MPK3 should be avoided to circumvent repelling. Accordingly, agrobacteria have evolved strategies to co-opt induction of this kinase. MPK3 phosphorylates the host protein VIP1 and thereby triggers cyto-nuclear translocation of this bZIP transcription factor (Djamei et al., 2007). VIP1, which enters the nucleus *via* interaction with importin alpha (Citovsky et al., 2004) subsequently induces expression of defense genes such as *PR1* (pathogenesis-related protein 1) (Djamei et al., 2007; Pitzschke et al., 2009b; Pitzschke and Hirt, 2010a). Agrobacteria, on the other hand, hijack VIP1 as a shuttle for nuclear import of the T-complex (Citovsky et al., 2004). A number of plant species lack putative VIP1 homologs; yet these species *are* transformable. This apparent paradox was solved by the discovery and characterization of virulence factor VirE3. VirE3 functionally replaces the "shuttle" function of VIP1, thus ensuring nuclear import of the T-DNA (Lacroix et al., 2005). In contrast to VIP1, VirE3 is not a transcription factor and is therefore unlikely to (directly) induce defense gene expression. VirE3 may thus be an attractive target for biotechnological approaches.

VIP1 as transcriptional regulator

A random-DNA-selection-assay (RDSA) enabled the identification of putative VIP1 target sequences. The DNA consensus motif recognized by VIP1 (VRE—VIP1 response element) was found to be enriched in promoters of stress-responsive genes (Pitzschke et al., 2009b). Notably, this motif does not resemble known regulatory DNA elements. *In vivo,* VIP1 directly binds to VRE sites in the promoter of *MYB44* (Pitzschke et al., 2009b), a stress-related transcription factor (Jung et al., 2008; Persak and Pitzschke, 2013). Importantly, this binding occurs in a stress-dependent manner that correlated with the MPK3 activation profile (Pitzschke et al., 2009b). Through binding to VRE sites, VIP1 might directly regulate expression of another stress-responsive gene, *thioredoxin Trxh8*. In protoplast cotransfection experiments, VIP1 triggered the expression of the pathogen-responsive PR1 gene (Djamei et al., 2007). However, this *PR1* induction is likely an indirect effect. The *PR1* promoter is devoid of VRE sites; and *PR1* is known as a late stress-responsive gene, in contrast to the early and transient nature of MPK3 activation and VIP1 cyto-nuclear translocation. A very recent report (Lacroix and Citovsky, 2013) provides a deeper insight into the VRE-VIP1 mechanism. In agreement with the original study (Pitzschke et al., 2009b), VIP1 bound VRE *in vitro*, and VIP1-VRE binding strongly correlated with transcriptional activation levels *in vivo*. Presence of the agrobacterial F-box protein VirF did not affect VIP1-VRE binding *in vitro*. In contrast, coexpression of *virF* markedly decreased VIP1 transcriptional activation ability *in vivo*. The most likely explanation for this effect is that *in vivo*, VirF prevents VRE induction by triggering proteasomal degradation of VIP1 (Lacroix and Citovsky, 2013). In fact, agrobacteria have learned to control VIP1 abundance by abusing the host proteasome machinery (see below). Being aware of the ongoing host-pathogen arms race, it is tempting to speculate that VIP1 may not only turn on expression of host defense genes. Instead, agrobacteria *may* benefit from one or more VIP1-induced gene products involuntarily provided by the plant. Discovering the VIP1-targetome seems a highly rewarding undertaking. Screening of the *Arabidopsis* genome for promoters enriched in VRE and related motifs isolated by RDSA (Pitzschke

et al., 2009b) could be a first step in that direction (Pitzschke, unpublished).

Overexpression studies in tobacco have shown that VIP1 also promotes transformation efficiency in heterologous systems (Tzfira et al., 2002). The cross-species functionality of VIP1 as transcription factor was further documented in a rather non-conventional expression system: protoplasts from red leaves of poinsettia (*Euphorbia pulcherrima*). Polyethylenglycol-mediated cotransfection experiments showed that VIP1 efficiently induces VRE-mediated gene expression (Pitzschke and Persak, 2012). For this transactivation to occur neither a tissue context, chloroplasts nor external stimuli are required.

In its unquestionable key role in *Agrobacterium*-mediated transformation, VIP1 presents an attractive target for manipulation. It appears feasible to uncouple the T-complex-vehicle from the defense-gene-inducer function. Experiments with a C-terminally truncated VIP1 variant have shown that full-length VIP1 is required for stable, but not for transient transformation (Li et al., 2005a). The transgenesis-enhancing effect most likely derives from VIP1 acting as mediator between host nucleosomes and T-DNA/VirE2 complexes. Therefore, replacing critical residues rather than deleting certain domains/peptides seems a more purposeful approach. Indeed, mutation of Lys212, located in the bZIP domain, rendered VIP1 fully incapable of transactivating the *PR1* promoter or a synthetic VRE promoter (Pitzschke et al., 2009b).

THE SCF PROTEASOMAL MACHINERY, VirF AND VBF

Many biological processes, including host-pathogen interactions, are controlled by SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complexes. These complexes mediate the proteasomal degradation of specific target proteins. The F-box protein contained in SCF complexes confers substrate specificity (Lechner et al., 2006).

Although prokaryotes lack SCF complexes, F-box-encoding genes are found in some pathogenic bacteria. The translocation of F-box effectors appears to be a wide-spread "infection strategy." Pathogens secrete F-box proteins into their hosts to abuse the SCF machinery, resulting in high infection rates. However, F-box effectors are intrinsically unstable proteins which are rapidly degraded by the host proteasome pathway (Magori and Citovsky, 2011b). The Citovsky laboratory uncovered yet another level of agrobacterial cleverness and callousness: Destabilization of the agrobacterial F-box protein VirF is counteracted by the bacterial effector, VirD5 (Magori and Citovsky, 2011a). As if this was not enough, agrobacteria also exploit additional host factors to maximize infection: Diverse pathogens, including *Agrobacterium*, induce expression of VBF (VIP1-binding factor), a host-encoded F-box protein. VBF can functionally replace the agrobacterial VirF in regulating VIP1 and VirE2 protein levels (Zaltsman et al., 2010b). Analogous to VirF, VBF destabilizes VirE2 and VIP1, most likely *via* SCF-mediated proteasomal degradation (Zaltsman et al., 2010a). A very recent study extends on this finding and highlights the importance of VBF at the final stage of T-DNA pre-integration (Zaltsman et al., 2013). As reported earlier, T-complexes can be reconstituted from ssDNA and VirE2 *in vitro* (Zupan et al., 1996). Its tight packaging by VirE2 molecules shields the ssDNA from the outside and makes it inaccessible to degradation by exogenously added DNAse. In the presence of extracts from wild type, but not from *VBF* antisense plants, this "shielding effect" was found to be rapidly lost. Thus, VBF-mediated un*coating* of the T-complex indeed results in *unmasking* of the T-DNA (Zaltsman et al., 2013).

Micro-bombardment studies in *N. benthamiana* leaves have disclosed a cytoplasmic-nuclear distribution of VBF. In contrast, VBF/VIP1 complexes occur exclusively in the nucleus. Based on these observations, VBF may have additional functions in the cytoplasm, besides acting in T-complex disassembly in the nucleus, (Zaltsman et al., 2010b). Alternatively, VBF may re-locate upon pathogen attack (similar to VIP1). If this—currently hypothetic—scenario was true, a straight-forward question arises. Is VBF distribution phosphorylation-dependent; is it controlled by MAPKs? At least *in silico*, such scenario appears possible (Pitzschke, unpublished). MAPKs phosphorylate their targets at serine or threonine residues adjacent to a proline. A kinase interaction motif [KIM; R/K-x2-6-I/Lx(/L)], known to be recognized by mammalian MAPKs (Tanoue and Nishida, 2003), assists MAPK binding also in substrate proteins of plant MAPKs (Schweighofer et al., 2007). The VBF protein sequence contains one Ser-Pro dipeptide motif as well as one KIM (position 164-171) (**Figure 1**). Pathogen-activated MAPK(s), such as MPK3, *may* phosphorylate residue Ser17 and thereby initiate VBF nuclear translocation.

THE ROLE OF PLANT HORMONES IN TRANSFORMATION AND TUMOR FORMATION

A plethora of developmental and stimulus-triggered responses are signaled *via* phytohormones. Auxin is involved in essentially all aspects of plant growth and development (Benjamins and Scheres, 2008; Ljung, 2013). Ethylene controls fruit ripening and plant senescence. It also mediates biotic stress and numerous other environmental responses (Merchante et al., 2013). Abscisic acid controls seed germination, stomatal movement

VBF AT1G56250
MMMLPEACIANILAFTSPADAFSSSEVSSVFRLAGDSDFVWEKFLPSDYKSLISQSTDHHWN
ISSKKEIYRCLCDSLLIDNARKLFKINKFSGKISYVLSARDISITHSDHASYWSWSNVSDSR
FSESAELIITDRLEIEGKIQTRVLSANTRYGAYLIVKVT**K**GAYG**L**D**L**VPAETSIKSKNGQIS
KSATYLCCLDEKKQQMKRLFYGNREERMAMTVEAVGGDGKRREPKCRDDGWMEIELGEFETR
EGEDDEVNMTLTEVKGYQLKGGILIDGIEVRPKT

FIGURE 1 | Arabidopsis VBF protein sequence. A peptide matching the consensus motif for MAPK interaction [R/K-x2-6-I/Lx(/L)], and a putative MAPK phosphorylation site are highlighted.

and is tightly connected with diverse abiotic and biotic stress responses (Nakashima and Yamaguchi-Shinozaki, 2013). Salicylic acid (SA), jasmonate and ethylene primarily act in biotic stress protection. There is ample evidence for the existence of substantial crosstalk between plant hormone defense pathways (De Torres Zabala et al., 2009; Robert-Seilaniantz et al., 2011a; Boatwright and Pajerowska-Mukhtar, 2013). These reports highlighted the importance of the plant's need to dynamically balance absolute and relative levels of phytohormones. A complex and comprehensive review on plant hormones and pathogen response was published very recently (Denance et al., 2013).

Agrobacteria largely shift the "hormone balance" in their infected hosts. This effect on endogenous growth regulators will ultimately lead to agrobacterium-induced tumor formation. An elaborate study provided an insight into *Agrobacterium*-induced phytohormonal changes, and it allowed the researchers to separate tumor-dependent and-independent host responses. Lee et al. (2009) examined the physiological changes and adaptations during tumor development provoked by an oncogenic strain (C58) or a disarmed derivate (GV3101), which only lacks the T-DNA but not the Vir factors (VirD2, VirE2, VirE3, VirF) (Holsters et al., 1980). The oncogenic strain was found to cause much stronger host responses than the disarmed strain. The authors monitored the kinetics of *Agrobacterium*-induced concentration changes of plant hormones, including SA, ethylene, jasmonic acid and indole-3-acetic acid (IAA, the most important auxin). In parallel, they assessed transcriptional changes, with a focus on hormone biosynthesis genes. At the early stage of infection, IAA and ethylene started to accumulate, while later, after T-DNA integration, primarily SA levels increased.

In the subsequent sections particular attention is given to the roles of auxin and SA in the agrobacterium/plant interaction.

AUXIN

Auxin-controlled processes are tightly linked to the intracellular auxin gradient. As reviewed recently (Korbei and Luschnig, 2011), this asymmetric hormone distribution arises from polar deployment and intracellular trafficking of auxin carriers. The stability and activity of these auxin transport proteins, in turn, is controlled by a number of post-translational modifications (Lofke et al., 2013; Rahman, 2013).

Upon its perception by a small number of F-box proteins, auxin rapidly induces the expression of two types of transcriptional regulators, encoded by the aux/IAA and ARF (auxin response factor) gene families. In fact, each physiological response might result from the combinatorial interaction between individual members of these two families (Kim et al., 1997). ARFs directly induce or repress the transcription of their target genes that contain auxin responsive elements in the promoter. By binding to their partner ARFs, aux/IAA proteins keep ARFs in an inactive state. In the presence of auxin, this inhibition is released by degradation of the aux/IAA protein. Recent comprehensive reviews on these principles of auxin responses can e.g., be found in (Korbei and Luschnig, 2011; Lofke et al., 2013; Rahman, 2013).

Several plant pathogens interfere with auxin signaling. This interference can occur at several levels. For instance, *Pseudomonas syringae* was shown to alter *Arabidopsis* auxin physiology *via* its type III effector protein AvrRpt2 (Cui et al., 2013). In this scenario, AvrRpt2 promotes auxin response by stimulating the turnover of aux/IAA proteins, the key negative transcriptional regulators in auxin signaling. Furthermore, some *P. syringae* strains were found to produce auxin themselves (Glickmann et al., 1998).

miR393 as regulator of auxin signaling and bactericide synthesis

Agrobacteria employ an impressive strategic repertoire to manipulate host auxin levels and signal transduction. First, auxin is one of the T-DNA products introduced by oncogenic *A. tumefaciens* (Weiler and Schroder, 1987). Because auxin stimulates cell growth and gall formation, T-DNA-based auxin biosynthesis serves the pathogen directly in remodeling its host. Attacked host plants, on the other hand, try to evade or at least restrict this remodeling. They employ a gene silencing-based mechanism involving production of a particular micro RNA. *miR393* targets three major auxin receptors (F-box proteins TIR1, AFB2, AFB3) and contributes to antibacterial resistance (Navarro et al., 2006). Increased levels of *miR393* were found in C58-infiltrated zones, but not in areas infiltrated with the disarmed control (Pruss et al., 2008). *miR393* appears to be a versatile instrument to keep pathogen invasion in check. *miR393* expression is induced by the PAMP-derived peptide flg22 (Robert-Seilaniantz et al., 2011b). Notably, flagellin sequences from *Agrobacterium* (as well as *Rhizobium*) are exceptionally divergent from this PTI-triggering conserved 22-amino-acid motif (Felix et al., 1999). *Arabidopsis* plants overexpressing *miR393* have a higher resistance to biotrophic pathogens (Robert-Seilaniantz et al., 2011b). The authors showed that miR393/auxin-related resistance is due to interference with another hormone pathway, SA. Generally, auxin and SA act as negative and positive regulators of plant defense, respectively (Denance et al., 2013). These opposing effects are largely due to the repressive effect of auxin on SA levels and signaling, although auxin also represses defense in an SA-pathway-independent manner (Kazan and Manners, 2009; Mutka et al., 2013). As proposed by (Robert-Seilaniantz et al., 2011b), *miR393* represses auxin signaling and thereby prevents auxin from antagonizing SA signaling. Infection studies with auxin signaling mutants furthermore indicated that the auxin-regulated transcription factor ARF9 induces accumulation of camalexin, but represses accumulation of glucosinolate (Robert-Seilaniantz et al., 2011b). Compared to camalexin, glucosinolates are considered more effective protectants against biotrophic invaders. Therefore, *miR393*-related stabilization of ARF9 in inactive complexes may present a means to shift camalexin toward glucosinolate production. Whether *miR393* synthesis upon agrobacterial attack "only" serves to repress auxin-related callus growth or whether it has additional functions in the defense remains to be established. As noticed recently, naturally high contents of glucosinolates *per se* are no obstacle to transformation. *Tropaeolum majus*, a glucosinolate-rich plant of the order Brassicales, is transformed by agro-infiltration of leaves (GV3101, disarmed strain) to high efficiency (Pitzschke, 2013b).

Besides camalexin and glucosinolates, plants produce various other secondary metabolites to defend themselves against biotrophic pathogens. Agrobacteria can defy at least one major

group of bactericides. Several phenolic compounds are enzymatically converted by the agrobacterial protein VirH; and a *virH2* mutant was found to be more susceptible to growth inhibition by these substances (Brencic et al., 2004).

One member of the bactericidal polyamines deserves special attention, putrescine. A recent study (Kim et al., 2013) documented that putrescine accumulation is controlled by MAPK signaling involving MPK3 and MPK6. In *Arabidopsis*, *ADC* genes, encoding key enzymes for putrescine biosynthesis, are induced by infection with *P. syringae*. *adc*-deficient mutants are impaired in *P. syringae*-induced *PR1* expression. Disease susceptibility in these mutants can be recovered by exogenous putrescine. *ADC* transcript and putrescine levels are elevated in transgenic *Arabidopsis* plants expressing a constitutively active MAPK3/6 regulatory kinase in the wild-type background. In the *mpk3* or *mpk6* mutant background, however, this effect is largely reduced. An earlier study in tobacco had shown that plants accumulate putrescine derivatives also to combat agrobacterial infection. Auxin likely is involved in this response (Galis et al., 2004). It remains elusive whether *P. syringae*- and *A. tumefaciens*-induced putrescine synthesis are mediated by a common MPK3/MPK6 signaling pathway.

SALICYLIC ACID

Plants produce SA in response to pathogen attack or microbial elicitors. Mutants with constitutively elevated SA levels are generally more resistant toward biotrophic pathogens (Boatwright and Pajerowska-Mukhtar, 2013). Previously, SA was shown to attenuate *A. tumefaciens*-induced tumors (Yuan et al., 2007; Anand et al., 2008). Additional experimental data documented that the antagonism of auxin to SA responses (see above) is reciprocal. SA represses expression of several auxin-related genes. Moreover, by stabilizing Aux/IAA proteins, SA inhibits auxin responses (Wang et al., 2007). Elevated SA levels were observed in *Arabidopsis* stalks during later stages (>6 dpi) of agrobacterial infection, indicating defense activation. This response was provoked by both the oncogenic (C58) and the disarmed strain (GV3101) (Lee et al., 2009). However, *Arabidopsis* stems infected with C58 contained higher levels of SA, which further increased in 35-day-old tumors. The authors (Lee et al., 2009) also found that high SA levels in mutant plants (*npr1, cpr5*) prevented tumor development, while low levels promoted it (*nahG, eds1, pad4*). One specific role of SA in the *Agrobacterium*-plant interaction is its inhibitory effect on *vir* gene expression, which is accomplished by shut-down of the *vir* regulon (Yuan et al., 2007). What is more, SA indirectly interferes with pathogen multiplication by activating the expression of quormone-degrading enzymes (Yuan et al., 2007). In summary, SA appears to counteract agrobacterial invasion at several levels. It represses *vir* regulon genes (Yuan et al., 2007; Anand et al., 2008) and induces quormone-quenching genes (Yuan et al., 2007). Furthermore, SA antagonises auxin responses (Wang et al., 2007) and acts as antimicrobial agent (Gershon and Parmegiani, 1962). Interestingly, SA accumulation in *Agrobacterium*-infected *Arabidopsis* stalks was not accompanied by the induction of SA-responsive pathogenesis-related genes (3 h, 6 d, 35 dpi tested) (Lee et al., 2009). This effect is different from what is known from other plant-pathogen interactions and from pharmacological studies. Generally, in pathogen-infected plants, elevated SA synthesis triggers PR gene expression. Likewise, *PR* genes are induced by exogenous application of SA or its analog BTH (Lawton et al., 1996). Despite the lack of *PR* gene induction, SA does play a role in agrobacterial infection, as evidenced by the altered tumor size in SA-deficient/accumulating mutants (Yuan et al., 2007; Lee et al., 2009). Apparently, *A. tumefaciens* cannot prevent SA accumulation, but it can suppress some SA-related defense responses. As suggested by (Lee et al., 2009), abnormally high SA levels in the host may have overextended the agrobacterial control machinery.

A recent comprehensive survey of *Arabidopsis* transcriptome profiling data (including diverse stress treatments and biotic stress signaling mutants *sid2, npr1, coi1, ein2*) provided a deeper insight into the SA/PR gene relation (Gruner et al., 2013). In *P syringae*-treated *Arabidopsis, PR1* expression fully depends on (isochorismate-synthase1) ICS1-mediated SA biosynthesis and on (non-expressor of PR1) NPR1-mediated downstream signaling. *PR1* is not induced by exogenous hydrogen peroxide, abscisic acid or flg22, and it is independent of jasmonic acid and ethylene signaling (Gruner et al., 2013).

The small set of genes induced by *Agrobacterium* (strain C58: 35genes; strain GV3101: 28 genes) (Lee et al., 2009) is in striking contrast to the high number (948) of elicitor-responsive (EF-Tu-derived peptide elf26) transcripts. Agrobacteria clearly dampen host responses (Lee et al., 2009). This dampening is not restricted to the transcriptional level. Histological analysis (using diaminobenzidine) revealed that agrobacteria efficiently repressed H_2O_2 accumulation in wounded stalks over several days post-infection. The agrobacterial interference with the host's redox-regulatory machinery is also mirrored by the differential expression of several oxidative-stress-related genes (Ditt et al., 2001; Veena Jiang et al., 2003; Lee et al., 2009). By repressing H_2O_2 production agrobacteria may also avoid activation of ROS-dependent defense genes. Given the known sensitivity of any living cell to reactive oxygen species (ROS), the blocking of accumulation appears an agrobacterial strategy to protect both itself and its living food source, i.e., the host.

PLANT ATTEMPTS TO REPRESS ONCOGENE EXPRESSION

Plants exhibit an admirable perseverance in their battle against microbial manipulation. Even *after* unsuccessful attempts to escape *Agrobacterium*-induced genetic re-programming, the host cell does not surrender. Instead, transformed cells employ gene silencing mechanisms to limit the levels of T-DNA-derived transcripts. Evidence for the involvement of post-transcriptional gene silencing had been provided in a pioneering work by Dunoyer et al. (2006). Small interfering RNAs (siRNAs) directed against T-DNA oncogenes (*tryptophan 2-monooxygenase* and *agropine synthase*) were detected in *Nicotiana benthamiana* leaves 3 days after infiltration with virulent agrobacteria. Additional experiments in *Arabidopsis* further stressed the importance of gene silencing as a disease-limiting strategy. RNA interference-deficient mutant plants (*rdr6*, lacking a RNA-dependent RNA polymerase) were found to be hypersusceptible to agrobacterial infection, as evidenced by extensive tumor formation (Dunoyer et al., 2006). The researchers also conducted infection studies in leaves and stems of *Nicotiana bethamiana* carrying a post-transcriptionally-silenced

reporter gene (green fluorescent protein, GFP). This approach enabled them to show that the siRNA protection strategy against T-DNA genes is efficient only at early stages of infection: Strong green fluorescence, high *GFP* mRNA concentrations and low siRNA concentrations were detected specifically in young tumors. Later in the infection process, the pathogen takes command. By specifically inhibiting siRNA synthesis, agrobacteria induce an anti-silencing state—thereby ensuring oncogene expression and tumor maturation (Dunoyer et al., 2006).

A more recent study furthermore documented that DNA methylation also plays a critical role in the regulation of T-DNA transcript levels (Gohlke et al., 2013). The authors compared the methylation pattern of mock- and *Agrobacterium*-inoculated *Arabidopsis* inflorescence stalks on a genome-wide level. Four-week-old tumors, arising from inoculation with the oncogenic *A. tumefaciens* strain C58 contained a globally hypermethylated genome. Intriguingly, a specifically low degree of methylation was observed in T-DNA-derived oncogenes (*Ipt IaaH, IaaM*). Data obtained from experiments with DNA methylation mutants lead to the conclusion that crown gall formation and oncogene expression correlate with the unmethylated state and, consequently that hypermethylation is a strategy to inhibit plant tumor growth.

RECALCITRANCE TO *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Agrobacterium naturally has a wide host range in plants, primarily dicot species. Driven by the demand for higher yields and improved stress tolerance the accessibility to transformation has become a prime issue in crop science. Despite intensive research it is still poorly understood why some plant species can be transformed easily, while others are recalcitrant to *Agrobacterium*-mediated transformation. Transformation methods of model plants and important crop species are frequently updated, documenting the striving for simpler, more robust and more efficient protocols (reviewed in e.g., Pitzschke, 2013a). These protocols primarily focus on optimizing the conditions of *Agrobacterium*—explant co-incubation. Here, duration, light conditions and the concentration of supplemented acetosyringone and plant hormones are key parameters.

One central message emerges from enumerable transformation studies. The outcome of co-cultivation is primarily determined by the timing and intensity at which host defense responses are activated. Understanding the molecular language of the plant—*Agrobacterium* dialogue is therefore of substantial interest both to basic research and agricultural science.

