

## EMBO Member's Review

# New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation

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***Agrobacterium tumefaciens* causes tumour formation in plants. Plant signals induce in the bacteria the expression of a range of virulence (Vir) proteins and the formation of a type IV secretion system (T4SS). On attachment to plant cells, a transfer DNA (T-DNA) and Vir proteins are imported into the host cells through the bacterial T4SS. Through interaction with a number of host proteins, the Vir proteins suppress the host innate immune system and support the transfer, nuclear targeting, and integration of T-DNA into host cell chromosomes. Owing to extensive genetic analyses, the bacterial side of the plant–*Agrobacterium* interaction is well understood. However, progress on the plant side has only been achieved recently, revealing a highly complex molecular choreography under the direction of the Vir proteins that impinge on multiple processes including transport, transcription, and chromosome status of their host cells.**

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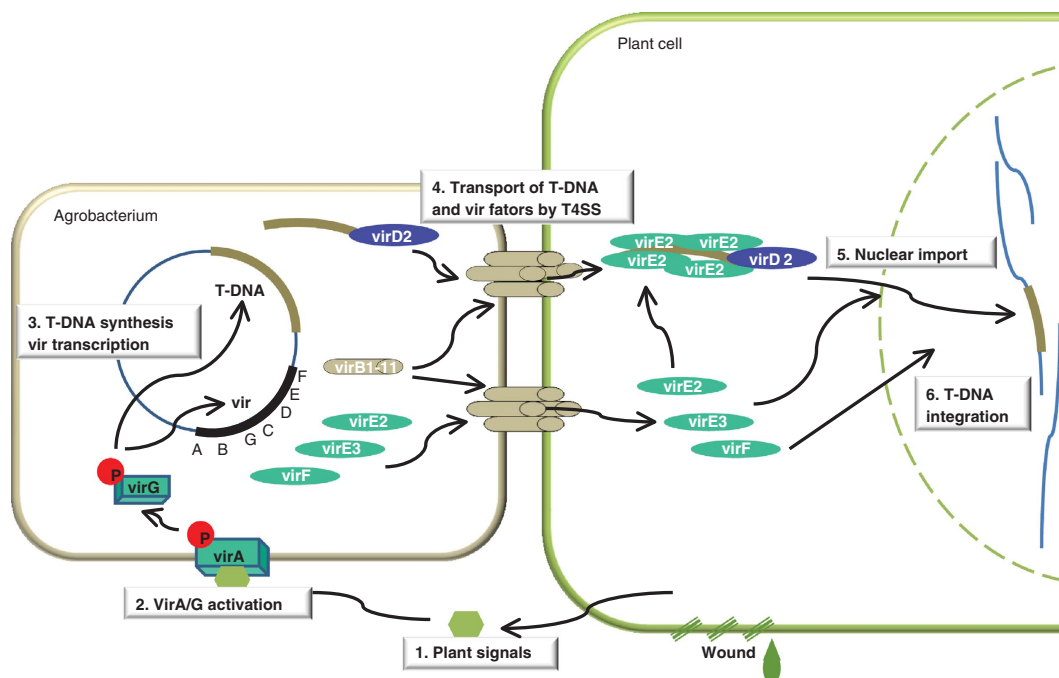
## Introduction

*Agrobacterium* species are known as the only organisms capable of interkingdom gene transfer. This soil-borne Gram-negative bacterium is a broad-host range plant pathogen, which initiates tumour formation on most dicotyledonous and some monocotyledonous species (DeCleene and DeLay, 1976). Such tumours do not require the continuous presence of the bacteria for proliferation (White and Braun, 1942), showing that the plant cells have been transformed

genetically. The factors required for tumour formation are encoded on a large tumour-inducing (Ti) plasmid of virulent *Agrobacterium* strains. The Ti plasmid also serves as a source for the transfer DNA (T-DNA), a DNA region that is imported into plant cells and integrated into the host chromosomal DNA—resulting in genetic manipulation of the host. The expression of T-DNA-encoded bacterial genes in the host cell results in the production of enzymes that catalyse the synthesis of plant hormones, which are responsible for tumour growth and the formation of novel amino-acid-sugar conjugates, termed as opines. As opines can serve as carbon and sometimes nitrogen sources for *Agrobacterium* to the exclusion of most other microorganisms, they provide a selective advantage for this species (Tempé and Petit, 1982). The capacity for gene transfer into plants has been used to develop *Agrobacterium tumefaciens* as a vector for genetic manipulation. Engineered DNA segments of interest, which are first cloned into the T-DNA region of ‘disarmed’ plasmids, are then introduced into *Agrobacterium* and subsequently transferred into plants. From these disarmed plasmids, the genes responsible for tumourous growth have been removed, ensuring that the transformed cells can be regenerated into fertile plants that transmit the engineered DNA to their progeny (Hooykaas and Schilperoort, 1992; Newell, 2000). By these means, the host range of *Agrobacterium* has been extended to include other bacterial species as well as fungi and even some mammalian cells (Lacroix *et al*, 2006). Under laboratory conditions, normally recalcitrant plants (Ishida *et al*, 1996; Hiei *et al*, 1997; Chen *et al*, 2006), fungi (Bundock *et al*, 1995; Abuodeh *et al*, 2000), and even human cells (Kunik *et al*, 2001; Tzfira *et al*, 2006) can be transformed by *Agrobacterium*. *Agrobacterium*-mediated transformation serves as an important model system for studying host–pathogen recognition and delivery of macromolecules into target cells. The interaction between *Agrobacterium* and plant cells can be divided into several steps: recognition, virulence (Vir) gene expression, attachment to the host cell, targeting of Vir factors and T-DNA into the host cell, and chromosomal T-DNA integration (Figure 1). On chemical recognition of plant-derived compounds, *Agrobacterium* Vir gene expression is induced, which is followed by the physical interaction between bacterium and plant cells. A bacterial transfer machinery is subsequently produced and assembled to import the *de novo* produced T-DNA strand along with a number of Vir factors into the host cell. Once inside the plant cell, the T-DNA is translocated into the nucleus, in which it integrates into the host chromosome. On expression of T-DNA genes, plant cells are re-programmed for tumour growth and production of opines.

