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Authors: Hwang, Hau-Hsuan, Yu, Manda, and Lai, Erh-Min

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Agrobacterium-mediated plant transformation: biology and applications

Hau-Hsuan Hwang[b,2], Manda Yu[a], and Erh-Min Lai[a,1]

[a]Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan, 115
[b]Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan, 402
Address correspondence to
[1]emlai@gate.sinica.edu.tw,
[2]hauhsuan@dragon.nchu.edu.tw

Plant genetic transformation heavily relies on the bacterial pathogen *Agrobacterium tumefaciens* as a powerful tool to deliver genes of interest into a host plant. Inside the plant nucleus, the transferred DNA is capable of integrating into the plant genome for inheritance to the next generation (i.e. stable transformation). Alternatively, the foreign DNA can transiently remain in the nucleus without integrating into the genome but still be transcribed to produce desirable gene products (i.e. transient transformation). From the discovery of *A. tumefaciens* to its wide application in plant biotechnology, numerous aspects of the interaction between *A. tumefaciens* and plants have been elucidated. This article aims to provide a comprehensive review of the biology and the applications of *Agrobacterium*-mediated plant transformation, which may be useful for both microbiologists and plant biologists who desire a better understanding of plant transformation, protein expression in plants, and plant-microbe interaction.

INTRODUCTION

Agrobacterium tumefaciens is a soil phytopathogen that naturally infects plant wound sites and causes crown gall disease via delivery of transferred (T)-DNA from bacterial cells into host plant cells through a bacterial type IV secretion system (T4SS). Through the advancement and innovation of molecular biology technology during the past few decades, various important bacterial and plant genes involved in tumorigenesis were identified. With the help of more comprehensive knowledge of how *A. tumefaciens* interacts with host cells, *A. tumefaciens* has become the most popular plant transformation tool to date. Any gene of interest can now easily be used to replace the oncogenes in the T-DNA region of various types of binary vectors to perform plant genetic transformation with *A. tumefaciens*. *Arabidopsis,* the most-studied model plant with powerful genetic and genomic resources*,* is readily transformable by *A. tumefaciens* for stable and transient transformation in several ecotypes tested, although variable transformation efficiencies in different accessions were observed.

The *Agrobacterium*-plant interaction is a complex process that can be divided into several functional steps, which have been reviewed extensively (Bhattacharya et al., 2010; Gelvin, 2010b, 2012; Lacroix and Citovsky, 2013; Pitzschke, 2013; Christie et al., 2014). In this article, we summarize the major achievements in the field in two main areas: first, the key genes and molecular events of *Agrobacterium*-plant interactions; and second, current status and methods of *Agrobacterium*-mediated stable and transient transformation methods. The aim of this article is to provide an overview for scientists with microbiology or plant background to have a better understanding for the other side, which could help them pursue their research interests in a more comprehensive way.

THE BIOLOGY OF AGROBACTERIUM-PLANT INTERACTIONS AND T-DNA TRANSFORMATION

The initial research interest in the genus *Agrobacterium* resulted from the search for the causative agent of plant diseases including crown gall, cane gall, and hairy root. *Agrobacterium tumefaciens* harboring the tumor-inducing (Ti) plasmid induces galls on roots and crowns of numerous dicot angiosperm species and some gymnosperms; whereas *Agrobacterium rhizogenes* harboring the root-inducing (Ri) plasmid induces abnormal root production on host plants. The neoplastic tumor-like cell growth is induced by expression of the oncogenes residing in the transferred-DNA (T-DNA) transported from these bacteria into the plant nucleus and integrated into the plant genome.

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The *A. tumefaciens*-mediated plant genetic transformation process requires the presence of two genetic components located on the bacterial Ti-plasmid. The first essential component is the T-DNA, defined by conserved 25-base pair imperfect repeats at the ends of the T-region called border sequences. The second is the virulence (*vir*) region, which is composed of at least seven major loci (*virA, virB, virC, virD, virE, virF*, and *virG*) encoding components of the bacterial protein machinery mediating T-DNA processing and transfer. The VirA and VirG proteins are two-component regulators that activate the expression of other *vir* genes on the Ti-plasmid. The VirB, VirC, VirD, VirE and perhaps VirF are involved in the processing, transfer, and integration of the T-DNA from *A. tumefaciens* into a plant cell. Figure 1 shows the major steps of the *Agrobacterium*-mediated plant transformation process. The current knowledge and recent findings regarding the key events of *Agrobacterium*-mediated plant transformation process are reviewed in the following sections. Table 1 summarizes the major transformation steps and known plant factors participating in these steps.

Attachment of *A. tumefaciens* to plant cells

Bacterial attachment to host cells is a crucial early step in disease development for many plant and animal pathogens. The isolation of *A. tumefaciens* mutants unable to bind to plant cells led to the identification of several chromosomal virulence (*chv*) genes, *chvA, chvB,* and *pscA,* required for the attachment processes. *chvA, chvB,* and *pscA* are involved in the synthesis, processing, and export of cyclic β-1,2-glucan and other sugars (Thomashow et al., 1987; Zorreguieta et al., 1988; Cangelosi et al., 1989; O'Connell and Handelsman, 1989). These mutants are either avirulent or highly attenuated in virulence under many inoculation conditions (Douglas et al., 1982; Douglas et al., 1985; Matthysse, 1987). During attachment, the *A. tumefaciens* cells synthesize cellulose fibrils that entrap large numbers of bacteria at the wounded sites (Matthysse et al., 1981; Matthysse, 1983). The cellulose deficient *A. tumefaciens* mutant is still able to attach to the carrot cell surface but cannot form aggregates (Matthysse, 1983). These mutants are virulent but are more susceptible to being washed off the plant cells (Matthysse et al., 1981; Matthysse,

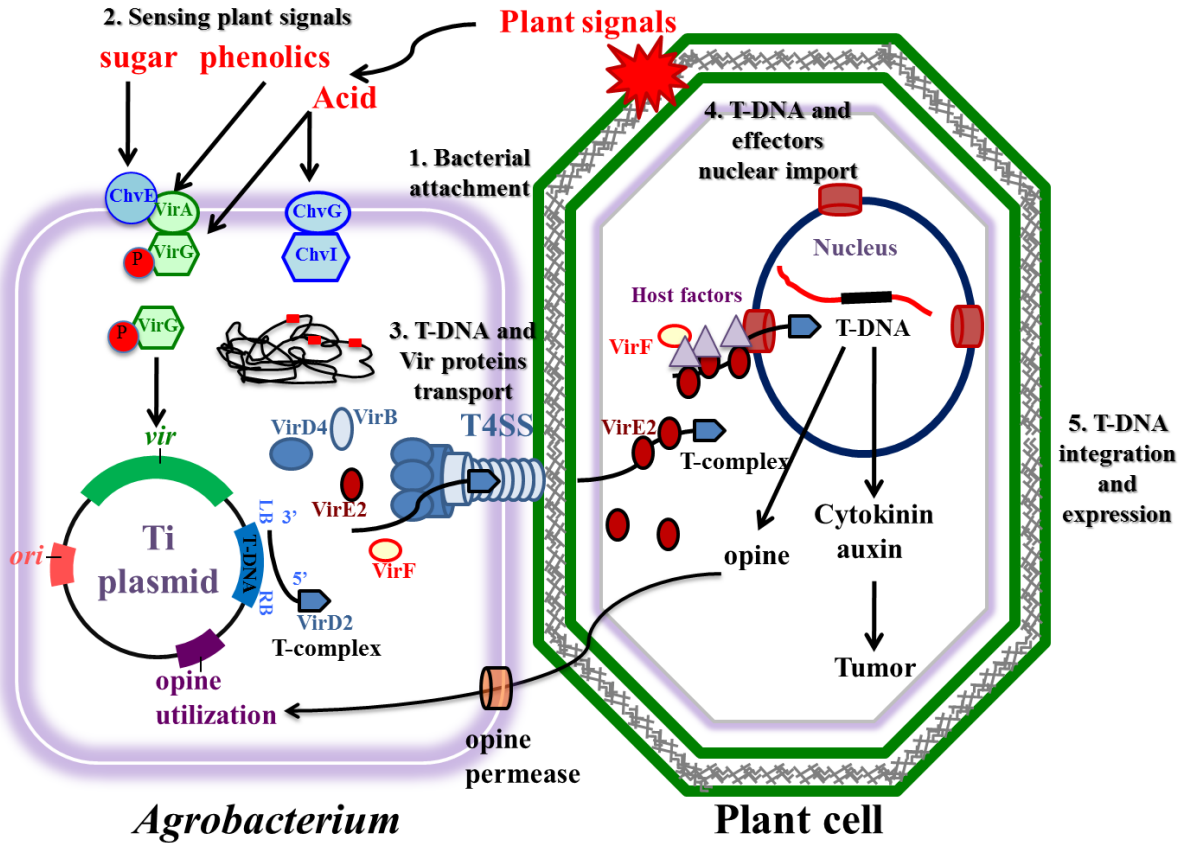


Figure 1. Major steps of the *Agrobacterium tumefaciens*-mediated plant transformation process.

(1) Attachment of *A. tumefaciens* to the plant cells. **(2)** Sensing plant signals by *A. tumefaciens* and regulation of virulence genes in bacteria following transduction of the sensed signals. **(3)** Generation and transport of T-DNA and virulence proteins from the bacterial cells into plant cells. **(4)** Nuclear import of T-DNA and effector proteins in the plant cells. **(5)** T-DNA integration and expression in the plant genome.

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Table 1. Plant factors involved in *Agrobacterium*-mediated plant transformation process

Known or possible functions	Plant factors	AGI locus identifier[1]	References
Attachment of *A. tumefaciens* to plant cells	Vitronectin	N/A	Wagner and Matthysse, 1992
	PVN1 (plant vitronectin-like protein 1)	N/A	Zhu et al., 1993; Zhu et al., 1994
	AGP17 (RAT1, arabinogalactan protein)	At2g23120	Zhu et al., 2003b; Gaspar et al., 2004
	CSLA (RAT4, cellulose synthase-like A9)	At5g03760	Zhu et al., 2003a, b
	MTF1 (Myb family transcription factor)	At2g40970	Sardesai et al., 2013
	AT14A (similar to integrins)	At3g28290	Sardesai et al., 2013
	Plant hormone compounds (salicylic acid, SA)	N/A	Anand et al., 2008; Yuan et al., 2007a; Yuan et al., 2007b
Plant signals sensed by *A. tumefaciens* and/or regulating *vir* gene expression	Phenolic compounds	N/A	Stachel et al., 1985
	Sugar compounds	N/A	Stachel et al., 1985; Ankenbauer and Nester, 1990; Shimoda et al., 1993; Doty et al., 1996; Hu et al., 2012
	HDMBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one, maize seedling root exudate)	N/A	Zhang et al., 2000
	Plant hormone compounds (auxin, cytokinin, salicylic acid, and ethylene)	N/A	Liu and Nester, 2006; Anand et al., 2007; Yuan et al., 2007a; Yuan et al., 2007b; Nonaka et al., 2008; Hwang et al., 2010; Hwang et al., 2013b
	Acidity	N/A	Li et al., 2002; Liu et al., 2005; Yuan et al., 2007a; Hu et al., 2012; Wu et al., 2012; Heckel et al., 2014
T-DNA/virulence protein transport or initial contact of *A. tumefaciens* to plants	RTNLB1 (reticulon-like protein B-1)	At4g23630	Hwang and Gelvin, 2004
	RTNLB2 (reticulon-like protein B-2)	At4g11220	Hwang and Gelvin, 2004
	RTNLB4 (reticulon-like protein B-4)	At5g41600	Hwang and Gelvin, 2004
	RAB8B (small GTPase)	At3g53610	Hwang and Gelvin, 2004
Cytoplasmic trafficking and nuclear import of T-DNA and effector proteins	ROC1 (cyclophilin proteins, peptidyl-prolyl cis-trans isomerase CYP1)	At4g38740	Deng et al., 1998
	PP2C (type 2C protein phosphatase, ABI1)	At4g26080	Tao et al., 2004
	KAPα/IMPA-1 (Karyopherin protein, importin alpha isoform 1 involved in nuclear import)	At3g06720	Ballas and Citovsky, 1997; Bhattacharjee et al., 2008
	IMPA-2 (importin alpha isoform 2)	At4g16143	Bhattacharjee et al., 2008; Lee et al., 2008
	IMPA-3 (importin alpha isoform 3)	At4g02150	Bhattacharjee et al., 2008; Lee et al., 2008
	IMPA-4 (importin alpha isoform 4)	At1g09270	Bhattacharjee et al., 2008; Lee et al., 2008
	importin β-3	N/A	Zhu et al., 2003a
	VIP1 (VirE2-interacting plant protein 1)	At1g43700	Tzfira, 2001; Tzfira et al., 2002
	VIP2 (VirE2-interacting plant protein 2)	At5g59710	Tzfira, 2001; Tzfira et al., 2002; Anand et al., 2007
	MPK3 (mitogen-activated protein kinase 3)	At3g45640	Djamei et al., 2007
	Microtubules and kinesin	N/A	Zhu et al., 2003a; Salman et al., 2005
	Microfilament and ACT2 (actin gene)	At3g18780	Zhu et al., 2003a; Yang et al., 2017
	ACT7 (actin gene)	At5g09810	Zhu et al., 2003a
	CAK2M kinase (cyclin-dependent kinase-activating kinases)	N/A	Bako et al., 2003

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Table 1. (continued)