Studies that compare different cultivars of the same species are particularly informative, and one such study shall be mentioned here. Transformation efficiencies between rice cultivars differ greatly. The indica variety lags far behind the japonica cultivars. A comparative study of the two cultivars in transient and stable transformation assays revealed that the lower transformation efficiency in indica rice was mainly due to less-efficient T-DNA integration into the host genome (Tie et al., 2012). Microarray analyses (1, 6, 12, and 24 h post-infection) revealed major differences in the *Agrobacterium*-induced changes in transcriptome profiles of the two cultivars. These differences were most pronounced at the early stages of infection (within the first 6 h). The authors observed an overall stronger response in the indica cultivar (Zs), with several genes being repressed, and they postulated that some of these genes may be required for the transformation process. From this study, one may conclude that (1) although T-DNA integration represents a late step in the transformation process, the "decision" that leads to failure or success is made early. This decision is made in a narrow time window, since many Zs-specific transcripts are repressed only transiently (at the 1 OR 6 h time-point only). (2) Agrobacteria manage to actively prevent repression of integration-assisting genes in the susceptible cultivar. Among others, gene ontology (GO) annotations "stress-responsive" and "lipid transport" are overrepresented in the group of indica-specific transcripts. The lower T-DNA integration efficiency in the indica cultivar may also be attributable to the specific repression of genes related to DNA damage repair. This assumption is in good agreement with the importance of the host DNA repair machinery in T-DNA integration reported earlier (Li et al., 2005b; Citovsky et al., 2007).

THE ROLE OF REACTIVE OXYGEN SPECIES IN RECALCITRANCE

A promising approach for converting hitherto non-transformable plant species is to determine the basis of this recalcitrance. Poor transformation rates can have entirely different reasons. As outlined above, bacterial and host factors contribute and need to be well-balanced. In pro- and eukaryotic organisms alike, ROS play important roles in the transmission of information. ROS- and MAPK signaling in plants is strongly inter-connected (Pitzschke and Hirt, 2009; Meng and Zhang, 2013). Because high ROS levels trigger cell death, their targeted stress-dependent production serves host organisms to restrict pathogen spread. Inappropriate ROS concentration or distribution can therefore be a barrier to successful transformation. For instance, recalcitrance in *Hypericum perforatum* (St. John's wart; medicinal herb), cell cultures was found to be due to an early oxidative burst, which killed 99% of the co-cultivated agrobacteria within 12 h of infection. Interestingly, the oxidative burst only affected agrobacterial viability but did not trigger plant apopotosis (Franklin et al., 2008). Antimicrobial factors likely also have a negative effect on transformation efficiency and agrobacterial viability in *H. perforatum*. A 12-fold increase in xanthone levels was observed in *H. perforatum* cells 1 day after infection. Increased xanthone levels correlated with an elevated antimicrobial and antioxidative competence. On the basis of these observations one may conclude that the plant can divert its antioxidant capacity to prevent itself, but not the invader, from oxidative damage.

One known agrobacterial factor determining oxidative resistance levels is the ferric uptake regulator Fur. A *fur*-deficient mutant was found to be hypersensitive to H_2O_2 and to have reduced catalase activity (a H_2O_2-detoxifying enzyme). Agrobacterial *fur* mutants were also compromized in tumorigenesis on tobacco leaves (Kitphati et al., 2007). Similarly, *A. tumefaciens* mutants in the *RirA* gene (*rhizobial iron regulator*; repressor of iron uptake) exhibited a peroxide-sensitive phenotype and were impaired in tumor formation on tobacco. In addition, induction of the virulence genes *virB* and *virE* was reduced in *rirA* mutants (Ngok-Ngam et al., 2009). Furthermore, *A. tumefaciens* mutants affected in oxidative stress tolerance

Agrobacterial strategies

Turn host defense steps into advantage
Benefit from stress-induced MAPK pathway to trigger activation of the VIP1 vehicle

Abuse host molecules
Hijack VIP1 for nuclear import of T-complex
Make use of VIP1 properties (chromatin affinity, histone interaction) to target the T-DNA to the host´s chromatin

Repress defense gene expression

Protect transferred factors from destruction
Mask T-DNA with VirE2 proteins to avoid nucleolytic cleavage
VirD5 stabilises (intrinsically unstable) VirF

Ensure oncogene expression to drive tumor formation
Inhibit synthesis of oncogene-targeting siRNAs
Repress methylation of T-DNA-encoded oncogenes

Counteract host-derived killer substances
VirH detoxifies (bacteriostatic) phenolics
Inhibit ROS production

Force plants to co-operate
Introduce oncogenes and trigger auxin production
→ to promote callus growth
→ as defense-repressing agent

Functional mimicry of host proteins
VirE3 mimics VIP1 in the T-complex nuclear import function
VirF mimics VBF as part of the SCF proteaasome machinery
→ to facilitate release of T-DNA for integration into the plant genome
→ to prevent VIP1-induced defense gene expression

Plant strategies

Sense microbial elicitors as "non-self" to raise the alarm
Elevate expression of receptor proteins

MAPK cascade signallng to transduce AND amplify the stress information
→ to activate and translocate stress-responsive transcription factors, incl. VIP1
→ to trigger putrescine synthesis

Express defense-related genes

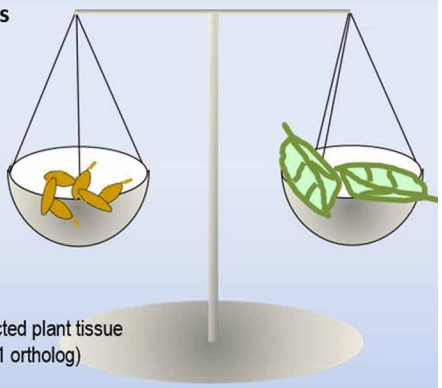
Degrade bacterial factors
Nucleolytic cleavage of T-DNA
SCF-mediated proteasomal degradation of VirD, VirE2

Prevent or repress oncogene expression
Express *miR393* to shut down auxin receptors
Produce siRNAs for post-transcriptional silencing of oncogenes
DNA methylation to repress gene expression in tumors

Kill the invader
Produce antimicrobial compounds
Produce reactive oxygen species

Salicylic acid as as multi-purpose weapon
→ SA attenuates tumorigenesis
→ SA shuts down expression of *vir* regulon
→ SA activates quormone-quenching genes
→ SA has antimicrobial activity *in vitro*
→ SA blocks auxin signaling by stabilising aux/IAA proteins

Biotechnological strategies to improve transformation efficiencies



1. Reported strategies
Use ornithine lipid-deficient agrobacterial strain (potato)
VIP1 overexpression (tobacco; *Arabidopsis*)
Myo-inositol-free medium and cold pre-treatment (perennial ryegrass)
Expression of *P. syringae* effector *AvrPto* prior to agroinfiltration (*Arabidopsis*, transient)
Add L-glutamine to protect agrobacteria from bactericidal polyphenols (tea)

2. Promising strategies (hypothetical)

Manipulate the microbe
Use alternatives to acetosyringone as pre-infection stimulant
Use *A. tumefaciens* strain with higher ROS-tolerance to enhance bacterial viability in infected plant tissue
Develop agrobacterial strains with hyperactive VirE3 (esp. for plant species lacking a VIP1 ortholog)

Manipulate the host
Pre-expose plants to a mild stress ("priming") to enhance agrobacterium-induced MAPK activation and thus VIP1 nuclear translocation
→ Chance: more efficient nuclear import of the T-complex / → Risk: stronger defense response

Transient repression of MAPK activation (pharmacologically) to minimize defense responses

Co-transform an optimized variant of VIP1, with the following characteristics:
→ cyto-nuclear translocation - (like wild-type VIP1)
→ full-length (needed for stable transformation) – (like wild-type VIP1)
→ manipulated DNA binding site (e.g. Lys212Thr replacement) to avoid stress gene induction

Manipulate VBF activity/kinetics to unmask the T-DNA *after* nuclear T-complex import

FIGURE 2 | The molecular arms race between host and microbe in Agrobacterium-mediated plant transformation. The activities of both partners need to be well-balanced for successful transformation. Numbers in brackets refer to the corresponding sections in the manuscript.

have been characterized, e.g., *mbfA* (membrane-bound ferritin) (Ruangkiattikul et al., 2012).

The above examples document the vital importance of ROS balancing for both invader and invaded cell. It is tempting to speculate that, the reduced tumor formation in the *fur*/tobacco and *rirA*/tobacco interaction is caused by the poor viability of agrobacteria in a ROS-rich environment of infected host cells. Such a scenario would be in analogy to the situation in *H. perforatum* (Franklin et al., 2008), At this point, concerted efforts of microbiologists and plant biologists are needed to systematically define the proportion and identity of ROS-related agrobacterial factors playing a limiting role in plant transformation.

Another recalcitrant species of agricultural importance that has attracted attention is grapevine (*Vitis vinifera*). Proteomic profiling in grapevine calli grown in the absence or presence of agrobacteria allowed identification of 38 differentially expressed proteins (Zhao et al., 2011). ROS scavenging enyzmes were down-regulated in co-cultivated cells (ascorbate peroxidase, tocopherol cyclase). The authors concluded that low transformation rates and extensive necrosis in *A. tumefaciens*-treated grapevine derive from an impaired ROS scavenging system and an over-activation of apoptotic/hypersensitive response pathways.

APPROACHES TO OVERCOME RECALCITRANCE

Because strong and prolonged host defense responses generally correlate with reduced transformation success (**Figure 2**), external attenuation of these responses may be a means to improve transformation efficiencies. The experimental approaches that can be taken to manipulate host defenses are as manifold as the defense strategies themselves. The problem can be tackled from different sides: (1) by using modified agrobacterial strains that elicit a weaker defense, as e.g., shown in a study on potato (Vences-Guzman et al., 2013); (2) by modifying the composition of plant media and/or growth conditions to keep defense levels low, e.g., Zhang et al. (2013a); (3) by transient and targeted manipulation of the plants non-self-recognition machinery (see below); (4) by counteracting the effect of antimicrobial substances. This strategy proved successful in tea, where L-glutamine was found to overcome the bactericidity of polyphenols (Sandal et al., 2007).

In an innovative study Tsuda and colleagues demonstrated how detailed knowledge on plant-microbe interactions can be employed for successful transformation. *AvrPto* encodes an effector protein from the bacterial plant pathogen *Pseudomonas syringae*. The protein suppresses plant immunity by interfering with plant immune receptors. The *AvrPto* gene was placed under the control of a dexamethasone-inducible promoter. In transgenic *Arabidopsis* plants carrying the inducible construct, dexamethasone pre-treatment largely improved transformation in agro-infiltrated leaves (Tsuda et al., 2012).

An entirely different "pre-treatment strategy" proved successful in perennial ryegrass (*Lolium perenne* L.) (Zhang et al., 2013a). Stable transformants were obtained at an impressively high rate (84%), and 60% of the transgenic calli were regenerated into green plantlets. This was achieved by combining two strategies, while either treatment alone had little effect (10–20% transformation efficiency): (1) Myo-inositol, a component of many standard media, was removed from the callus culture medium. (2) A cold shock pre-treatment was applied prior to agrobacterial infection.

Myo-inositol levels in plants are primarily controlled by a specific oxygenase, which catalyses the first step in the conversion of this sugar into plant cell wall polysaccharides (Endres and Tenhaken, 2009). The basis of the effect observed by Zhang and colleagues is still largely elusive. It appears that myo-inositol acts in different ways and at multiple levels: omission of myo-inositol promoted *Agrobacterium* binding to the cell surface. It also repressed H_2O_2 production in infected tissue. One indirect consequence of ROS production, callus browning, could furthermore be suppressed when including the cold pre-treatment (Zhang et al., 2013a). Worthwhile questions are: Does growth of cold-pre-treated calli on myo-inositol-free medium alter cell wall composition to support agrobacterial attraction, invasion and/or survival in *L. perenne* cells? If so, what is the critical difference? Can such favorable cell wall characteristics be imitated to facilitate agrobacterial transformation of other recalcitrant species?

CONCLUSIONS

The molecular battle between agrobacteria and plants is impressive, instructive and challenging (**Figure 2**). Impressive, because the arms race takes so many forms. Instructive, because discoveries from *Agrobacterium*-plant interaction studies may drive progress in other fields of microbe-host association research. Challenging, because the external conditions that permit or prohibit transformation including transgene expression are diverse, and the balance needs to be determined empirically. The current state of research provides substantial breeding ground for plant scientists to search for this balance in their favorite species in a more targeted manner.

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Plant responses to *Agrobacterium tumefaciens* and crown gall development

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Agrobacterium tumefaciens causes crown gall disease on various plant species by introducing its T-DNA into the genome. Therefore, *Agrobacterium* has been extensively studied both as a pathogen and an important biotechnological tool. The infection process involves the transfer of T-DNA and virulence proteins into the plant cell. At that time the gene expression patterns of host plants differ depending on the *Agrobacterium* strain, plant species and cell-type used. Later on, integration of the T-DNA into the plant host genome, expression of the encoded oncogenes, and increase in phytohormone levels induce a fundamental reprogramming of the transformed cells. This results in their proliferation and finally formation of plant tumors. The process of reprogramming is accompanied by altered gene expression, morphology and metabolism. In addition to changes in the transcriptome and metabolome, further genome-wide ("omic") approaches have recently deepened our understanding of the genetic and epigenetic basis of crown gall tumor formation. This review summarizes the current knowledge about plant responses in the course of tumor development. Special emphasis is placed on the connection between epigenetic, transcriptomic, metabolomic, and morphological changes in the developing tumor. These changes not only result in abnormally proliferating host cells with a heterotrophic and transport-dependent metabolism, but also cause differentiation and serve as mechanisms to balance pathogen defense and adapt to abiotic stress conditions, thereby allowing the coexistence of the crown gall and host plant.

Keywords: plant defenses, phytohormones, morphological adaptions, metabolomic changes, epigenetics

INTRODUCTION

Agrobacterium tumefaciens causes crown gall disease on a wide range of host species by transferring and integrating a part of its own DNA, the T-DNA, into the plant genome (Chilton et al., 1977). This unique mode of action has also made the bacterium an important tool in plant breeding. After attachment of *Agrobacterium* to plant cells and expression of multiple virulence (vir) genes, several effector proteins, together with T-DNA, are transported into the plant cell by a type-IV-secretion system (Thompson et al., 1988; Ward et al., 1988, 2002; Kuldau et al., 1990; Shirasu et al., 1990; Beijersbergen et al., 1994). Plant factors assist with T-DNA integration into the plant genome (Gelvin, 2000; Mysore et al., 2000; Tzfira et al., 2004; Magori and Citovsky, 2012). After integration, expression of the T-DNA-encoded oncogenes iaaH, iaaM, and ipt induces biosynthesis of auxin and cytokinin (Morris, 1986; Binns and Costantino, 1998). Increased levels of these phytohormones result in enhanced proliferation and formation of crown galls. Despite the transfer of bacterial proteins into the plant cell, most *Agrobacterium* strains do not elicit a hypersensitive response (HR), which is associated with rapid and localized death of cells (Staskawicz et al., 1995). Such a response often occurs when plants are challenged by bacterial pathogens and serves to restrict the growth and spread of pathogens to other parts of the plant. Accordingly, no systemic, broad-spectrum resistance response throughout the plant (systemic acquired resistance, SAR) is induced. Within the first several hours of co-cultivation, pathogen defense response pathways are activated more or less strongly depending on the plant system and *Agrobacterium* genotype used for infection (Ditt et al., 2001, 2006; Veena et al., 2003; Lee et al., 2009). Defense responses become stronger during crown gall development. Furthermore, the physiological behavior of the transformed cells changes drastically. In contrast to the articles which focus on the molecular mechanism utilized by the bacterium to transform the plant cell, here we review the latest findings on the responses of the host plant and in the crown gall to *Agrobacterium* infection. Special attention is paid to the role of gene expression regulation, phytohormones, and metabolism.

HOST RESPONSES TO *Agrobacterium tumefaciens* BEFORE T-DNA TRANSFER

PATHOGEN DEFENSE

The recognition of microbial pathogens plays a central role in the induction of active defense responses in plants. The conserved flagellin peptide flg22 is recognized by the receptor kinase FLS2 and induces the expression of numerous defense-related genes to trigger resistance to pathogenic bacteria (Gómez-Gómez et al., 1999, 2001; Zipfel et al., 2004; Chinchilla et al., 2006). However, the genus *Agrobacterium* fails to induce this type of rapid and general defense response because of an exceptional divergence in

the N-terminal conserved domain of flagellin (Felix et al., 1999). When comparing early gene expression changes after infection with the virulent *Agrobacterium* strain C58 with application of the bacterial peptide elf26 (after 1 and 3 h, respectively), dampening of host responses becomes apparent with *Agrobacterium* treatment. The elf26 peptide, a highly conserved motif of one of the most abundant proteins in microbes recognized by the receptor kinase EFR, is a fragment of the elongation factor Tu (EF-Tu). EF-Tu triggers innate immunity responses associated with disease resistance in *Arabidopsis* (Kunze et al., 2004). While treatment with pure elf26 induces gene expression changes of 948 *Arabidopsis* genes (Zipfel et al., 2006), only 35 genes are induced after infection with the virulent *Agrobacterium* strain C58, suggesting that the bacterium somehow neutralizes the response to elf26 by the host plant (Lee et al., 2009). It should be mentioned that the *Arabidopsis* ecotype and age (seedling vs. adult stalk) used in the studies may also account for some of the differences in defense response.

Concerning the transcriptional activation of genes involved in early plant defense responses, several studies have come to different conclusions. *Ageratum conyzoides* cell cultures showed differential expression of defense genes as early as 24 h post infection with a non-oncogenic hypervirulent *Agrobacterium* strain (Ditt et al., 2001). In tobacco suspension cultures infected with different *Agrobacterium* strains, transcription of defense genes increased within 3–6 h, but started to decrease with the onset of T-DNA-transfer (Veena et al., 2003). A study using suspension-cultured cells of *Arabidopsis* did not show changes in transcript levels within 4 to 24 h but activation of defense genes 48 h after infection (Ditt et al., 2006). When agrobacteria are inoculated at the base of wounded *Arabidopsis* stems just very few defense genes are activated 3 h post infection compared to uninfected wounded stems (Lee et al., 2009). In contrast to cell cultures, the latter experimental setup does neither require phytohormone pre-treatment nor virulence gene induction prior to infection. Phytohormone pre-treatment of the cell culture systems of the earlier studies may alter host cell defense responses. Thus, discrepancies between these studies probably result from the different plant inoculation systems used. Nevertheless, agrobacteria can abuse host defense responses for T-DNA delivery. The mitogen-activated protein kinase MPK3 phosphorylates the *Arabidopsis* VIP1 protein, inducing VIP1 relocalization from the cytoplasm to the nucleus. Nuclear localization of VIP1 increases T-DNA transfer and transformation efficiency (Djamei et al., 2007).

PHYTOHORMONES

Agrobacteria produce auxin and cytokinin themselves in order to modulate plant responses (**Figure 1A**). These phytohormones have been determined in the cells as well as cultivation medium (Morris, 1986). It was postulated that biosynthesis of the phytohormones is catalyzed by enzymes of the T-DNA encoded oncogenes, as transcripts and proteins of these genes were detected in agrobacterial cells (Schröder et al., 1983; Janssens et al., 1984). Pronounced amounts of auxin have been determined in the virulent *Agrobacterium* strain C58 and at lower levels also in plasmidless and T-DNA depleted strains (Liu and Kado, 1979; Kutáèek and Rovenská, 1991). More recent data have confirmed the latter results (Lee et al., 2009). The finding that a strain without a Ti-plasmid still can make auxin implies localization of genes also outside of the Ti-plasmid. However, this assumption is not supported by sequencing data for strain C58 (Wood et al., 2001). Genes known to be involved in auxin biosynthesis seem to be encoded only by the T-DNA of the Ti-plasmid. Recently, these authors determined the presence of iaaH and iaaM transcripts by PCR in *Agrobacterium* cells of strain C58 and confirmed the earlier findings. It remains to be proven whether these genes are responsible for auxin production or if auxin is synthesized by a different mechanism in *Agrobacterium* cells. The mechanism for cytokinin biosynthesis by agrobacteria is far better understood. In nopaline utilizing *Agrobacterium* strains cytokinin is produced in high amounts by the Ti-plasmid encoded *trans*-zeatin synthesizing (tzs) enzyme of which the gene is located in the vir regulon (Akiyoshi et al., 1985, 1987; Hwang et al., 2010). A substantial smaller source for cytokinin production is isopentenylated transfer RNA (tRNA) catalyzed by the chromosomal-encoded enzyme tRNA:isopentenyltransferase (MiaA) present in all *Agrobacterium* strains (Gray et al., 1996).

Earlier studies have shown that pre-treatment of explants with either auxin alone or both auxin and cytokinin increase T-DNA transfer efficiency and stable transformation (Krens et al., 1996; Chateau et al., 2000) as well as crown gall growth (Gafni et al., 1995). In this respect, *Agrobacterium* produced phytohormones play a role at very early time points of infection (**Figure 1A**), before T-DNA-encoded enzymes catalyze synthesis of cytokinin and auxin in the transformed host cell. Concerning the mechanism causing an increase in susceptibility it was speculated that phytohormones induce plant cell division and that the cell cycle phase influences agrobacterial attachment and stable transformation. It seems likely that phytohormone-mediated modification of the physiological state of the cell increases competence for T-DNA transformation and integration. More recent investigations addressed the question about the molecular mechanism and the signaling pathways by which these phytohormones influence host cell susceptibility. Transcriptome microarray data from 3 h after inoculation of *Agrobacterium* strain C58 into *Arabidopsis* stems revealed that the genes known to be involved in phytohormone-dependent signaling are not induced in host cells at this very early time point of infection before transfer of the T-DNA (Lee et al., 2009). It has been shown that indole-3-acetic acid (IAA) has an impact on agrobacterial virulence by inhibiting vir gene induction and growth of agrobacteria (Liu and Nester, 2006). However, this effect was observed with relatively high concentrations of auxin (25–250 μM). In *Agrobacterium* cells the total (free and conjugated) IAA content is 0.3 ± 0.1 μM and in *Arabidopsis* stems 3 h after inoculation with strain C58 it is 2.1 ± 1 μM, whereas in *Arabidopsis* crown galls the content is ca. 10 times higher (17.3 ± 8.8 μM) due to the expression of the T-DNA encoded iaaH and iaaM genes and their enzyme activity (own data and Thomashow et al., 1986). Application of 1 μM IAA, a concentration found in wounded and uninfected *Arabidopsis* stems (0.8 ± 0.2 μM), stimulated growth of *Agrobacterium* cells, whereas growth stimulation vanished at

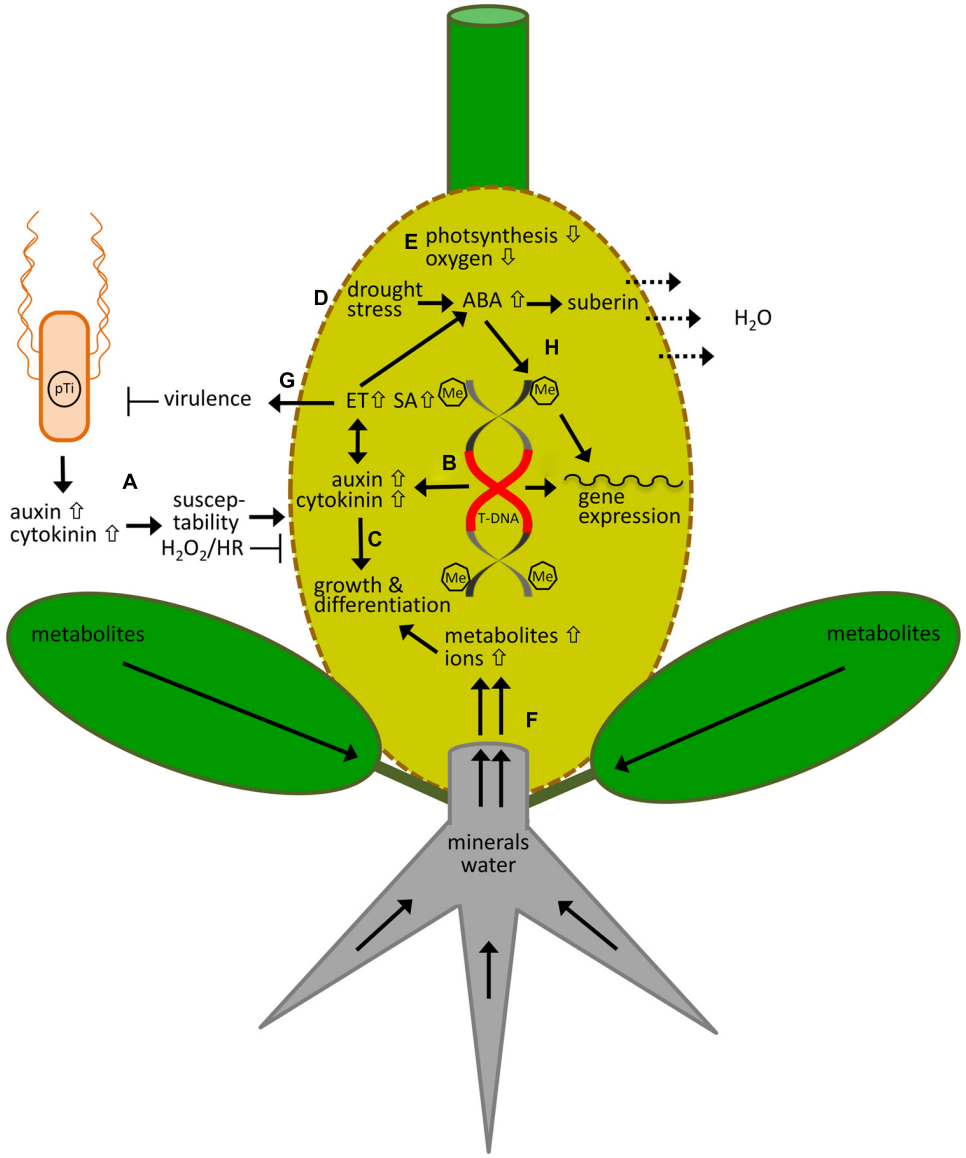

FIGURE 1 | Responses of the model plant *Arabidopsis thaliana* to *Agrobacterium tumefaciens* and crown gall development. (A) Virulent (pTi) agrobacteria cells themselves produce and release cytokinin and auxin, which increase host susceptibility and inhibit hydrogen peroxide production (H_2O_2) and hypersensitive response (HR) at initiation of infection. **(B)** After integration of the bacterial T-DNA into the plant genome, cytokinin and auxin is synthesized by T-DNA encoded enzymes and accumulate inside the tumor. **(C)** This causes massive changes in the gene expression pattern, resulting in metabolomic and morphological adaptations that are necessary for tumor growth and differentiation. **(D)** Loss of water is minimized by drought stress protecting mechanism, which causes an increase in the levels of the stress hormone ABA, and ABA-dependent suberization of cells to prevent water loss. Evaporation of water (H_2O) from the disrupted crown gall surface drives the flow of water and minerals into crown galls. **(E)** Because photosynthesis is down-regulate the oxygen levels are low, the tumor produces C and N compounds heterotrophically and gains energy mainly anaerobically by alcoholic fermentation. **(F)** Consequently the developing tumor becomes a metabolic sink for the host plant, which accumulates metabolites produced by source leaves and minerals taken up by the roots. **(G)** Auxin and cytokinin also cause an increase in ethylen (ET) which together with salicylic acid (SA) inhibits agrobacterial virulence. **(H)** ABA also induces DNA methylation of the plant genome, thereby regulating gene expression of drought-stress responsive genes. Overall, the crown gall genome becomes hypermethylated (Me) after *Agrobacterium* infection and possibly contributes to the strong changes in gene expression during tumor growth. The oncogenes of the T-DNA remain unaffected by methylation of the plant genome.