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**Figure 1** Overview of the *Agrobacterium*–plant interaction. 1. Plant signals induce 2. VirA/G activation and thereby 3. T-DNA synthesis and *vir* gene expression in *Agrobacterium*. 4. Through a bacterial type IV secretion system (T4SS) T-DNA and Vir proteins are transferred into the plant cell to assemble a T-DNA/Vir protein complex. 5. The T-DNA complex is imported into the host cell nucleus in which 6. the T-DNA becomes integrated into the host chromosomes by illegitimate recombination.

## Recognition of plant cells as host by *Agrobacterium*

*Agrobacterium* strains are widely distributed in the soil. Moreover, most isolates do not contain a Ti plasmid and are capable of living independently of a plant host. Yet, as tumour-produced opines are a specific food source for *Agrobacterium*, the capacity of *Agrobacterium* strains to induce such tumours is a clear selective advantage. However, because plant transformation is a complex process and energetically demanding, Vir gene expression must be carefully regulated. The identification of *vir* genes, which are required for virulence, but lie outside the T-DNA (Klee *et al.*, 1983; Stachel and Nester, 1986), was a major step towards understanding the transformation process. With the exception of *virA* and *virG*, the *vir* genes were found to be essentially silent unless the bacteria are cultured with plant cells (Stachel and Nester, 1986). Although *vir* gene induction depends on molecules exuded by the plant, attachment to plant cells is necessary for transformation and is mediated by chromosomally encoded *Agrobacterium* genes (Lippincott and Lippincott, 1969; Douglas *et al.*, 1982). Thus, host recognition by *Agrobacterium* resulting in transformation is composed of two independent processes: Vir gene activation and attachment to the host cell.

### *Agrobacterium* Vir gene expression

The *vir* gene activation by plant factors requires two genes, *virA* and *virG* (Stachel and Nester, 1986), which are constitutively expressed at a basal level, but can become highly induced in a feed-forwards manner (Winans *et al.*, 1988). The *virA* and *virG* genes encode a two-component phospho-relay

system in which VirA is a membrane-bound sensor and VirG is the intracellular response regulator (Wolanin *et al.*, 2002). On signal sensing, the histidine kinase VirA activates VirG through transferring its phosphate to a particular aspartate of VirG, thereby activating VirG to function as a transcription factor. Phosphorylated VirG then binds at specific 12 bp DNA sequences of the *vir* gene promoters (*vir* boxes), thereby activating transcription (Brencic and Winans, 2005).

The signals perceived by VirA are phenols, aldose monosaccharides, low pH, and low phosphate (Palmer *et al.*, 2004; Brenic and Winans, 2005). Phenols are indispensable for *vir* gene induction, whereas the other signals sensitise VirA to phenol perception, for example sugars allow induction of the VirA/VirG system at much lower phenol concentrations and increase the response several-fold (Shimoda *et al.*, 1990). The identification of phenols, such as acetosyringone, as inducers of *vir* gene expression was achieved through analysis of root exudates and leaf protoplasts (Stachel *et al.*, 1985). Acetosyringone is now routinely used for enhancing the efficiency of *Agrobacterium*-mediated plant transformation. The capability of the VirA/VirG system to recognise a diversity of phenols and sugars is a likely explanation for the broad-host range exhibited by *Agrobacterium*.

### Plant entry sites for *Agrobacterium*

In nature, *Agrobacterium* attacks mainly wounded tissue (Braun, 1952). A wound site may simply be a portal of entry, but other specific processes specifically occurring at these sites are likely to facilitate transformation: wound-secreted compounds such as phenols and sugars induce *vir* gene expression. In addition, the latter act as chemotactic attractants of *Agrobacterium*. Thus, wound-specific features

such as high activity of the phenylpropanoid pathway, low pH, and sugars associated with cell wall synthesis/wound repair correlate with enhanced transformation frequency and efficiency (Baron and Zambryski, 1995). Although transformation can also occur in unwounded plants—with *Agrobacterium* cultures grown in pre-induction medium (Escudero and Hohn, 1997)—it seems that *Agrobacterium* has optimised the VirA/VirG system to respond to signals from wound sites. Cell division activity at the wound sites is thought to be equally important for transformation (Braun, 1952). However, cells in the root elongation zone were found to be the most highly transformable (Yi *et al.*, 2002). Cells of this non-meristematic zone are not undergoing a normal cell cycle, but endoreduplication.