Known or possible functions	Plant factors	AGI locus identifier[1]	References
Integration and/ or expression of T-DNA	AtLIG4 (DNA ligase IV)	At5g57160	Ziemienowicz et al., 2000; Friesner and Britt, 2003; van Attikum et al., 2003; Zhu et al., 2003a; Nishizawa-Yokoi et al., 2012; Park et al., 2015
	KU80	At1g48050	van Attikum et al., 2001; Friesner and Britt, 2003; Gallego et al., 2003; Li et al., 2005b; Nishizawa-Yokoi et al., 2012; Jia et al., 2012; Mestiri et al., 2014; Park et al., 2015
	KU70	At1g16970	van Attikum et al., 2001; Li et al., 2005b; Nishizawa-Yokoi et al., 2012; Jia et al., 2012; Mestiri et al., 2014; Park et al., 2015
	MRE11 (meiotic recombination 11)	At5g54260	van Attikum et al., 2001; Jia et al., 2012;
	XRCC1 (homolog of X-ray repair cross complementing 1)	At1g80420	Mestiri et al., 2014; Park et al., 2015
	XRCC2 (homolog of X-ray repair cross complementing 2)	At5g64520	Mestiri et al., 2014; Park et al., 2015
	XRCC4 (homolog of X-ray repair cross complementing 4)	At3g23100	Vaghchhipawala et al., 2012; Park et al., 2015
	XPF/RAD1/UVH1 (ultraviolet hypersensitive 1)	At5g41150	Nam et al., 1998; Mestiri et al., 2014; Park et al., 2015
	PARP1 (poly(ADP-ribose) polymerases 1)	At2g31320	Jia et al., 2012; Park et al., 2015
	HTA1 (histone H2A)	At5g54640	Nam et al., 1999; Mysore et al., 2000a, b; Yi et al., 2002, 2006; Zhu et al., 2003a; Tenea et al., 2009
	HTR11 (histone H3-11)	At5g65350	Zhu et al., 2003a; Tenea et al., 2009
	VIP1 (VirE2-interacting plant protein 1)	At1g43700	Tzfira, 2001; Tzfira et al., 2002; Li et al., 2005a; Loyter et al., 2005; Lacroix et al., 2008
	VIP2 (VirE2-interacting plant protein 2)	At5g59710	Tzfira, 2001; Tzfira et al., 2002; Anand et al., 2007
	HDA1/HDA19 (histone deacetylase 1/ histone deacetylase 19)	At4g38130	Zhu et al., 2003a; Crane and Gelvin, 2007; Gelvin and Kim, 2007
	HDA2/HDT1 (histone deacetylase 2A)	At3g44750	Zhu et al., 2003a; Crane and Gelvin, 2007; Gelvin and Kim, 2007
	HD2B/HDT2 (histone deacetylase 2B)	At5g22650	Zhu et al., 2003a; Crane and Gelvin, 2007; Gelvin and Kim, 2007
	SGA1/ASF1B (anti-silencing function 1B)	At5g38110	Crane and Gelvin, 2007
	FAS1 (fasciata 1/nucleosome/ chromatin assembly factor group B/ chromatin assembly factor-1 (CAF-1) subunit)	At1g65470	Endo et al., 2006
	FAS2 (fasciata 2/nucleosome/ chromatin assembly factor group B/ chromatin assembly factor-1 (CAF-1) subunit)	At5g64630	Endo et al., 2006
	ASK1/SKP1 (SKP1 homologue 1)	At1g75950	Schrammeijer et al., 2001; Zaltsman et al., 2010a, b, 2003; Anand et al., 2012; Tzfira et al., 2002, 2004
	ASK2 (SKP-like 2)	At5g42190	Schrammeijer et al., 2001; Zaltsman et al., 2010a, b, 2003; Anand et al., 2012; Tzfira et al., 2002, 2004
	ASK10 (SKP-like 10)	At3g21860	Schrammeijer et al., 2001; Zaltsman et al., 2010a, b, 2003; Anand et al., 2012; Tzfira et al., 2002, 2004
	SGT1a (suppressor of the G2 allele of SKP1)	At4g23570	Anand et al., 2012
	SGT1b/EDM1 (Enhanced downy mildew 1)	At4g11260	Anand et al., 2012
	VBF (VIP1-binding F-box protein)	At1g56250	Ditt et al., 2006; Zaltsman et al., 2010a, b, 2013; Wang et al., 2014; Niu et al., 2015
	TEBICHI/POLQ (DNA polymerase theta, Pol θ)	At4g32700	van Kregten et al., 2016

[1]N/A: not available

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1983). Thus, the major role of cellulose fibrils may be in aiding attachment of *A. tumefaciens* to the plant cell.

Aside from the cellulose and cyclic β-1,2-glucan, *A. tumefaciens* produces an additional exopolysaccharide, unipolar polysaccharide (UPP), that participates in bacterial attachment (Tomlinson and Fuqua, 2009; Li et al., 2011; Xu et al., 2012; Heindl et al., 2014). The unipolar polysaccharide helps *A. tumefaciens* cells attach to the plant surfaces in a polar fashion because the UPP is mainly produced at the cell pole. The UPP is rarely observed in *A. tumefaciens* planktonic cells or in colonies on solid media, suggesting that the production of UPP is a signature of *A. tumefaciens* permanent attachment (Li et al., 2011; Xu et al., 2012; Xu et al., 2013). The UPP consists of at least two types of sugars, N-acetylglucosamine and N-acetylgalactosamine (Tomlinson and Fuqua, 2009; Xu et al., 2012). The *A. tumefaciens* UPP biosynthetic mutant *uppE* is defective in both bacterial attachment and biofilm formation, supporting the idea that the UPP plays an important role in bacterial attachment (Xu et al., 2012; Heindl et al., 2014). Interestingly, while *A. tumefaciens* attachment to plant tissues often leads to biofilm formation and is required for virulence, *A. tumefaciens* strains lacking UPP or impaired in biofilm formation remain efficient in T-DNA transfer. Thus, biofilm formation and UPP-mediated bacterial attachment may not play a role during *Agrobacterium* T-DNA translocation process, or their importance may not be discernible using the current infection assays under laboratory conditions.

Plant factors are also required for bacterial attachment to the plant cell surface. One such surface factor may belong to a vitronectin protein family. In animal cells, vitronectin, a component of the extracellular matrix, is utilized as a specific receptor by several pathogenic bacterial strains (Paulsson, and Wadstrom, 1990). Human vitronectin and anti-vitronectin antibodies both inhibited the binding of *A. tumefaciens* to cultured plant cells (Wagner and Matthysse, 1992). Further, *A. tumefaciens chvB*, *pscA*, and *att* mutants that are unable to bind to plant cells exhibit reduced binding to vitronectin (Wagner and Matthysse, 1992). Plant proteins immunologically related to the animal substrate adhesion molecule vitronectin, named PVN1 (plant vitronectin-like protein 1), have been identified in tobacco (Zhu et al., 1993; Zhu et al., 1994). PVNI is localized in the cell wall and may mediate cell adhesion (Zhu et al., 1994). However, whether PVN1 is a receptor for *Agrobacterium* binding remains to be investigated.

Nam et al. (1997) identified several *Arabidopsis* ecotypes that are deficient in binding *A. tumefaciens* and are resistant to *A. tumefaciens* root mediated transformation. Two of these ecotypes, Bl-1 and Petergof, showed lower transient transformation efficiency than a more susceptible ecotype, suggesting that the transformation process was blocked at step(s) prior to integration. Further, microscopic analysis revealed that fewer bacteria bound to the root surface of B1-1 and Petergof than to a highly transformable ecotype such as Aa-0. In addition, Nam et al. (1999) identified three *Arabidopsis* T-DNA insertion mutants in the ecotype Ws with resistance to *Agrobacterium* transformation (*rat* mutants). The root explants of *rat1*, *rat3,* and *rat4* mutants are deficient in binding *A. tumefaciens*. *RAT1* encodes an arabinogalactan protein (AtAGP17, At2g23120) that is found in the extracellular matrix associated with the plant cell wall (Gaspar et al., 2004). Interestingly, although the *Arabidopsis rat1* and *rat3* mutants and the Bl-1 and Petergof ecotypes are recalcitrant to *A. tumefaciens* root-mediated transformation, they are transformed as well as are the corresponding wild-type plants and highly transformable ecotypes using a flower vacuum infiltration method (Mysore et al., 2000a). These results suggested that plant proteins needed for transformation of somatic tissues may not be required for the transformation of the female gametophyte. In addition, a genetic screen to identify *Arabidopsis* hyper-susceptible to *A. tumefaciens* transformation (*hat*) mutants uncovered the heterozygous mutant *mtf1-1*, in which the mRNA transcript of the *MTF1* (myb family transcription factor, At2g40970) gene exhibited more than a 12-fold reduction as compared to wild-type plants (Sardesai et al., 2013). The increased *A. tumefaciens* attachment to roots and improved stable and transient transformation efficiencies in *mtf1-1* suggested that the MTF1 played a negative role in mediating the *A. tumefaciens* transformation process. Interestingly, the presence of *A. tumefaciens*-secreted cytokinin caused decreased *MTF1* gene expression via the cytokinin response regulator ARR3-mediated signaling pathway. Because gene expression of *AT14A* (At3g28290), encoding a putative transmembrane receptor for a cell adhesion molecule, was increased in the *mtf1* mutant and the *at14a* mutant showed decreased transformation rates and *A. tumefaciens* attachment to plant roots, AT14A might be an anchor point for *A. tumefaciens* binding (Sardesai et al., 2013).

Sensing and regulation of virulence genes of *A. tumefaciens* in response to plant signals

In nature, wounded plant tissues secrete a wide range of chemical compounds that can function as chemotactic agents to attract *A. tumefaciens* to the wounded sites for infection in plants. Wounded plants secrete sap with a characteristic acidic pH (5.0 to 5.8) and high content of different phenolic compounds, including lignin and flavonoid precursors. While these compounds can be used to ward off microbial attachment, these conditions stimulate *A. tumefaciens* virulence gene expression. The best studied and most effective *vir* gene inducers are monocyclic phenolics such as 3,5-dimethoxy acetophenone (acetosyringone, AS), as shown by induction of *vir::lacZ* fusions (Stachel et al., 1985). Several other environmental conditions are also important for optimal *vir* gene induction, including acidity, low phosphate, temperature, and sugars. However, phenolics are the only signal that is absolutely required (Brencic et al., 2003; Gao and Lynn, 2005; McCullen and Binns, 2006; Lin et al., 2008; Subramoni et al., 2014).

A. tumefaciens uses the VirA/VirG two-component regulatory system to regulate *vir* gene induction (Stachel et al., 1986; Jin et al., 1990a, 1990b, 1990c). To initiate the signaling pathway, plant phenolics interact either directly or indirectly with the transmembrane sensory protein VirA. Although two chromosomally encoded proteins, p10 and p21, but not VirA, are able to bind the phenolic signal *in vitro* (Lee et al., 1992), genetic evidence strongly suggests that VirA directly senses the phenolic compounds required for *vir* gene activation (Lee et al., 1995; Lee et al., 1996). ChvE, a chromosomally encoded glucose/galactose binding protein, interacts with VirA and enhances *vir* gene activation by binding to sugars (Shimoda et al., 1993; Doty et al., 1996). *A. tumefaciens* also uses the ChvE protein to recognize and bind various plant sugars that may help the bacterium to establish its

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host range (Hu et al., 2012). In addition, the affinity of the ChvE protein for sugar acids is increased at low pH, suggesting a link between the sugar and acidity response in *A. tumefaciens* (Hu et al., 2012). *A. tumefaciens* utilizes the ChvG/ChvI two-component system to activate the transcription of *virG* and induces expression of several other bacterial genes (Mantis and Winans, 1992; Chang and Winans, 1996), including genes involved in chemotaxis and motility, several chromosome-encoded virulence genes, succinoglycan biosynthesis genes, and the gene cluster encoding the type VI secretion system (T6SS) (Yuan et al., 2007a; Wu et al., 2008; Wu et al., 2012). Recent studies further identified ExoR as a periplasmic negative regulator acting upstream of the ChvG sensor kinase to repress acid-inducible gene expression (Wu et al., 2012; Heckel et al., 2014) and uncovered a role for the T6SS in interbacterial competition activity during the plant colonization process (Ma et al., 2014). These data strongly suggested that these acid-inducible genes may play a role in *A. tumefaciens* survival and competitive fitness during *Agrobacterium*-plant interactions.

Because phenolic molecules are bacteriostatic at high concentration, *A. tumefaciens* also harbors cytochrome P450-like enzymes such as VirH2 to detoxify a phenolic *vir* gene inducer, ferulic acid (Kalogeraki et al., 1999; Brencic et al., 2003), suggesting that *virH* may be involved in the detoxification of harmful phenolics secreted by the wounded plants (Kanemoto et al., 1989). On the other hand, plant factors can also act as inhibitors of the *A. tumefaciens* sensory machinery. A major organic exudate of maize seedling roots, 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (HDMBOA), has been demonstrated to provide innate immunity and hinder successful *A. tumefaciens* infection of maize plants (Zhang et al., 2000); an o-imidoquinone decomposition intermediate, (3Z)-2,2-dihydroxy-N-(4-methoxy-6-oxocyclohexa-2,4-dienylidene) acetamide, is a specific inhibitor of signal perception in *A. tumefaciens* (Maresh et al., 2006; Lin et al., 2008). These inhibitors may account for the recalcitrance of some plants to *Agrobacterium*-mediated transformation.

Several studies revealed that two plant hormones, auxin and cytokinin, not only contribute to tumor growth, but also affect agrobacterial physiology and gene expression (Liu and Nester, 2006; Hwang et al., 2010; Hwang et al., 2013b). It has been shown that IAA, the plant hormone auxin overproduced upon T-DNA integration, affects bacterial growth and inhibits *vir* gene induction by competing with phenolic inducers for interaction with the VirA protein. It has been proposed that relatively low auxin levels may promote transformation during the initial stages of *A. tumefaciens* infection, whereas the higher level of auxin produced in the crown gall tissues may prevent further transformation by inhibiting bacterial growth and virulence. In addition to auxin, *A. tumefaciens*-secreted cytokinin also affects bacterial virulence by regulating *vir* promoter activity and bacterial growth (Hwang et al., 2010, 2013b). Other studies also indicate that the plant defense molecule salicylic acid (SA) influences the *A. tumefaciens* infection process by inhibiting the expression of *vir* genes, bacterial growth, bacterial attachment to plant cells, and virulence (Anand et al., 2008; Yuan et al., 2007a, 2007b). The plant defense roles of SA against bacterial infections are further supported by genetic studies showing that mutant plants with SA overproduction are resistant to *A. tumefaciens* infection, whereas mutant plants with lower SA accumulation have a higher percentage of tumor formation (Anand et al., 2008; Yuan et al. 2007b; Lee et al., 2009). Another plant hormone, ethylene, can repress *vir* gene expressions in *A. tumefaciens* but shows no significant inhibitory effects on bacterial growth and population size (Nonaka et al., 2008). In an *Arabidopsis* ethylene-insensitive mutant, the *A. tumefaciens*-mediated transformation efficiency increased (Nonaka et al., 2008). In summary, intricate temporal and spatial regulation and a delicate balance of various plant hormones likely play important roles in modulating *A. tumefaciens* infection efficiency (Gohlke and Deeken, 2014; Subramoni et al., 2014).