10 μM and higher IAA concentrations (personal communication, J. Ludwig-Mueller, Technical University Dresden, Germany). It is known that the effect of auxin is strongly dose dependent with a growth promoting effect at low concentrations and an inhibitory effect at high concentrations, which slightly varies dependent on the plant and tissue type. One may speculate that at initiation of infection, the relatively low auxin levels of agrobacterial cells and/or of wounded plant tissue stimulate growth of agrobacteria, whereas the higher concentrations produced in the crown gall inhibit virulence as well as growth of *Agrobacterium*. Such an

antagonistic auxin effect would promote transformation of the host cell at the beginning of the infection process and inhibit agrobacterial virulence and growth to prevent further transformation events in developing crown galls. In contrast to auxin, the role of cytokinin signaling in plant susceptibility is well known. Recently, it has been shown that cytokinin secreted by *Agrobacterium* controls virulence via bacterial cell growth and vir gene expression at early stages of the infection process (Hwang et al., 2010). Some, but not all plant species showed a cytokinin-dependent increase in transformation efficiency (Hwang et al., 2013). *Agrobacterium*-derived cytokinin not only acts on bacterial physiology but also influences host gene expression via the classical cytokinin-dependent signaling pathway including cytokinin receptors and the phosphotransfer cascade (Sardesai et al., 2013). Activation of this signaling cascade through agrobacterial-derived cytokinin results in inhibition of gene expression of the *Arabidopsis* MYB family transcription factor, MTF1 (Sardesai et al., 2013). MTF1 turned out to be a negative regulator of transformation susceptibility by blocking expression of the integrin-like protein At14a, a plant membrane receptor. At14A serves as anchor points for bacterial attachment at the host cell surface. Thus, at early stages of infection agrobacterial auxin and cytokinin manipulates plant phytohormone signaling pathways to prepare the host cell for transformation.

In addition to auxin and cytokinin, plant defense signaling involves a network of interconnected pathways in which salicylic acid (SA) and jasmonic acid (JA) together with ethylene (ET) function as essential signaling molecules (Kunkel and Brooks, 2002). Exogenous application of the plant defense molecule SA to *Agrobacterium* cells inhibited expression of vir genes including tzs, bacterial growth, bacterial attachment to plant cells and virulence (Yuan et al., 2007; Anand et al., 2008). However, at initiation of infection (3 h post infection) neither SA nor JA levels nor the genes of these signaling pathways are elevated in *Agrobacterium*-infected *Arabidopsis* tissues (Lee et al., 2009). At this time point only the level of 1-amino-cyclopropane-1-carboxylic acid (ACC), an ET precursor, is increased in the presence of both virulent and disarmed *Agrobacterium* strains, but not expression of marker genes of the ET-dependent defense-signaling pathway. Inoculation of melon (*Cucumis melo*) explants with *Agrobacterium* also increases ET production (Ezura et al., 2000). ET is known to trigger plant auxin production due to increased expression of plant genes involved in auxin biosynthesis (Stepanova et al., 2005). Auxin enhances host susceptibility whereas plant ET production has a negative effect on agrobacterial virulence. Application of ACC reduces *Agrobacterium*-mediated gene transfer to melon explants whereas addition of aminoethoxyvinylglycin, an inhibitor of ACC synthase, increased it (Ezura et al., 2000). A reduction in transformation efficiency results from suppression of vir gene expression, but not *Agrobacterium* growth (Nonaka et al., 2008). The promoting effect of low auxin concentrations on agrobacterial growth and the inhibiting effect of ET on virulence illustrates that both, *Agrobacterium* and the host plant control host cell transformation. Taken together, at early stages of the infection process, cytokinin and auxin produced by *Agrobacterium* cells have a promoting effect on transformation efficiency, which is in part counteracted by the inhibitory effect of host plant-derived ET and SA on agrobacterial virulence. Thus, the correct phytohormone balance decides on the success of infection.

HYPERSENSITIVE RESPONSE

Examination of early events in pathogenesis has demonstrated that virulent *Agrobacterium* does not induce HR in *Arabidopsis* (**Figure 1A**; Lee et al., 2009). Moreover, *Agrobacterium* is able to suppress HR induced by *Pseudomonas syringae pv. phaseolus* in plants (Robinette and Matthysse, 1990). This suppression is dependent on the activity of the *iaaH* and *iaaM* oncogenes which encode enzymes for auxin synthesis, since several *Agrobacterium* transposon mutants in the *iaa* genes failed to inhibit a HR. Likewise, transcription of several genes involved in oxidative stress signaling are only induced by the oncogenic, but not the T-DNA-depleted *Agrobacterium* strain (Lee et al., 2009). Production of H_2O_2 precedes HR, which is degraded via a chromosomally encoded catalase of *Agrobacterium* (Xu and Pan, 2000). H_2O_2 acts both as a local trigger for the programmed cell death and as a diffusible signal for the induction of cellular protectant genes in surrounding cells (Levine et al., 1994). Apart from its signaling functions, H_2O_2 is also involved in toughening of cell walls in the initial stages of plant defense by cross-linking of cell wall structural proteins (Bradley et al., 1992). Accumulation of H_2O_2 is prevented only at the early stages of agrobacterial infection, but proceeds in the course of tumor development (Lee et al., 2009).

HOST RESPONSES TO CROWN GALL DEVELOPMENT

MORPHOLOGICAL ADAPTATIONS

Development of crown galls is accompanied by profound changes in the gene expression profile, metabolism, and morphology. The uncontrolled synthesis of auxin and cytokinin by cells transformed with a T-DNA of tumorigenic Ti-plasmids drives tumor development, while the auxin to cytokinin ratios determine the crown gall morphology (**Figure 1B**). In the early days of studies about the molecular basis of crown gall development it was observed that mutations in the *tmr* locus encoding *ipt* cause rooty crown galls and those in the *tms* loci coding for *iaaH* and *iaaM* induce shooty phenotypes (Garfinkel et al., 1981; Akiyoshi et al., 1983; Barry et al., 1984; Buchmann et al., 1985; Black et al., 1994). A recent study on the T-DNA locus Atu6002 of strain C58 indicated that when the encoding protein C is expressed, it increases host cell sensitivity to auxin (Lacroix et al., 2013). In addition to the T-DNA-encoded genes, the expression of several host genes involved in auxin and cytokinin metabolism and signaling are expressed in crown galls (Lee et al., 2009). Cytokinin and auxin together with ET are known to be essential for growth of crown gall tumors and differentiation of cell types with different morphology and function (**Figure 1C**). Particularly, ET has been shown to be essential for the formation of vascular tissue and crown gall tumor development (Aloni et al., 1998; Wächter et al., 1999, 2003; Ullrich and Aloni, 2000). Application of the ET synthesis inhibitor aminoethoxyvinyl-glycine prevents vascularization in castor bean (*Ricinus communis*) stems and inhibits tumor growth completely (Wächter et al., 2003). When the ET-insensitive tomato (*Lycospersicon esculentum*) mutant, *never ripe*, is infected with virulent *Agrobacterium* cells it does not develop

tumors despite integration and expression of the T-DNA encoded oncogenes for auxin and cytokinin biosynthesis (Aloni et al., 1998). Thus, neovascularization is a prerequisite for crown gall development.

Growth and expansion of crown gall tumors cause disruption of the epidermal cell layer and thereby loss of guard cells and an intact cuticle. Accordingly, expression of genes involved in cutin biosynthesis is downregulated (Deeken et al., 2006). As a disrupted surface area provides access for pathogens and leads to uncontrolled loss of water for the host plant, the crown gall surface has to be sealed. This is achieved by differentiating a periderm-like surface layer (Efetova et al., 2007). The polymerization of suberin monomers involves peroxidases for which H_2O_2 is the electron donor. Thus, H_2O_2 produced in crown galls functions in strengthening of cell walls rather than in induction of a HR. The stimulus for inducing suberization is drought stress-mediated ABA signaling (**Figure 1D**). Drought stress signaling seems to play a central role in crown gall development. ABA accumulates in crown galls in high amounts and transcription of a set of drought and/or ABA-inducible genes is elevated (Mistrik et al., 2000; Veselov et al., 2003; Efetova et al., 2007). ABA synthesis is triggered by ET as demonstrated by the application of various inhibitors of ET or ABA biosynthesis and the use of ET-insensitive or ABA-deficient tomato mutants (Hansen and Grossmann, 2000). Among the genes which play a role in drought stress protection of crown gall tumors is *FAD3,* encoding a fatty acid desaturase. The *fad3-2* mutant with impaired biosynthesis in α-linolenic acid (C18:3) develops much smaller crown gall tumors particularly in low but not high relative humidity (Klinkenberg et al., 2014). Elevated levels of C18:3 were found in the phospholipid fraction of *Arabidopsis* crown gall tumors and maintain membrane integrity under drought stress conditions. In addition to gene expression changes, crown galls accumulate high amounts of osmoprotectants, such as proline (Pro), gamma aminobutyric acid (GABA), and alpha-aminoadipinic acid. The retarded tumor growth in *abi* and *aba* mutant plants underlines the importance of an ABA-mediated drought stress-signaling pathway in crown gall development (Efetova et al., 2007).

NUTRIENT TRANSLOCATION AND METABOLISM

Expression profiles of genes involved in energy metabolism, such as photosynthesis, mitochondrial electron transport, and fermentation together with physiological data revealed that *Arabidopsis* tumors produce C and N compounds heterotrophically and gain energy mainly anaerobically by alcoholic fermentation (**Figure 1E**; Deeken et al., 2006). The change from autotrophy to heterotrophy reduces the oxygen level in crown gall tumors thereby inducing expression of hypoxia-sensitive genes, such as *SAD6.* This gene encodes a stearoyl-acyl carrier protein desaturase, which belongs to a class of enzymes known to catalyze the first step in fatty acid desaturation, an oxygen-dependent process. Despite limited oxygen availability in crown galls, SAD6 provides the monounsaturated fatty acid, oleic acid, for membrane phospholipids (Klinkenberg et al., 2014). Thus, expression of SAD6 maintains fatty acid desaturation under hypoxic conditions.

Crown gall tumors primarily use organic carbon and nitrogen for growth and are therefore a strong sink for the host plant. Metabolites and minerals have to be provided by the host plant and translocated into the crown gall tumor (**Figure 1F**). The mechanisms of nutrient translocation and their accumulation have been studied on crown gall tumors by applying cytological staining, eletrophysiological, and $^{14}CO_2$ tracer techniques as well as a viral movement protein (Marz and Ullrich-Eberius, 1988; Malsy et al., 1992; Pradel et al., 1999). Solutes enter the crown gall tumor via vascular tissue, which is connected to that of the host plant and consists of phloem for the transport of assimilates and xylem for water and minerals (Aloni et al., 1995; Deeken et al., 2003). Assimilates are produced by source leaves and are apoplastically and symplastically unloaded from the phloem in crown gall tumors. High apoplastic invertase activity indicated that sucrose is unloaded apoplastically (Malsy et al., 1992). After cleavage of sucrose by sucrose-degrading enzymes, hexoses can be taken up via hexose transporters into tumor cells. *Arabidopsis* crown galls show elevated expression of several genes encoding sucrose degrading enzymes and a monosaccharide transporter (Deeken et al., 2006). In addition, a high-affinity hexose transporter has been isolated from meristematic tobacco cells transformed with a tumor inducing T-DNA and was characterized as energy independent hexose uptake transporter (Verstappen et al., 1991). Application of the membrane impermeable fluorescent probe, carboxyfluorescein (CF) to source leaves and transient expression of the GFP-labeled potato virus X (PVX) coat protein (CP), exclusively exploiting plasmodesmata for distribution, demonstrated the existence of a symplastic transport pathway between the phloem and tumor cells (Pradel et al., 1999). Both reporters show extensive cell-to-cell movement in the parenchyma of crown gall tumors but not in uninfected stem tissues of different plant species ranging from symplastic (*Curcubita maxima*) to apoplastic loaders (*R. communis, Nicotiana benthamiana*). The disrupted and enlarged surface of the crown gall tumor drives water and mineral translocation into crown gall tumors since the evaporation rate of crown galls exceeds that of leaves and non-infected stems (Schurr et al., 1996; Wächter et al., 2003). The periderm-like layer of suberized cells that covers the crown gall surface provides a considerable diffusion resistance against water vapor, but it is not an impermeable barrier for water (**Figure 1D**; Kolattukudy and Dean, 1974; Vogt et al., 1983; Schreiber et al., 2005). Cations and anions are taken up into the tumor cells through the function of membrane-localized channels and transporters expressed in the crown gall (Deeken et al., 2003). Potassium channel mutants with impaired crown gall growth underline the importance of optimal nutrient supply for growth.

DEFENSE RESPONSES

Gamma aminobutyric acid and Pro not only serve as osmoprotectants in drought-stress related processes of the host plant, but have also an impact on *Agrobacterium* virulence (Haudecoeur et al., 2009a,b). GABA produced in crown gall tumors can be taken up by *Agrobacterium* cells and causes a delay in accumulation of 3-oxo-octanoylhomoserine lactone (OC8HSL) and Ti plasmid conjugation. GABA activates the AttKLM operon of which the AttM lactonase degrades the quorum sensing signal, OC8HSL, thereby turning on quorum quenching to protect the host plant

against infections with bacterial pathogens (Yuan et al., 2008). However, Pro interferes with the import of GABA and thereby prevents GABA-induced degradation of the bacterial quorum sensing signal OC8HSL. Thus, Pro antagonizes the GABA-induced degradation of OC8HSL and therefore may be used by the pathogen to by-pass the GABA-based host plant defense.

In addition to growth and developmental processes regulated by auxin and cytokinin, crown gall biology also involves pathogen defense signaling pathways. Hormones such as SA, JA, and ET are the primary signals inducing defense responses (López et al., 2008). In *Arabidopsis* crown galls the levels of SA and ET, but not JA, are elevated (**Figure 1G**). JA has no obvious impact on crown gall tumor development, as the development on *Arabidopsis* JA-insensitive mutants is wildtype-like (Lee et al., 2009). SA and ET contents together with the expression of pathogen-related marker genes of the SA- and ET-dependent signaling pathways increase with accumulation of the T-DNA-encoded iaa and ipt transcripts. Thus, auxin and/or cytokinin seem to be important for defense signaling in crown gall tumors, since the non-tumorigenic *Agrobacterium* strain which contains a disarmed pTiC58 does not induce expression of marker genes of the SA- and ET-dependent signaling pathways (Lee et al., 2009). It is known that high levels of auxin and cytokinin stimulate ET synthesis and its accumulation in crown galls (Goodman et al., 1986; Aloni et al., 1998; Johnson and Ecker, 1998; Vogel et al., 1998; Wächter et al., 1999). In contrast to ET, the classical marker genes of the SA-dependent signaling pathways are not induced most likely as a result of the high auxin content, which has been shown to inhibit SA responses to avoid the induction of SAR (Robert-Seilaniantz et al., 2011). Despite the lack of induction of SA-dependent defense signaling, *Arabidopsis* mutant plants with high SA levels strongly reduce while those with low SA levels promote tumor growth (Lee et al., 2009). Instead of inducing host defense pathways, high SA levels act directly on oncogenic agrobacteria by inhibiting vir gene expression and thereby reducing agrobacterial virulence (Yuan et al., 2007; Anand et al., 2008). Besides SA-mediated inhibition of *Agrobacterium* virulence, SA activates the AttKLM operon, just like GABA does, to down regulate quorum sensing in *Agrobacterium* (Yuan et al., 2008). Thus, activation of quorum quenching by auxin, SA, and GABA, is part of the plant defense program against *Agrobacterium* in the developing crown gall. In addition to SA, ET and IAA also inhibit the vir regulon and T-DNA transfer into plant cells (**Figure 1G**; Ezura et al., 2000; Nonaka et al., 2008). Thus, the interaction between the host plant and *Agrobacterium* is very much based on phytohormone cross talk which provides a balance between pathogen-defense by the host and crown gall development promoted by *Agrobacterium*.

EPIGENETIC PROCESSES IN DNA INTEGRATION, ONCOGENE EXPRESSION, AND CROWN GALL DEVELOPMENT

EPIGENETIC CHANGES ASSOCIATED WITH T-DNA INTEGRATION AND ONCOGENE EXPRESSION

Epigenetic changes that affect chromatin structure play an important role in regulating a wide range of cellular processes. Histones for example are subject to post-translational modification including acetylation, phosphorylation, methylation, and ubiquitination. These modifications may influence crown gall development on different levels, either by affecting chromatin structure and DNA integration or by influencing gene expression in the host tissue. Up-regulation of several members from the core histone gene families after *Agrobacterium* infection indicates that they are important for the transformation process (Veena et al., 2003). For example, *Arabidopsis* mutants lacking histone H2A are defective in T-DNA integration (Mysore et al., 2000). In addition, a truncated version of VIP1, an *Arabidopsis* protein proposed to interact with the T-DNA-protein-complex (T-complex), which is not able to interact with histone H2A, strongly decreases *Agrobacterium* tumorigenicity (Li et al., 2005). As this decrease is most likely due to a reduced T-DNA integration efficiency, this suggests that association of the VIP1 with the host chromatin is critical for integration of the T-DNA. One hypothesis of how epigenetic information affects DNA integration is that chromatin modifications surrounding double-strand breaks (DSBs) of the DNA can be recognized by the T-complex. The resulting chromatin-T-complex may then bring T-DNA into close proximity to DSBs and facilitate its integration by the DSB repair pathway (Magori and Citovsky, 2011). Alternatively, histones may also enhance transformation by protecting incoming DNA from nuclease digestion during the initial stages of transformation. Indeed, overexpression of several histone genes in *Arabidopsis* results in higher amounts of transferred DNA and increased transient transgene expression in transformed cells (Tenea et al., 2009). Other epigenetic modifications like DNA methylation do not correlate with the T-DNA integration pattern, suggesting that T-DNA integration occurs without regard to this type of modification (Kim et al., 2007). Concerning post-translational modifications of histones, RNA-mediated knockdown of two histone deacetylases (HDT1 and HDT2) decreases *Agrobacterium*-mediated transformation efficiency of *Arabidopsis* root segments (Crane and Gelvin, 2007). Histone deacetylation functions in chromatin compaction and transcriptional repression (Strahl and Allis, 2000). Therefore, the observed effect on transformation may either be a result of effects on chromatin structure or gene expression of plant factors involved in the integration process. Histone deacetylation may also influence DNA integration by affecting DSB repair, as several histone deacetylases are critical for the DNA repair process in yeast (Munoz-Galvan et al., 2013).

After T-DNA is integrated into the plant genome, the host plant often silences transgenes. Gene silencing can occur by two different mechanisms. Transcriptional gene silencing (TGS) is a result of promoter inactivation while post-TGS (PTGS) occurs when the promoter is active but the mRNA fails to accumulate. DNA methylation of promoter sequences is frequently associated with inactivation of transgenes (Linne et al., 1990; Matzke and Matzke, 1991; Kilby et al., 1992). Screening of a large collection of transgenic *Arabidopsis* lines with single T-DNA copies including a pNOS-*NPTII* reporter gene has shown that promoter methylation is required but not sufficient for transcriptional inactivation (Fischer et al., 2008). Silencing only occurs when the plants, challenged by the silencer transgene, also provide an RNA signal. Concerning local features of the host genome affecting gene silencing, repeats flanking the site of integration seem to promote inactivation whereas flanking genes rather attenuate it. RNA

silencing is triggered only if the transcript level of a transgene surpasses a gene-specific threshold, suggesting that the inactivation is part of plant defense mechanism corresponding to excessively transcribed genes (Schubert et al., 2004).

Apart from the down-regulation of transgenes that are integrated into the plant genome along with the T-DNA, the T-DNA itself may also be subject to modification by the plant silencing machinery. The first comprehensive analysis of T-DNA methylation revealed that methylation can occur in different plant tumor lines induced by *Agrobacterium*. At least one T-DNA copy in each tumor genome remained unmethylated, thereby allowing oncogene expression and crown gall proliferation (Gelvin et al., 1983). Experiments using the demethylating agent 5-azacytidine indicates that methylation negatively correlates with gene expression in plant tumors (Hepburn et al., 1983). A more recent study on T-DNA methylation in crown gall tumors induced on *Arabidopsis* stems demonstrates that the oncogene sequences are only methylated to a very low degree (Gohlke et al., 2013). The two intergenic regions, which serve as promoters for expression of the oncogenes *iaaH*, *iaaM,* and *ipt,* are completely unmethylated in *Arabidopsis* crown galls. As the gene products of these oncogenes are essential for an increase in levels of cytokinin and auxin, they are always actively transcribed in crown gall tumors of *Arabidopsis* stems (Deeken et al., 2006). The low degree of T-DNA methylation in crown galls suggests that this is a prerequisite to maintain the expression levels of oncogenes required for tumor formation. Indeed, induction of DNA oncogene methylation by production of double-stranded RNAs is sufficient to repress oncogene transcription and prevent tumor development (Gohlke et al., 2013).