### Host cell attachment by *Agrobacterium*

As T-DNA and proteins are transferred from *A. tumefaciens* into plant cells, an intimate association between pathogen and host cell is a prerequisite for transformation. Quantitative-binding assays have revealed a non-specific interaction that is readily removed and a specific interaction (Neff and Binns, 1985). The specific attachment of *A. tumefaciens* to plant cells is not dependent on the Ti plasmid (Douglas *et al.*, 1982; Neff and Binns, 1985). Instead, it is facilitated by the chromosomally encoded bacterial genes *chvA*, *chvB*, and *pscA* (*exoC*), which are involved in the synthesis and/or localisation of periplasmic  $\beta$ -1,2 glucan (reviewed in McCullen and Binns, 2006).

Early studies revealed that the exposure of *A. tumefaciens* cells to soluble pectic plant cell wall fractions decreases both the specific binding of *Agrobacterium* to plant cells and tumour-induction frequencies (reviewed in Gelvin, 2000), suggesting the presence of as yet elusive *Agrobacterium* receptor-like components. Possible candidates are BTI-domain proteins that had been isolated from a screen for VirB2-interacting proteins. Owing to its transient increase immediately after *Agrobacterium* infection and its preferential localisation to the periphery of root cells, a direct contact of BTI1 with the *Agrobacterium* T-pilus in the initial interaction of *Agrobacterium* with plant cells has been proposed (Hwang and Gelvin, 2004).

Genomic studies are beginning to provide new insight into possible plant molecules involved in the attachment process. A number of *Arabidopsis* mutants have been isolated that are recalcitrant to *Agrobacterium* transformation (*rat* mutants) (Zhu *et al.*, 2003) and it was shown that *Agrobacterium* can no longer bind efficiently to some *rat* mutants. One well-characterised mutant is affected in the gene encoding a cell wall arabinogalactan protein to which bacteria bind poorly (Nam *et al.*, 1999; Zhu *et al.*, 2003). Further analysis of these mutants should help to unravel the recognition process and physical interaction of *Agrobacterium* and host cells.

### *Agrobacterium* secretion of T-DNA and Vir proteins into plant cells

After *vir* gene activation and attachment of *Agrobacterium* to plant cells, a transporter complex formed by VirB proteins and VirD4 enables Vir proteins and T-DNA to cross the inner bacterial membrane, the peptidoglycan layer, and outer membrane, as well as the plant host cell wall and membrane.

The VirB complex belongs to the class of type IV secretion systems (T4SS), which are found across a broad range of Gram-negative bacteria and are involved in the conjugative transfer of plasmids between bacteria as well as the translocation of Vir factors from pathogens to host cells during infection (Cascales and Christie, 2003).

The VirB complex is composed of at least 12 proteins: VirB1–11 and VirD4 and is required for virulence. The proteins associate with the cell envelope and form a multi-subunit envelope-spanning structure (Christie *et al.*, 2005). The bacterial factors transported into host cells by the VirB complex include the VirD2-T-DNA, VirE2, VirE3, VirF, and VirD5 (Vergunst *et al.*, 2005). VirD2 nicks the T-DNA at the 25-nucleotide long repeats that border the T-DNA and VirD2, then becomes covalently bound to the 5'-end of the T-DNA. VirD2 seems to be transported with the T-strand into the plant cell, in which it is involved in nuclear import and integration of the T-DNA into the host genome (Gelvin, 2003). VirE2 is a single-stranded DNA-binding protein that can coat the length of the T-strand *in vitro* (Christie *et al.*, 1988; Citovsky *et al.*, 1992). It likely interacts with T-DNA in the plant cell cytoplasm and also has functions in nuclear import and integration (Gelvin, 2003). Intriguingly, the VirB/D4 complex can not only transport Ti-derived T-DNA, but also the broad-host range plasmid RSF1010 to plants or other *Agrobacterium* species, showing that conjugative intermediates must also be substrates (Buchanan-Wolloston *et al.*, 1987; Beijersbergen *et al.*, 1992).

### Import of *Agrobacterium* Vir factors into host cells

The *A. tumefaciens* *virB*-encoded T4SS transports substrates across the bacterial cell envelope. Certain C-terminal motifs were found to be required for the export of targeted substrates. These export signals mediate the interaction of substrates with the T4SS. The C-termini of VirF, VirE2, and VirE3 are sufficient to mediate transport of fusion proteins to plants (Vergunst *et al.*, 2000). The minimal size of VirF required to direct protein translocation to plants is the C-terminal 10 amino acids (Vergunst *et al.*, 2005), from which the minimal consensus sequence R-X(7)-R-X-R-X-R required for substrate secretion by the VirB complex could be derived (Vergunst *et al.*, 2005).