Transport of T-DNA and virulence proteins via type IV secretion system (T4SS)

Virulence gene expression leads to the production of a single-stranded T-DNA, termed the T-strand, which is then transported into the host cell. VirD1 and VirD2 proteins function together as a site- and strand-specific endonuclease which binds to the supercoiled Ti plasmid at the T-DNA borders, relaxes it and nicks the bottom T-DNA strand between the third and fourth bases of the T-DNA borders (Stachel et al., 1986; Yanofsky and Nester, 1986; Albright et al., 1987; Jayaswal et al., 1987; Stachel et al., 1987; Wang et al., 1987; Filichkin and Gelvin, 1993). This process results in the generation of single-strand T-DNA molecules (T-strand) covalently attached to VirD2 at the 5' end of the T-strand at the right border nick (Herrera-Estrella et al., 1988; Ward and Barnes, 1988; Young and Nester, 1988; Howard et al., 1989). Recently, three VirD2-binding proteins (VBP) from *A. tumefaciens* were identified by pull-down assays using AS-treated *A. tumefaciens* crude extract (Guo et al., 2007a). These proteins are functionally redundant, but removal of all three *vbp* genes in *A. tumefaciens* affects bacterial virulence on *Kalanchoe* plants (Guo et al., 2007a). The VBP1 protein not only interacts with the VirD2-bound T-strand, but also interacts with three components of the Type IV secretion system (T4SS), VirD4, VirB4, and VirB11, via two independently binding domains (Guo et al., 2007b). The VBP may form a dimer in the *A. tumefaciens* cytoplasm and recruit the VirD2-bound T-strand to the T4SS apparatus through interactions with the VirD4 proteins (Guo et al., 2007b; Padavannil et al., 2014).

T-DNA export requires the *vir*-encoded T4SS for delivery across the bacterial envelope and the plasma membrane of the host plant cell (Zechner et al., 2012; Bhatty et al., 2013; Chandran, 2013; Christie et al., 2014). This *A. tumefaciens* T4SS consists of 12 proteins (VirB1-B11 and VirD4), which form two functional components: a filamentous T-pilus and a membrane associated transporter complex that is responsible for translocating substrates across both bacterial cell membranes (Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009a; Waksman and Fronzes, 2010; Wallden et al., 2010). This transport complex can be further subdivided into four functional subassemblies: the VirD4 coupling protein as substrate receptor, an inner-membrane translocase (VirB3-B4-B6-B8-B11), an outer-membrane core complex (VirB7-B9-B10), and an extracellular T-pilus (VirB2-B5) (Christie et al., 2014). While it is clear that each of the VirB2-11 and VirD4 proteins is essential for T-DNA and effector transport, it remains unknown whether the extracellular T-pilus is part of the translocation channel.

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The VirD4 protein is an integral inner membrane protein with ATPase activity functioning as a coupling protein responsible for presentation of DNA and protein substrates to the VirB transporter. Studies using a transfer DNA immunoprecipitation (TrIP) assay revealed that the transferring T-DNA substrates make close contacts with the VirD4 protein, further supporting the function of VirD4 as the substrate receptor (Cascales and Christie, 2004a). Genetic studies of the *virD4* mutants also revealed that DNA substrate binding of VirD4 protein and delivery to VirB11 both activate the ATP hydrolysis activities of VirD4 and VirB11. These signals may cause a conformational change of VirB10 which plays an important role in regulating DNA substrate transfer through the T4SS apparatus opening and assembly (Atmakuri et al., 2004; Cascales and Christie, 2004b; Cascales et al., 2013).

VirB4 is an ATPase and contains a Walker A nucleotide triphosphate binding motif essential for virulence (Christie et al., 1989; Fullner et al., 1994). The VirB4 ATPase also directly participates in T-pilus biogenesis. VirB4 can interact with the VirB2 T-pilin and induce changes in the VirB2 membrane topological state via an ATP-dependent mechanism, suggesting that the VirB4 ATPase may function as a dislocase to mediate membrane extraction of pilin monomers during pili biogenesis (Kerr and Christie, 2010; Wallden et al., 2010). In addition, VirB11 may function with VirB4 to coordinate a structural change in VirB2 pilin protein and mediate VirB2 binding to other T4SS components, which in turn affects pilus polymerization (Kerr and Christie, 2010). Accumulating genetic studies and recent structural studies support a model in which VirB4 dimers or homomultimers contribute both structural information for the assembly of a trans-envelope channel competent for DNA transfer and an ATP-dependent activity for configuring this channel as a dedicated export machine (Fronzes et al., 2009a; Durand et al., 2010, 2011; Kerr and Christie, 2010; Li et al., 2012; Pena et al., 2012; Wallden et al., 2012). In addition, the TrIP assay results showed that translocating DNA substrates first interact with the VirD4 receptor, followed by VirB11 ATPase, suggesting VirB11 may function together with VirB4 and deliver the T-DNA substrate to the transfer channel of the T4SS (Cascales and Christie, 2004a; Bhatty et al., 2013; Chandran, 2013; Christie et al., 2014).

Recent electron microscopy imaging has revealed the structure of purified VirB3-10 complex encoded by R388 T4SS, in which VirB3, VirB4, and likely VirB5, VirB6, and VirB8 are part of the inner membrane complex (Low et al., 2014). This result is consistent with a predicted inner-membrane translocase complex consisting the VirB3, VirB4, VirB6, VirB8, and VirB11 based on subcellular localization, protein-protein interaction, and biochemical studies (Christie et al., 2014). This study also revealed the outer membrane complex consisting of VirB7-B9-B10, which is in agreement with previous cryoelectron microscopy and crystallography of purified VirB7, VirB9, and VirB10 proteins encoded from pKM101 (Chandran et al., 2009; Fronzes et al., 2009b). This outer-membrane core complex contains 14 copies each of VirB7, VirB9, and VirB10; and is inserted in both the inner and outer membranes (Fronzes et al., 2009a, 2009b). The structure of the core complex is cylindrical and contains two layers, the inner (I) and outer (O) layers (Fronzes et al., 2009b; Wallden et al., 2012; Bhatty et al., 2013). Upon sensing ATP energy utilization by the VirD4 and VirB11 ATPases, the VirB10 protein conformation changes and may help regulate the O layer hole opening to allow substrate transfer through the T4SS channel (Cascales and Christie, 2004b). While the majority of T4SS components can be assembled into subcomplexes when expressed in a heterologous *E. coli* system, an alpha-crystallin-type small heat shock protein, HspL was shown to be important for stability of several VirB proteins and efficient T-DNA transfer and tumorigenesis (Tsai et al., 2009). HspL expression is induced by AS in response to VirB protein expression and functions as a VirB8 chaperone (Tsai et al., 2009, 2010). Moreover, overexpression of HspL in *A. tumefaciens* enhanced transient transformation efficiencies of both susceptible and recalcitrant *Arabidopsis* ecotypes. *A. tumefaciens* virulence during heat shock was also considerably improved by over-expression of the HspL, revealing the importance of the VirB8 chaperone HspL during *A. tumefaciens* infections (Hwang et al., 2015).

The extracellular T-pilus consists of major subunit VirB2 and minor subunit VirB5, which is located at the pilus tip (Lai and Kado, 1998; Schmidt-Eisenlohr et al., 1999; Aly and Baron, 2007). Genetic and environmental studies show that each *virB* gene is required for T-pilus biogenesis; however, only the *virB2* gene is required for VirB2 pro-pilin processing and peptide cyclization (Eisenbrandt et al., 1999; Lai et al., 2000, 2002). Non-polar *virB* mutants abolish T-pilin transport and T-pilus biogenesis, and support a model in which the VirB-specific transporter is not only used for translocating the T-complex, but also for exporting cyclic T-pilin subunits to the bacterial cell surface, perhaps as a scaffold to facilitate efficient assembly of the T-pilus (Lai and Kado, 2000; Christie et al., 2014). However, several amino acid substitutions in the T4SS components VirB6, VirB9, VirB10, and VirB11 have been demonstrated to block the polymerization of VirB2 pilin to form the extracellular T-pilus but do not significantly affect substrate transfer. These “uncoupling” mutations still require intracellular VirB2 for substrate transfer (Zhou and Christie, 1997; Sagulenko et al., 2001; Jakubowski et al., 2003, 2005, 2009; Banta et al., 2011; Garza and Christie, 2013). Wu et al. (Wu et al., 2014a) further generated several uncoupling *virB2* mutants with single amino acid substitutions in the VirB2 protein that retain wild-type levels of tumor formation on tomato stems but show reductions in transient transformation efficiencies of *Arabidopsis* seedlings. Taken together, these results suggest the auxiliary role of the filamentous T-pilus in optimizing the *A. tumefaciens* transformation efficiency of certain plant species or infection conditions.

Biochemical studies show that VirB5 co-fractionates as a minor component in pilus preparations and contributes to T-pilus assembly (Schmidt-Eisenlohr et al., 1999). Additionally, immunogold staining demonstrated that VirB5 is located at the tips of the cell-bound T-pili and the ends of detached T-pili, suggesting the VirB5 might be involved in host-bacteria contact (Aly and Baron, 2007). This notion is supported by two lines of evidence. One is that the VirB5 homolog CagL is localized on the T4SS pilus tip of *Helicobacter pylori* and functions in binding host human cells, likely by interacting with the integrin receptor (Kwok et al., 2007; Backert et al., 2008). Evidence that the CagL protein, the VirB5 homologue in the *H. pylori,* functions as a specialized adhesin binding to the human integrin ß1 and may activate the downstream host cell response, suggested a putative host cell receptor for the bacterial T4SS protein (Kwok et al., 2007; Backert et al., 2008). However, whether *A. tumefaciens* VirB5 also serves as an adhesin remains to be determined. Secondly, addition of

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exogenous VirB5 or expression of secreted VirB5 in the tobacco transgenic plant was able to enhance T-DNA expression (Lacroix and Citovsky, 2011). Interestingly, the VirB5 protein interacts with the cytokinin biosynthetic enzyme Tzs (*trans*-zeatin synthesizing) protein in yeast and *in vitro* (Aly et al., 2008). The *tzs* gene in the nopaline strains of *A. tumefaciens* shares significant homology with the *ipt* gene, is located outside the T-DNA region and is not incorporated into the host plant genome during infection (Akiyoshi et al., 1985, 1987; Beaty et al., 1986; Powell et al., 1988). The loss of Tzs protein expression and *trans*-zeatin secretion by the *tzs* mutants correlates with reduced stable and transient transformation efficiencies on *Arabidopsis* roots, suggesting the Tzs, by synthesizing *trans*-zeatin at early stage(s) of the infection process, modulates plant transformation efficiency by *A. tumefaciens* (Hwang et al., 2010; Hwang et al., 2013b). Because Tzs was detected in the *A. tumefaciens* membrane fraction (Lai et al., 2006; Aly et al., 2008) and a functional T4SS is required for Tzs localization on the cell surface (Aly et al., 2008), Tzs may function extracellularly, likely at the interface of the *Agrobacterium*-plant interaction. Since AT14A has partial sequence similarity to the animal integrin receptor, has been shown to mediate the cell wall-plasma membrane-cytoskeleton continuum in *A. thaliana* cells (Lu et al., 2012), and is critical for *A. tumefaciens* attachment to plant roots (Sardesai et al., 2013), it is plausible to hypothesize that T-pilus tip protein VirB5 may interact with AT14A for *A. tumefaciens* binding to the plant cell surface.

VirB1 may have two functions in the VirB transporter. The amino-terminal region of VirB1 contains a signal peptide and a motif homologous to that of lytic transglycosylases and lysozymes (Mushegian et al., 1996; Baron et al., 1997), suggesting that VirB1 may function to prepare sites in the bacterial envelope for transporter assembly by localized lysis of the peptidoglycan layer. The *virB1* non-polar mutant is highly attenuated in virulence and cannot assemble T-pili (Berger and Christie, 1993; Lai and Kado, 2000), suggesting that pilus assembly across an intact peptidoglycan layer is inefficient or unstable. VirB1 is processed to a 73-residue, carboxy-terminal fragment termed VirB1*. The secreted VirB1* may play a direct role in T-pilus assembly, either by stabilizing VirB5 or by mediating VirB2-VirB5 interaction (Llosa et al., 2000; Zupan et al., 2007). VirB5 may directly interact with VirB2 or confer its effect by means of other proteins, e.g., VirB7. Energy for T-pilus assembly may be provided by VirB11, and it may be transduced to the pilus assembly complex in the outer membrane via VirB6 and VirB7 (Krall et al., 2002; Christie et al., 2005). This current view of the T4SS structure is in accordance with the T-DNA transfer route in the T4SS that was proposed based on the results of TrIP assays (Cascales and Christie, 2004a). The T-DNA substrate contacts six of the 12 components of the T4SS machine, including the VirD4 receptor, followed by the VirB11 ATPase, VirB6/VirB8, and VirB2 pilin/VirB9 proteins (Cascales and Christie, 2004a). However, whether the VirB2 protein in contact with T-DNA is part of T4SS translocation channel or filamentous pilus has not been determined.

A. tumefaciens cells attach to plant cells at the bacterial poles and several VirB proteins, as well as VirD4, were observed at cell poles, suggesting that the VirB/D4 transfer apparatus localizes at the poles (Matthysse, 1987; Kumar and Das, 2001; Kumar et al., 2002; Jakubowski et al., 2004; Judd et al., 2004, 2005). However, recent studies using very high resolution deconvolution microscopy suggested that the *A. tumefaciens* VirB/D4 transfer apparatus forms helically arranged foci around the bacteria cells to help make intimate lateral contact with plant cells, which might maximize substrate transfer through the T4SS (Aguilar et al., 2010, 2011; Cameron et al., 2012). Several possible functions could be assigned to the T-pilus. First, the T-pilus could serve as a conduit for several components needed for virulence, including the T-pilin subunit, VirE2 proteins and single-stranded T-DNA that is piloted by the covalently linked VirD2 protein (Cascales and Christie, 2004a; Cascales et al., 2013). Second, the T-pilus could serve as a bridge to bring the bacterium and the host cell into close proximity while T-DNA is transferred into the host cell through T4SS translocation channel. Kelly and Kado (2002) described a presumably coiled thread-like interconnection with an average width of approximately 30 nm between *A. tumefaciens* and *Streptomyces lividans* cells during *A. tumefaciens* transformation. This interconnecting structure is dependent on *virB* genes and appears only under the same conditions as those required for T-pilus formation. One possible model derived from this observation is that the T-pilus may retract and subsequently draw the bacterial cell into sufficiently close contact with the target host cells for T-DNA transfer to occur.