EPIGENETIC MODIFICATIONS IN THE CROWN GALL GENOME

Analysis of *Agrobacterium*-infected inflorescence stalks allowed monitoring of gene expression in the crown gall tumor at later developmental stages and revealed massive changes in its transcriptome (Deeken et al., 2006). A large part of the *Arabidopsis* genome (about 22% of genes) was found to be expressed differentially between crown galls and mock-infected stems. Of these genes, a slightly higher percentage was found to be down-regulated in crown galls (12%) compared to up-regulated genes (10%). Distinct expression changes occur at genes pivotal for energy metabolism, such as those involved in photosynthesis, mitochondrial electron transport, and fermentation. This reflects the induced host cell changes from an auxotrophic, aerobic metabolism to a heterotrophic, transport-dependent, sugar-dependent anaerobic metabolism (see Nutrient Translocation and Metabolism).

Considering that a high percentage of the *Arabidopsis* genome is differentially regulated in crown gall tumors, transcriptional reprogramming probably occurs on several levels. For example, the transcript levels of several transcription factor families (MYB, bHLH, bZIP, AP domain) change after *Agrobacterium* infection (Ditt et al., 2006; Sardesai et al., 2013), thereby inducing a tumor-specific gene expression pattern. Gene expression may also be regulated by epigenetic mechanisms like chromatin modification or DNA methylation. Apart from modifications which play a role during T-DNA integration and silencing of oncogenes (see Epigenetic Processes in DNA Integration, Oncogene Expression and Crown Gall Development), DNA methylation of plant genes can also influence tumor growth (**Figure 1H**). Indeed, 8% of protein-coding genes are differentially methylated in crown galls compared to mock-infected stems, with an overall tendency toward being hypermethylated (Gohlke et al., 2013). Depending on the position of DNA methylation, different effects on the gene expression levels are observed. In agreement with trends observed for DNA methylation changes in *Arabidopsis* (Zhang et al., 2006), increased methylation at transcription start and end sites has a negative impact on gene expression, while the two processes are positively correlated in the transcribed region. Mapping of DNA methylation in tumors revealed hypomethylation in the upstream regions of genes as well as hypermethylation in transcribed regions. Both of these may, in turn, influence gene expression and contribute to the tumor-specific expression pattern. Not surprisingly, pathways that are associated with tumor development like genes associated with cell division, biotic stress, and redox regulation are differentially methylated. Changes in the methylation pattern also have an impact on tumor growth, as *Arabidopsis* mutants in *de novo* methylation pathways promote crown gall development. Intriguingly, callus induction, which like crown gall development is also associated with dedifferentiation of plant cells, is increased in the methyltransferase mutant *cmt3* (Berdasco et al., 2008). In addition, treatment with the methyltransferase inhibitor 5-acacytidine results in increased callus formation. Recently, the DNA methylation pattern has been extensively studied in calli from *Populus trichocarpa* and *Oryza sativa*. In *Oryza sativa* calli, hypermethylation was detected compared to wild-type plants (Stroud et al., 2013). Gene bodies are hypermethylated in *Populus trichocarpa* calli compared to explants, while promoter methylation is reduced (Vining et al., 2013). Consistent with the methylation pattern in crown galls, DNA hypermethylation seems to be a general feature of a dedifferentiated status.

An attempt to identify internal plant signals which may influence DNA methylation suggests that high levels of ABA induce DNA methylation of promoter sequences (**Figure 1H**; Gohlke et al., 2013). Therefore, this phytohormone may at least partly be responsible for the methylation pattern found in crown galls. It is tempting to speculate that ABA induces DNA methylation as a response to abiotic stresses such as drought stress acclimation due to the increased water loss in crown gall tumors (Schurr et al., 1996). Possibly, ABA signaling pathways are interconnected with methylation processes in crown galls, as has been suggested for *Physcomitrella patens* (Khraiwesh et al., 2010). In the future, it would be interesting to analyze ABA knockout mutants concerning their methylation pattern in order to map ABA-induced methylation changes in a comprehensive manner and thereby improve our understanding of the connection between the different pathways. In addition, other phytohormones would also be interesting to study regarding their influence on the DNA methylation pattern in crown galls, as they display not only increased levels of ABA, but also of cytokinin, auxin, ET, and JA (Veselov et al., 2003; Lee et al., 2009).

SUMMARY AND OUTLOOK

At the beginning of infection, sensing of *Agrobacterium* does not induce a strong defense response of the host plant. *Agrobacterium*

rather exploits defense responses to increase host susceptibility for transformation and host signaling pathways to promote bacterial growth. In crown galls, however, pathogen defense pathways are considerably activated and inhibit *Agrobacterium* virulence. Accordingly, the host plant is able to limit the number of further T-DNA transformation events and to control the growth dimension of crown galls, which represent a strong metabolic sink for the host plant. Metabolic and morphological adaptations accompany the development of crown galls and generate an import-oriented tissue. The heterotrophic metabolism together with anaerobically gain of energy requires translocation of metabolites, water and minerals from the plant into the proliferating crown gall tissue. As a basis for nutrient translocation the vascular tissue needs to differentiate and the disrupted and suberized crown gall surface provides the driving force for nutrient flow. In fact, the suberized surface minimizes water loss, but still allows enough evaporation of water. Membrane integrity is maintained under the low oxygen and elevated ROS levels in crown galls by adaptation of lipid metabolism. The transcriptional changes underlying the physiological changes are partially caused by differential DNA methylation of the crown gall genome. In conclusion, both *Agrobacterium* infection and crown gall growth are highly regulated processes, which are accompanied by pathogen defense of the host and counter-defense launched by *Agrobacterium*. This regulation takes place on different levels including epigenetic control of gene expression, changes in phytohormone content as well as metabolic and morphological adaptions.

Despite the fact that the *Agrobacterium*-plant-interaction has been studied since more than 100 years and is most likely one of the best-known pathogen-host-relationships, there are still some questions left, which one may aim to answer. In addition to the one raised about the role of phytohormones other than ABA on DNA methylation in crown gall development, another one would be about the molecular mechanisms of how *Agrobacterium* cells produce auxin and how auxin increases host susceptibility for transformation. Furthermore, the status and type of plant cell susceptible for T-DNA integration is as yet unknown. The knowledge about the cellular identity sensitive for transformation will improve our understanding of transformation recalcitrant plant species. Moreover, differentiation processes in crown galls do not follow the usual patterning, unlike the situation in plant organs where developmental patterning underlies a precise spatiotemporal expression of signals and their cognate receptors. Since the original/typical developmental program seems to be overruled, crown gall tumors provide a unique opportunity for studying the molecular and biochemical mechanisms underlying cellular de-differentiation as well as differentiation processes. Not all of the questions raised may be easy to address, as some require sophisticated techniques, which at first have to be developed and established. However, invention of new techniques will benefit the entire scientific community as they have done before when *Agrobacterium* became the biotechnological tool for generation of genetically modified plants.

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REVIEW
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Unmasking host and microbial strategies in the *Agrobacterium*-plant defense tango

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Coevolutionary forces drive adaptation of both plant-associated microbes and their hosts. Eloquently captured in the Red Queen Hypothesis, the complexity of each plant–pathogen relationship reflects escalating adversarial strategies, but also external biotic and abiotic pressures on both partners. Innate immune responses are triggered by highly conserved pathogen-associated molecular patterns, or PAMPs, that are harbingers of microbial presence. Upon cell surface receptor-mediated recognition of these pathogen-derived molecules, host plants mount a variety of physiological responses to limit pathogen survival and/or invasion. Successful pathogens often rely on secretion systems to translocate host-modulating effectors that subvert plant defenses, thereby increasing virulence. Host plants, in turn, have evolved to recognize these effectors, activating what has typically been characterized as a pathogen-specific form of immunity. Recent data support the notion that PAMP-triggered and effector-triggered defenses are complementary facets of a convergent, albeit differentially regulated, set of immune responses. This review highlights the key players in the plant's recognition and signal transduction pathways, with a focus on the aspects that may limit *Agrobacterium tumefaciens* infection and the ways it might overcome those defenses. Recent advances in the field include a growing appreciation for the contributions of cytoskeletal dynamics and membrane trafficking to the regulation of these exquisitely tuned defenses. Pathogen counter-defenses frequently manipulate the interwoven hormonal pathways that mediate host responses. Emerging systems-level analyses include host physiological factors such as circadian cycling. The existing literature indicates that varying or even conflicting results from different labs may well be attributable to environmental factors including time of day of infection, temperature, and/or developmental stage of the host plant.

Keywords: immunity, pathogen, *Agrobacterium*, hormones, actin, trafficking, temperature, light

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Overview

At its most basic, any form of immunity must distinguish between self, or beneficial, and harmful non-self interactions. In animals, adaptive immunity is delegated to specialized immune cells that undergo selection to recognize new pathogens and mount specific, targeted defenses more rapidly during a second attack. This adaptive immunity is only possible because innate immunity, consisting of evolutionarily ancient, non-specific and rapidly mobilized defenses, staves off – or

eliminates – the pathogen while adaptive immunity is activated. Innate immune responses are triggered by highly conserved "pathogen-associated molecular patterns," or PAMPs, that are widely shared within distinct classes of pathogens, such as bacteria or fungi. Due to their structural and sequence-level evolutionary conservation, PAMPs are often found in non-pathogenic microbes as well and hence are also called microbial-associated molecular patterns, or MAMPs. These PAMPs/MAMPs elicit a set of defense mechanisms tailored to the type of microbe perceived (Ausubel, 2005).

Plants lack specialized immune cells, but have a robust and sophisticated system of innate defenses. Extra- and intra-cellular receptors detect microbial presence and trigger signal transduction pathways that lead to immediate physiological changes. These include the production of damaging reactive oxygen species (ROS), extracellular alkalization, Ca^{2+} fluxes, callose deposition, seedling growth inhibition, stomate closure, and localized programmed cell death [hypersensitive response (HR); Boller and Felix, 2009]. One of the earliest signal transduction events regulating these responses is the mitogen-activated protein kinase (MAPK) pathway, which modulates defense gene expression (Asai et al., 2002). Hormone biosynthesis genes, notably for salicylic acid (SA), jasmonate (JA) and ethylene, are among those expressed, and play a crucial role in plant defenses. These hormones can induce systemic defenses throughout the plant to slow or prevent the pathogen from spreading beyond the infection site. They also upregulate hormone-specific defense genes further downstream and in their volatile forms, can even signal to neighboring plants (Shulaev et al., 1997).

Two arms of the plant innate immune system control the selective activation of these responses: effector-triggered immunity (ETI) and PAMP (or pattern)-triggered immunity (PTI; also called MTI, or MAMP-triggered immunity). Unlike PAMPs or MAMPs, effectors are secreted only by specific bacterial strains and are highly polymorphic (Spoel and Dong, 2012). Effectors are generally virulence-promoting factors that typically suppress host defenses. Particular plant genomes encode R proteins that specifically recognize and bind bacterial effectors to reduce their efficacy, decreasing plant susceptibility (Jones and Dangl, 2006).

A unifying theme of this review is the interplay between *Agrobacterium tumefaciens* and the defense responses mounted by its hosts. *Agrobacterium*, the causative agent of crown gall disease, is best known for its ability to genetically transform host plants by delivering a portion (the "T-DNA") of its tumor-inducing (Ti) plasmid or a foreign DNA construct that has been inserted into a Ti plasmid-derived vector (Lacroix and Citovsky, 2013a). A number of studies (e.g., Ditt et al., 2005; Zipfel et al., 2006; Tie et al., 2012; Zhang et al., 2013b) in a range of host species including rice, ryegrass, and *Arabidopsis thaliana* indicate that *Agrobacterium*-elicited defenses limit transformation efficiency. However, unlike several other well-characterized phytopathogens, *Agrobacterium* does not appear to incite an HR on most host species. Consistent with this observation, there is growing evidence that *Agrobacterium* modulates host defenses, at least in part by regulating hormone accumulation, although the mechanism(s) mediating this host manipulation are not yet known. Given the relative paucity of published studies on the defenses affected by *Agrobacterium*, our goal here is to synthesize for the reader those facets of the host response that appear most relevant to *Agrobacterium* infection. Because there are no known *Agrobacterium*-encoded effectors or cognate host R proteins, we focus primarily on PAMP-triggered immune signaling and its downstream consequences. Nonetheless, in light of recent arguments questioning the distinction between PTI and ETI (see PTI and ETI: A False Distinction?), we briefly consider ETI as well before reviewing in detail the hormonal regulatory pathways (particularly SA) that appear to be a critical target for agrobacterial counter-defense strategies. Finally, we highlight both host physiological and environmental factors that may impact the outcome of the host-*Agrobacterium* interaction.

Pathogen Elicitors and Host Recognition/Response Systems

PAMP Perception and PTI

PAMP-triggered immunity is elicited by highly conserved molecular features, such as bacterial flagellin. These patterns are specific epitopes derived from molecular structures that are essential for microbial fitness. In general, evolutionary selective pressure prevents the loss or modification of the PAMPs and, in theory, distinguishes PAMPs from host-specific pathogen-derived effectors. As universal harbingers of microbial presence, known PAMPs predictably include a number of cell wall components such as peptidoglycan, lipopolysaccharides, and fungal chitin (Felix et al., 1999; Gust et al., 2007; Miya et al., 2007; Erbs et al., 2008; Thomma et al., 2011). The two best-characterized bacterial PAMPs are peptides derived from flagellin (flg22) and the elongation factor EF-Tu (elf18; Felix et al., 1999; Kunze et al., 2004). Flagellin and more generally, pathogen motility, play key roles in pathogenesis, as chemotaxis and entry into the host are often essential early in infection (Josenhans and Suerbaum, 2002). EF-Tu is the most abundant protein found in many bacteria experiencing rapid growth (Furano, 1975) and is released into the extracellular space upon disruption of bacterial cell membrane integrity (Zipfel et al., 2006; Nicaise et al., 2009).

PAMPs and MAMPs are perceived by the extracellular domains of plant pattern recognition receptors (PRRs), typically receptor-like kinases that trigger downstream kinase-dependent signaling pathways. No intracellular PRRs have yet been found (Thomma et al., 2011). In *Arabidopsis*, the flagellin receptor FLS2 and the EF-Tu receptor (EFR) detect subnanomolar concentrations of flg22 and elf18, respectively (Chinchilla et al., 2006; Zipfel et al., 2006). The presence of EFR only in *Brassicaceae* (Boller and He, 2009) suggests that it is evolutionarily younger than FLS2 (Nekrasov et al., 2009; Saijo et al., 2009). The demonstrated success in conferring resistance to *Agrobacterium* and other pathogens by introducing the *EFR* gene from *Arabidopsis* into *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) raises the possibility of engineering broad-spectrum bacterial resistance by heterologous expression of PRRs in vulnerable crops (Lacombe et al., 2010).

Significantly, some pathogens exhibit enough divergence in their PAMP sequences to avoid host detection (Boller and Felix, 2009). In particular, the *Arabidopsis* FLS2 is unable to perceive flagellin from *Agrobacterium* (Bauer et al., 2001). Instead, EFR appears to be the key determinant of susceptibility to *Agrobacterium* in *Brassicaeceae*. Co-inoculation with the elicitor peptide elf18 reduces transformation efficiency of the bacterium, while an *efr* mutant plant line exhibits enhanced susceptibility to infection and transgene transformation (Zipfel et al., 2006). *Agrobacterium* cell-wall derived peptidoglycans do elicit defense responses, including rapid increases in ROS and extracellular pH in *Arabidopsis*, albeit at much reduced levels as compared to those from the necrotrophic pathogen *Xanthomonas campestris* pathovar *campestris*; this difference may reflect the agrobacterial requirement to maintain host viability for successful transformation (Erbs et al., 2008).

Effectors and ETI

Since microbes unwittingly elicit PTI with passive expression of conserved microbial patterns, successful infection often requires the secretion of virulence effectors to subvert those defenses (Jones and Takemoto, 2004). While many effectors act enzymatically, others decrease plant defenses by increasing transcription of genes that further down-regulate defense activation genes (Chisholm et al., 2006). Still other effectors, including the *Pseudomonas syringae* pv. *tomato* DC3000 Type III secretion system (T3SS)-delivered avirulence factors AvrPto and AvrPtoB, directly target PRRs for inactivation and suppress PTI signaling events, thus increasing host susceptibility to the incoming pathogen (Abramovitch et al., 2006; He et al., 2007; Zipfel, 2009).

Predictably, many plant hosts have evolved a second branch of immunity called ETI to detect these virulence-promoting effectors via polymorphic nucleotide-binding leucine-rich repeat (NB-LRR) or extracellular leucine-rich repeat (eLRR) proteins encoded by *R* genes (Jones and Dangl, 2006). ETI is also known as "gene-for-gene immunity" because R proteins have evolved to specifically detect and recognize particular pathogenic effectors (Chisholm et al., 2006). Some R proteins can also indirectly recognize the changes in host proteins targeted by pathogen effectors, a phenomenon initially articulated as the "guard hypothesis" (Jones and Takemoto, 2004). *R* gene-mediated resistance results in severe host defense activation, including a HR, or apoptosis at the infection site, in an effort to limit the pathogen's spread throughout the plant and hence the development of disease (He et al., 2007).

Unlike MAMPs, effectors are not required for microbe survival, and are thus under strong evolutionary pressure to mutate and evade host plant detection by R proteins. This force similarly drives *R* genes to mutate to more successfully detect effectors. The "four phased 'zigzag' model" (Jones and Dangl, 2006) maps the dance between pathogen attack and plant defense. In this model, the plant's detection of PAMPs triggers PTI, which is dampened by the pathogen's secretion of effectors. These effectors (sometimes referred to as Avr proteins) are then recognized by plant R proteins, increasing plant defense via ETI, until mutated effectors can evade plant detection and successfully hamper plant defense activation (Jones and Dangl, 2006). The co-evolution or Red Queen-like relationship between effectors and R proteins drives the 'zigzag' as the plant or the pathogen temporarily gains the upper hand.

As noted above, there are no reports to date of a classic gene-for-gene mechanism of resistance in any host species to *Agrobacterium*. There is, however, mounting evidence that the pathogen has the capability to disable or dampen defenses. Ditt et al. (2006) reported no difference in gene expression between *Agrobacterium*-infected and mock-infected *Arabidopsis* at 4–24 h post-infection (hpi), although their microarray analysis did reveal distinct sets of up-regulated defense genes, as well as down-regulated cell-proliferation genes, at 48 hpi. In contrast, other studies (e.g., Veena et al., 2003; Lee et al., 2009; Zhang et al., 2015) uncovered a variety of alterations in the host transcriptome at earlier time points. Given the rapidity of the basal defenses described here, it seems likely that Ditt et al. (2006) missed many of the changes in gene expression that may well have returned to pre-infection levels by 4 hpi. Using subtractive hybridization and macroarray analysis for expression profiling, Veena et al. (2003) noted that defense gene induction in tobacco BY2 suspension cells was suppressed at 30–36 hpi by a strongly virulent *Agrobacterium* strain but not by a Ti plasmid-deficient strain, although early defense gene induction (3–6 hpi) appeared to be largely similar between the two. Consistent with the observed capacity of the pathogen to suppress accumulation of ROS within 3 h of infection (Lee et al., 2009), a catalase-deficient mutant is highly attenuated for virulence (Xu and Pan, 2000). Somewhat paradoxically, Anand and Mysore (2013) found that RAR1, a plant protein required for R-gene mediated resistance to fungal pathogens, contributes to efficient *Agrobacterium* transformation. Finally, hormones produced by *Agrobacterium* block the HR that would normally result from subsequent infection with *P. syringae* pv. *phaseolicola* (Robinette and Matthysse, 1990) and enhance host susceptibility by suppressing expression of an infection-inhibiting transcription factor (Sardesai et al., 2013). Compared to the plethora of data on effectors produced by other pathogens, especially those with T3SS, we still know very little about the mechanisms *Agrobacterium* employs to thwart host defenses. The examples cited here hint at a diversified portfolio of strategies that may fail to conform to the canonical effector-R gene-mediated duel for dominance (Anderson et al., 2010). Instead, it seems reasonable to hypothesize that molecules delivered via the Ti-plasmid and/or chromosomally borne secretion systems in *Agrobacterium* could promote host transformation by repressing defense activation (or inducing expression of defense repressors). Similar to ETI, such *Agrobacterium*-derived saboteurs might in turn induce additional host responses.

PTI and ETI: A False Distinction?

Several lines of evidence have recently called into question the notion of clear temporal, evolutionary and structural distinctions between PTI and ETI (Qi et al., 2011; Thomma et al., 2011). For example, the *Arabidopsis* proteins RPM1, RPS2, and RPS5 were identified as R proteins that indirectly detect effector activity on the plant protein RIN4 (Jones and Takemoto, 2004), a regulator of PAMP-triggered responses (Kim et al., 2005). Qi et al. (2011) recently demonstrated that RPM1, RPS2, and

RPS5 also physically interact with FLS2 to trigger PTI. Moreover, certain PAMPs such as Ax21 and Pep-13 from *Xanthomonas oryzae* pv. *oryzae* and the *Phytophthora* species, respectively, are narrowly distributed and conserved in only a few strains of pathogens, characteristics classically attributed to effectors rather than PAMPs (Thomma et al., 2011).

Transcriptome analysis of inoculated plants also indicates that PAMP- and ETI may regulate similar sets of defense responses (Tsuda and Katagiri, 2010). Navarro et al. (2004) found overlap between *Arabidopsis* flg22-induced genes and their *P. syringae* effector-induced tobacco orthologs, though the extent of overlap was weak and diminished over time. Parallels in the early stages of the response indicate that both PAMPs and effectors may initially trigger a common signaling mechanism(s). Indeed, Boller and Felix (2009) have argued that there exists a single, convergent innate immune system whose kinetics and strength of response are fine-tuned depending on the type of perceived ligand (e.g., flg22). From this perspective, responses are ligand-dependent and an elicitor cannot be categorized as a PAMP or an effector based solely on its evolutionary conservation or role in virulence.

Events Downstream of PRR-Mediated Perception of Bacterial Pathogens, and their Exploitation by *Agrobacterium*

Signal Transduction Cascades and Early Downstream Responses

Despite differences between FLS2 and EFR in mechanisms of biogenesis and desensitization to be discussed below, stimulation of *Arabidopsis* seedlings by flg22 or elf18 shows striking similarities in physiological responses and in the genes induced or repressed (Zipfel et al., 2006). For example, both peptides rapidly induce extracellular alkalization (Felix et al., 1999; Kunze et al., 2004), which would be predicted to enhance host resistance by attenuating the acid-dependent activation of the *Agrobacterium* virulence genes required for T-DNA transfer (Lacroix and Citovsky, 2013a). One mechanism that explains these overlapping responses is the use of a shared signaling pathway, which has been explored at two non-mutually exclusive levels: that of an adaptive co-receptor, and that of a shared MAPK signaling cascade.