C-terminal fusions of VirE2 blocked its translocation to host cells (Vergunst *et al.*, 2000). Accordingly, insertion of a FLAG tag at the C-terminus of VirE2, or truncation of the C-terminal 18 amino acids of VirE2, renders the protein non-functional in *A. tumefaciens*, while not affecting its capability to bind single-stranded DNA (Simone *et al.*, 2001). However, overexpression of such VirE2 C-terminal mutant derivatives in transgenic plants confers susceptibility to transformation by an *A. tumefaciens* *virE2*-deficient strain, suggesting that the mutations disrupted a region of amino acids required for translocation, such as a secretion signal.

In an elegant experimental approach, using fusion proteins of VirE2 or VirF to the Cre recombinase, the transport of these proteins in the absence of T-DNA has been studied (Vergunst *et al.*, 2000). The experiments were designed in such a way that the transport of Cre-VirE2 or Cre-VirF fusions into host plant cells results in a recombination event conferring kanamycin resistance to host tissues.

Thus, it could be shown that the transport of bacterial factors is dependent on the VirB/VirD4-complex. In addition, VirE3 and the C-terminus of VirD5 were found to mediate substrate targeting into host cells (Schrammeijer *et al.*, 2003; Vergunst *et al.*, 2005). VirE3 may function in the plant to aid nuclear localisation of VirE2 (Lacroix *et al.*, 2005), and VirD5 may function as transcription factor in the plant cells (Schrammeijer *et al.*, 2000).

### Host cell entry of *Agrobacterium* factors

It is presently unclear how the Vir proteins and the T-DNA protein complex traverse the host cell wall and membrane barriers. In T4SS-mediated plasmid transfer, the pilus enables the interaction between donor and recipient, followed by the fusion of outer membranes in a mating junction (Schroder and Lanka, 2005). The mechanism by which the transferred conjugal intermediate traverses the bacterial wall and inner membrane is not known. Even less is known about VirB-mediated transfer across host cell barriers. The enormous host range transformed by *Agrobacterium* suggests that the specificity of host-pathogen interaction required to breach the host cell wall and membrane barriers may be less important than expected.

### Targeting of *Agrobacterium* T-DNA into the host cell nucleus

Once inside the plant cell, the T-DNA must find its way into the nucleus. Several *Agrobacterium* Vir proteins, as well as a number of plant proteins, seem to be involved in this process (Figure 2). The proteins VirD2 and VirE2 contain plant-active nuclear localisation signal (NLS) sequences. VirD2, which is covalently linked to the 5'-end of the T-DNA, contains two NLS regions, both of which can direct chimeric proteins to the nucleus. Sterical considerations suggest that the bipartite NLS in the carboxy-terminus of VirD2 might be biologically important for nuclear targeting of the T-DNA complex (Tinland *et al.*, 1995).

VirE2 protein contains two separate bipartite NLS regions that can target fusion reporter proteins to plant nuclei (Citovsky *et al.*, 1992, 1994). Fluorescently labelled single-stranded DNA coated with VirE2 and microinjected into plant cells localises to the nucleus, whereas naked single-stranded DNA remains in the cytoplasm (Zupan *et al.*, 1996).

In agreement with a function of VirD2 and VirE2 in T-DNA nuclear guidance, deletion of the VirD2 bipartite NLS resulted in almost complete loss of transformation (Rossi *et al.*, 1993), indicating that VirE2 NLS domains cannot compensate for the loss of the VirD2 NLS. Furthermore, VirD2 and VirE2 proteins were shown to be necessary for nuclear targeting of *in vitro* synthesised T-complexes in permeabilised HeLa cells (Ziemienowicz *et al.*, 1999).

As shown by Shurvinton *et al.* (1992), the C-terminal NLS of VirD2 is essential for virulence, but not for intrabacterial T-strand production. This NLS does not contribute to targeting of the T-DNA to the nucleus (Shurvinton *et al.*, 1992; Rossi *et al.*, 1993; Mysore *et al.*, 1998), and it was suggested that deletion of the VirD2 NLS may alter the structure of the VirD2 protein such that it can still nick the T-DNA border, but may fail to pass through the T4SS or the nuclear pore (Mysore *et al.*, 1998). VirE2 might provide nuclear targeting in the

absence of VirD2 NLS. In support of this notion, Gelvin (1998) observed that an *A. tumefaciens virE2 virD2ΔNLS double mutant was able to form tumours on VirE2-producing transgenic tobacco, but not on wild-type tobacco, and suggested that the NLS of VirE2 could have a function in directing T-DNA to the nucleus.*