So far, limited knowledge exists about what kind of plant proteins might be involved in the initial contact of *A. tumefaciens* T4SS components with host cell surfaces. Two kinds of plant proteins that interact with VirB2 protein were identified. The first class is composed of three related proteins with unknown function, RTNLB1 (reticulon-like protein B-1, At4g23630), RTNLB2 (At4g11220), and RTNLB4 (At5g41600) proteins; the second class is an AtRAB8B GTPase (At3g53610). The level of RTNLB1 protein is transiently increased immediately after *A. tumefaciens* infection. Overexpression of *RTNLB1* in transgenic *Arabidopsis* results in plants that are hypersusceptible to *Agrobacterium*-mediated transformation while transgenic *RTNLB* and *AtRAB8* antisense and RNA interference *Arabidopsis* plants are less susceptible to transformation by *A. tumefaciens* than are wild-type plants. These data suggested that the three RTNLB proteins and AtRAB8 are involved in the initial interaction of *A. tumefaciens* with plant cells (Hwang and Gelvin, 2004).

Cytoplasmic trafficking and nuclear import of T-DNA and effector proteins in plant cells

The VirD2 protein attaches to the 5’ end of the T-strand and serves as a pilot protein to guide the T-DNA from the bacteria into the plant cell. In addition to T-strands, several virulence effector proteins can be exported from the bacterial cells independently of each other and of the T-DNA. In addition to VirD2, VirE2, VirD5, VirE3, VirF, MobA, and Atu6154 in *A. tumefaciens*, and the GALLS protein in *A. rhizogenes,* have been shown to be transferred from bacteria into plant cells via the *A. tumefaciens* transfer apparatus, using the Cre recombinase reporter assay for translocation (CRAfT) (Vergunst et al., 2000, 2003, 2005). C-terminal clusters of positively-charged amino acids (specifically an Arg-x-Arg motif) of T4SS effectors function as the secretion signal for the *A. tumefaciens* transfer apparatus. In addition to genetic evidence, split-GFP studies directly visualized VirE2 protein

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translocation in plant and yeast cells (Li et al., 2014b; Sakalis et al., 2014). However, active movement of linear and filament VirE2 and transgene expression only occur in tobacco cells but not in yeast cells, suggesting that active VirE2 trafficking is important for efficient T-DNA expression. Because translocated VirE2 forms the filamentous structure in the absence of T-DNA, the associations and assistance of host plant cell proteins may be required for VirE2 movement (Li et al., 2014b; Sakalis et al., 2014). Indeed, using split-GFP technology to visualize VirE2 movement in agroinfiltrated *N. benthamiana* leaf epidermal cells, recent studies showed that VirE2 is transported into plant cells via clathrin-mediated endocytosis (Li and Pan, 2017) and trafficked via an ER/actin network that is powered by myosin XI-K (Yang et al., 2017).

Both VirD2 and VirE2 proteins contain plant-active nuclear localization signal (NLS) sequences that are believed to be involved in T-DNA import into the nucleus. By the yeast interaction trap (two-hybrid) approach, several plant proteins that interact with either VirD2 or VirE2 proteins were identified (Ballas and Citovsky, 1997; Deng et al., 1998; Bako et al., 2003; Tao et al., 2004; Bhattacharjee et al., 2008). Two VirD2-interacting proteins, ROC1 (peptidyl-prolyl cis-trans isomerase CYP1, At4g38740) and the CyP A proteins of *Arabidopsis,* belong to a large cyclophilin family of peptidyl-prolyl *cis-trans* isomerases, which are highly conserved in plants, animals, and prokaryotes (Deng et al., 1998). The VirD2 domain interacting with the cyclophilins is in the central region of VirD2 protein, which is distinct from the nuclear localization signal domains (Deng et al., 1998). Although the biological role of cyclophilins in *A. tumefaciens* infection is unclear, they were proposed to maintain the proper conformation of VirD2 within the host cell cytoplasm and/or nucleus during T-DNA nuclear import and/or integration (Deng et al., 1998; Bako et al., 2003). Another cellular factor encoded by a tomato protein phosphatase 2C (PP2C) interacted with the VirD2 NLS region (Tao et al., 2004). An *Arabidopsis abi1* (At4g26080) mutant showed higher sensitivity to *A. tumefaciens* transformation than did the wild-type plant, whereas over-expression of PP2C in tobacco protoplasts inhibited nuclear import of a ß-glucuronidase-VirD2 NLS fusion protein (Tao et al., 2004). These data suggested that phosphorylation of the VirD2 protein near the NLS region may influence the import of the T-complex.

The VirD2 protein was also found to interact with an *Arabidopsis* protein, AtKAPα/IMPA-1 (At3g06720), which belongs to a large karyopherin family known to mediate nuclear import of NLS-containing proteins (Ballas and Citovsky, 1997). AtKAPα-VirD2 interactions, which occur both in the yeast two-hybrid system and *in vitro*, require the presence of the VirD2 carboxyl-terminal NLS (Ballas and Citovsky, 1997). There are nine importin α members in *Arabidopsis.* The IMPA-2 (At4g16143), IMPA-3 (At4g02150), and IMPA-4 (At1g09270) show interactions with VirD2 and VirE2 with different strengths in yeast, *in vitro,* and in plants (Bhattacharjee et al., 2008; Lee et al., 2008). In addition, IMPA-1 interacts with the VirE2 protein in the plant cytoplasm and IMPA-4 interacts with VirE2 predominantly in the plant nucleus (Lee et al., 2008). Stable and transient transformation assay results showed that an *IMPA1*, *IMPA2*, or *IMPA3* T-DNA insertion mutant remains susceptible to *Agrobacterium*-mediated transformation, whereas the *IMPA4* T-DNA insertion mutant is resistant to *Agrobacterium*-mediated root transformation (Bhattacharjee et al., 2008). The resistant phenotype of the *impa4* mutant could be complemented by the *IMPA4* gene but could not be complemented by other importin α genes under the control of their native promoters, which might be due to the different promoter expression profiles of importin α genes. Additionally, an *Arabidopsis* T-DNA insertion mutant of the importin β-3 exhibits lower *Agrobacterium*-mediated transformation efficiencies, suggesting that importin β-like transportins might be also involved in *Agrobacterium*-mediated transformation (Zhu et al., 2003b). Collectively, these data indicate that after the T-DNA and Vir proteins enter the plant cells, the host cell nuclear import machinery may be hijacked to mediate the nuclear targeting of T-DNA (Gelvin, 2010b, 2012; Lacroix and Citovsky, 2013).

Yeast two-hybrid assays identified two VirE2-interacting plant proteins, VIP1 (At1g43700) and VIP2 (At5g59710) (Tzfira et al., 2001, 2002; Anand et al., 2007). The VIP1 protein shows homology to a class of basic leucine zipper (bZIP) proteins, and is known to localize to the cell nucleus (Tzfira et al., 2001). *VIP1* anti-sense transgenic tobacco plants exhibit significantly reduced nuclear import of GUS-VirE2 but not of GUS-VirD2, suggesting a role for VIP1 in VirE2 nuclear import (Tzfira et al., 2001). The early stage of T-DNA expression is blocked in *VIP1* anti-sense tobacco transgenic plants, whereas the over-expression of VIP1 in transgenic tobacco plants enhances the early steps of the *A. tumefaciens* infection process (Tzfira et al., 2001, 2002). Further, the evidence of VIP1 interacting with VirE2–ssDNA complexes *in vitro* (Tzfira et al., 2001) with VirE2 and importin **α** to form a ternary complex (Citovsky et al., 2004) suggested that VIP1 might function as an adaptor between VirE2 and the conventional nuclear import machinery of plant cells. Nuclear targeting of the VIP1-VirE2 complex could be triggered by the phosphorylation of VIP1 on serine-79 by the mitogen-activated protein kinase 3 (MPK3, At3g45640) (Djamei et al., 2007). Substitution of the serine-79 to alanine, mimicking the non-phosphorylated form of VIP1, resulted in the cytoplasmic localization of VIP1 (Djamei et al., 2007). These data led to the proposed 'Trojan horse' model, which suggested that *A. tumefaciens* infection activates MPK3-mediated phosphorylation of VIP1 in plant cells, and thereby promotes VIP1-VirE2-ssDNA complex trafficking to the plant nucleus (Djamei et al., 2007). However, a recent study by Shi et al. (2014) indicated that transformation efficiencies of the *Arabidopsis vip1* mutant and the *VIP1* overexpression transgenic plants were not altered when infected by numerous tested *A. tumefaciens* strains. Furthermore, overexpression of several forms of VIP1 did not alter the subcellular localization of VirE2 (Shi et al., 2014). The conflicting results from these VIP1 studies might be due to different plant systems, experiment setups, and/or analysis methods (Tzfira et al., 2001; Li et al., 2005a; Shi et al., 2014). Thus, a modified model of VirE2-VIP1 interactions in *A. tumefaciens* infection process has been proposed, in which the nuclear import of T-DNA is largely affected by the VirD2, and VirE2 may play an accessory structural role to facilitate T-DNA targeting to the plant nucleus. VirE2 proteins may interact with VIP1 in the cytoplasm regions and prevent some endogenous VIP1 from activating plant defense genes, thus promoting *A. tumefaciens* infection (Pitzschke, 2013; Shi et al., 2014).

As described above, *A. tumefaciens* may utilize the host cell nuclear import system to help T-DNA and effector proteins to enter plant nucleus. The plant cytoskeleton may also play a role in T-complex cytoplasmic trafficking. Salman et al. (2005) utilized

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single-particle-tracking methods to follow the movement of fluorescently labeled VirE2–ssDNA complexes in a cell-free system. Chemical disruptions of microtubule networks and dynein motors blocked VirE2 complex movement, suggesting the possible involvement of microtubules in *A. tumefaciens* T-complex nuclear targeting (Salman et al., 2005; Tzfira, 2006). However, cytoskeleton inhibitor treatment together with real time VirE2 trafficking imaging experiments in *N. benthamiana* demonstrated that cytoplasmic movement of VirE2 does not require microtubules but does rely on actin powered by myosin (Yang et al., 2017). The involvement of actin in VirE2 trafficking is also supported by the findings that *Arabidopsis* mutant plants impaired in actin genes (*ACT2,* At3g18780 and *ACT7,* At5g09810) expressed in the roots were resistant to stable and transient transformation, suggesting the involvement of the actin cytoskeleton in *Agrobacterium*-mediated transformation (Zhu et al., 2003b). In addition, both VirD2 and VirE2 proteins interact with filamentous actin in an *in vitro* cosedimentation assay. Inhibitors of the actin cytoskeleton lowered the *A. tumefaciens* transformation efficiency of tobacco BY-2 cells (Gelvin, 2012).

Integration into the plant genome and expression of the T-DNA

T-DNA integration into the plant cell genome is the final step of the *Agrobacterium*-mediated transformation process. Unlike other mobile DNA elements such as transposons and retroviruses, the T-DNA does not encode enzymatic activities required for integration. Thus, T-DNA insertion into the plant DNA must be mediated by proteins transported from *A. tumefaciens* itself, namely VirD2 and VirE2, and/or host cell factors.

VirD2 might play a dual role in the T-DNA integration process, ensuring both its fidelity and efficiency. The integration of the 5' end of the T-strand into plant DNA is generally precise. This may result from the linkage of VirD2 to the 5' end of the T-strand, which provides protection from exonucleases inside the plant cells (Durrenberger et al., 1989; Tinland et al., 1995; Rossi et al., 1996). A region in the C-terminal portion of VirD2 called the ω domain is implicated in the integration process (Shurvinton et al., 1992; Tinland et al., 1995; Mysore et al., 1998). However, it is not clear how exactly the ω domain of VirD2 is involved in T-DNA integration because the pattern of integration of the T-DNA is not affected by deletion of the ω sequence (Shurvinton et al., 1992; Tinland et al., 1995; Bravo-Angel et al., 1998; Mysore et al., 1998). Involvement of VirD2 in protecting the integrity of the right border was also demonstrated in a fungal transformation system (Michielse et al., 2004); this study further showed that VirE2 similarly protects the left end of the T-DNA, while VirC2 helps ensure correct processing. Importantly, VirC2 is also required for single-copy integration in this system (Michielse et al. 2004).

A report by Bako et al. (2003) demonstrated that VirD2 interacts with, and is phosphorylated both *in vitro* and in alfalfa cells by, the nuclear CAK2Ms kinase, a conserved plant ortholog of cyclin-dependent kinase-activating kinases. VirD2 is also found in tight association with the TATA box-binding protein (TBP) *in vitro* and in *Agrobacterium*-transformed *Arabidopsis* cells (Bako et al., 2003). The TBP protein is implicated in the control of transcription-coupled repair (TCR), which ensures preferential and effective removal of DNA lesions from transcribed genes (Bako et al., 2003). CAK2Ms isolated by immunoprecipitation phosphorylate cyclin-dependent kinase (CDK2) and the C-terminal domain (CTD) of the RNA polymerase II largest subunit, which may serve as a TBP-binding platform (Bako et al., 2003). The functional conservation of TBP and CDK-activating protein kinase (CAK2) orthologs in eukaryotes indicates that these nuclear VirD2-binding factors may provide a link between T-DNA integration and transcription-coupled repair.