The strong similarity in downstream responses led to a search for a common signaling element shared by EFR and FLS2. In particular, LRR receptor kinases belonging to the somatic-embryogenesis receptor-like kinase (SERK) family, particularly the Brassinosteroid receptor-associated kinase 1 (BAK1), have emerged as candidate co-receptors of EFR and FLS2. Using a reverse genetic screen to identify flg22-insensitive mutants, Chinchilla et al. (2007) discovered *bak1* mutants that were largely deficient in their flg22- and early elf18-induced ROS production but also showed a reduced response to brassinolide. Immunoprecipitation of MYC-tagged BAK1 *in vitro* in the presence of flg22 provided evidence for ligand-triggered formation of a FLS2-BAK1 complex. In a parallel experiment Heese et al. (2007) isolated the flg22-induced FLS2 complex and used MS/MS peptide sequencing to identify the co-immunoprecipitated BAK1 (also called SERK3). Roux et al. (2011) coimmunoprecipitated EFR with BAK1 and additionally showed that other SERK family members interact with EFR as well as with FLS2. Their results indicated that EFR can form strong complexes with several SERK family members (SERK1, SERK2, BAK1, and BKK1), while FLS2 complexes most strongly with BAK1, implying that functional redundancy between the SERK family members explains the observation that EFR is less dependent than FLS2 on BAK1 (Chinchilla et al., 2007; Roux et al., 2011; Schwessinger et al., 2011). Further experiments revealed that *bak1* null mutants are also impaired in their responses to lipopolysaccharides and peptidoglycans. Thus, BAK1 appears to act broadly as a co-receptor (Heese et al., 2007; Schulze et al., 2010). BAK1 also enhances FLS2 and EFR-mediated signaling by *trans*-phosphorylating the cytoplasmic kinase BIK1, a positive regulator of PTI shown to directly induce ROS production (Monaghan and Zipfel, 2012; Kadota et al., 2014). The recently identified BAK1-interacting receptor-like kinase BIR2 negatively regulates PTI by sequestering sub-pools of BAK1 from PRR interactions in the absence of a PAMP ligand, thereby controlling the availability of BAK1 to engage with its PRR partners (Halter et al., 2014a,b). The discovery of other critical co-receptors (e.g., BAK1-LIKE1, or BKK1), as well as other positive (e.g., SUPPRESSOR-OF-BIR1, or SOBIR1) and negative (e.g., BIR1) LRR-receptor-like kinase regulators has led to an appreciation of the cross-phosphorylation events that occur within an entire signaling complex, rather than an isolated BAK1-PRR interaction (Liebrand et al., 2014).

As its name implies, BAK1 was originally discovered to complex with a brassinosteroid receptor, BRI1 (Nam and Li, 2002). Brassinosteroids are plant hormones involved in cell growth and elongation, as well as in developmental processes including senescence (Clouse, 2011). *A priori*, it appeared plausible that a limiting pool of BAK1 could be responsible for the phenomenon of PAMP-triggered seedling growth inhibition, if FLS2/EFR-BAK1 complex formation competed with BR-mediated growth signals. However, elegant work by the Zipfel lab refuted this theory by showing brassinolide could inhibit PAMP-triggered responses without interfering with FLS2-BAK1 complex formation or downstream signaling (Albrecht et al., 2012). A forward genetic screen for elf18-insensitive mutants uncovered a new *bak1* mutant allele, *bak1-5*. Unlike the *bak1-4 bkk1-1* null mutant, the *bak1-5 bkk1-1* mutant shows far fewer pleiotropic effects and, in particular, normal brassinosteroid signaling and cell death control (Schwessinger et al., 2011). *bak1-5* is still severely impaired in characteristic PAMP-triggered responses, as well as in the ability to transphosphorylate BIK1 (Schwessinger et al., 2011).

Ligand binding to PRRs activates both MAPK and calcium-dependent protein kinase (CDPK) signaling cascades (Boudsocq et al., 2010; Tena et al., 2011). Asai et al. (2002) elucidated one such pathway, demonstrating that MEKK1, MKK4/5, and MPK3/6 mediate downstream responses upon flg22 perception. Chitin, peptidoglycan, and elf18 were also shown to induce MAPK activation. Interestingly, low concentrations of flg22 and elf18 act additively upon extracellular alkalization and MAPK activation, while high concentrations saturate both responses (Zipfel et al., 2006); these data indicate that a shared pool of MAP kinases may exist downstream of PAMP detection, although it

remains unclear whether the MAPKs themselves are responsible for limiting the amplitude of defense responses. Upon reaching a duration and magnitude threshold, the MAPK cascade protein activation interfaces with calcium-activated pathways to activate further defense responses (Tena et al., 2011), including regulation of defense hormone synthesis, metabolite synthesis, stomatal closure, and antimicrobial compound synthesis (Meng and Zhang, 2013).

Another response mediated by the MAPK pathway upon PAMP activation is the induction of early defense genes such as *WRKY29* and *FRK1* (Asai et al., 2002). WRKY29 is part of the WRKY superfamily of transcription factors that affect pathogen defenses, wounding, senescence, and trichome development by interacting with W-box motifs (TTGAC) in the promoter regions of a large variety of target genes (Maleck et al., 2000). These regulatory proteins are characterized by a DNA-binding motif whose amino acid sequence (WRKYGQK) gives the family its name (Eulgem et al., 2000). The WRKY superfamily is very large, and WRKY proteins tend to interact and work in redundant and antagonistic roles depending on the type of pathogenic attack (Xu et al., 2006). While many WRKY factors promote resistance, many others suppress basal defenses to prevent deleterious effects on the host. In at least some cases, the same WRKY proteins that down-regulate PAMP-induced responses are inactivated upon R protein recognition of their cognate effectors during ETI, thus derepressing defense mechanisms (Kim et al., 2008). The complexity of the WRKY network is further embellished by multiple positive and negative feedback loops and feed-forward modules (Taj et al., 2014).

Given the complexity of the receptor network and the centrality of the MAPK pathway to PTI, it should come as no surprise that multiple pathogen-derived effectors target these various components. A complete catalog of such effectors is beyond the scope of this review (for a recent review, see Deslandes and Rivas, 2012). As one example, HopAI1 is delivered by the T3SS of *P. syringae* into the host cytoplasm, where it interacts physically with MPK3 and MPK6, deactivating the pathway via dephosphorylation (Zhang et al., 2007). Likewise, the *P. syringae* effector HopAO1 suppresses PAMP-induced resistance by reversing the ligand-induced tyrosine phosphorylation of EFR required for signal transduction (Underwood et al., 2007; Macho et al., 2014). The afore-mentioned *P. syringae* effector AvrPtoB physically associates with the FLS2/BAK1 complex, and acts as a ubiquitin ligase to target FLS2 for degradation (Gohre et al., 2008). In a classic tit-for-tat strategy, the host resistance protein Pto is able to inactivate the effector's ligase domain, thus thwarting the pathogen's attempt to block host immunity (Ntoukakis et al., 2009).

Exploitation of Early Host Defenses by *Agrobacterium*

Although there is no evidence that *Agrobacterium* can suppress the initial recognition by EFR and/or any associated co-receptors, both the downstream phosphorylation cascade and specific WRKY proteins are important targets for subversion by this pathogen. Within 5 min of exposure of *Arabidopsis* seedlings to *Agrobacterium*, the key defense modulators MPK3, MPK4, and MPK6 are phosphorylated (Djamei et al., 2007). One of the substrates of MPK3 is the stress-responsive transcription factor VIP1 (Pitzschke et al., 2009), which was initially identified as interacting with the *Agrobacterium* virulence protein VirE2 (Tzfira et al., 2001). Since MPK3-catalyzed phosphorylation of VIP1 results in its translocation to the nucleus, Djamei et al. (2007) proposed that nuclear localization of the interacting single-stranded DNA binding protein VirE2 would neatly serve to deliver the associated T-DNA as well. In this "Trojan horse" model, *Agrobacterium* co-opts the MAPK-mediated defense pathway it has triggered to ensure nuclear entry of its transgene "gift." However, a very recent study from Shi et al. (2014) called this model into question by showing that under their experimental conditions neither the location of VirE2 nor host susceptibility to transformation correlate with, respectively, subcellular localization or abundance of VIP1. These authors' alternative model posits that instead of shuttling the T-DNA to the nucleus, VirE2 promotes tumorigenesis by sequestering the low-abundance VIP1 in the cytoplasm, thus serving as a true effector to dampen the activity of this host defense-related transcription factor.

Expression of the *VIP1* gene is repressed in roots (but not in shoots) by WRKY17, a negative regulator of host defenses that may function to prevent over-reactive responses to pathogens. Mutant *Arabidopsis* deficient in *WRKY17* exhibit elevated levels of *Agrobacterium*-mediated transformation (Lacroix and Citovsky, 2013b), although they are more resistant than wild-type plants to *P. syringae* (Joumot-Catalino et al., 2006). This discrepancy between the responses of these two bacteria to a *wrky17* mutant illustrates a prevailing observation that enhanced resistance to one pathogen can correlate with elevated susceptibility to another, a phenomenon often attributed to shifts in the antagonistic SA–jasmonic acid balance discussed in Section "Salicylic Acid" below (Joumot-Catalino et al., 2006). More generally, the difference serves as a cautionary note about the potential pitfalls of predicting precise defense-related outcomes for different pathogens, even if dealing with the same host species.

In an intriguing twist, *Agrobacterium* has very recently been shown to exploit the WRKY network by co-opting several members of this transcription factor family to drive expression of a key gene on the T-DNA. The three major *Agrobacterium*-derived cancer-causing transgenes encode enzymes that direct the production of the phytohormones auxin and cytokinin (Lacroix and Citovsky, 2013a). Zhang et al. (2015) discovered that although the auxin-production genes *IaaH* and *IaaM* are constitutively expressed in *Arabidopsis*, the promoter for the cytokinin synthesis gene *Ipt* contains several W-boxes and is activated by the mutually interacting trio of WRKY18, WRKY40 and WRKY60. *WRKY40* and *WRKY60* are induced within 2 h of *Agrobacterium* infection, while *WRKY18* is turned on slightly later. WRKY40 binds directly to the *Ipt* promoter, and its ability to activate expression is synergistically enhanced by auxin. Predictably, mutants deficient in any of the three *WRKY* genes form smaller tumors than wild-type plants (Zhang et al., 2015). These three host factors apparently normally function to dampen host defenses to bacterial and fungal pathogens, perhaps as part of the plant's feedback mechanism to prevent the deleterious effects

of over-reaction to a pathogen (Pandey et al., 2010). The findings of Zhang et al. (2015) reveal that *Agrobacterium* has evolved to capitalize on this host self-defense strategy. Given the complex and varied repertoire of steps choreographed by the large WRKY superfamily, it seems likely that other family members may also be unwitting partners in the Agrobacterial thrust for control of its host's metabolism.

Contributions of Cytoskeletal Dynamics and Membrane Trafficking to the Regulation of Defense Responses

Genetic dissection of the molecular mechanisms underlying PTI and ETI has led to several illuminating discoveries linking plant immunity to intracellular trafficking and cytoskeletal dynamics. Both of these fundamental cell biology processes are critical and well-documented facets of mammalian innate immune responses and bacterial pathogenesis (reviewed in Day et al., 2011), but represent under-studied and exciting new areas of research with respect to plant defenses. In particular, recent findings specific to EFR are likely to be of potential interest to researchers interested in the host responses to *Agrobacterium*.

Actin Dynamics

The efficiency of *Agrobacterium*-mediated transient transformation is reduced in actin-deficient roots and in cultured tobacco cells treated with actin microfilament or myosin light chain kinase inhibitors (Gelvin, 2012). Furthermore, exposure of *Arabidopsis* cotyledons to *Agrobacterium* causes an increase in actin filament density, without a concomitant change in the bundling of those filaments, within 6–9 h post-inoculation (Henty-Ridilla et al., 2013). These data are consistent with a growing appreciation for the involvement of actin dynamics in plant defenses. In light of recent discoveries in other host–pathogen interactions summarized here, the effects of *Agrobacterium*-induced changes in the host cytoskeleton on the progression of the infection is a question that warrants further investigation.

Changes in actin dynamics have been associated with both PTI and ETI in *Arabidopsis*. Susceptibility to *P. syringae* pv. *tomato* is increased upon pharmacological disruption of host actin filaments (Henty-Ridilla et al., 2013; Kang et al., 2014). Among the consequences that contribute to this outcome are defects in the actin-dependent protein transport and movements of endocytic vesicles required for immune system function (Kang et al., 2014). The *P. syringae* effector HopW1 promotes virulence by reducing the density of the actin filament network (Kang et al., 2014). Independently, Henty-Ridilla et al. (2013) also noted a change in actin bundling late in the infection only with a T3SS-competent pathogenic strain of this bacterium and that was hence attributed to effector-triggered events.

Using reverse genetics, Tian et al. (2009) identified the *Arabidopsis* actin depolymerizing factor 4 (ADF4) as essential for effector-specific HR. The sensitive phenotype of an *adf4* mutant could be partially rescued by exogenous application of an actin depolymerizing agent, supporting the claim that actin dynamics *per se* are required. In contrast with fungal and oomycete infections, in which ADF4 contributes to a block in pathogen entry, ADF4-dependent protection against the bacterial pathogen is linked to MAPK signaling (Tian et al., 2009; Porter et al., 2012). Taken together, the observation that ablation of *ADF4* specifically compromises resistance conferred by recognition of one specific bacterial effector, yet results in a reduction in expression of a PTI-specific target gene, provides additional evidence for coordinated regulation of PTI and ETI (Porter et al., 2012).

Subsequent experiments documented transient PTI-associated increases in actin filament density in *Arabidopsis* cotyledons as early as 3 h after challenge with bacterial or fungal pathogens, and with flg22 or chitin but not elf26; the flg22-induced changes required FLS2 as well as the co-receptors BAK1 and BIK1 (Henty-Ridilla et al., 2013). In contrast, in hypocotyls, high spatial and temporal resolution microscopic imaging of cortical actin filament architecture revealed EFR/BAK1/BIK1- and ADF4-dependent changes in single filament turnover within minutes following elf26 treatment (Henty-Ridilla et al., 2014). These changes lead to a rapid increase in the stability and hence overall number of actin filaments. Significantly, several hallmarks of elf26-triggered, but not chitin-triggered, PTI are dependent on ADF4 function; callose deposition and transcriptional changes downstream of CDPKs are disrupted in the *adf4* mutant, while MAPK-mediated responses to elf26 are unaffected. These findings strongly implicate ADF4 as an elf26-specific mediator of actin rearrangements and other downstream innate immune responses.

Among the other potential roles for actin dynamics in mediating host resistance is the regulation of stomate closure (Day et al., 2011). These openings serve as an important portal for bacterial entry into the host apoplast (Zeng et al., 2010). Both PAMPs (through their cognate PRRs) and effectors can trigger stomatal closure as an early line of host defense (Melotto et al., 2006; Zeng et al., 2010), and alterations in the arrays of actin filaments within the guard cells are associated with changes in stomate aperture (Gao et al., 2008; Day et al., 2011). To our knowledge, there is no published information on the stomatal response to *Agrobacterium*, although the elf18 peptide from *E. coli* can trigger closure in *Arabidopsis* (Zeng and He, 2010). As discussed below (see Pathogen Manipulation of Plant Hormone Responses), certain *P. syringae* strains have the ability to suppress closure (Melotto et al., 2006). Given that the guard cell transduction pathway mediating closure involves SA (Zeng and He, 2010), an early target of *Agrobacterium* intervention (see Modulation of Host Hormonal Responses by *Agrobacterium*), it seems plausible that *Agrobacterium* also has the ability to thwart the stomatal barrier.

Membrane Trafficking

Membrane trafficking has also emerged as having important consequences for defense signaling. Within 1 h of ligand binding, FLS2, but not EFR, is internalized by endocytosis; *bak1-4* mutants bind flg22 with wild type-like efficiency but are inhibited in endocytosis of the bound ligands (Chinchilla et al., 2007).

This cytoskeletal-dependent process results in localization of the PRR to late endosomal compartments called multi-vesicular bodies (Robatzek et al., 2006; Spallek et al., 2013). Ligand-bound endocytosed FLS2 is ultimately degraded, but this turnover is significantly slower than the internalization, leading to accumulated intracellular pools of activated receptor (Robatzek et al., 2006). In mammalian innate immunity, the analogous PAMP-responsive Toll-like receptors initiate certain signaling pathways from the endosome (McGettrick and O'Neill, 2010); whether the internal flg22-FLS2 pools are similarly functional is an open and potentially important question (Smith et al., 2014). Genetic ablation of the endosomal sorting complex required for transport (ESCRT-1) reduces FLS2 re-localization without affecting overall endocytic trafficking; the mutant plants are significantly more susceptible to colonization by *P. syringae* and are defective in flg22-induced stomatal closure, but not in other flg22-triggered responses such as oxidative burst, MAPK activation or callose deposition (Spallek et al., 2013). Pharmacological interference with the molecular machinery responsible for intracellular trafficking provided an additional approach to experimentally decouple various flg22-elicited responses. Collectively, the data to date suggest that ligand-triggered internalization and degradation of FLS2 desensitizes cells, potentially mitigating the costs associated with constitutive activation of FLS2-mediated defenses (Smith et al., 2014).

Genetic screens for EF-Tu-insensitive mutants and for FLS2-interacting partners uncovered several proteins involved in endoplasmic reticulum quality control and trafficking. Mutants deficient in the former process specifically abrogate EFR-mediated, but not FLS2-dependent, responses by abolishing accumulation of EFR (Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). At the same time, alterations in reticulon-like proteins that interact with FLS2 impair its accumulation at the plasma membrane and consequently FLS2-dependent signaling; FLS2 retention in the ER and its glycosylation, but not its stability, are affected (Lee et al., 2011). The dependence of EFR but not FLS2 on endoplasmic reticulum-associated folding factors for biogenesis is consistent with the notion that EFR may have evolved more recently than FLS2 and thus may not yet have undergone selection for high protein stability (Nekrasov et al., 2009; Saijo et al., 2009). It remains to be seen, however, whether this relative instability could also have mechanistic significance if, for example, different EFR conformers activate different, and separable, branches of the downstream signaling pathway (Saijo et al., 2009).

Unexpectedly differential fates for the two closely related receptors were also discovered through a yeast two-hybrid screen for BAK1 interactors. The results of this screen led to the finding that FLS2, but not EFR, is subject to polyubiquitination by PUB12 and PUB13, two E3 ubiquitin (Ub) ligases that are activated by BAK1-mediated phosphorylation. Upon flg22 binding to FLS2, the constitutively assembled PUB12/PUB13/BAK1 complex is recruited to FLS2. BAK1 then phosphorylates and thereby activates the Ub ligases, which rapidly ubiquitinate FLS2 but not BAK1 or BIK1. FLS2 ubiquitination appears to be unlinked to its internalization, but as expected modulates the ability of FLS2 to confer immunity; a *pub12/pub13* double mutant exhibits significantly higher resistance to *P. syringae* challenge (Lu et al., 2011).

These differences between FLS2- and EFR-centered intracellular events underscore again the need for caution when extrapolating from other, better-characterized plant pathogens to *Agrobacterium*. Despite the existence of multiple PAMPs and hence the possibility of functionally redundant or additive modes of elicitation by a given pathogen, at least some facets of the defenses induced in *Arabidopsis* by *P. syringae* appear to rely solely on FLS2 (Zeng and He, 2010). Conversely, as noted previously, *Agrobacterium* is detected primarily through EFR. In certain endoplasmic reticulum-quality control mutants in which EFR-mediated signaling is not completely abolished, the downstream outputs (MAPK signaling, callose deposition, and ROS production) are uncoupled, i.e., only partially and differentially impaired. Significantly, the characterization of these mutant plant lines has led to the proposal that there may be differences between FLS2 and EFR in the order of these post-recognition defense events (Lu et al., 2009).

Hormone Regulation of Systemic and Local Plant Immunity

Plant hormones typically act as mediators between external input and internal responses on both a systemic and intracellular level, often by influencing developmental processes. Their wide-ranging effects require tight control, as evidenced by the extensive antagonistic and synergistic cross-regulation that occurs among them. Three plant hormones, ethylene, JA, and SA are known as classic defense regulators, though other abiotic and developmentally induced hormones, including auxin, cytokinins, abscisic acid and giberellins, are also thought to be involved (Robert-Seilaniantz et al., 2011). In the sections below, we summarize briefly the roles of each of the three major defense hormones, before expanding on the pathways downstream of SA that, based on existing data, are most likely to represent potential foci of subversion by *Agrobacterium*.

Ethylene

Ethylene is a gaseous olefin that is critical for plant developmental processes including fruit ripening, senescence, and leaf abscission (Schaller, 2012). It accumulates in response to herbivore damage and mechanical wounding and is believed to fine-tune the balance between JA and SA-induced defenses (described below), as it can alternately reinforce or repress either in a context-dependent manner. *ein2* and *ein1* mutants, which overproduce ethylene but are characterized by ethylene insensitivity, exhibit increased disease susceptibility compared to wild-type plants (Guzmán and Ecker, 1990; Boutrot et al., 2010; Mersmann et al., 2010). Several lines of evidence indicate that ethylene contributes significantly to PTI. First, expression of the *FLS2* receptor gene is controlled by the binding of an ethylene response transcription factor, EIN3, to its promoter. Conversely, flg22 treatment induces both *EIN3* expression and ethylene synthesis (Felix et al., 1999; Chen et al., 2009; Boutrot et al., 2010). Thus, ethylene may play a role in regulating the positive feedback of *FLS2* accumulation

observed in response to EF-Tu and flg22 stimulation (Zipfel et al., 2006; Robert-Seilaniantz et al., 2011). Significantly, although *ein2* mutants are less sensitive to elf18 as well as flg22, the mechanisms underlying these dependencies are different; in contrast to *FLS2*, both *EFR* expression and accumulation of the encoded receptor are unaffected by the *EIN2* deficiency (Tintor et al., 2013). Ethylene signaling is also required for the FLS2-triggered oxidative burst (Mersmann et al., 2010). Furthermore, MPK6, activated by FLS2-triggered PTI, has a role in stabilizing ACS2 and ACS6, enzymes necessary for ethylene biosynthesis (Liu and Zhang, 2004). These results suggest a close connection between the initial events that trigger PTI and ethylene-induced plant immunity (Liu et al., 2013).

Jasmonate

Jasmonate is a lipid-derived signaling molecule ubiquitous to plants, animals, fungi, and some algae (Thaler et al., 2012). In plants, JA has roles in root growth inhibition, tuber formation, touch-mediated responses, flower development, and senescence (Wasternack, 2007). JA synthesis occurs via a linoleic acid precursor in response to herbivores and mechanical wounding, and both JA- and ethylene-dependent responses are required for resistance to necrotrophic pathogens. Some genes regulated by jasmonic acid are also regulated by ethylene, so many discussions of hormonal defense pathways tend to invoke JA and ET pathways together. For example, at least one transcription factor, ERF1, requires both hormones to activate defense gene transcription (Glazebrook, 2005). Moreover, EIN2, a necessary component of the ET-induced pathway can itself restore both ethylene and jasmonic acid signal responses in ethylene insensitive plants (Alonso et al., 1999).

Salicylic acid

The phenolic acid SA can be synthesized through two major pathways in plants, including one that uses phenylalanine as a precursor (Seyfferth and Tsuda, 2014). However, defense response-induced SA is typically only synthesized through the isochorismate (ICS) pathway, and a key enzyme (ICS1), encoded by the gene *SID2*, is often a target for SA regulation by downstream transcription factors (Wildermuth et al., 2001; Wang et al., 2006). The conservation of ICS1 in algae and bacteria suggests that the pathway may have originated from a plastid through an evolutionary endosymbiotic event (Wildermuth et al., 2001). Such a relationship could explain one way that certain pathogens, e.g., *P. syringae*, have evolved mechanisms to subvert plant hormone-mediated defenses. A well-known example is the bacterial NahG hydrolase that metabolizes SA. SA is critical for the induction of systemic acquired resistance, or SAR (Vlot et al., 2009). As initially described more than half a century ago, SAR results in enhanced resistance to secondary infection in locations far from the primary site of pathogen exposure (Ross, 1961). *nahG*-expressing plants have long been used in plant immunity research because they are unable to accumulate SA, induce SAR or express the pathogenesis-related (*PR*) genes that act as hallmarks and mediators of SAR. It should be noted, however, that pleiotropic effects caused by *nahG* overexpression have been reported (Heck et al., 2003). Similarly, *sid2-2* mutant plants with a defective ICS1 enzyme are unable to induce PR1 (Wildermuth et al., 2001) and conversely, exogenous application of SA or its functional analogs is sufficient to trigger PR gene expression (White, 1979).

The bioactive form of SA can only act on a local level or through phloem transport. Long-distance SAR induction is mediated by the volatile and biologically inactive form, methyl SA; interestingly, the extent to which SAR requires MeSA is light-dependent (discussed in more detail below). Perhaps the most intriguing recent discovery in this field is the existence of "trans-generational" SAR, in which enhanced resistance was observed in the progeny of primed hosts, even after a biotic stress-free generation. This epigenetic phenomenon is due to changes in histone modification and DNA methylation state, rather than hormone levels (Luna et al., 2012). Future exploration of this imprinting might prove fruitful in the development of disease-resistant seed stock.