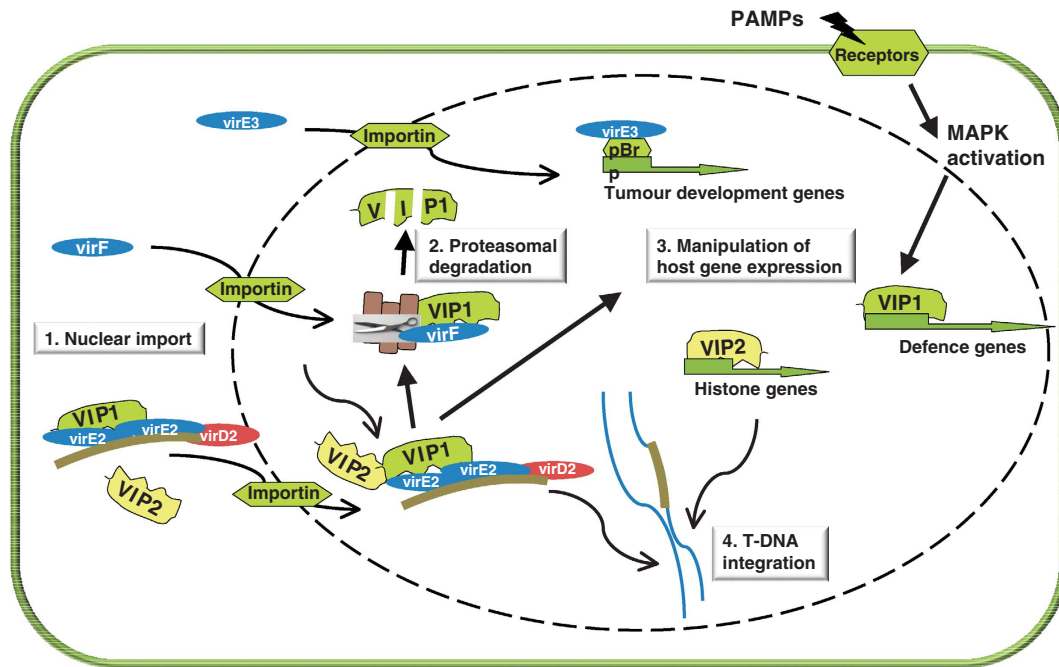
Several studies suggest additional functions of VirE2 as transmembrane DNA transporter. VirE2 can insert itself into artificial membranes and form channels. These channels can facilitate the efficient transport of ssDNA through membranes (Dumas *et al.*, 2001; Duckely *et al.*, 2005). Indeed, as shown by biophysical experiments and particle-bombarded tobacco cells transiently expressing VirE2 fusion protein, VirE2 seems to actively pull ssDNA into the host (Grange *et al.*, 2008).

Despite the prominent function of VirE2 in the transformation process, some strains of *Agrobacterium rhizogenes* lacking this protein can still transfer T-DNA efficiently. This is achieved through GALLS proteins (Hodges *et al.*, 2004, 2006, 2009). Interestingly, despite their dissimilarity to VirE2, GALLS protein restored pathogenicity to *virE2* mutant *A. tumefaciens* (Hodges *et al.*, 2004). The GALLS gene encodes for two proteins: full-length GALLS and a C-terminal domain that initiates at an internal in-frame start codon. Full-length GALLS protein contains domains for ATP binding, nuclear localisation, and type IV secretion (Hodges *et al.*, 2006). In plant cells, interaction of GALLS-FL with VirD2 was observed (Hodges *et al.*, 2009). On the basis of these findings, as well as the nuclear localisation of GALLS-FL and its predicted helicase activity, the authors proposed that GALLS-FL may pull T-strands into the nucleus.

VirF has been implicated in the degradation of host cell factors during infection. In the host nucleus, VirF, in concert with the host proteasome machinery, is believed to mediate degradation of the T-DNA complex, thus facilitating the release of the T-DNA and its subsequent chromosomal integration (Schrammeijer *et al.*, 2001; Tzfira *et al.*, 2004a, b).

### Functions of Vir proteins in T-DNA integration

Relatively little is known about the precise mechanism of T-DNA integration into the plant genome or the function specific proteins have in this process. The major mode foreign DNA integrates in plants is by illegitimate recombination or non-homologous end-joining; and T-DNA integrates into plant chromosomes by a similar mechanism (Paszowski *et al.*, 1988; Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991). An alternative model proposes that the initial invasion of plant DNA by the T-DNA is also of importance for integration (Meza *et al.*, 2002). In addition, analysis of T-DNA-integration sites suggests the involvement of microhomologies in the integration process (Pelczar *et al.*, 2004). Measurements of the relative amounts of transient versus stable expression of reporter genes in *Agrobacterium*-infected plant cells suggests that most T-DNA is not stably integrated into chromosomal DNA (Nam *et al.*, 1997). Although T-DNA enters the nucleus as a single-stranded molecule, much of the T-DNA likely becomes double stranded, because the conversion to a transcriptionally competent form requires the synthesis of a complementary DNA to the T-strand (Narasimhulu *et al.*, 1996). It is not yet clear whether the T-DNA integrates



**Figure 2** Molecular details of *Agrobacterium* and host factors involved in T-DNA transfer and host responses. 1. Nuclear import of the T-DNA complex along with other Vir proteins. 2. VIP1 becomes activated by PAMP-triggered MAPK activation, but undergoes proteasomal degradation through VirF. 3. Manipulation of host gene expression by Vir proteins helps 4. T-DNA integration and tumour development as well as suppression of defence responses.