Ziemienowicz et al (2000) demonstrated that VirD2 protein does not possess general ligase activity using an *in vitro* ligation assay. Enzyme(s) present in plant extracts, likely DNA ligase, and *E. coli* T4 DNA ligase were able to ligate the 5' end of T-DNA from an artificial T-DNA-VirD2 complex to a partly double-stranded oligonucleotide (Ziemienowicz et al., 2000). An *Arabidopsis* orthologue of the yeast and mammalian DNA ligase IV gene termed AtLIG4 (At5g57160) is required for the repair of DNA damage because seedlings of the *Atlig4* mutant are hyper-sensitive to the DNA-damaging agents methyl methanesulfonate and X-rays (Friesner and Britt, 2003; van Attikum et al., 2003). However, using tumorigenesis and germline transformation assays, the *Arabidopsis Atlig4* mutant is not impaired in T-DNA integration. Thus, DNA ligase IV may not be required for T-DNA integration in certain plant tissues. Similarly, the *ku80 Arabidopsis* mutant plant is hyper-sensitive to the DNA-damaging agent methyl methane sulphonate and has a reduced capacity to carry out non-homologous end joining recombination (Gallego et al., 2003). *ku80* (At1g48050) mutant plants show no significant defect in the efficiency of floral dip transformation of plants with *A. tumefaciens* (Friesner and Britt, 2003; Gallego et al., 2003). However, in other studies, contradictory data indicated that *Arabidopsis ku80* or *ku70* (At1g16970) mutant plants and rice plant lines down-regulated in *ku70*, *ku80*, or *lig4* showed different transformation responses (van Attikum et al., 2001; Friesner and Britt, 2003; Gallego et al., 2003; Li et al., 2005b; Jia et al., 2012; Nishizawa-Yokoi et al., 2012; Vaghchhipawala et al., 2012; Mestiri et al., 2014; Park et al., 2015). These discrepancies might be the result of different techniques and different plant tissues used to examine transformation efficiency, or they may reveal more complex and redundant pathways for T-DNA integration mechanisms during *A. tumefaciens* infections (Tzfira et al., 2004a; Citovsky et al., 2007; Gelvin, 2010a, 2010b; Magori and Citovsky, 2012; Lacroix and Citovsky, 2013).

The contradictory conclusions for the involvement of NHEJ for T-DNA integration implied that an alternative DNA repair pathway might be responsible for T-DNA integration. A recent breakthrough showed that T-DNA integration in *A. thaliana* requires the polymerase theta (Pol θ, At4g32700) (van Kregten et al., 2016). This discovery was inspired by the similar signature of double-strand breaks (DSBs) of T-DNA integration with other systems. A kind of genome scar 'filler DNA' is usually found in the site of T-DNA integration as well as the Pol θ-mediated error-prone DSB repair in mammalian cells (Mateos-Gomez et al., 2015). Pol θ mutant plants *tebichi/polq* showed resistant to T-DNA integration but were susceptible to *Agrobacterium*-mediated transient transformation demonstrated via both floral dip and root transformation. Based on the knowledge of molecular action of Pol θ, it has been proposed that T-DNA could target the random DSBs in the genome and the microhomology between the ends of T-DNA and

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the DSBs is required for the integration of T-DNA. Enhanced understanding of the mechanism of T-DNA integration may allow researchers to improve transgene targeting by introducing artificial breaks in the plant genome, which ideally will lead to specific integration of DNA fragments in a designated location (van Kregten et al., 2016).

Identification of additional cellular factors involved in the T-DNA interaction comes from genetic approaches. Nam et al. (1999) identified several *Arabidopsis rat* mutants deficient in T-DNA integration. One mutant, *rat5*, which harbors a mutation in the *H2A* histone gene (*HTA1*, At5g54640), is deficient in stable but not transient T-DNA expression, suggesting the involvement of HTA1 in T-DNA integration (Mysore et al., 2000b). The *rat5* mutant plant is highly recalcitrant to stable transformation by root inoculation. However, the mutant plant is efficiently transformed by flower vacuum infiltration (Mysore et al., 2000a). The highest levels of *HTA1* gene expression are detected in the elongation zone of the root, a region that is most susceptible to transformation (Yi et al., 2002). The *HTA1* promoter activity is induced by wounding or by *A. tumefaciens* infections (Yi et al., 2006). The *HTA1* cDNA not only complements the *rat5* mutant, but was also shown to increase transient and stable transformation rates of wild-type plants, indicating that histone H2A-1 may play an role in transformation (Mysore et al., 2000b; Yi et al., 2006; Tenea et al., 2009). Tenea et al. (2009) demonstrated that overexpression of *Arabidopsis* cDNAs encoding seven tested histone H2A (*HTA*), histone H4 (*HFO*), and histone H3-11 (*HTR11*, At5g65350) genes increased both *Agrobacterium*-mediated transformation and transgene expression; whereas none of the seven tested histone H2B (*HTB*) cDNAs, nor other tested histone H3 (*HTR*) cDNAs, increased transformation. Overexpression of *HTA, HFO,* or *HTR11* cDNA increased expression of incoming single-stranded or double-stranded forms of a *gusA* gene in plant cells. These results suggested that several histone proteins may enhance transgene expressions within the plant cells and therefore may increase *A. tumefaciens* infection efficiency (Tenea et al., 2009; Gelvin, 2010b).

The VirE2-interacting protein, VIP1, may also participate the T-DNA integration step, possibly by creating a link to the host cell chromatin component histone protein. VIP1 shows interaction with plant histone H2A-1 *in vivo*, and with purified *Xenopus* core histones H2A, H2B, H3 and H4 *in vitro* (Li et al., 2005a; Loyter et al., 2005). In addition, the VIP1 protein associates with purified plant nucleosomes *in vitro* (Lacroix et al., 2008). VIP1 is also able to provide links between plant nucleosomes with VirE2 and with a synthetic minimal T-complex *in vitro* by forming quaternary nucleosome-VIP1-VirE2-ssDNA complexes (Lacroix et al., 2008). Another VirE2-interacting protein, VIP2, is a basic domain/leucine zipper motif-containing protein. Virus-induced gene silencing of *VIP2* in *Nicotiana benthamiana* and the *Arabidopsis vip2* mutant are both defective in T-DNA integration but not in transient T-DNA expression. The transcriptome analyses of *Arabidopsis vip2* mutant revealed that expression of several histone genes was decreased, suggesting VIP2 may function as a transcriptional regulator to affect histone gene expression, thereby participating in the T-DNA integration step indirectly (Anand et al., 2007). When the T-DNA is inserted into the host chromosomal DNA wrapped in chromatin, the chromatin structure may need to be remodeled or removed to accommodate external T-DNA insertions. Several genetic studies indicated that T-DNA integration may be influenced by several chromatin proteins, including histone deacetylase (*HDA1*/*HDT19*, At4g38130; *HDA2/HDT1*, At3g44750; *HD2B/HDT2*, At5g22650), and two subunits of the chromatin assembly factor 1 (*FAS1,* At1g65470; *FAS2,* At5g64630) (Zhu et al., 2003b; Endo et al., 2006; Crane and Gelvin, 2007; Gelvin and Kim, 2007).

After the T-complex enters the plant nucleus, the associated bacterial virulence proteins and host proteins may need to be removed from the T-DNA to allow efficient T-DNA integration. The disassembly of the T-complex is likely mediated by targeted proteolysis, which is accomplished by the VirF protein and the plant ubiquitin proteasome complex (Tzfira et al., 2004b; Zaltsman et al., 2010a, 2010b). *virF* mutant bacterial strains show weak virulence on some plant species, including *Nicotiana glauca* and tomato, but can efficiently transform other plant species, suggesting the VirF protein may function as a host-range factor (Melchers et al., 1990; Regensburg-Tuïnk and Hooykaas, 1993). The *virF* mutant can be complemented by expressing VirF in transgenic plants, showing that the VirF protein functions in plant cells (Regensburg-Tuïnk and Hooykaas, 1993). VirF is an F-box protein and interacts with three host Skp-1 related proteins, ASK1 (At1g75950), ASK2 (At5g42190), and ASK10 (At3g21860) proteins (Schrammeijer et al., 2001). Both F-box proteins and Skp1 proteins are core components of the SCF (Skp1-Cullin-F-box protein) family of E3 ubiquitin ligases and serve to mediate targeted protein destabilization by the 26S proteasome. The VirF protein can interact with the VIP1 protein and mediate VirE2 protein degradation in the presence of VIP1 in yeast and plant cells, suggesting the VirF-mediating protein degradation is important for efficient transformation (Tzfira et al., 2004b). The involvement of members of the host SCF-E3 ligase complex were further deciphered by gene silencing and gene expression studies (Anand et al., 2012). The SCF-E3 ligase is composed of four different proteins: an F-box protein that recruits substrates; SKP1 that functions as an adaptor protein; Cullin (CUL1); and a RING-finger protein (RBX1) that interacts with both Cullin and E2 conjugating enzymes (Anand et al., 2012; Magori and Citovsky, 2012). Microarray analysis results showed that only *ASK1*, *ASK2*, and *ASK20*, among 21 *Arabidopsis SKP1-LIKE (ASK)* genes, are induced after *A. tumefaciens* infections. The *Arabidopsis ask1* and *ask2* mutants showed lower *A. tumefaciens* infection efficiencies, suggesting the SKP1 proteins may be positively involved in *A. tumefaciens* infection (Anand et al., 2012; Magori and Citovsky, 2012). However, the transformation rates of both *RBX1* and *CUL1c* gene silenced *N. benthamiana* were not significantly affected (Anand et al., 2012). The *Arabidopsis SGT1a* (suppressor of the G2 allele of SKP1, At4g23570) and *SGT1b* (At4g11260) genes that encode accessory proteins interacting with the SCF-complex were induced following *A. tumefaciens* infection (Anand et al., 2012). Only the *sgt1b* mutant showed lower tumor formation efficiencies on roots when infected with *A. tumefaciens,* suggesting the potential role of SGT1 in the *A. tumefaciens*-plant interaction (Anand et al., 2012). Another plant SCF-E3 ligase complex member, the VIP1-binding F-box protein (VBF, At1g56250), is able to substitute for VirF function in plants and its expression is also induced by *A. tumefaciens* infection (Ditt et al., 2006; Zaltsman et al., 2010b). The VBF protein interacts with VIP1 and targets VIP1 and VIP1-VirE2 complexes for proteasomal destabilization via the SCF^{VBF}

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complex (Zaltsman et al., 2010b). Additionally, the involvement of the VBF protein in uncoating a synthetic single-stranded DNA packaged by VirE2 was demonstrated in a cell-free proteasomal degradation system (Zaltsman et al., 2013). This proteasomal uncoating of synthetic minimal T-complexes is dependent on the presence of functional VBF and VIP1 proteins; and is inhibited by a known selective inhibitor of proteasomal activity, MG132 (Lacroix and Citovsky, 2013; Zaltsman et al., 2013).

The VirF protein may have multiple functions during *A. tumefaciens* infection, in addition to mediating the host cell ubiquitin-proteasome system (UPS) to unmask the T-complex and help T-DNA become integrated into the host genome (Gelvin, 2010a; Magori and Citovsky, 2012; Lacroix and Citovsky, 2013; Pitzschke, 2013). As noted above, upon *A. tumefaciens* infection, the VIP1 protein is phosphorylated by the mitogen-activated protein kinase 3 (MPK3) and functions as a transcription factor that induces expression of several stress-responsive genes (Djamei et al., 2007; Pitzschke, 2013). *A. tumefaciens* may utilize VirF to repress the VIP1-mediated host defense responses via VIP1 protein degradation. At the same time, premature or excessive degradation of VirE2-VIP1 protein complexes by VirF might also hinder T-DNA nuclear import and integration. In order to work against host cell UPS-mediated VirF degradation, *A. tumefaciens* exports another effector protein, VirD5, into plant cells; this protein interacts with VirF and protects it from rapid degradation by the defensive action of the host UPS (Magori and Citovsky, 2011). Similarly, VirD5 interacts with the *Arabidopsis* VIP1 protein and is able to form a ternary complex of VirD5-VIP1-VirE2 (Wang et al., 2014). VirD5 can compete with VBF for VIP1 binding and help stabilize VIP1 and VirE2 proteins, supporting the idea that the VirD5 protein may target T-complex to prevent its degradation in the plant cell nucleus (Wang et al., 2014). Finally, the VirE3 protein can be transferred into plant cells (Vergunst et al., 2000, 2003, 2005; Schrammeijer et al., 2003). Interestingly, VirE3 may interact with VirE2 and expression of VirE3 in VIP1 antisense tobacco plants can complement both nuclear import of VirE2 and transformation susceptibility (Lacroix et al., 2005). Thus, the *A. tumefaciens* may use the VirE3 effector protein as a substitute for VIP1 in plants to facilitate VirE2 nuclear targeting during infection. Collectively, these examples illustrate how the *A. tumefaciens*-mediated plant transformation process represents an impressive arms race, in which the plant cells try to fight off pathogen infections while the pathogen utilizes bacterial effectors to evade or outweigh plant defense responses.

APPLICATIONS OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Stable transformation

A. tumefaciens can genetically transform various host cells including numerous dicot and some monocot angiosperm species and gymnosperms (De Cleene and De Ley, 1976). In addition, *A. tumefaciens* can transform non-plant organisms, including fungi, algae, sea urchin embryos, human cells, and the gram positive bacterium *Streptomyces lividans* (Bundock et al., 1995, 1999; Piers et al., 1996; de Groot et al., 1998; Kunik et al., 2001; Kelly and Kado, 2002; Hooykaas, 2004; Kumar et al., 2004; Pelczar et al., 2004; Michielse et al., 2005; Bulgakov et al., 2006; Lacroix et al., 2006). In order to perform effective and large-scale functional genomic studies, highly efficient and simple genetic transformation methods for the studied organisms are crucial. The *A. tumefaciens*-mediated transformation method has several advantages over other transformation methods; it is easy to use, relatively inexpensive, and generally results in low copy number and well-defined DNA insertions into the host cell chromosome (Koncz et al., 1994; Pawlowski and Somers, 1996; Hansen et al., 1997; Enríquez-Obregón et al., 1998; Shou et al., 2004; Travella et al., 2005; Zhang et al., 2005; Gao et al., 2008). The traditional transformation methods such as protoplast transformation by electroporation, microinjection, or polyethylene glycol fusion are not as well-suited for production of transgenic plants, because the regeneration of plants from protoplasts is time-consuming and low efficiency (Fromm et al., 1985, 1986; Uchimiya et al., 1986; de la Peña et al., 1987; Newell, 2000; van den Eede et al., 2004; Banta and Montenegro, 2008). Microprojectile bombardment, also called biolistics, is the most important alternative to Ti plasmid DNA delivery systems for plants (Sanford, 2000). Spherical gold or tungsten particles are coated with DNA, accelerated to high speed with a particle gun, such that they penetrate into plant tissues (Kikkert et al., 2004). Microprojectile bombardment can be used to introduce foreign DNA into various plant tissues of a wide range of plant species. The gene of interest needs not to be cloned into a specialized transformation vector in order to be transformed into plant cells (Herrera-Estrella et al., 2005). However, the bombardment process tends to cause multiple-copy DNA insertions and the loss of molecular integrity of the DNA, and there are limitations on the size of the DNA. The complex DNA integration patterns usually result in genetic instability and/or epigenetic silencing of the transgene in the transgenic plants (Newell, 2000; Kikkert et al., 2004; Lorence and Verpoorte, 2004; van den Eede et al., 2004; Altpeter et al., 2005; Herrera-Estrella et al., 2005). Due to these limitations and drawbacks of physical and chemical transformation methods, the *A. tumefaciens*-mediated gene transfer method is still the most frequently used and most popular method for generation of transgenic plants and genetically modified crops (Table 2).