The ethylene, SA and JA hormonal pathways work with other defense responses to orchestrate different defenses against biotrophic and necrotrophic pathogens (Glazebrook, 2005). Since biotrophic pathogens acquire sustenance from live plant tissue and nectrophic pathogens feed on dead plant tissue, the HR responses that result in plant cell apoptosis would provide resistance against biotrophic pathogens and encourage necrotrophic pathogen growth. Generally speaking, the SA-dependent pathway is elicited in defenses against biotrophic pathogens such as *Agrobacterium*, while as mentioned above, the ET/JA-dependent pathways are activated in the presence of necrotrophic pathogens (Glazebrook, 2005). However, more detailed multi-mutant analysis has revealed that all three hormonal pathways positively regulate defenses induced by both necrotrophic and biotrophic pathogens to different degrees (Tsuda et al., 2009). These data suggest a model in which an unspecialized and highly interconnected network may enable plants to maximize survival upon simultaneous attack by multiple pathogens on a single plant (Bar-Yam et al., 2009).

Evolutionary evidence suggests that JA–SA antagonism dates to the last common ancestor of land plants, although this antagonism may have arisen multiple times. In animals, an SA-derivative (acetyl salicylic acid, or aspirin) also inhibits JA-like prostaglandins, blocking platelet aggregation and pain transmission (Thaler et al., 2012). In plants, low concentrations of JA and SA can synergistically increase levels of *PR1* gene expression, but high levels work antagonistically, inducing ROS production and cell death. The regulatory mechanisms mediating this antagonism are complex, involving proteins (e.g., MAPK4) that suppress SA signaling but are required for the JA-induced pathway, and others (PAD4 and EDS1) that act to repress the JA/ET pathways while simultaneously increasing the SA-induced defense pathway (Loake and Grant, 2007). Like JA, the ethylene signal transcription factor EIN3 can also decrease SA levels by directly binding to the *SID2* promoter sequence and down-regulating gene expression (Chen et al., 2009).

NPR1 Mediates SA-Responsive Modulation of Defenses

NPR1 is a critical mediator of SA action and nexus of SA/JA crosstalk. This positive regulator of SA-induced defenses is

responsible for SA-responsive transcriptional reprogramming on a global genomic level through both direct and indirect mechanisms. At the same time, NPR1 acts through a negative feedback loop to limit SA accumulation (Shah, 2003). The *NPR1* (non-expresser of PR genes 1) gene was first identified through a screen of mutants deficient in SAR-induced PR gene expression (Cao et al., 1994). Further genetic screening for SA-insensitive mutants repeatedly revealed only mutants of *npr1* alleles, suggesting that either SA directly regulates NPR1 or that there are functionally redundant signaling factors between SA recognition and NPR1 activation (Dong, 2004). NPR1 expression occurs at a low constitutive level and does not change dramatically upon SA induction or pathogen infection. Its overexpression confers a protective effect but does not correlate with constitutive expression of *PR* genes (Ryals et al., 1997; Cao et al., 1998; Durrant and Dong, 2004), suggesting that NPR1 is primarily regulated post-transcriptionally.

Cytosolic NPR1 is held inactive as oligomers and released NPR1 monomers translocate to the nucleus, where they associate with the TGA family of transcription factors; this complex binds to SA-responsive *cis*-acting promoter elements to induce PR gene expression (Dong, 2004). How SA activates NPR1 remains an intriguing, if challenging, open question. Much of what we know thus far comes from the Dong and Després labs, whose mechanistic studies have slowly unraveled the mechanisms of NPR1 activation over the past decade. One recent study demonstrated that two NPR-family proteins, NPR3 and NPR4, bind SA with differing affinities and act as adaptors for proteasomal degradation of NPR1. In a yeast two-hybrid system, exogenous SA promoted NPR1-NPR3 association while disrupting the interactions between NPR1 and NPR4 (Fu et al., 2012). These and other data led these authors to propose a model in which basal levels of NPR1 are modulated upon ETI by the gradient of SA that develops in and around the site of infection. In this model, high SA concentrations in the center of the HR lesion facilitate NPR3-mediated degradation of NPR1, allowing programmed cell death to proceed, while lower SA levels at the margins enable NPR1 accumulation which inhibits the spread of the HR and promotes SAR. In the absence of pathogen challenge, the high-affinity binding of NPR4 with SA relieves the constitutive NPR4-directed turnover of NPR1 that prevents inappropriate defense activation. Other data and models, however, argue that NPR1 binds SA directly, leading either to a conformational change that induces oligomer disassembly and thereby releases auto-inhibition of NPR1 (Wu et al., 2012) or to diminished inhibitory interactions between NPR1 and the negative defense regulator NIMIN2 (Maier et al., 2011). Additionally, sequential interactions with various members of the NIMIN protein family may enable NPR1 to respond to differential concentrations of SA, preventing inadvertent PR activation (Hermann et al., 2013).

While SA-dependent activation of NPR1 seems to be a critical node in SAR regulation, *NPR1* expression may itself be modulated by other defense genes. Its promoter contains W-boxes for WRKY binding (Yu et al., 2001), and conversely, NPR1 directly targets and upregulates expression of many *WRKY* genes. Elucidation of NPR1 targets has proven difficult because NPR1 requires SA activation, and simple transcriptome analysis of SA-induced cells would yield many indirect or non-NPR1 targets. Using a transgenic plant line expressing *NPR1* under glucocortecoid-inducible promoter control and comparing SA-induced changes in the transcriptomes of a protein synthesis-inhibited and a non-inhibited sample, Wang et al. (2006) identified candidate genes that were directly regulated by NPR1. Of these, mutant analysis revealed both positive (e.g., WRKY18) and negative (e.g., WRKY58) regulators of SAR. NPR1 also targets WRKY70 and its functional homolog, WRKY54, which both act as positive regulators of SA-mediated gene expression and resistance, but repress SA biosynthesis through ICS1, thus regulating SA/JA cross-talk.

Pathogen Manipulation of Plant Hormone Responses

Pathogens may modulate host hormone responses to increase the likelihood of successful infection or, as in the case of biotrophic pathogens, to create a more favorable environment for long-term survival. One of the most direct examples of modulation comes from the hemibiotrophs *P. syringae* pv. *tomato* (*Pst* DC3000) and pv. *maculicola* ES4326, which repress SA accumulation in *Arabidopsis* through a JA analog and phytotoxin, coronatine (Bereswill et al., 1994; Kloek et al., 2001; Brooks et al., 2005). Mechanistically, coronatine upregulates NAC transcription factors. In guard cells, this prevents the stomatal closure defense response (Melotto et al., 2006), while in neighboring leaf cells, it suppresses *ICS1* and induces the SA-metabolizing genes *SAGT1* and *BSMT1* (Zheng et al., 2012). Thus, certain strains of *Pseudomonas* take advantage of the plant's natural hormone JA, and its antagonistic relationship to SA, by producing an effector that mimicks JA, thereby suppressing SA-mediated host defenses necessary for establishing local and systemic bacterial resistance (Brooks et al., 2005).

Modulation of Host Hormonal Responses by *Agrobacterium*

Several lines of evidence indicate that *Agrobacterium* also engages in hormonal dueling to attenuate host defense responses, although this story is far from fully resolved. Extensive transcriptome profiling of JA/SA/ethylene and auxin-induced genes in *Agrobacterium*-inoculated *Arabidopsis* stems over three timepoints (3 hpi, 6 dpi, 35 dpi) revealed that a number of auxin and ethylene-signaling genes were upregulated in response to the virulent *Agrobacterium* C58 strain, while only a few auxin-related genes responded to inoculation with the avirulent T-DNA deficient *Agrobacterium* strain GV3101 (Lee et al., 2009). Since ethylene has been demonstrated to suppress *Agrobacterium* virulence, the increase in host ethylene levels upon infection may contribute to the plant's ability to combat agrobacterial infection at a relatively early stage (Nonaka et al., 2008; Lee et al., 2009). Indeed, although *Agrobacterium* lacks the ACC deaminase used by other plant-associated bacteria to enzymatically cleave the ethylene precursor, engineering the bacteria to express this enzyme enhances transformation efficiency (Nonaka and Ezura, 2014).

In contrast, the relationship between *Agrobacterium* infection and SA-mediated defenses defies simple characterization based on known paradigms for SA activity. Tomato seedlings respond to *Agrobacterium* exposure by altering which pathway is utilized for SA synthesis (Chadha and Brown, 1974). On tobacco leaves, 48 h of exposure to *Agrobacterium* is sufficient to dampen the SA production elicited by subsequent *P. syringae* inoculation, and confers resistance both to *P. syringae* colonization (Rico et al., 2010) and to tobacco mosaic virus by a mechanism that is at least partially SA-dependent (Pruss et al., 2008). Interestingly, neither of these effects requires Ti-plasmid encoded functions. In *Arabidopsis* stems, SA accumulates in C58-inoculated plants at 6 days, but not at 3 h, post-infection, suggesting a role for SA in regulating late defenses (Lee et al., 2009). In contrast, an earlier study had found that within an hour after bacterial attachment, SA accumulation is reduced by 40% and *PR* genes are down-regulated in *Arabidopsis* roots infected with the avirulent strain GV3101 (Gaspar et al., 2004). The *Agrobacterium*-triggered activation of MPK4 as rapidly as 5 min post-inoculation (Djamei et al., 2007) may be one of the ways this plant response is attenuated, as MPK4 has been shown to negatively regulate both pathogen-induced SA accumulation and ROS production (but not callose deposition; Berriri et al., 2012). Since SA directly inhibits expression of the *Agrobacterium* virulence genes required for T-DNA delivery (Yuan et al., 2007; Anand et al., 2008), down-regulation of SA accumulation in the first hour(s) of infection would enable the bacterium to initiate the transformation process. Indeed, tumor growth is significantly higher in mutant plants with low endogenous SA levels (*nahG*, *eds1*, *pad4*), and lower in mutant plants with high endogenous SA levels (*npr1*, *cpr5*; Yuan et al., 2007; Anand et al., 2008; Lee et al., 2009). Paradoxically, stems from *sid2* mutants have been reported to exhibit wild-type amounts of tumor formation (Lee et al., 2009). Furthermore, neither *ICS1* expression nor *PR1* gene expression (typically induced by elevated SA levels) is affected by either virulent or avirulent agrobacterial strains at the time points examined, although genes implicated in SA methylation and other *PR* genes are upregulated after T-DNA integration (Lee et al., 2009).

Taken together, these data implicate ethylene and an NPR1-independent function for SA in protecting the host against *Agrobacterium*, likely in part by attenuating the bacterial virulence machinery at early stages of the infection process (Yuan et al., 2007; Anand et al., 2008; Lee et al., 2009). Conversely, by manipulating hormonally regulated plant defense pathways, *Agrobacterium* is able to confer resistance to subsequent pathogen challenge.

Host Physiology Influences Defense and Hormonal Pathways

Circadian Effects

Given that both hormones and light control many aspects of plant life, it is not surprising that the defense and hormonal pathways interact and crosstalk with the plant circadian clock in a variety of ways (Robertson et al., 2009). In *Arabidopsis*, *P. syringae* infections during morning and midday elicit higher SA levels and greater defense responses than in the evening (Griebel and Zeier, 2008). The circadian-regulated PHT4;1 (phosphate transporter 4;1), also named ANTR1, acts upstream of the SA-induced pathway (Wang et al., 2011). JA accumulates in a circadian-regulated fashion that coincides with the feeding patterns of *Trichoplusia ni* insects, allowing *Arabidopsis* an advantage over herbivores by perpetually mounting a timely resistance against attack (Goodspeed et al., 2012). Ethylene production peaks at midday in *Arabidopsis;* the *ACS* genes necessary for ethylene biosynthesis are both circadian- and light-regulated, although mutants with aberrant ethylene production exhibit normal circadian control over cycles of growth (Thain et al., 2004). Finally, circadian gating of light-responsive, hormone-triggered stomata opening (Robertson et al., 2009) may have important implications for pathogen entry.

The circadian clock also affects hormone-independent defense pathways, although this is less well-studied. Circadian clock component transcription factors CCA1 (circadian clock associated 1) and LHY (late elongated hypocotyls) directly regulate defense activation independent of SA-induced defense responses (Schaffer et al., 1998; Wang and Tobin, 1998; Zhang et al., 2013a). A number of defense-related genes including *PHT4;1* and the *PCC1* (pathogen and circadian controlled) gene, which plays a role in defenses specifically against virulent oomycetes, are under CCA1 transcriptional control (Sauerbrunn and Schlaich, 2004; Wang et al., 2014). CCA1 and LHY synergistically affect both PTI and ETI, in part by regulating stomatal control. Significantly, *P. syringae* infection or the PAMP flg22 feed back to affect the circadian clock, thus demonstrating cross-talk between innate defenses and the circadian clock (Zhang et al., 2013a).

Involvement of the circadian clock in defenses enhances the plant's ability to anticipate potential infection and to mount defenses accordingly (Eriksson and Millar, 2003). In *Arabidopsis*, the circadian clock modulates PTI-related responses to anticipate dawn infection of *P. syringae* pv. *tomato* DC3000 by up-regulating levels of downstream responders, including the MKK4/5-MAPK3/6-WRKY22 protein module and WRKY29 (Bhardwaj et al., 2011). Similarly, in the case of the fungal pathogen *Hyaloperonospora arabidopsidis*, circadian-regulated defense gene activation increases at dawn and decreases at dusk, consistent with the fact that *H. arabidopsidis* releases spores at dawn (Wang et al., 2011). As these examples illustrate, it is clear that plants with functional circadian rhythms may have an advantage in combating pathogenic attack by anticipating pathogen infection and priming defenses at peak-infection times to maximize growth time and resources (McClung, 2011). There are as yet no published reports on the impact of day length or time-of-day of inoculation on the outcome of *Agrobacterium* infection. The data reviewed here suggest, however, that such effects would not be surprising, and would point to the importance of early defenses in determining the success of the agrobacterial assault.

Developmental Stage of the Plant

More mature hosts are often more resistant than younger plants, a phenomenon that has been referred to generically as age-related resistance (ARR). A variety of forms of resistance are

encompassed by this term; they vary with host species and with pathogen, and can be either broad-spectrum or specific to one pathogen, pathovar, or strain. Lasting resistance emerges at multiple major life cycle transitions, such as juvenile to adult, flowering or upon the onset of senescence, although neither flowering nor senescence *per se* appears to be required or sufficient for ARR (Carella et al., 2015). In some cases, race-specific ARR reflects the induction of R genes at a particular point in the plant's development (Develey-Riviere and Galiana, 2007). Hormonal pathways, central as they are to various developmental processes, also contribute to ARR. For example, although ARR is distinct from SAR, SA accumulation is required (Kus et al., 2002), and both developmental and pathogen modulation of SA may be key determinants of ARR (Carella et al., 2015). The developmental transition from susceptibility to resistance includes the accumulation of anti-microbial compounds, possibly SA itself, in the intercellular fluids (reviewed in Carella et al., 2015), although the mechanisms of resistance differ between *Arabidopsis* and tobacco (Kus et al., 2002; Develey-Riviere and Galiana, 2007). Given the apparent centrality of SA in the *Agrobacterium*-host interchange, these observations raise the possibility that the outcome of *Agrobacterium* infection may be exquisitely sensitive to ARR, and that different labs could obtain distinctly non-concordant results depending on the precise developmental stage of the host at the time of infection.

Environmental Influences on Plant Defenses and Microbial Virulence

Light-Dependent Effects

Though the circadian clock is entrained by the presence and absence of light to regulate plant defenses, light stimulus can itself affect plant responses to pathogen encounters (Roden and Ingle, 2009). The strength of both *PR* gene induction and the HR response depend on the presence of light (Genoud et al., 2002; Chandra-Shekara et al., 2006). Moreover, the time-of-day dependence of defenses is at least partially due to the availability of light and phytochrome photoperception during local and systemic defense induction, independent of any circadian-regulated stomatal control (Griebel and Zeier, 2008). For example, both accumulation and activation of the radish-derived defense regulator Raphanusanin require light (Moehninsi et al., 2014). More generally, Sano et al. (2014) recently showed that 30% of the genes up-regulated upon flg22 perception require light, and specifically photoelectron flow, for that induction; several of those genes are involved in SA biosynthesis, and flg22-treated plants accumulated more SA in the light than in the dark. In this study plants were illuminated for 4 h prior before the flg22 elicitation. Overall, there was significant overlap between the pool of flg22-responsive genes and those that were light-dependent or light-repressed.

Thus, the success of a pathogenic infection may be partially determined by the prior (or subsequent) light exposure, and hence time of day of the inoculation, which can impact laboratory research experiments on plant defenses (Roden and Ingle, 2009). Perhaps the clearest illustration of this principle to date is the influence of light on the requirements for SAR signaling. Several lines of evidence in multiple host species have implicated methyl salicylate (MeSA), a volatile form of SA, as the signal molecule that is translocated through the phloem to distal leaves, where it is converted to active SA (Park et al., 2007). However, Attaran et al. (2009) showed that *A. thaliana* mutant lines deficient in methyl SA production are nonetheless able to mount SAR in leaves distal to the site of pathogen inoculation. Liu et al. (2011) resolved this apparent conflict by discovering that MeSA is essential for SAR development in plants infected late in the day but not in the morning; the key determinant was shown to be the length of time of light exposure subsequent to the inoculation.

While light stress induces excess excitation energy (EEE) relative to that needed for normal physiological photosynthetic activity, pathogen infection can also induce EEE by affecting photosynthetic rates. Moreover, EEE-induced plant responses share similarities to defense responses including increases in ROS, SA-induced signaling through EDS1 and PAD4, and programmed cell death. Interestingly, plants acclimated to high intensities of light also display high levels of resistance against pathogens, similar to the effect of SAR in resistance to secondary infections (Roden and Ingle, 2009).

Light also seems to have an effect on the virulence of some pathogens. In *Agrobacterium*, the presence of constant light during co-cultivation with host plants correlates with higher levels of T-DNA transfer, potentially by affecting attachment to plant cells and/or plant cell's ability to take up the *Agrobacterium* T-DNA (Zambre et al., 2003). Additionally, in the *Dendrobium* orchid and *Agrobacterium* model, synthesis of the virulence gene inducer coniferyl alcohol is stabilized in the presence of light (Nan et al., 1997). Paradoxically, the presence of light has been reported to decrease *Agrobacterium* accumulation of flagellar proteins FlaA and FlaB, reducing bacterial motility, attachment to plant cells, and bacterial virulence on cucumber (Oberpichler et al., 2008).

Temperature-Dependent Effects

In addition to light, SA-mediated responses and susceptibility to pathogen attack are affected by temperature. Cheng et al. (2013) recently demonstrated that PTI, as measured by expression of the MAPK target genes *WRKY29* and *FRK1* in response to flg22, is most activated in *Arabidopsis* at temperatures between 23 and 32°C (with an optimum of 28°C). In contrast, activation of ETI and resultant cell death by inducible expression of a bacterial effector transgene peaked at 16°C and was significantly less pronounced at 28°C or higher. In tobacco, the failure of the HR or SAR at elevated temperatures is correlated with a lack of *PR* gene induction, which can be overcome by exogenous application of SA (Yalpani et al., 1991). The preferential activation of ETI at low ambient temperatures correlates well with the temperature range at which bacterial secretion systems and effector production are optimally functional (Cheng et al., 2013). In the case of *Agrobacterium*, we and others have shown that elevated temperature (28°C) prevents biogenesis of the VirB/VirD4 Type IV secretion system responsible for export of the T-DNA and several virulence proteins (Banta et al., 1998; Baron et al., 2001). Conversely, bacterial proliferation, and hence synthesis of PAMP elicitors, thrive at the

elevated temperature range at which the PTI is most responsive (Cheng et al., 2013).

Areas Ripe for Future Research

In conclusion, the success of pathogenic infection on plants results from a complex network of interwoven interactions among host recognition/response systems, plant hormonal pathways, circadian rhythms, light perception, and other plant-specific events such as host developmental stage, as well as bacterial and fungal virulence factors. For practical as well as historical reasons, much of the research to date on plant defenses has focused on a rather limited subset of pathogens and host species. The great majority of these investigations have utilized leaf tissue and/or seedlings as a model system. Yet as the plant tissue exposed to the remarkable microbial richness of the rhizosphere, roots represent perhaps the most relevant site for studying many *in situ* host–microbe interactions (De Coninck et al., 2014). Recent work in the Ausubel lab has confirmed that *Arabidopsis* roots mount complex, highly choreographed, tissue-specific responses to PAMP/MAMP elicitation (Millet et al., 2010). Some of these responses are ethylene-dependent, and some are suppressed by coronatine mimicking of JA but, unlike in leaves, in a manner that is independent of SA–JA antagonism. Significantly, these authors detected no root response to the EF-TU-derived elicitor elf26 (Millet et al., 2010), implying that this host lacks a critical mode of surveillance for *Agrobacterium* in the pathogen's primary habitat.

Given that defense gene expression patterns are not always conserved in timing or magnitude among different host tissues, future efforts will be needed to explore the similarities and differences between the canonical model systems and the other "native" settings in which chance or deliberate host–microbe encounters occur. In nature, of course, every such encounter is perturbed by countless bystander microbes, some hoping to "cut in" to the dance while others are merely milling around, crowding the dance floor. While some bystanders may suppress host defenses, others induce defenses, priming the host for enhanced resistance to subsequent pathogen exposure. Indeed, this resistance forms the basis for one common assay for the capacity of a pure elicitor to incite basal defenses. The nature of the activated defenses will almost certainly vary depending on the partners tested, but many such studies to date have relied upon a single bacterial model, *P. syringae*. Deepening the pool of well-characterized host defense-pathogen relationships to include *Agrobacterium* will likely uncover previously uncharacterized PAMPs and their cognate receptors, as well as novel mechanisms by which the microbes suppress or thwart their hosts' responses.

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Horizontal gene transfer from *Agrobacterium* to plants

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Most genetic engineering of plants uses *Agrobacterium* mediated transformation to introduce novel gene content. In nature, insertion of T-DNA in the plant genome and its subsequent transfer via sexual reproduction has been shown in several species in the genera *Nicotiana* and *Linaria*. In these natural examples of horizontal gene transfer from *Agrobacterium* to plants, the T-DNA donor is assumed to be a mikimopine strain of *A. rhizogenes*. A sequence homologous to the T-DNA of the Ri plasmid of *Agrobacterium rhizogenes* was found in the genome of untransformed *Nicotiana glauca* about 30 years ago, and was named "cellular T-DNA" (cT-DNA). It represents an imperfect inverted repeat and contains homologs of several T-DNA oncogenes (Ng*rolB*, Ng*rol*C, NgORF13, NgORF14) and an opine synthesis gene (Ng*mis)*. A similar cT-DNA has also been found in other species of the genus *Nicotiana*. These presumably ancient homologs of T-DNA genes are still expressed, indicating that they may play a role in the evolution of these plants. Recently T-DNA has been detected and characterized in *Linaria vulgaris* and *L. dalmatica*. In *Linaria vulgaris* the cT-DNA is present in two copies and organized as a tandem imperfect direct repeat, containing Lv*ORF2*, Lv*ORF3*, Lv*ORF8*, Lv*rolA*, Lv*rolB*, Lv*rolC*, Lv*ORF13*, Lv*ORF14*, and the Lv*mis* genes. All *L. vulgaris* and *L. dalmatica* plants screened contained the same T-DNA oncogenes and the *mis* gene. Evidence suggests that there were several independent T-DNA integration events into the genomes of these plant genera. We speculate that ancient plants transformed by *A. rhizogenes* might have acquired a selective advantage in competition with the parental species. Thus, the events of T-DNA insertion in the plant genome might have affected their evolution, resulting in the creation of new plant species. In this review we focus on the structure and functions of cT-DNA in *Linaria* and *Nicotiana* and discuss their possible evolutionary role.