through single-strand invasion of locally denatured plant DNA (Tinland, 1996), or whether the extrachromosomal double-stranded T-DNA is the substrate for integration. As VirD2 is covalently linked to the T-DNA strand, it likely has some function in the integration process: VirD2 can mediate site-specific cleavage and reversal of it, site-specifically only (Pansegrau *et al.*, 1993). Ligation of T-DNA to plant DNA is rather mediated by host proteins. VirD2 failed to facilitate *in vitro* ligation-integration reactions in which T-DNA was ligated to a model target sequence, whereas this reaction did take place in the presence of plant extracts (Ziemienowicz *et al.*, 1999). The integration of the 5' end of the T-strand into plant DNA is generally precise and only a few 5' nucleotides are usually deleted on T-DNA integration into the plant genome (Tinland *et al.*, 1995). This may result from the protection from exonucleases that VirD2 offers to the capped 5' T-strand end, as mutations in VirD2 result in imprecise ligation and deletion of the 5' end of the T-DNA to plant chromosomal DNA (Tinland *et al.*, 1995). Moreover, the  $\omega$  domain of VirD2, a conserved region outside the NLS, is important for tumorigenesis (Shurvinton *et al.*, 1992; Mysore *et al.*, 1998), but has only minor effects on transient T-DNA expression (Narasimhulu *et al.*, 1996).

The *virE2* mutants are extremely attenuated in virulence (Stachel and Nester, 1986). As VirE2 can function as a gated pore for the passage of ssDNA (Dumas *et al.*, 2001; Duckely *et al.*, 2005; Grange *et al.*, 2008), the severe attenuation of *virE2* mutant *Agrobacterium* strains might be explained by a defect in nuclear host transport. However, the integrated T-DNA molecules transferred from *virE2* mutant *Agrobacterium* strains also exhibit extensive deletions corresponding to the 3' ends of the T-strand (Rossi *et al.*, 1996), suggesting that nucleolytic protection of the T-strand is also a major function of VirE2.

## Plant factors and defence responses involved in *Agrobacterium* tumour formation

Although certainly unintentionally, the host plant actively participates in *Agrobacterium* transformation. This 'assistance' occurs at several levels: Vir protein/T-DNA import, dissociation of the Vir/T-DNA complex, T-DNA integration, and re-programming of gene expression for tumour development. A number of host factors that are exploited by *Agrobacterium* to achieve transformation have been identified. Major progress has been made through yeast-two-hybrid (Y2H) screens for identifying host proteins that interact with *Agrobacterium* Vir proteins (Ballas and Citovsky, 1997; Deng *et al.*, 1998; Hwang and Gelvin, 2004). Another important progress was achieved through large plant mutant screens (Zhu *et al.*, 2003; Crane and Gelvin, 2007).

## Functions of plant proteins in T-DNA nuclear import

VirD2 was reported to interact with several members of the Arabidopsis importin  $\alpha$  and cyclophilin families *in vitro* and in the Y2H system (Koncz *et al.*, 1989; Deng *et al.*, 1998; Bako *et al.*, 2003; Bhattacharjee *et al.*, 2008). Importins  $\alpha$  are NLS-binding proteins of the nuclear import machinery that specifically interact with the bipartite NLS region of VirD2 and may thus facilitate its nuclear import (Ballas and Citovsky, 1997). Deng *et al.* (1998) identified an Arabidopsis cyclophilin that interacts in Y2H experiments with a central domain of VirD2. As some cyclophilins have peptidyl-prolyl isomerase activity, the authors speculated that this protein might serve as a chaperonin to hold VirD2 in a transfer-competent conformation during T-strand trafficking through the plant cell.

However, this hypothesis is put into question by a recent study in which the cyclophilin-binding domain of VirD2 was found to be dispensable for virulence (van Kregten *et al.*, 2009).

Furthermore, VirD2 is subject to post-translational modification. Nuclear targeting of VirD2 is apparently controlled through phosphorylation of a serine residue close to the bipartite NLS (Tao *et al.*, 2004). Alanine substitution of this residue resulted in the predominantly cytoplasmic localisation of a  $\beta$ -glucuronidase (GUS)-VirD2 NLS fusion protein. Moreover, Tao *et al.* (2004) identified DIG3, a type 2C serine/threonine protein phosphatase, which negatively affects nuclear import of a GUS-VirD2 NLS fusion protein.