The transformation methods can be categorized into two types: transient transformation and stable transformation. For transient transformation, T-DNA integration into the host genome is not required, and T-DNA expression usually lasts for a few days (Wroblewski et al., 2005; Marion et al., 2008; Jones et al., 2009; Kim et al., 2009; Li et al., 2009; Tsuda et al., 2012; Wu et al., 2014b). It presents useful advantages, in that it allows results of experimental treatments to be observed in a short period of time, as discussed below. On the other hand, stable transformation is a long process that often requires established tissue culture techniques to promote whole plant growth from the transformed cells or tissues. For stable transformation, the T-DNA must integrate in the host cell genome, so that it is subsequently passed on to the next generation (Bent, 2006; Gelvin, 2006; Tague and Mantis, 2006; Rivero et al., 2014).

In order to utilize pathogenic *A. tumefaciens* to generate transgenic plants, several obstacles need to be overcome. First, the oncogenes from the wild-type T-DNA are removed to eliminate the pathogenicity of the bacterium, and the strain becomes nonpathogenic (disarmed) without affecting its ability to transfer

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Table 2. Summary of different plant transformation methods

Method	Advantages	Disadvantages
Agrobacterium tumefaciens-mediated transformation	Minimal equipment and facility required and less expensive; easy to set up and use; generally low transgene copy number; minimal DNA rearrangements; fewer undesired mutations-somaclonal variation; relatively higher stability of transferred gene	Several elite monocot crops, legumes, and woody plants are recalcitrant to *A. tumefaciens*-mediated transformation
Microprojectile bombardment	A wide range of plants and tissues can be used; an excellent and efficient system for transient expression; simultaneous delivery of large numbers of different genetic elements; effective system for organelle transformation	Multiple copies of DNA insertions; fragmentation and loss of molecular integrity of DNA insertions; possible emergence of chimeric plants
Microinjection	Only few successful reports in rye, barley, and other plants	Limited to protoplasts or few plant cell types; requirement for highly skilled personnel; difficult to regenerate viable plants
Electroporation	An alternative system to obtain transient expression in plants; convenient, simple, and fast	Limited to protoplasts or few plant cell types; difficult to regenerate viable plants
Polyethylene glycol (PEG)-mediated gene transfer	Direct gene transfer to cells circumvents the host range limitation; easy to perform with relatively low cost	Limited to protoplasts or few plant cell types; difficulties and slow process in regeneration of viable plants
Pollen tube pathway	Elimination of tissue culture systems and is not limited by the ability to regenerate from transformed plant tissues, cells or protoplasts; only few successful reports in rice, maize, cotton, soybean, wheat, and other plants	Difficulty in establishing standard protocols and low success rates; only works with plants that flower and readily produce seeds; can only be performed during the flowering period

T-DNA. Second, the gene of interest and selection markers for transgenic plants need to be introduced into the T-DNA, and molecular biology tools are needed for DNA cloning *in vitro*. The Ti plasmid size is large and usually low-copy number, which makes isolation and cloning of the Ti plasmid quite challenging. In order to overcome these difficulties, most research teams utilize a binary vector system, in which the T-DNA region is carried on a broad-host range replicon and the *vir* genes required for T-DNA transfer are located on the disarmed Ti-plasmid (Barton et al., 1983; de Framond et al., 1983; Hoekema et al., 1983; Zambryski et al., 1983; Bevan, 1984). This binary vector system has provided the plant research community enormous versatility and flexibility, and has enabled an upsurge in the production of transgenic plants.

With advances in recombinant DNA technology, T-DNA binary vectors have evolved over the past years to be highly developed and more specialized for different purposes (Hellens et al., 2000; Chung et al., 2005; Komari et al., 2006; Lee and Gelvin, 2008; Mehrotra and Goyal, 2012; Anami et al., 2013). T-DNA-based binary vectors generally consist of several features, including (1) the T-DNA right and left border repeat sequences that define the T-DNA region; (2) selectable marker genes to function in bacteria (*E. coli* and *A. tumefaciens*) and in plants; (3) restriction endonuclease sites within the T-DNA to allow insertion of one or more gene(s) of interest; (4) origin(s) of replication to allow plasmids to replicate in bacteria. In order to identify the transformed cells or plants, it is essential to be able to detect the foreign DNA that has been integrated into plant chromosome. Various antibiotic or herbicide resistance genes have been inserted into the T-DNA region of the binary vectors and used for transgenic plant selection. The neomycin phosphotransferase (NPTII) gene for kanamycin resistance and hygromycin phosphotransferase (HPT), conferring resistance to hygromycin, are the most widely used selectable marker gene systems in plants. Several other frequently used selection systems utilize other antibiotics, including chloramphenicol, gentamicin, streptomycin, bleomycin, blasticidin; herbicides, including phosphinothricin, gluphosinate, glyphosate, bromoxynil; or metabolic markers, including acetolactate synthase, threonine dehydratase, phosphomannose isomerase (Gheysen et al., 1998; Newell, 2000; Banta and Montenegro, 2008; Lee et al., 2008; Lee and Gelvin, 2008; Anami et al., 2013). Furthermore, in plant gene functional studies and other quantification studies, the use of reporter genes allows transformed cells to be quantified. The frequently used reporter gene products, such as ß-D-glucuronidase (GUS), both firefly and bacterial luciferases (LUC), green fluorescent protein (GFP) and other fluorescent proteins, can be detected in transformed plant tissues. The GUS activity in the transformed plant tissues can be observed by the blue staining after hydrolysis of the 5-bromo-4-chloro-3-indolyl ß-D-glucuronic acid; or can be quantitatively assayed by a fluorometric analysis that requires hydrolysis of the 4-methylumbelliferyl ß-D-glucuronide. The GFP protein is a useful *in vivo* marker to examine gene expression or recombinant protein localization, because it can be excited with either ultraviolet or blue light without addition of any substrates or cofactors.

Based on the agrobacterial chromosomal background and the resident Ti plasmids, the commonly used disarmed *A. tumefaciens* strains originated from two representative wild isolates: C58 (Lin and Kado, 1977) and Ach5 (Kovacs and Pueppke, 1994). The *A. tumefaciens* strains AGL1, EHA101, EHA105, and GV3101(pMP90), have the nopaline type C58 chromosomal background and the modified Ti plasmids from either the pTiBo542 or the pTiC58 plasmids (Hood et al., 1986; Koncz and Schell, 1986; Lazo et al., 1991). The *A. tumefaciens* strain LBA4404 has the octopine type Ach5 chromosomal background and the octopine-type Ti plasmid pAL4404 derived from the pTiAch5 plasmid (Ooms et al., 1982). Genetic background and modified Ti plasmid

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combinations of the disarmed *A. tumefaciens* strains noticeably affect the bacterial host range and the transformation efficiencies of various plant species (Hwang et al., 2010; Hwang et al., 2013a) (Table 3).

The host species *A. thaliana* is characterized by several traits, including small plant size, short generation time, large quantity of seeds, relatively small genome size, and ease of transformation by *A. tumefaciens*, which has led it to become the model system for plant biologists to address questions of basic and applied technology. Several *Agrobacterium*-mediated transfer methods for *A. thaliana* were developed using various *A. thaliana* ecotypes, plant tissue types, bacterial strains, binary vectors, and selection marker (Lloyd et al., 1986; Feldmann and Marks, 1987; Sheikholeslam and Weeks, 1987; Valvekens et al., 1988; Akama et al., 1992; Clarke et al., 1992; Huang and Mā, 1992; Sangwan et al., 1992; Chang et al., 1994). Here, two major stable transformation methods of *A. thaliana* are described as follows.

Table 3. Frequently used disarmed *Agrobacterium* strains

Strain Name	Chromosome background	Ti-plasmid types	Antibiotic Resistance[1]	Reference
LBA4404	Ach5	a disarmed octopine-type Ti plasmid pAL4404	Rm^R	Hoekema et al., 1983
EHA101	C58	a disarmed agropine-type Ti plasmid pEHA101 (pTiBo542ΔT-DNA)	Rm^R, Km^R	Hood et al., 1986
EHA105	C58	a disarmed agropine-type Ti plasmid pEHA105 (pTiBo542ΔT-DNA)	Rm^R	Hood et al., 1993
C58C1	C58	Cured of Ti plasmid	Rm^R	Deblaere et al., 1985
C58C1(pTiB6S3ΔT, pCH32)	C58	a disarmed octopine-type Ti plasmid pTiB6S3ΔT-DNA and a helper plasmid pCH32	Rm^R, Cb^R, Tc^R	McBride and Summerfelt, 1990
GV3101	C58	Cured of Ti plasmid	Rm^R	Holsters et al., 1980
GV3101(pMP90)	C58	a disarmed nopaline-type pTiC58ΔT-DNA	Rm^R, Gm^R	Koncz and Schell, 1986
A136	C58	Cured of Ti plasmid	Rm^R	Watson et al., 1975
AGL-1	C58	a disarmed pTiBo542ΔT-DNA	Rm^R, Cb^R	Lazo et al., 1991
C58-Z707	C58	a disarmed nopaline-type pTiC58-Z707	Km^R	Hepburn et al., 1985
NTL4(pKPSF2)	C58	a disarmed chrysopine-type pTiChry5ΔT-DNA	Em^R	Palanichelvam et al., 2000

[1]Cb, carbenicillin; Em, erythromycin; Gm, gentamicin; Km, kanamycin; Rm, rifampicin, Tc: tetracycline.

Stable transformation using root explants

Root explant is one of the plant tissues that is frequently used to genetically transform *A. thaliana* (van den Elzen et al., 1985; Valvekens et al., 1988; Akama et al., 1992; Clarke et al., 1992; Kemper et al., 1992; Czako et al., 1993; DeNeve et al., 1997; De Buck et al., 1998, 2000, 2009). There are several advantages of root explants for genetic transformation by *A. tumefaciens. A. thaliana* root cells are competent for regeneration, can be easily acquired in large quantities, and can be maintained in sustained cultures (Valvekens et al., 1988; Sangwan et al., 1992; Czako et al., 1993). Furthermore, a relatively low percentage of transformants show polyploidy when root explants are used for *A. tumefaciens* transformation, in comparison to leaf transformation or direct gene transfer methods, such as microprojectile bombardment (Altmann et al., 1994). Additionally, root transformations of *Arabidopsis* tend to result in higher percentage of single T-DNA insertions when compared with leaf-disc transformations (Grevelding et al., 1993). The root transformation assay also provides a reproducible and quantitative assay system for plant biologists to examine transformation efficiencies of somatic cells (Gelvin, 2006). Several studies have utilized quantitative root transformation assays to determine virulence of different *A. tumefaciens* strains and relative transformation efficiencies of different *A. thaliana* mutants or ecotypes. Based on the results obtained from these root transformation assays, bacterial and plant proteins that are involved in *Agrobacterium*-mediated plant transformation process can be identified and characterized; several of these were described above (Nam et al., 1997, 1998, 1999 Bundock et al., 1999; Mysore et al., 2000a; Zhu et al., 2003a, 2003b; Tsai et al., 2009; Gelvin, 2010a, 2010b; Hwang et al., 2010; Tsai et al., 2010; Gelvin, 2012; Hwang et al., 2013b, 2015).

It is known that different factors affect transformation efficiency, including plant growth temperature, plant-bacteria co-incubation time and temperature, light intensity and periods, addition of AS, pretreatment with phytohormones, different *A. thaliana* ecotypes, different *A. tumefaciens* helper strains, and other factors (Vergunst et al., 1998; Zambre et al., 2003; Gelvin, 2006). The optimum growth temperature for *Arabidopsis* is 25°C. It is recommended that night temperature can be 2 to 4°C lower than the day temperature. During co-cultivation of plant root explants with *A. tumefaciens*, the transformation efficiencies and plant regeneration frequencies were relatively higher at 25°C in comparison

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to 21°C (Vergunst et al., 1998). On the other hand, although *A. tumefaciens* grows well at 28°C, accumulation of some of the VirB proteins constituting the T4SS T-DNA delivery machinery, as well as virulence on the host species *Kalanchoe*, is strongly compromised at this temperature (Fullner and Nester 1996; Banta, et al. 1998; Baron et al., 2001); these data indicate that pre-induction with AS (see below) should be performed at 19-21°C. Furthermore, *A. tumefaciens* can lose virulence when grown at 37°C due to loss of the Ti plasmid in the bacteria (Hamilton and Fall, 1971; Kerr, 1971). Light conditions during *Agrobacterium*-mediated plant transformation also affect transformation efficiencies significantly. The transformation efficiency is higher under continuous light than under a 16 hour light/8 hour dark photoperiod (Vergunst et al., 1998; Zambre et al., 2003). Another important factor for obtaining high transformation frequency is addition of 50-200 μM AS, a natural wound response molecule, to the *A. tumefaciens* culture prior to or during the co-incubation period in order to fully induce virulence gene expression in bacteria (Sheikholeslam and Weeks, 1987; Vergunst et al., 1998). It has also been suggested that coincubation periods should not exceed two days in order to avoid overgrowth of bacteria (Vergunst et al., 1998; Gelvin, 2006). After bacteria and root explant coincubation, different antibiotics, such as carbenicillin, cefotaxime, vancomycin HCl, or timentin, may be added into the selection media to eliminate *A. tumefaciens*. Timentin is an antibacterial combination consisting of the semisynthetic antibiotic ticarcillin disodium and the β-lactamase inhibitor clavulanate potassium and has frequently been used in *A. thaliana* root transformation methods (Nam et al., 1997, 1998, 1999; Vergunst et al., 1998; Bundock et al., 1999; Mysore et al., 2000a; Zhu et al., 2003a).