Keywords: *Agrobacterium*, T-DNA, horizontal gene transfer, *Nicotiana*, *Linaria*

INTRODUCTION

Horizontal gene transfer (HGT) takes place widely in prokaryotes, where its ecological and evolutionary effects are well-studied (Koonin et al., 2001). Comparative and phylogenetic analyses of eukaryotic genomes show that considerable numbers of genes have been acquired by HGT. Gene acquisition by HGT is therefore a potential creative force in both eukaryotic and prokaryotic genome evolution. However, mechanisms of HGT are poorly understood in the Eukaryota in comparison to gene transfer among the Procaryotae. The persistence of horizontally transferred genes in some organisms may confer selective advantages (Koonin et al., 2001; Richardson and Palmer, 2007). Most examples of HGT in higher plants involve the transfer of chloroplast or mitochondrial DNA and have been the subject of numerous reviews (Dong et al., 1998; Richardson and Palmer, 2007). There are few descriptions of horizontal transfer of nuclear genes between species. One example is transfer of the gene that codes for the cytosolic enzyme phosphoglucose isomerase predicted to have occurred between *Festuca ovina* and some species from the genus *Poa* (Ghatnekar et al., 2006; Vallenback et al., 2008, 2010). Evidence of gene transfer from bacteria to the nuclei of multi-cellular eukaryotes is rare (Richards et al., 2006; Acuna et al., 2012). HGT from bacteria to plants has been restricted to *Agrobacterium rhizogenes* and representatives of genera *Nicotiana* and *Linaria*, and represents some of the most recent transfers in evolution (White et al., 1983; Intrieri and Buiatti, 2001; Matveeva et al., 2012; Pavlova et al., 2013).

A. rhizogenes, and the related bacterium *A. tumefaciens*, transform a wide variety of host plants by transferring a segment of the large tumor-inducing plasmid, called T-DNA, into host cells (White et al., 1982; Otten et al., 1992; Veena et al., 2003; Tzfira and Citovsky, 2006; Vain, 2007). The T-DNA is integrated through non-homologous recombination into the host cell genome where it is expressed. Expression of T-DNA genes results in the formation of hairy roots or crown galls, that are transgenic tissues, formed on a non-transgenic plant. This phenomenon is called "genetic colonization," one of the examples of the host-parasite relationship (Tzfira and Citovsky, 2006). It is unclear whether or not colonized plants have received benefits from such colonization, however, we could expect that it is beneficial in some cases since there are footprints of HGT from *Agrobacterium* to plants in the genomes of several present day plant species.

T-DNA IN *NICOTIANA GLAUCA*

In early investigations of *Agrobacterium* mediated transformation of plants, most researchers assumed that there was no significant homology to the T-DNA in untransformed plant genomes. White et al. (1982) attempted to detect pRiA4b T-DNA sequences in the genome of *Nicotiana glauca*, transformed in laboratory conditions by *Agrobacterium rhizogenes* strain A4. Southern analysis detected a fragment of pRiA4 in the transgenic tissue. Surprisingly, a hybridization signal was also detected in uninfected tissues of *N. glauca*. Further analysis confirmed the presence of DNA homologous to T-DNA in the *N. glauca* genome. This homologous DNA was referred to as "cellular T-DNA" (cT-DNA) (White et al., 1983).

Furner et al. (1986) investigated *Nicotiana glauca* plants, collected in geographically separated territories. Southern analyses showed the presence of cT-DNA in all studied varieties of *N. glauca*. Sequencing of the *N. glauca* cT-DNA demonstrated that it was organized as an imperfect inverted repeat. The left arm of cT-DNA, containing *rolB* and *rolC* homologs (Ng*rolB* and Ng*rolC*L) was more extended than the right arm, which contained only the *rolC* homolog (Ng*rolC*R). The coding sequences of Ng*rolB* and Ng*rolC*R were found to contain early stop codons.

Subsequent analysis of the nucleotide sequence of this cT-DNA identified open reading frame 13 (ORF13) and ORF14 homologs in both the left and right arms, called NgORF13L, NgORF14L, NgORF13R, and NgORF14R, respectively (Aoki et al., 1994).

In 2001 Suzuki et al. characterized *A. rhizogenes* strain MAFF301724 and described a new opine synthase gene (mikimopine synthase gene *mis*). A part of the *mis* gene displayed strong homology to distal fragments of *N. glauca* cT-DNA, called Ng*mis*L and Ng*mis*R, respectively (Suzuki et al., 2002). Suzuki et al. (2002) suggested that the complete cT-DNA region of *N.glauca* is comprised of the 7968 bp left arm and 5778 bp right arm that were derived from the T-DNA of a mikimopine Ri plasmid similar to pRi1724. The level of nucleotide sequence similarity between the left and right arms is greater than 96% and the gene order is conserved suggesting a duplication event. The structure of the *N.glauca* cT-DNA is summarized in **Figure 1**. Since cT-DNA has been identified in all studied varieties of *N. glauca* (Furner et al., 1986), it is reasonable to suggest that the transformation event occurred before the formation of this species. This suggests that other related species may contain cT-DNA.

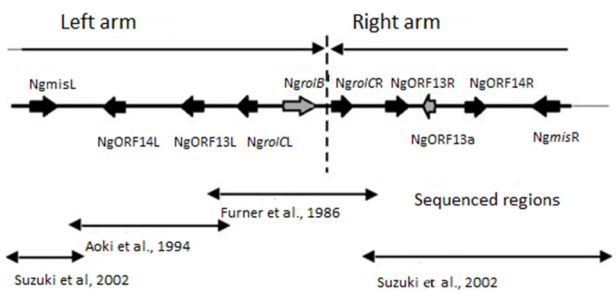

FIGURE 1 | Structure of cT-DNA in the *Nicotiana glauca* genome (based on Suzuki et al., 2002). The cT-DNA and its flanking regions are indicated. Lines with a single arrowhead indicate the imperfect inverted repeat. Lines with arrowheads at both ends indicate regions sequenced by each of three groups.

T-DNA IN OTHER *NICOTIANA SPECIES*

The genus *Nicotiana* is one of the largest genera in the Solanaceae and contains 75 species that are characterized by a wide range of variations among their floral and vegetative morphology (Clarkson et al., 2004). The different *Nicotiana* species evidence interspecific crosses which complicates *Nicotiana* phylogeny. Goodspeed hypothesized that there are two distinct lineages in *Nicotiana* which arose from two ancestral pre-petunioid and pre-cestroid lineages. He supposed that the base chromosome number of the genus was 12 and stressed the role of doubling and hybridization in *Nicotiana* evolution. Goodspeed divided *Nicotiana* into three sub-genera *Rustica, Tabacum*, and *Petunioides* and 14 sections (Goodspeed, 1954). Since then, the number of subgenera of *Nicotiana* has remained constant, while the number and composition of the sections has been revised (Clarkson et al., 2004). 75% of tobacco species originate from the Americas and 25% of species are from Australia (Goodspeed, 1954; Clarkson et al., 2004).

Identification of T-DNA in *N. glauca* raises two questions: what other *Nicotiana* species contain cT-DNA, and what was the pattern of dissemination within the group?

To answer the first question Furner et al. (1986) examined the genomes of 17 species of the genus *Nicotiana*. Using Southern analyses he showed that only six species from the subgenera *Rustica* and *Tabacum* contained sequences homologous to the *rol* genes of *Agrobacterium rhizogenes*. These species are *N. glauca*, *N. otophora, N. tomentosiformis, N. tomentosa, N. benavidesii, N. tabacum*. Examination of T-DNA- like sequences in *N. tabacum* has shown that it contains a *rolC* homolog and two ORF13 homologs (t*rolC*, tORF13-1 *and* tORF13-2, respectively) (Meyer et al., 1995; Frundt et al., 1998). Intrieri and Buiatti studied the distribution and evolution of *Agrobacterium rhizogenes* genes in the genus *Nicotiana.* Forty two species representing all *Nicotiana* sections were examined for the presence of *rolB*, *rolC*, ORF13, and ORF14 homologs in their genomes. T-DNA-like sequences detected were compared with each other and with contemporary sequences of *Agrobacterium*. The results demonstrated the presence of at least one T-DNA gene in each of 15 *Nicotiana* species representing all three subgenera. All currently available data on the distribution of cT-DNA among *Nicotiana* species are summarized in **Table 1**.

It is important to note, that there are some inconsistencies among the data by Furner et al. (1986) and Intrieri and Buiatti (2001). For example, Intrieri and Buiatti (2001) showed that T-DNA is present in *N. debneyi* and *N. cordifolia*. Furner et al. (1986) found no T-DNA in these species. This contradiction requires additional studies.

Thus, to date, T-DNA was found in every *Nicotiana* subgenus which include species, native to America and Australia (Goodspeed, 1954).

Phylogenetic analyses were performed by Intrieri and Buiatti (2001) to compare nucleotide sequences of cT-DNA in several *Nicotiana* species with the T-DNA of *Agrobacterium*.

Table 1 | Distribution of T-DNA-like sequences among *Nicotiana* species.

	Section*	Species	T-DNA genes	Sequence Acc#	References
Rustica	*Paniculatae*	*N.glauca* *	+*(rolB-mis)*	X03432.1; D16559.1 AB071334.1; AB071335.1	1, 2, 3, 4**
		N.paniculata	–		2, 5
		N.knightiana	–		2, 5
		N.solanifolia	–		5
		N.benavidesii	+*(rolC)*	n/a***	2, 5
		N.cordifolia	+*(rolB-ORF14)*	AF281252.1 AF281248.1 AF281244.1	2, 5
		N.raimondi	–		5
	Rusticae	*N.rustica*	–		2, 5
Tabacum	*Tomentosae*	*N.tomentosa*	+*(ORF13-mis)*	n/a	2, 4
		N.tomentosiformis	+*(rolC-mis)*	AF281249.1 AF281245.1 AF281241.1	2, 4,5
		N.otophora	+*(rolC-ORF14)*	AF281250.1 AF281247.1 AF281243.1	2, 5
		N.setchelli	+*(rolC)*	n/a	2
	Nicotiana	*N.tabacum*	+*(rolC-mis)*	AF281246.1 AF281242.1	2,4,5
Petunioides	*Undulatae*	*N.glutinosa*	–		2, 5
		N.undulata	–		5
		N.arentsii	+*(rolC)*	n/a	5
	Trigonophyllae	*N.trigonophylla*	–		5
	Sylvestris	*N.sylvestris*	–		5
	Alatae	*N.langsdorffi*	–		2, 5
		N.alata	–		5
		N.longiflora	–		5
		N.forgetiana	–		5
		N.sanderae	–		5
		N.plumbaginifolia	–		5
	Repandae	*N.nesophila*	–		5
		N.stocktonii	–		5
		N.repanda	–		5
		N.nudicaulis	–		5
	Noctiflorae	*N.noctiflora*	–		5
		N.petunioides	–		5
	Petunioides	*N.acuminata*	*(rolC)*	n/a	5
		N.pauciflora	–		5
		N.attenuata	–		5
		N.miersii	+*(rolB)*	n/a	5
	Bigelovianae	*N.bigelovi*	+*(rolB)*	n/a	5
	Polydiclae	*N.clevelandi*	–		5
	Suaveolentes	*N.umbratica*	–		5
		N.debneyi	+*(rolC)*	AF281251.1	5
		N.gossei	+*(rolC)*	n/a	5
		N.rotundifolia	–		5
		N.suaveolens	+*(rolC)*	n/a	5
		N.exigua	+*(rolC)*	n/a	5
		N.goodspeedii	–		5

*Nicotiana sections from Knapp et al. (2004) and N.glauca section is from Goodspeed (1954); ** 1, White et al., 1983; 2, Furner et al., 1986; 3, Aoki et al., 1994; 4, Suzuki et al., 2002; 5, Intrieri and Buiatti, 2001; ***n/a, not available.

The following species were used in the analyses and represented all three subgenera: *N. cordifolia* (subgenus *Rustica* sec. *Paniculatae*); *N. tomentosiformis* and *N. otophora* (subgenus *Tabacum*), *N. tomentosiformis* (participated in *N. tabacum* speciation together with *N. sylvestris*); *N. glauca* used to be included in the subgenus *Rustica* sec. *Paniculatae* (Goodspeed, 1954), but later it was moved to the sec. *Noctiflorae* of the subgenus *Petunioides* (Knapp et al., 2004); and *N. debneyi*

[*Suaveolentes*, an Australian section of *Nicotiana*, and a polyploid species of the subgenus *Petunioides* (Knapp et al., 2004)].

Analysis of nucleotide sequences revealed that *N. cordifolia* and *N. glauca rolB, rolC*, ORF13, and ORF14 genes show a high level of sequence similarity (93.5–98.5%). These data indicate that *N. cordifolia* and *N. glauca* are related species and are consistent with the proposal of Goodspeed (1954) that both species should be included in subgenus *Rustica* sec. *Paniculatae*. Similar clustering was found between the representatives of the subgenus *Tabacum*. Sequence similarities were lower between *Rustica* and *Tabacum* species, ranging from 66.3 to 68.6% for *rolC* and from 70.2 to 82.9% for ORF13 but was higher for ORF14 (94–97%). Surprisingly, the petunioid *N. debneyi rolC* gene demonstrates high sequence similarity (93.4%) with the *N. glauca rolC* gene, but lower similarity (around 67%) with those found in species belonging to the subgenus *Tabacum*. It was speculated that the polyploid species *N. debneyi* got cT-DNA from an ancestor of sec. *Paniculatae*. The homologies suggest that the *Nicotiana rol* genes parallel *Nicotiana spp*. evolution, being divided into two clusters, one that includes *N. glauca, N. cordifolia*, and *N. debneyi*, the second comprising species from the subgenus *Tabacum* (Intrieri and Buiatti, 2001).

Present day cT-DNA genes clustered with each other, making it difficult to predict which Ri-plasmid would be the source of the cT-DNA in each *Nicotiana* species. Since the pace of evolution differs between bacteria and plants, and since the pRi T-DNAs may have undergone rearrangements with each other (Moriguchi et al., 2001), it is difficult to define which ancient T-DNA was the origin of cT-DNA in different *Nicotiana* species using such phylogenetic analysis (Tanaka, 2008).

Another option for exploring the origin of cT-DNA is opine typing which was performed by Suzuki et al. (2002). They identified opine gene homologs in *N. glauca* (Ng*misL and* Ng*misR*, respectively) and screened 12 *Nicotiana* species for *mis* homologs using Southern blot hybridization. The analyses included five species from the subgenus *Rustica* (*N. glauca, N. benavidesii, N. paniculata, N. knightiana, N. rustica*), five species from the subgenus *Tabacum* (*N. tomentosa, N. tomentosiformis, N. otophora, N. tabacum and N. glutinosa*), and two species from the subgenus *Petunioides* (*N. langsdorfii and N. sylvestris*). Homologs of gene *mis* were detected in the genomes of *N. glauca, N. tomentosa*, *N. tomentosiformis* and *N. tabacum*, however, the size of the hybridized fragments was different between *N. glauca* and species in the subgenus *Tabacum* and the hybridization pattern in *N. tomentosa* was different from that of the two species in the subgenus *Tabacum* (*N. tabacum* and *N. tomentosiformis*). Since T-DNA fragments of *N. tabacum* were identical to those of *N.tomentosiformis* and were not detected in the genome of *N. sylvestris*, the *mis* gene of *N. tabacum* likely came from *N. tomentosiformis*.

Suzuki et al. (2002) sequenced DNA in *N. glauca* adjacent to the cT-DNA. To investigate regions adjacent to the cT-DNA in other species, Southern hybridization was carried out using either a DNA fragment outside the left or right arms of the cT-DNA of *N. glauca* as a probe. All examined *Nicotiana* genomes showed the presence of sequences homologous to both sides of the cT-DNA suggesting that these are original sequences existing in the genomes of *Nicotiana* plants. Similar size fragments were found in most species of the subgenus *Rustica* and *Tabacum*, which likely represent subgenus-specific restriction fragments. Interestingly, the signals using NgL and NgR as probes fell into the same fragment in the genomes of *N. tomentosa*, *N. tomentosiformis*, *N. tabacum*. Although the same DNA sequences bordering the cT-DNA in *N. glauca* were found in the genome of these three species, the sequences were not contiguous to the cT-DNA therefore the location of the cT-DNA in *N. glauca* is different from that in the species of the subgenus *Tabacum*.

Thus, the phylogenetic analysis undertaken by Intrieri and Buiatti (2001), and the study of opine genes and T-DNA integration sites performed Suzuki et al. (2002), suggest that there have been no less than two acts of *Agrobacterium* mediated transformation in the evolution of *Nicotiana* species (**Figure 2**). While the comparison of DNA sequences and the detection of *mis* homologs clearly demonstrates that the origin of the cT-DNA in *N. glauca*, *N. tabacum*, *N. tomentosa,* and *N. tomentosiformis* is derived from a mikimopine-type Ri plasmid similar to pRi1724, the origins of the cT-DNA in other species are still unknown.

EXPRESSION OF *NICOTIANA* cT-DNA GENES

pRi transgenic plants exhibit a specific phenotype (dwarfing, loss of apical dominance, increased root mass, and decreased rate of fertilization) (Tepfer, 1984). However, *Nicotiana* species that contain cT-DNA in their genomes show no such phenotype. Are these genes expressed and functional, or they are pseudogenes?

Early experiments by Taylor et al. (1985) did not detect transcripts of the cT-DNA genes in *Nicotiana glauca*. Other researchers were able to detect transcripts of Ng*rolB*, Ng*rolC*, NgORF13, NgORF14 in callus tissues of *N. glauca* (Aoki and Syono, 1999a) as well as in genetic tumors of F1 *N. glauca* × *N. langsdorffii* hybrids, but not in leaf tissues of the same hybrid (Ichikawa et al., 1990; Aoki et al., 1994). Northern analyses of *N. tabacum* oncogenes showed that a *trolC* transcript accumulated in shoot tips and young leaves. The expression pattern of tORF13 was similar to t*rolC*, however, tORF13 expression was detected in flowers (Meyer et al., 1995; Frundt et al., 1998).

Intrieri and Buiatti studied transcription of the cT-DNA genes using RT-PCR in a number of species, using *N. langsdorffii* as a negative control. For their analyses authors used leaves from young *in vitro*-grown plantlets and hormone autotrophic (habituated) callus tissues grown on a hormone-free cultural medium as previously described by Bogani et al. (1985).

In this study *rolB* transcripts were found to be present in all cases in habituated callus tissues, but not in leaves; *rol*C was expressed in calli as well as in leaves of *Rustica* species (*N. glauca* and *N. cordifolia*), and only in calli in species representing the subgenus *Petunioides*; no expression was demonstrated to occur in species from the subgenus *Tabacum*. ORF13 and 14 mRNA was always detected in calli and ORF13 transcripts were found in leaf tissues of *N. tabacum* and *N. tomentosiformis*.

Aoki and Syono (1999b, 2000) analyzed the function of Ng*rol* genes by transforming leaf explants of *N. tabacum* and *N. debneyi* with *A. tumefaciens* that harbored either a *rolB-rolC*-ORF13-ORF14 fragment from pRi or cT-DNA of *N. glauca*. Nearly all of the leaf segments inoculated with pRi fragment developed

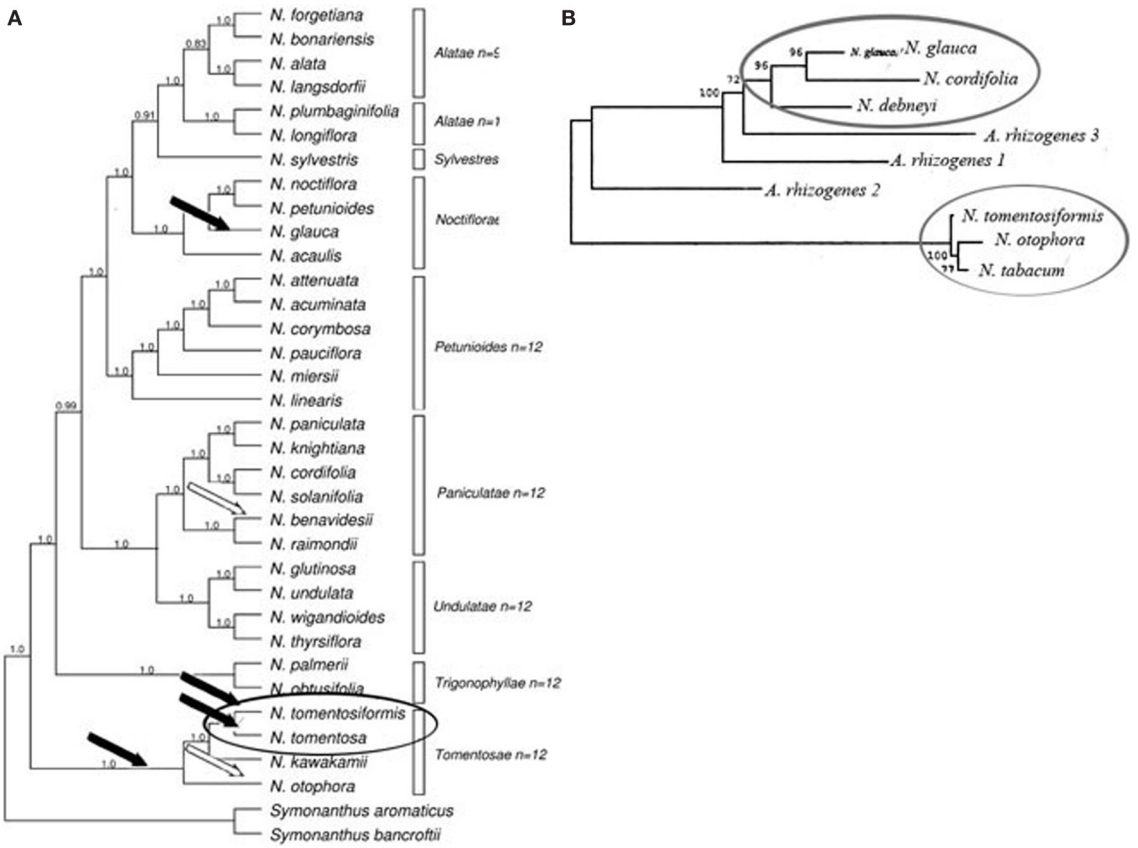

FIGURE 2 | Phylogenetic analysis of *Nicotiana* (Clarkson et al., 2004), using ITS and chloroplast sequences together with T-DNA marker. **(A)** Bayesian analysis of diploids only combined dataset (plastid and ITS). Consensus of 40,001 trees with posterior probabilities shown above branches. Bars indicate *Nicotiana* sections according to Knapp et al. (2004), Black and white arrows indicate the deduced insertion events by mikimopine-type or unknown opine-type pRi T-DNAs correspondingly (Suzuki et al., 2002). **(B)** Phylogenetic analysis of *rolC,* gene (Intrieri and Buiatti, 2001) by neighbor-joining method. Ovals show results of possible independent transformation events.

hairy roots. No significant root growth, however, appeared on the explants treated with *A. tumefaciens* that harbored Ng cT-DNA.

A comparison of the nucleotide sequences of Ng*rolB* and Ri*rolB* indicates that these oncogenes have different length coding regions. Each ORF starts at the same position, but Ng*rolB* ends at early termination codon 633 bp from the initiation site. The authors suggested that *N. glauca* plants do not exhibit the hairy-root phenotype due to the truncation of the Ng*rolB* reading frame.

A comparison of the DNA sequences of Ng*rolC* and Ri*rolC* indicates that the reading frame of Ng*rolC* begins and terminates at the same positions as Ri*rolC*. *Nicotiana tabacum* leaf disks were transformed with the P35S-Ng*rolC* chimeric construction, yielded transformants that expressed a dramatically dwarfed phenotype, probably because of the reduced length of internodes. The leaves of these P35S-Ng*rolC* transgenic plants were lanceolated and pale green, with floral organs that were thin and small. These characteristics were identical to the phenotype of the P35S-Ri*rolC* transgenic plants, described earlier (Schmülling et al., 1988). Transgene expression was detected only in transformants that demonstrated these characteristic morphological shifts, no transcripts were detected in leaf tissues from a comparable T0 plant demonstrating a normal phenotype (Aoki and Syono, 1999b).