A biochemical approach has led to the identification of a VirD2-interacting kinase, CAK2M (Bako *et al.*, 2003). VirD2 interacts with and is phosphorylated by CAK2M *in vivo*; CAK2M might correspond to the kinase that regulates nuclear import of VirD2 (Gelvin, 2000). Another substrate of CAK2M is RNA polymerase II large subunit (RNAPolII CTD), a factor that is responsible for recruiting TATA-box-binding proteins (TBP) to actively transcribed regions. Comparative sequence analysis of insert junctions and target sites suggested a preferential integration of the T-DNA into promoters of transcribed chromatin domains (Koncz *et al.*, 1989; Mayerhofer *et al.*, 1991; Brunaud *et al.*, 2002; Szabados *et al.*, 2002). Moreover, experiments in *Agrobacterium*-transformed *Arabidopsis* cells revealed an association of VirD2 with TBP, one of the most conserved nuclear proteins in eukaryotic cells (Nikolov *et al.*, 1992) in transformed *Arabidopsis* cells (Bako *et al.*, 2003). A hypothetical scenario suggested by the authors is that TBP or CAK2M may target VirD2 to the CTD, thereby controlling T-DNA integration. However, an alternative explanation for TBP-VirD2 interaction might have to be found. A genome-wide analysis of T-DNA-integration sites in *Arabidopsis* performed under non-selective conditions does not support the earlier concept of preferential T-DNA integration into transcriptionally active regions. Instead, Kim *et al.* (2007) found that T-DNA integration occurs rather randomly and that the earlier reported enrichment of such integration sites in gene-rich or transcriptionally active regions of chromatin is due to the selection pressure applied for recovery of T-DNA insertions. However, the results leading to non-selected T-DNA-integration events could not be verified, as sequence analysis was presented only and transgenic plants could not be recovered because of the special experimental design. O'Malley *et al.* (2007) developed a novel PCR-based method for high throughput sequencing the T-DNA/genomic DNA junction of 150 000 T-DNA insertional mutants. The analysis of this library should provide detailed information on the sequence requirements for T-DNA integration at a large scale.

Two VirE2-interacting proteins, VIP1 and VIP2, have been isolated and characterised. Most data on VIP1 originate from experiments in tobacco cells (Tzfira *et al.*, 2001, 2002). Elevated levels of VIP1 enhance *Agrobacterium* susceptibility and transformation efficiency, as shown by larger calli formed in infected VIP1 overexpressing plants (Tzfira *et al.*, 2002). Further interaction experiments led to the suggestion that VIP1 functions as a bridge between VirE2 and the plant importin  $\alpha$ -1 (IMPa-1), thereby facilitating nuclear import of VirE2 and its associated T-DNA (Tzfira *et al.*, 2002).

## Nuclear transport of the T-DNA complex

VIP1 functions in the shuttling of the T-DNA complex into the host cell nucleus, but this function can be partially complemented for by *Agrobacterium* VirE3, which, similar to VIP1, was found to be capable of binding to VirE2 and IMPa-1 (Lacroix *et al.*, 2005). *In vitro*, VIP1 forms ternary complexes with VirE2 and ssDNA (Tzfira *et al.*, 2001). Analysis of an *Arabidopsis vip1-1* mutant, which produces a truncated VIP1 protein, revealed that the C-terminal region of the protein is required for stable transformation, but dispensable for transient transformation (Li *et al.*, 2005a). VIP1 is a mobile protein, which undergoes cytoplasmic-nuclear trafficking in a stress-dependent manner (Djamei *et al.*, 2007). The other VirE2-interacting protein, VIP2, shows nuclear localisation (Tian *et al.*, 2004). The most characteristic feature of VIP2 is an NOT domain (negative on TATA-less), rendering VIP2 a putative transcriptional repressor protein (Anand *et al.*, 2007). Virus-induced gene silencing of VIP2 in *Nicotiana benthamiana* and characterisation of the *Arabidopsis vip2* mutant revealed that VIP2 is required for stable, but not for transient, transformation. As shown by Y2H and bimolecular fluorescence complementation (BiFC) studies, VIP2 does not only interact with VirE2, but also with VIP1 (Anand *et al.*, 2007). Microarray analysis revealed a major impairment of the transcriptional response of *vip2* to *Agrobacterium* in comparison with wild-type plants. Moreover, as many as 52 histone/histone-associated genes are constitutively repressed in *vip2* mutants (Anand *et al.*, 2007). Together, these observations prompted the authors to suggest that the recalcitrancy of *vip2* mutants to *Agrobacterium* infection and the decreased transformation efficiency are due to impaired *Agrobacterium*-responsive gene induction and constitutive histone gene repression.

Data on some aspects of VirE2 are still somewhat controversial. Although Ballas and Citovsky (1997) reported specific interaction of VirE2 with *Arabidopsis* importin IMPa-1 and nuclear localisation of VirE2, other studies showed a predominant cytoplasmic VirE2 localisation (Bhattacharjee *et al.*, 2008; Grange *et al.*, 2008) and interaction of VirE2 with several importin isoforms *in planta* (Bhattacharjee *et al.*, 2008). Moreover, *impa-4*, but not other importin mutants, is recalcitrant to transformation. This deficiency can be overcome through ectopic overexpression of heterologous importin isoforms (Bhattacharjee *et al.*, 2008).

A possible explanation for the differences in reported VirE2 localisation might be found in the stress-dependent subcellular translocation of the VirE2-interacting protein VIP1. Stress-triggered phosphorylation of VIP1 mediates VIP1 nuclear localisation and virulence presumably by directing VirE2 to the nucleus (Djamei *et al.*, 2007). Thus, under stress conditions such as those occurring in cell bombardment, phosphorylated VIP1 may pull the otherwise cytoplasmic VirE2 into the nucleus.