Different opine-type *A. tumefaciens* strains show different degrees of virulence on different plant species, partly because the *tzs* and v*irF* genes exist in only one type of Ti-plasmid. It has been suggested that nopaline-type *A. tumefaciens* strains are preferable for transformation of *A. thaliana* and other plants from the *Brassicaceae* family (Nam et al., 1997; Gelvin, 2006; Hwang et al., 2013a). Similarly, different wild-type *A. thaliana* ecotypes show different competence for *A. tumefaciens* transformation (Nam et al., 1997; Chateau, 2000; Mysore et al., 2000a; Hwang et al., 2013a); ecotypes Aua/Rhon (Aa-O), Bensheim/Bergstrasse (Be-O), Wassilewskija (Ws), Columbia (Col-O), and C-24 are more susceptible to *A. tumefaciens* root transformation (Nam et al., 1997). Furthermore, competence of several *A. thaliana* ecotypes can be enhanced when the explants are pre-cultured on media containing the phytohormones auxin and cytokinin (Valvekens et al., 1988; Sangwan et al., 1991, 1992; Hwang et al., 2013b; Sardesai et al., 2013).

Stable transformation by floral dipping

The transformation and regeneration of plants is a vital tool in both basic and applied plant biology research fields. The typical plant transformation method includes delivery of target gene into plant cells by *A. tumefaciens* transformation, followed by selection and regeneration of transgenic plants from the transformed cells through tissue culture techniques. Established plant tissue culture systems and sterile conditions are essential to regenerate plants. Suitable plant growth regulators are added to help regeneration of transgenic plants. However, several difficulties exist in the typical molecular transformation method. First, some *A. thaliana* ecotypes, such as Antwerpen (An-1), Angleur (Ang-0), Bologna (Bl-1), Blanes/Gerona (Bla-2), Calver (Cal-0), and Dijon-G, and mutant lines, are resistant to transformation and/or regeneration (Nam et al., 1997; Chateau, 2000; Mysore et al., 2000a; Hwang et al., 2013a). In addition, regeneration of whole plants from transformed somatic cells sometimes leads to somatic mutations. The presence of phytohormones in the tissue culture during regeneration also increases the chance of somatic mutations (Bent, 2000; Bent, 2006; Tague and Mantis, 2006). In order to resolve these difficulties, several plant transformation methods have been established and improved (Feldmann and Marks, 1987; Bechtold et al., 1993; Chang et al., 1994; Katavic et al., 1994; Clough and Bent, 1998; Richardson et al., 1998; Martinez-Trujillo et al., 2004; Zhang et al., 2006). Feldmann and Marks (1987) first reported that imbibition of *A. thaliana* germinating seeds with an appropriate *A. tumefaciens* strain can generate transformed progeny in the next generation. This method was applied to provide the first insertion mutagenized lines of *A. thaliana*. Another whole plant transformation method was presented by Chang et al. (1994) and by Katavic et al. (1994) in which reproductive inflorescences were cut off and the wounded sites were inoculated with *A. tumefaciens*. The treated plants were grown to maturity, harvested, and the progeny screened for transformants under selection. These methods avoid tissue culture steps and the resulting somaclonal variation, and a relatively short time is needed to acquire transformed progeny. However, the frequency of obtaining transformants in the next generation is highly variable and sometimes inconsistent for routine use.

An improved *in planta* transformation method was later proposed by Bechtold et al. (1993) in which the uprooted *A. thaliana* plants were vacuum infiltrated with the appropriate *A. tumefaciens* culture, infiltrated plants were then immediately replanted, and harvested seeds were finally screened for successful transformants. This method was developed to preclude altogether the requirement for plant tissue culture or regeneration steps (Bechtold et al., 1993; Bechtold and Pelletier, 1998). However, the removal and replanting of plants constrain the usefulness of this method. It was later discovered that submergence of inflorescences in the early flowering stages in a *A. tumefaciens* suspension is sufficient to obtain successful transformants (Clough and Bent, 1998). Furthermore, the vacuum infiltration process can be eliminated; simple dipping of developing floral tissues into the bacterial suspension is enough to acquire transformants in the next generation (Clough and Bent, 1998; Richardson et al., 1998; Bent, 2000). *A. tumefaciens* strains are first cultured in rich media with appropriate antibiotic selection until stationary phase (OD_{600}= 0.8-1.0). The *A. tumefaciens* cultures are then collected by centrifugation and the pellet is resuspended in a solution containing 5% sucrose and the surfactant Silwet L-77 (Clough and Bent, 1998; Bent, 2000). Both sucrose and surfactant 0.05% Silwet L-77 are important for the success of the floral dip method (Clough and Bent, 1998; Richardson et al., 1998; Bent, 2000; Bent, 2006). However, Silwet L-77 can be toxic to plants at high concentrations, and 0.02% L-77 or as low as 0.005% L-77 can be used in the floral dip method. Repeated applications of *A. tumefaciens* can enhance the transformation efficiency (Clough and Bent, 1998; Martinez-Trujillo et al., 2004). After bacterial inoculation, covering

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plants for 1 day to maintain humidity also increase the transformation rates (Bent, 2000; Bent, 2006). Spraying of the *A. tumefaciens* is a useful alternative to transform *Arabidopsis,* and this method may provide the possibility of *in planta* transformation of plant species that are too large for dipping or vacuum infiltration (Chung et al., 2000). With these floral dip or spray methods, 0.1-3.0% of the seeds is typically found to be transgenic. This floral dip method is simple and can be easily performed by nonspecialists, which avoids extensive labor and the issues and equipment associated with regeneration. This method has been successfully used on a large number of *A. thaliana* ecotypes and mutants that might be resistant to transformation by other methods targeting somatic cells or tissues (Mysore et al., 2000a). The most striking contribution of this method is the high-throughput generation of *A. thaliana* T-DNA insertion mutant lines (Alonso et al., 2003; Alonso and Stepanova, 2003). Plant researchers can now easily obtain various T-DNA insertion mutants for most *Arabidopsis* genes from various stock centers (Table 4) (Scholl et al., 2000; Pan, 2003; Li et al., 2014a).

In the *A. thaliana* vacuum infiltration method, most of the primary transformants are hemizygous at any T-DNA insertion site, suggesting that *A. tumefaciens* transformation may occur late in the floral development, possibly after the divergence of male and female gametophyte cell lineages (Bechtold et al., 1993; Bent, 2000; Bent, 2006). It was later discovered that the majority of transgenic plants result from transformation of the female gametophyte; this conclusion is based on the following observations (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000; Bechtold et al., 2003): First, no transformed progeny were obtained after inoculation of *A. tumefaciens* on pollen donor plants, while 0.48% of transformed progeny were produced after inoculation of *A. tumefaciens* on pollen recipient plants (Desfeux et al., 2000). Second, various promoters fused to a GUS marker gene were used to observe sites of delivery of T-DNA (Ye et al., 1999; Bechtold et al., 2000; Desfeux et al., 2000). GUS staining was mainly observed in the ovules of the flowers and only rarely in pollen, suggesting that ovules are the primary target for *A. tumefaciens* transformation (Ye et al., 1999; Desfeux et al., 2000). In *A. thaliana* flowers, the gynoecium is an open and vase-like structure, which can fuse to form closed locules almost three days prior to anthesis. Based on this observation, the timing of *A. tumefaciens* infection is important to allow *A. tumefaciens* to enter the interior of the gynoecium prior to locule closure and ensure successful transformation (Desfeux et al., 2000). Third, most of the transformants contain T-DNA that is derived from the maternal chromosome set, based on a genetic linkage study (Bechtold et al., 2000). The floral dip or infiltration transformation methods have been successfully applied to wheat, radish, *Medicago truncatula,* and some other members from the Brassicaceae, such as pakchoi (*Brassica campestris*), Shepherd's purse (*Capsella bursa-pastoris*), *Lepidium campestre*, and *Arabidopsis lasiocarpa* (Liu et al., 1998; Trieu et al., 2000; Curtis and Nam, 2001; Tague, 2001; Wang et al., 2003; Inan, 2004; Agarwal et al., 2008; Bartholmes et al., 2008; Zale et al., 2009; Lenser and Theißen, 2014). Other bacteria, including *Ensifer adhaerens*, *Rhizobium* sp. NGR234, *Sinorhizobium meliloti*, and *Mesorhizobium loti*, contained the foreign *vir* genes and T-DNA regions on separate plasmids and have been used for floral dip transformation of *A. thaliana* (Broothaerts et al., 2005; Wendt et al., 2012). Recently, the *Rhizobium etli* CFN42 genome analysis results showed that it has functional and high homologs of *vir* genes in its p42a plasmid. The *R. etli* can both stably and transiently transform tobacco plants when the T-DNA region is introduced into the *R. etli* on a binary vector (Lacroix and Citovsky, 2016). Although these bacteria are not naturally able to transform plants because they lack native T-DNA and/or *vir* genes, most of them belong to *Rhizobiaceae* family, specifically the *Alpha-Proteobacteria* class.

The clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein (Cas) technology is a recent advance and powerful tool for genome editing in eukaryotic cells. The technique utilizes *Streptococcus pyogenes* Cas9 endonuclease and single chimeric guide RNA (sgRNA) to generate DNA double-stranded breaks in targeted genome sequences. It has been successfully used to edit genomes in animal systems (Cong et al., 2013; Hwang et al., 2013c; Wang et al., 2013) and was recently applied in plants as well. CRISPR can create genome-edited transgenic plants if a stable transformation approach (such as floral dip) is used (Zhang et al., 2015). In summary, the floral dip or infiltration transformation method has now become the most important and frequently used *Agrobacterium*-mediated transformation method in *Arabidopsis* research.

Transient transformation

In general, months are required to generate a transgenic line, and even longer if a homozygous genotype is desirable. *Agrobacterium*-mediated transient transformation is a rapid alternative to assay gene function, promoter behaviour and protein function when generation of a stable transgenic line is unnecessary. A binary vector carrying a reporter system (i.e. promoter-of-interest fused with a GUS/luciferase/fluorescence protein gene) or a fusion protein driven by a constitutive promoter (e.g. for subcellular localization, protein activity or protein-protein interaction study) can be naturally processed to generate single stranded T-DNA and delivered into the nucleus of the host. In the host nucleus, the single stranded T-DNA can be synthesized into double-stranded T-DNA, which is readily transcribed and translated into the reporter protein/fusion protein for analysis without genome integration. Transient transformation can be also used for gene silencing by expressing small interfering RNAs (siRNAs) and microRNAs (miRNAs) in plant tissues (McHale et al., 2013). As integration is not the main purpose in the transient system, a plant selectable marker is usually not required in the construct to be delivered. The drawback however, is the highly variable transformation efficiency for individual *Agrobacterium* strains, target plant species (including different *A. thaliana* ecotypes) and specific tissues. Although *A. thaliana* is not the easiest species to transiently transform, due to its importance in plant biology, extensive optimizations have been carried out. Below, we have summarized the reported methods (an overview can be seen in Table 5) that facilitate the transient transformation in *A. thaliana.*

Transient transformation in roots

Root explants of various *A. thaliana* ecotypes respond differently to *Agrobacterium*-mediated transient transformation. Some eco-

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Table 4. Selected *Arabidopsis* stock and database resources.

	Database/Center	Websites	Description	References
1	Arabidopsis Biological Resource Center (ABRC)/ The Arabidopsis Information Resource (TAIR)	https://www.arabidopsis.org/portals/mutants/stockcenters.jsp https://abrc.osu.edu/	Various seed and clone stocks can be searched and ordered from the ABRC stock center. Useful tools and resources for *Arabidopsis* researchers are provided through the website.	Huala et al., 2001; Garcia-Hernandez et al., 2002; Rhee et al., 2003; Swarbreck et al., 2008; Lameschet al., 2011
2	The European Arabidopsis Stock Centre/ The Nottingham Arabidopsis Stock Center (NASC)	http://arabidopsis.info/	Various seed and clone stocks can be searched and ordered from the NASC.	Scholl et al., 2000
3	AGRIKOLA	http://www.agrikola.org/index.php?o=/agrikola/main	Various RNAi knock-down lines for *Arabidopsis* can be searched by the website. Various seed and clone stocks can be ordered through the NASC	Hilson et al., 2004
4	The French National Institute for Agricultural research (INRA) Arabidopsis Resource Center for Genomics/ The Versailles Arabidopsis Stock Center	http://publiclines.versailles.inra.fr/	Various seed and clone stocks can be searched and ordered from the stock center.	
6	The Lehle Seeds Company	http://www.arabidopsis.com/	The private company sells *Arabidopsis* seeds and consumables.	
7	The RIKEN Biological Resource Center Experimental Plant Division (Japan)	http://epd.brc.riken.jp/en	Various seed and clone stocks can be searched and ordered from the stock center.	
8	Bielefeld University	http://www.gabi-kat.de/	Various seeds of *Arabidopsis* T-DNA insertion lines (GABI-Kat lines) and clone stocks can be searched and ordered through the websites.	Li et al., 2007; Kleinboelting et al., 2012
9	SIGnAL (Salk Institute Genomic Analysis Laboratory): T-DNA Express	http://signal.salk.edu/cgi-bin/tdnaexpress	Various T-DNA insertion lines of *Arabidopsis* can be searched through the website.	Alonso et al., 2003
10	GARNet	http://www.garnetcommunity.org.uk/resources/mutant-collections	Useful tools and resources for *Arabidopsis* researchers are provided through the website.	Beale et al., 2002

types such as Bl-1, Petergof, Cal-0, and Dijon-G are poorly transformed (Nam et al., 1997). Attachment assays revealed that the wild type *A. tumefaciens* virulent strain C58 binds poorly to roots of the Bl-1 and Petergof ecotypes, in contrast to highly efficient binding to roots of susceptible ecotypes Aa-0 and WS. This indicates that the binding ability of agrobacteria to the root tissue could be directly related to transformation efficiency. The *Arabidopsis rat4* mutant, with a deletion of the cellulose synthase-like gene *CSLA9* (At5g03760), showed a reduction in transient transformation efficiency (Zhu et al., 2003a). It has been shown that cellulose is not essential but important for efficient binding of *A. tumefaciens* cells to host cells for subsequent infection (Zhu et al., 2003a; Matthysse et al., 2005). The *CSLA9* promoter is specifically active in the elongation zone of roots, the root tissue that is most susceptible to *Agrobacterium*-mediated transient transformation (Zhu et al., 2003a). RTNLB1 (reticulon-like protein B-1, At4g23630), which was found to interact with the *A. tumefaciens* T-pilus protein VirB2, is important for stable and transient transformation efficiency of root explants (Hwang and Gelvin, 2004).