To compare the expression patterns between the Ng*rol* genes of N. glauca and the Ri*rol* genes of Agrobacterium rhizogenes, Nagata et al. (1996) carried out fluorometric and histochemical analyses of the tissues from transgenic genetic tumors, growing on the hybrid of Nicotiana glauca × N. langsdorffii (F1) that contained a beta-glucuronidase (GUS) reporter gene fused to the promoter of (Ng*rol*B, Ng*rol*C, Ri*rol*B, or Ri*rol*C. In all constructs they studied, significantly higher GUS activity was found in tumors than in the other organs (roots, stems, and leaves) of transgenic plants. The tendency toward higher GUS activities in tumors than in normal tissues seen with the Ri*rol*B and Ri*rol*C promoters was also seen with the Ng*rol*B and Ng*rol*C promoters. GUS activities from the rolB promoter expressed in normal F1 plants were, however, different from those seen from the rolC promoter. The expression of the Ri*rol*B and Ng*rol*B promoters in stems, roots, and leaves were 10–100 fold lower than in genetic tumors. Almost no activity was detected in leaves. By contrast, expression from the Ri*rol*C and Ng*rol*C promoters was only 1.5–10 fold lower than in genetic tumors and a significant activity was detected in leaves.

Histochemical analysis of transgenic normal Fl plant tissues showed that Ng*rolB*-GUS and Ng*rolC*-GUS, as well as Ri*rolB*-GUS and Ri*rolC*-GUS, had common tissue-specific expression patterns. Ng*rolB*-GUS normal Fl transgenic plant tissues displayed high GUS activity in the meristematic zones of roots and in the apexes of shoots. A similar pattern of staining was ob-served in the Ri*rolB*-GUS transgenic plants. In the case of Ng*rolC*-GUS and Ri*rolC*-GUS normal Fl transgenic plant tissues, GUS activity was observed primarily in the apices, vascular bundles of leaves, stems, and roots.

Expression of the *mis* gene homologs in *N. glauca* was detected by RT-PCR (Suzuki et al., 2002). It was shown that both homologs of the *mis* gene were amplified by RT-PCR using separate ortholog specific primers. These data support the hypothesis that the *mis* homologs are not pseudogenes. Transgenic plants transformed by the T-DNA of the wild type plasmid Ri1724 or by the *mis* gene alone, synthesize mikimopine in different organs (Suzuki et al., 2001), although no mikimopine accumulation was detected in wild-type *N. glauca* by paper electrophoresis. It is therefore likely that Ng*mis* homologs are transcribed at a very low level.

A full-length Ng*mis*R homolog was isolated and integrated into an expression vector in *Escherichia coli*. The purified Mis protein was able to catalyze synthesis of mikimopine from L-histidine and α-ketoglutaric acid in a reaction buffer supplemented with NADH as a co-factor (Suzuki et al., 2002).

Thus, the oncogenes of *Nicotiana* cT-DNA are expressed in different tissues of present day tobacco plants at a low level and are therefore not pseudogenes.

cT-DNA AND GENETIC TUMORS IN *NICOTIANA*

Genetic tumors appear in certain genotypes spontaneously without being induced by any detectable environmental factor, and the tumor state is hereditary. Spontaneous genetic tumors in *Nicotiana* were first reported by Tanaka (2008). They have been detected throughout the plant and in whole progeny populations of certain crosses of *Nicotiana* species (Kehr and Smith, 1954). In some hybrids, genetic tumors have been reported to be formed irregularly in some of the offspring or limited to certain organs of the plants (Smith, 1958). It has been proposed that certain genes appropriately combined in a hybrid promote the development of these genetic tumors (Naf, 1958; Ahuja, 1968). Naf (1958) divided *Nicotiana* species into two groups, so called "plus" and "minus" groups. The "plus" group consists mainly of the species of the section *Alatae* whereas the minus group contains species from several sections. Crosses between the species within "plus" or "minus" groups do not produce tumorous progeny, while crosses between species from "plus" and "minus" groups do. Ahuja (1968) hypothesized that the species belonging to the "plus" group have a gene or a locus defined as initiator (I) and the species belonging to the "minus" group have a number of genes or loci (ee) for tumor enhancement and expression. For tumor formation both I and ee loci must be present.

Fujita (1994) expect that most species belonging to the minus group contain cT-DNA and that its genes could somehow be associated with the formation of genetic tumors on the *Nicotiana* hybrids. However, since there are no reports showing a connection between the ee genes and cT-DNA genes so far, this promising hypothesis has not yet been validated (Tanaka, 2008). As already mentioned, Ng*rolB*, Ng*rolC*, NgORF13 and NgORF14 genes are transcribed in genetic tumors on *N. glauca* × *N. langsdorffii* F1 hybrids (Ichikawa et al., 1990; Aoki et al., 1994). Some of these genes function in several organs of non-tumorous hybrid plants, like their counterparts in pRi T-DNA (Nagata et al., 1995, 1996; Udagawa et al., 2004). As soon as tumorigenesis is initiated by aging or stress, these genes are active in the developing outgrowth in a regulated manner. This means that a high level of expression of Ng*rol* genes is correlated with tumor formation on an F1 hybrid. However, it has not been determined if the formation of tumors is caused by the expression of Ng*rol* genes (Tanaka, 2008). Moreover, the stem and leaf tissues of *Nicotiana* species accumulate transcripts of the Ng*rol* genes (Meyer et al., 1995; Frundt et al., 1998). These observations suggest that the expression of these Ng*rol* genes might be unrelated to the induction of tumors. Overexpression of Ng*rolC*, NgORF13, or tORF13 cause the proliferation of cells on carrot disks (Frundt et al., 1998), and morphological alterations of tobacco explants, similar to hairy root syndrome on transgenic plants (Aoki and Syono, 1999a,b). Therefore, cT-DNA oncogenes may be responsible for enhancing the development of genetic tumors (Tanaka, 2008).

It is widely discussed that phytohormones contribute to genetic tumor formation. The role of auxin and cytokinins in genetic tumor formation in *Nicotiana*, however, has been disputed. On the one hand, in the light-grown tissues of genetic tumors, indole acetic acid (IAA) was found to be the predominant auxin and its level increased during tumor initiation (Bayer, 1967; Ichikawa et al., 1989). On the other hand, in dark conditions endogeneous IAA remained at a constant, low level throughout the tumorigenetic process (Fujita et al., 1991). A higher cytokinin level was associated with tumorigenesis in tumor-prone hybrid tissues. While analyzing the role of cytokinins in *Nicotiana* genetic tumor formation, Feng et al. (1990) have shown that tumor formation of X-ray-induced non-tumorous mutants of *N. glauca* × *N. langsdorffii* was restored either by the insertion of the *A. tumefaciens ipt* gene, which encodes the key enzyme of cytokinin biosynthesis, or by the addition of cytokinin. Nandi et al. (1990) determined the profile of endogenous cytokinins in genetic tumors of *N. glauca* (Grah.) × *N. langsdorffii* (Weinm.) hybrids. They showed that while zeatin is predicted to be the predominant endogenous cytokinin in tissues of all ages, the genetic tumor tissue derived from this hybrid does not contain notably high endogenous cytokinin levels.

Since tumor growth may be caused not only by high concentrations of hormones, but also by enhanced sensitivity to them, this may explain the contradictory data on the content of hormones in tumors. Even if hormone levels are increased in tumors, it is not necessarily caused by the expression of the cT-DNA genes: *rol* genes can be regulated by hormones. For example, it was shown that expression of Ng*rolB* was induced by auxin, as was Ri*rolB*, probably through the presence of the auxin-responsive *cis*-element ACTTTA found in the promoters of Ng*rolB* and Ri*rolB* which is acted upon by the trans-factor NtBBF1 (Tanaka, 2008).

It is clear that further work will be needed to establish the relationship between cT-DNA oncogenes and genetic tumorigenesis in *Nicotiana*.

SEARCH FOR T-DNA-LIKE SEQUENCES IN OTHER SOLANACEAE SPECIES

The Solanaceae is a large angiosperm family containing many economically important crops. *A. rhizogenes* is known to infect species belonging to different Solanaceae genera. Intrieri and Buiatti (2001) attempted to identify T-DNA-like sequences in species belonging to genera *Cestrum*, *Petunia,* and *Solanum* (*C. parqui, C. foetidus, P. hybrida, S. tuberosa, S. melongena, C. annuum,* and *S. lycopersicon*) using the same screening procedure, as they did for *Nicotiana*. None of the species screened showed amplification by PCR and no hybridization was obtained using *A. rhizogenes* and *N. glauca* probes. Kulaeva et al. (2013) extended the analysis to species from genus *Solanum* looking for T-DNA-like sequences. The authors used TaqMan real-time PCR with degenerate primers and probes for *rolB, rolC,* ORF13, ORF14 to analyze the following species: *S. chmielewskii, S. esculentum var. cerasiforme, S. glabratum, S. habrochaites, S. peruvianum, S. pimpinellifolium*, *S. cheesmanii, S. parviflorum, S. chilense, S. acaule, S. ajanhuiri, S. albicans, S. andigenum, S. berthaultii, S. boyacense, S. boyacense, S. canarense, S. canarense, S. cardiophyllum, S. chacoense, S. chaucha, S. chocclo, S. curtilobum S. demissum, S. demissum, S. doddsii, S. dulcamara*, *S. fendleri, S. goniocalyx, S. hjertingii, S. hondelmanii, S. hougassi, S. jamesii, S. juzepczukii, S. kurtzianum, S. mamilliferum, S. phureja, S. pinnatisectum, S. pinnatisectum, S. polytrichon, S. riobambense*, *S. rybinii, S. sparsipilum, S. spegazzinii, S. stenotomum, S. stoloniferum, S. tarijense, S. tenuifilamentum, S. tuberosum, S. vernei, S. verrucosum, S. oplocense.* They used *N. tabacum* DNA as positive control and *N. langsdorffii* as negative control. Amplification of specific sequences was not detected in any of the tested species.

This data shows that the presence of cT-DNA is not a feature of whole Solanaceae family, but has only been described to date for members of the genus *Nicotiana*.

T-DNA IN OTHER DICOTYLEDONOUS FAMILIES

Given the documented occurrence of cT-DNA, it is reasonable to hypothesize that other plant species, outside of the family Solanaceae, would have been transformed by *Agrobacterium* and contain at least remnants of cT-DNA.

The existence of cT-DNAs in species outside of the family Solanaceae has been reported by several groups. Using Southern analyses, sequences similar to pRi T-DNA were found in the genomic DNA of normal carrot (*Daucus carota*) (Spano et al., 1982), field bindweed (*Convolvulus arvensis*) (Tepfer, 1982); and carpet bugleweed (*Ajuga reptans*) (Tanaka, 2008). These studies did not involve DNA sequencing so they were not able to confirm whether there is T-DNA in the analyzed species.

Prior work in our group (Matveeva et al., 2012) attempted to clarify whether fixed T-DNA is present in other species outside the genus *Nicotiana*, and to evaluate the evolutionary relevance of natural T-DNA transfer. We sought to quickly screen a large number of plant genomes for the presence of T-DNA from both *A. tumefaciens* and *A. rhizogenes* using a modification of TaqMan-based real-time PCR (Livak et al., 1995) that combines the positive features of PCR and DNA blot hybridization in a single reaction (Matveeva et al., 2006). The search was limited to dicotyledonous plants native to temperate zones (mild winter, warm summer, and sufficient rainfall) due to the common occurrence of *Agrobacterium* in soils under these climate conditions.

This work analyzed 127 dicotyledonous plant species belonging to 38 different families. Species included carrot (*Daucus carota*) and field bindweed (*Convolvulus arvensis)*, mentioned above. Each of the plants was screened for DNA sequences homologous to two different sets of T-DNA oncogenes: The first set included sequences homologous to *A. rhizogenes* oncogenes (*rolB, rolC*, ORF13, and ORF14), and the second contained sequences homologous to *A. tumefaciens* oncogenes (*tms1* and *tmr*). Plant DNA samples from 126 species did not display detectable fluorescent signals for the T-DNA genes from either *A. rhizogenes* or *A. tumefaciens*. However, DNA samples isolated from several plants of *L. vulgaris* gave a positive result. In contrast, amplification was not observed using primers for the *tms1* and *tmr* genes of *A. tumefaciens* (Matveeva et al., 2012).

The absence of T-DNA homologs in most of the plant species investigated leads us to the conclusion that HGT from *Agrobacterium* is a rare event in the plants. However, finding cT-DNA sequences in the genomes of species other plants than *Nicotiana* indicates that HGT from *Agrobacterium* to plants occurs outside of this genus.

It is interesting to note that the only examples of HGT demonstrated thus far occur in plants transformed by *A. rhizogenes*, but not *A. tumefaciens.* This may suggest that infection induced by *A. rhizogenes* is more efficient than that induced by *A. tumefaciens* (Tepfer, 1984).

cT-DNA IN THE GENOMES OF THE GENUS *LINARIA*

Sequences homologous to T-DNA oncogenes in *Linaria vulgaris*, were identified by real time PCR and named Lv*rolB,* Lv*rolC,* LvORF13, LvORF14. BLAST analyses demonstrated the highest level of sequence identity (93%) between Lv*rolC* and Ri*rolC* from the pRiA4 of *A. rhizogenes.* LvORF14 had the lowest similarity to the corresponding *Agrobacterium* oncogene (85%). A homolog of a gene for mikimopine synthase (*mis*) was also identified. To define the full extent of the cT-DNA integrated into *Linaria vulgaris* genome, a chromosome walking approach was performed to identify the upstream fragment of the Lv cT-DNA. This work indicated that the *L. vulgaris* genome contains two copies of cT-DNA which are organized as an imperfect direct repeat. Analysis of the cT-DNA copies demonstrated that both of them contain sequences similar to the following genes: ORF2, ORF3, ORF8, *rolA, rolB*, ORF 13, ORF1, and *mis*. The left side of the repeat contains additional sequence, homologous to part of the agrocinopine synthase (*acs*) gene. Analysis of the flanking regions of the Lv T-DNA was performed by real-time thermal asymmetrical interlaced (TAIL)-PCR with primers and probes to the Lv*mis* gene. The flanking plant DNA identified in this analysis was found to be similar to the Ty3/gypsy-like retrotransposon (Matveeva et al., 2012).

Samples of *L. vulgaris* were collected in the European part of Russia in Moscow, Voronezh and Krasnodar regions, and in the Asian part of Russia in the Novosibirsk, Tumen, and Chelyabinsk regions. The distance between the most western to the most eastern points was about 4000 km. The distance from the most northern to the most southern points was about 2000 km. Two to three plants were analyzed from each of these collection points. All of the samples contained T-DNA-like sequences, however, there was polymorphism among their nucleotide sequences (Matveeva et al., 2012).

Analysis of the sequences for both *L. vulgaris* homologs of *rolC* demonstrated that they contained intact open reading frames. The fragments corresponding to the coding regions of genes Lv*rolB*, LvORF13, LvORF14, and Lv*mis* contain several stop codons or frameshifts that alter the ORFs. An analysis of the expression of these genes was carried out in tissues from the internodes, leaves, and roots of 1 month old, *in vitro* aseptically grown plants using RT real-time PCR. No mRNA corresponding to Lv*rolB*, Lv*rolC*, LvORF13, LvORF14 and Lv*mis* genes was amplifiable from these samples, therefore, the *rol* genes do not appear to be transcribed in *L. vulgaris* (Matveeva et al., 2012).

Toadflaxes (*Linaria* Mill.) form the largest genus within the tribe *Antirrhineae*. *Linaria* includes about 150 species that are widely spread in the Palearctic region, but the representatives of the genus are the most variable in the Mediterranean basin. The origin of the genus has been placed in the Miocene era (Fernandez-Mazuecos and Vargas, 2011) predating the Messinian Salinity Crisis (Hsu et al., 1977). The latest classification of the genus *Linaria* accepts seven sections (*Linaria, Speciosae, Diffusae, Supinae, Pelisserianae, Versicolores, and Macrocentrum*) (Sutton, 1988).

Studies indicate that cT-DNA exists in a number of *Linaria* species belonging to the sections *Linaria* and *Speciosae* (Matveeva and Kosachev, 2013; Pavlova et al., 2013). No cT-DNA was detected in *Linaria* species outside these sections. It appears that *rolC* is concerved among studied *Linaria* species based on the sequencing analysis of *rolC* homologs in *L. genistifolia* subsp. *dalmatica* (sec. *Speciosae*) and *L. acutiloba* (sec. *Linaria*) (Matveeva and Kosachev, 2013; Pavlova et al., 2013).

Thus, HGT of T-DNA from *Agrobacterium* to plants is not limited to *Nicotiana* spp, it has also occurred in the genus *Linaria*. The *rolC* homolog is the most conserved gene among the cT-DNA genes in *Linaria* and *Nicotiana* spp. In both genera plants were transformed by a mikimopine strain of *A. rhizogenes.*

POSSIBLE FUNCTION OF T-DNA IN PLANT GENOMES

The existence of several independent acts of *Agrobacterium* mediated transformation of plants and the maintenance of the cT-DNA in plant genomes during the process of evolution propose, that T-DNA-like sequences may give some selective advantages to the transformed plants (Ichikawa et al., 1990; Matveeva et al., 2012).

Suzuki et al. (2002) mentioned two possible functions of cT-DNA: increasing root mass leading to tolerance to drought, and changing the biological environment, particularly the soil microbiome represented by root-associated bacterial populations. Increasing root mass would seem beneficial for tolerance to dry conditions. Hence, ancient transformed plants with increased root mass might have demonstrated increased tolerance to dry environments surviving in arid conditions (Tanaka, 2008). However, no phenotype of the hairy root disease is observed in *Nicotiana* and *L. vulgaris* plants. In contrast, *L. vulgaris* explants show *in vitro* a shooty phenotype and in representatives of both genera *rolB* is mutated. Among the oncogenes of pRi T-DNA, *rolB* gene function seems to be the most important for hairy root induction because transformation of plants by the Ri*rolB* gene alone can induce hairy root formation. In contrast to the pRi*rolB*, the Ng*rolB* gene alone or in combination with other *N. glauca* homologs of *A. rhizogenes* oncogenes did not induce adventitious roots (Aoki and Syono, 1999a,b).

Aoki and Syono (1999b) performed base substitutions at two nucleotide positions, using site-directed mutagenesis, with the aim of producing a full-length form of Ng*rol*B capable of stimulating adventitious root induction. Transgenic plants overexpressing this altered Ng*rolB* demonstrated typical morphogenetic abnormalities. This experiment shows the possibility that a functional *rolB* gene may have operated during early steps of the evolution of transgenic *Nicotiana*.

Identification and sequencing of the *mis* homologs in *Nicotiana* and *Linaria* suggests that the origin of their cT-DNA is probably the mikimopine Ri plasmid. The presence of this gene may be related to plant–microbe interactions. Oger et al. (1997, 2000) reported that producing opines in genetically modified plants alters their ecological environment, in particular, changing the soil microbiome and root-associated microbe populations. If the synthesis of opines were beneficial for a plant species (even at a low level, in a specific tissue, or at a specific stage of oncogenesis), it may impact the appearance of advantageous plant–bacterium interactions. Plants maintaining cT-DNA in the genome could potentially maintain certain species of microorganisms in their rhizosphere via the secretion of opines in the root zone. Such potentially beneficial bacteria in the rhizosphere may in turn influence the root microbiome and convey nutritional and/or defensive features.

Early flowering or a shift from biennial to annual lifecycle without vernalization can take place on pRi transgenic *Cichorium intybus* and *Daucus carota* plants (Limami et al., 1998). These flowering features are beneficial when propagating such transgenic plants over the untransformed parentals. When considering the adaptational potential of natural transformation, the authors focused on the occurrence of flowering in the absence of a cold treatment. Given the mobility of seeds by wind, animals, and water, it is likely that biennial varieties or ecotypes may be transported to the southern latitudes where annualism would be beneficial. However, *Nicotiana* and *Linaria* species are not biennials (Goodspeed, 1947; Sutton, 1988; Blanco-Pastor et al., 2012). In addition, cT-DNA containing *Linaria* species from the sections *Linaria* and *Speciosae* are perennial, while other sections contain annual species. It is interesting to note that the cT-DNA containing sections are found worldwide while other sections are in the Mediterranean region and the Pyrenees (**Table 2**) (Sutton, 1988). It is unclear if this observation is due to the rarity by which plants acquire permanent cT-DNA, or if its foundation is related

Table 2 | Major features of infrageneric taxa of the genus *Linaria* (according to Sutton, 1988).

Section	Habit	Distribution
Linaria	Perennial	Eurasia
Speciosae	Perennial	Europe
Diffusae	Annual or perennial	Mediterranean
Supinae	Annual or perennial	Mediterranean
Pelisserinae	Annual or perennial	Mediterranean
Versicolores	Annual or perennial	Mediterranean, Iberian Peninsula
Macrocentrum	Annual	Mediterranean

to some fitness benefit conferred by the cT-DNA. It can be speculated, however, that the ecological plasticity of species within the sections *Linaria* and *Speciosae* is somehow associated with the presence of cT-DNA in their genomes.

It would appear, therefore, that annualism is not related to natural transformation in *Nicotiana* and *Linaria.*

It is interesting to note that *rolC* is the most conserved gene among the cT-DNA oncogenes found in *Nicotiana* and *Linaria* (Intrieri and Buiatti, 2001; Mohajjel-Shoja et al., 2011). In some representatives of the *Nicotiana*, only *rolC* is able to encode a functional product (Mohajjel-Shoja et al., 2011). The same trend was observed for *Linaria* T-DNA-like sequences (Matveeva et al., 2012; Matveeva and Kosachev, 2013). The function of *rolC*, however, is poorly understood. It has been speculated that the product of *rolC* releases cytokinins from conjugates (Estruch et al., 1991). Other researchers demonstrated that the RolC protein participates in the processes of sucrose metabolism and/or transport (Nilsson and Olsson, 1997; Mohajjel-Shoja et al., 2011). RolC has also been proposed to promote somatic embryogenesis in plants (Gorpenchenko et al., 2006). Such data are consistent with a cytokinin function of the gene. Constitutive expression of *rolC* in cultured plant tissues activates secondary metabolism: the *rolC* gene alone increases production of tropane alkaloids, pyridine alkaloids, ginsenosides, and anthraquinones among others (Bulgakov et al., 1998, 2002; Palazon et al., 1998a; Bonhomme et al., 2000a,b; Bulgakov, 2008) and stimulates the expression of pathogenesis-related proteins (Kiselev et al., 2007). It is unclear how the *rolC* gene product mediates such pleiotropic effects, further biochemical characterization of RolC is required. This is complicated by the fact that *rolC* has no significant homology with any other genes (of prokaryotic or eukaryotic organisms) whose function is known (Bulgakov, 2008).

Activation of secondary metabolism in transformed cells may be due to the action of other *rol* genes (Chandra, 2012). Shkryl et al. (2008) studied the influence of *rol* genes products on secondary metabolism of *Rubia cordifolia.* They investigated *rol* genes individually and studied their combined action. They found that individual *rolA, rolB*, and *rolC* genes were able to stimulate biosynthesis of anthraquinones in transformed calli. The strongest anthraquinone—stimulating activity was detected for an *R. cordifolia* culture overproducing RolB where they saw a 15-fold increase of anthraquinone accumulation as compared to untransformed calli. The *rolA*- and *rolC*-expressing calli produced 2.8- and 4.3-fold higher amounts of anthraquinones, correspondingly. Palazon et al. (1998b) reported that the *rolA* gene stimulated production of nicotine.

Thus, increasing the amount of secondary metabolites is a characteristic of tissues where *rol* genes are expressed. This property can be useful for plants, because secondary metabolites may contribute to the resistance of plants to pests. It seems likely that a possible function cT-DNA is to mediate how plants interact with their environment by secreting opines and/or by changing the amounts of secondary metabolites. It will be essential to confirm such hypotheses through additional experimentation that might include silencing or excision experiments that are now possible using CRISPR technology (Qi et al., 2013).

The study of the long term impacts of HGT by *Agrobacterium* in plant lineages is in the early stages. However, we can note some trends:

- HGT of T-DNA from *Agrobacterium* to plants occurred in the evolution of several genera, at least *Nicotiana* (family Solanaceae) and *Linaria* (family Plantaginaceae);
- in both genera plants were transformed by a mikimopine strain of *A. rhizogenes*;
- a *rolC* homologe is the most conserved gene among the T-DNA genes in *Linaria* and *Nicotiana* spp;
- In *Linaria vulgaris* and *Nicotiana glauca* there are more than one copy of T-DNA per genome.

Continued studies of the genetic and biochemical effects of cT-DNA integration in naturally transgenic plants are important and will continue to provide insights into the impact of such rare acquisitions on plant evolution.

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