Agrobacterium rhizogenes is a relative of *A. tumefaciens* which cause adventitious root development at the site of infection (also known as “hairy root disease”) and can be manipulated to co-transfer the T-DNA borne on a binary vector into root cells (Limpens et al., 2004). *A. rhizogenes*-mediated transformation appears to be a fast and effective tool to study the function of genes involved in root biology. It has been employed in RNA interference (RNAi) studies in roots (Limpens et al., 2004) and the disarmed strain can also be used for transient expression at the

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Table 5. Summary of *Agrobacterium*-mediated transient transformation assays in plants

Method Type	Tissue/Reporter (genes)/Applications	Key findings	References
Agroinfiltration of adult leaves	Leaves of adult plants in vegetative stage/Expression of *GUS* reporter gene	No pre-induction or co-cultivation with acetosyringone is required, C58C1(pTiB6S3ΔT, pCH32) was the strain with the best efficiency	Wroblewski *et al*., 2005
		Expression of *nahG* increases the efficiency of *Agrobacterium*-mediated transient transformation	Rosas-Díaz *et al*., 2017
	Leaves of 4-week-old adult plants/*GUS*/ Quantitative analysis of *Agrobacterium*-mediated transient transformation efficiency	Use of *efr* mutant for enhanced transient expression	Zipfel *et al*., 2006
	Leaves of 6-week-old adult plants/*GUS*	Application of acetosyringone and 0.01% Triton X-100 with LBA4044 showed better transformation efficiency than C58C1(pTiB6S3ΔT, pCH32)	Kim *et al*., 2009
	Leaves of 4- to 5-week-old adult plants/ *GUS, CFP*/ subcellular localization, protein–protein interaction	AvrPto suppresses immune responses triggered by multiple receptors, more susceptible than *efr* mutant	Tsuda *et al*., 2012
	Leaves of 4-week-old adult plants/*GFP*	Pre-induction of *vir* genes by acetosyringone in AB-MES followed by infiltration solution containing acetosyringone	Mangano *et al*., 2014
Seedling transformation	Seedlings (4-day-old)/*GFP*/subcellular localization	(FAST)Vacuum infiltration of *Agrobacterium* directly into young Arabidopsis seedlings	Marion *et al* 2008
	Seedlings within 1-week-old/ fluorescent proteins /subcellular localization, protein-protein interaction	Use of surfactant Silwet L-77	Li *et al*., 2009
	Seedlings (4-day-old)/*GUS*/gene function analysis	(AGROBEST) Pre-induction of *vir* genes by acetosyringone, optimization with AB salts in plant culture medium buffered to pH 5.5 during *Agrobacterium* infection	Wu *et al*., 2014b
	Seedlings/*GFP, SYP121*/membrane transport analysis	Use of *Ubiquitin-10* promoter based bicistronic vector to co-express gene of interest and GFP	Chen *et al*., 2011.
Root transformation	Root segments/*GUS*/distinguish transient expression from T-DNA integration	Co-cultivation with *Agrobacterium* for 2 days followed by incubation in callus induction medium for 4 days/ Quantitative analysis of *Agrobacterium*-mediated transient transformation efficiency	Nam *et al*. 1997 Zhu *et al*., 2003a Hwang and Gelvin, 2004
	Seedlings/tagged fluorescence proteins/ study of root–rhizosphere interactions	Use of *Agrobacterium rhizogenes* strain MSV440 to effectively transform roots	Campanoni *et al*., 2007
RNA silencing via agroinfiltration	Seedlings at two- to three- leaf stage/ *AtPDS*/knocking down genes involved in metabolism and defence	Use of tobacco rattle virus(TRV)-based VIGS vector for gene silencing	Burch-Smith *et al*., 2006
	Seedlings/number of transformed seeds/ increased stable transformation rate	Transient down-regulation of the *AGO2* and *NRPD1a* increased stable transformation efficiency	Bilichak *et al.,*2014

root epidermis without affecting the root morphology (Campanoni et al., 2007).

Transient transformation in adult leaves via agrofiltration

Due to the anatomical specificity, root tissues may not be suitable for experiments related to defense, chloroplasts, or for protein purification. Vegetative tissues like leaves are more abundant and sacrificing the plant is not necessary. For species *Nicotiana benthamiana* and lettuce, which can reach ~80% mesophyll cells, it is possible to achieve nearly 100% transient transformation efficiency in some regions of the leaf (Wroblewski et al., 2005). In contrast, successful transient transformation in *A. thaliana* leaves by the same method can be challenging and depends on ecotype and *A. tumefaciens* strain. As mentioned above, most laboratory *A. tumefaciens* strains are derivatives of two wild type virulent strains, C58 and Ach5. For instance, GV3101(pMP90) from C58 and LBA4404 from Ach5 are routinely used for transient transformation of various plant species (Kim et al., 2009). Different *A. tumefaciens* strains produce variable opines: nopaline from pTiC58, octopine from pTiAch5, agropine from A281 and succinamopine from EHA105 (Dessaux et al., 1992; Frandsen, 2011). Although different opine-producing strains seem to work better in certain plant species, it remains unclear whether the specificity of opine utilization contributes to different transformation efficiencies in specific plant species. Strain C58 cured of pTiC58, also known as C58C1, harbouring the octopine-type pTiB6S3DT-DNA and a pCH32 helper plasmid is regarded as the best; in a study of more than 10 *A. tumefaciens* wild type and disarmed strains, it achieved the highest transient transformation efficiency of *A. thaliana*, lettuce and tomato leaves (Wroblewski et al., 2005).

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Phenolics have been reported to influence transient transformation efficiency. As is true for stable transformation, it is now routine to add AS during transformation. Pre-treating *A. tumefaciens* with AS before agroinfiltration of in *A. thaliana* leaves can also increase the transient transformation efficiency (Mangano et al., 2014). Detergents/surfactants are also used to improve cuticular penetration of fluid on leaf surfaces. Silwet L-77 is one of the widely used surfactants in transient transformation while Triton X-100 was also shown to significantly enhance the efficiency (Kim et al., 2009).

Plant defense responses also play a key role in limiting transient transformation. *Arabidopsis* senses *A. tumefaciens* elongation factor EF-Tu through a transmembrane Leucine-rich-repeat (LRR) receptor kinase, EFR (At5g20480), to trigger downstream immune responses. *A. thaliana efr* mutants fail to elicit defence responses effectively against *A. tumefaciens,* and as a result the mutants are more susceptible to transient transformation in agroinfiltrated *Arabidopsis* leaves, as assessed using GUS as a reporter (Zipfel et al., 2006). Effector AvrPto from the plant bacterial pathogen *Pseudomonas syringae* weakens the immunity of *Arabidopsis* by targeting multiple pattern recognition receptors (PRRs) (Hauck et al., 2003; Xiang et al., 2008). The effector was engineered into *A. thaliana*, such that expression was driven by a dexamethasone (DEX)-inducible promoter. In the transgenic line, AvrPto was expressed only when DEX was applied. Pre-treating the plant with DEX before *A. tumefaciens* infiltration suppressed the plant immunity and hence the transient expression efficiency was significantly enhanced (Tsuda et al., 2012). Salicylic acid (SA) plays an important role in defense against bacteria, a recent report shows that use of *nahG* (a bacterial SA hydroxylase) expressing *Arabidopsis* plants can also enhance the transient transformation efficiency in *Arabidopsis* leaves. (Rosas-Díaz et al., 2017)

Leaf transient transformation has been employed for virus-induced gene silencing (VIGS). VIGS uses viral vectors to generate double-stranded RNA of a gene of interest to be silenced. While performing VIGS in *Solanaceous* plant species, including *N. benthamiana*, tomato, pepper, potato and petunia is straightforward, it is relatively difficult in *A. thaliana* due to the inconsistent transformation efficiency. Optimization of Tobacco rattle virus (TRV)-based VIGS in *A. thaliana* involved minimal modification of the *N. benthamiana* protocol, but transformation has to be done in seedlings at the two- to three-leaf stage (Burch-Smith et al., 2006). Light intensity and temperature were shown to affect systemic movement of the silencing signal in transient agroinfiltration in *N. benthamiana*. High light intensities (≥ 450 $\mu E/m^2/s$) and temperatures (≥ 30°C) localized the silencing signal in the leaf tissue that was infiltrated. In contrast, lower light intensities with a constant temperature of 25°C supported strong systemic movement of the silencing signal (Patil and Fauquet, 2015). On the other hand, the transient VIGS technique can be applied to improve overall stable transformation efficiency. *A. thaliana AGO2* (At1g31280) and *NRPD1A* (At1g63020) are inhibitors of *Agrobacterium*-mediated transformation. Transient transformation with *AGO2* and *NRPD1a* silencing VIGS vectors by agroinfiltration of leaves prior to transformation with a gene of interest by floral dip was shown to increase the number of transgenic seeds by 6.0- and 3.5-fold, respectively (Bilichak et al., 2014).

Transient transformation in seedlings

Transient transformation of seedlings has the advantage of allowing analysis in the whole-plant cellular context, in contrast to localized agroinfiltration in adult leaves. Most seedling optimization has aimed to provide fast and convenient protocols for a preliminary analysis of uncharacterised genes and constructs. Vacuum infiltration was introduced to facilitate whole *A. thaliana* seedling adsorption of agrobacterial cells (Marion et al., 2008), while the Fast *Agrobacterium*-mediated seedling transformation (FAST) protocol optimized the cell density (OD_{600}=0.5) and concentration of Silwet L-77 (0.005%) without the need for vacuuming (Li et al., 2009). FAST was demonstrated to work for *Catharanthus roseus* seedling transformation. However, it is worth noting that the FAST method strongly induces host defence genes *Zct1* and *Orca3*; this should be taken into account in experimental design (Weaver et al., 2014). Optimization of *A. tumefaciens* culture conditions can also improve seedling transformation efficiency. The AGROBEST (*Agrobacterium*-mediated enhanced seedling transformation) method achieves high transient transformation efficiency in whole seedlings, allowing gain-of-function studies, by using a pH-buffered medium supplemented with AB salts and glucose. It has been shown that transient expression of GUS or LUC of Col-0 or *efr-1* seedlings was significantly enhanced when AGROBEST was used during *A. tumefaciens* inoculation (Wu et al., 2014b). Similar to what was observed for agroinfiltration in adult leaves (Wroblewski et al., 2005), C58C1(pTiB6S3ΔT-DNA, pCH32) achieves higher transient transformation efficiency than the wild type C58 virulent strain or the C58-derived disarmed strain GV3101(pMP90) in *A. thaliana* seedlings (Wu et al., 2014b). This study also showed an inverse association of root growth inhibition and transient expression efficiency, suggesting that C58C1(pTiB6S3ΔT-DNA, pCH32) may circumvent a plant defense barrier to enable high transient expression levels in *A. thaliana* seedlings. However, the genetic factors and mechanisms underlying the ability of this chimeric disarmed *A. tumefaciens* strain to achieve the highest transient transformation efficiency await future investigations. By expressing a gene of interest separately with a GFP marker in one bicistronic vector, fluorescent GFP signals are used to identify and locate the cells that are successfully transformed by the vector; in the same cells, the signals also indicates expression of the gene of interest. It has been demonstrated that the bicistronic vector transiently expressing *SYP121* (At3g11820) was able to rescue the *syp121* mutant inward K^+ channel current phenotype (Chen et al., 2011).

CONCLUSIONS AND FUTURE DIRECTIONS

From the discovery of *Agrobacterium tumefaciens* to the marketing of genetically modified (GM) crops, tremendous knowledge in plant gene function, cell biology, plant-microbe interaction and biotechnology has been unveiled. In particular, the optimized protocols for *A. thaliana* transformation have provided an irreplaceable tool for studying plant genetics by generation of random T-DNA insertion mutants. Seed stocks with various genomic disruptions by T-DNA insertion are available to order and have been used

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by plant scientists around the world. This enormous resource has greatly transformed plant research by streamlining studies of genes of interest. The convenience of *Agrobacterium*-mediated transformation in *A. thaliana* is also an ideal pilot scheme to test the effects of gene constructs before applying them in other plant species such as crops. At the same time, due to its role in plant applications, the biology of *A. tumefaciens* has been extensively studied. The most notable area is the study of the mechanism of T4SS, which mediates gene transfer from *A. tumefaciens* to plants. By comprehensively reviewing the functions of key genes involved in *Agrobacterium*-plant interactions and the transformation process including the recent breakthrough discovery of Pol θ in T-DNA integration, we hope to draw the attention of plant community to the importance of *A. tumefaciens* research and to the many unresolved questions about T-DNA translocation and integration process that await future studies.

Although *A. tumefaciens* is an excellent tool for dicot transformation, the efficiency in monocots, including in some important crop plants, is lagging far behind. Among the reasons for the poor efficiency may be the unique defense mechanisms in monocots and the absence of essential (efficient) components in monocots for T-DNA transfer and integration. One future direction could be investigating the defence signalling pathways in monocots against *A. tumefaciens* and introducing essential components into monocots. Combining the efficient transformation by *A. tumefaciens* with the CRISPR technique is also a growing trend in genome editing; this enables the removal of unwanted gene fragments such as antibiotic markers and non-essential foreign genes in the genome. The unmarked genome should help reduce public concerns about the potential toxicity of the current GM crops because of whole T-DNA integration. Apart from T4SS, *A. tumefaciens* possesses the T6SS which carries antibacterial activity for interbacterial competition; its secretion activity was shown to be suppressed when virulence proteins including T4SS were massively expressed (Wu et al., 2012). Some bacterial T6SS effectors are able to manipulate host immunity in animal systems, but there is no solid evidence to suggest that *A. tumefaciens* or other plant pathogens can use their T6SS effectors to manipulate host immunity in plants. Future efforts to identifying bacterial T6SS effectors targeting plants could be beneficial to enhance transformation efficiency. Given the increasing demands for plant engineering in medicine, environmental protection and food security, it is expected that *A. tumefaciens* will continue to play an important role in plant sciences, and understanding the biology of agrobacteria as well as its interactions with plants will remain essential, contributing to scientific developments throughout the plant domain.

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