VIP1 interacts with VirE2 and IMPa-1. Tzfira *et al.* (2002) found interaction between VIP1 and IMPa-1, but not between VirE2 with IMPa-1. They, therefore, suggested that VIP1 may serve as an adaptor molecule to facilitate the import of VirE2-bound T-strands into the nucleus. Bhattacharjee *et al.* (2008), however, found that VirE2 can directly bind all tested importin to isoforms (IMPa-1, -2, -3, -4, -7, and -9) in Y2H and

BiFC interaction studies. Combining own results and those of Tzfira *et al* (2002), Bhattacharjee *et al* (2008) proposed that VirE2 may use several cellular mechanisms for nuclear import, thereby creating additional opportunities for T-complex entry into the nucleus. This explanation seems plausible, particularly when considering that MPK3, the VIP1-nuclear-targeting kinase, is only transiently (5–15 min) activated on *Agrobacterium* contact (Djamei *et al*, 2007). VIP1-independent nuclear translocation of VirE2 may, therefore, be particularly relevant for securing T-DNA-complex entry over a prolonged period. Moreover, as VIP1, irrespective of its phosphorylation status, can bind IMPa-1 (Djamei and Pitzschke, unpublished) and VirE2 (Lacroix *et al*, 2008), it may still assist VirE2 nuclear translocation indirectly by guiding VirE2 to the nuclear periphery. Direct VirE2–IMPa-4 interaction would then accomplish nuclear VirE2 import. This assumption is in accordance with the results from Lee *et al* (2008). Particle bombardment in onion cells revealed VirE2–IMPa-1 protein complexes around the nucleus, but VirE2–IMPa-4 complexes exclusively within the nucleus.

## Functions of plant proteins in T-DNA integration

As T-DNA must interact with chromatin to integrate into plant chromosomal DNA, it is likely that altering chromatin conformation will affect T-DNA integration. Forward and reverse genetic approaches have been carried out to determine which chromatin proteins are important for transformation (Zhu *et al*, 2003; Crane and Gelvin, 2007). In this way, mutants were identified in or near various histone genes, histone acetyltransferase genes, histone deacetylase genes as *rat* mutants. Moreover, 340 stable *Arabidopsis* RNAi mutant lines were screened for *rat* phenotypes. These lines comprised 109 chromatin genes of 15 gene families, including bromodomain and chromodomain proteins, chromatin remodelling complexes, DNA methyltransferases, global transcription factors, histone acetyltransferases, histone deacetylases, histone H1, methyl-binding-domain proteins, MAR-binding filament-like proteins, nucleosome assembly factors, and SET-domain proteins. Silencing of 24 chromatin genes reproducibly resulted in some level of decreased transformation susceptibility. As T-DNA integrates into the plant genome by illegitimate recombination (Mayerhofer *et al*, 1991), plants deficient in DNA repair and recombination may be deficient in T-DNA integration. Such DNA metabolism mutants are likely to be hypersensitive to DNA-damaging agents such as UV and radiation and DNA-damaging drugs such as bleomycin. Sonti *et al* (1995) investigated a number of radiation-sensitive *Arabidopsis* mutants for transient and stable *Agrobacterium*-mediated transformation. Among these, *uvh1* and *rad5* mutants seemed to be resistant to stable, but not to transient transformation, as assessed by formation of kanamycin-resistant calli. However, an in-depth analysis by Nam *et al* (1998) confirmed stable transformation deficiency only for *rad5*, but not *uvh1* mutants. Although the latter did form less calli on kanamycin selection medium, tumour growth on non-selective medium as well as stable phosphotricin resistance were similar to wild type. Furthermore, results from these authors also suggest RAD5 to be involved in some step before T-DNA integration, such as T-DNA transfer or nuclear targeting (Nam *et al*, 1998).

In addition to *rad5*, some radiation-sensitive *Arabidopsis* ecotypes are also recalcitrant to *Agrobacterium*-mediated transformation. By examining almost 40 *Arabidopsis* ecotypes for susceptibility to root transformation by *Agrobacterium*, ecotype UE-1 was found to be both slightly radiation-hypersensitive and transformation-deficient (Nam *et al*, 1997). Further testing of the *rat* mutants revealed that 5 of the initial 21 *rat* mutants were integration deficient, as indicated by high transient, but low stable transformation efficiency. One of these mutants, *rat5*, contains an insertion in the 3' untranslated region of a *histone H2A* gene (Mysore *et al*, 2000). Although highly recalcitrant to stable transformation by root inoculation, *rat5* is efficiently transformed by flower vacuum infiltration. These results suggest that some factor(s) required for efficient transformation are present in the female gametophyte, but absent in root somatic tissue. As *rat5* plants can be complemented to transformation proficiency with the wild-type *RAT5 histone H2A* gene, the *rat5* mutant is haplo-insufficient (dosage-dependent). The function of histones in T-DNA transformation is further emphasised in a recent study by Tenea *et al* (2009), who tested the effect of overexpression of several histones on *Arabidopsis* transformation and transgene expression. After transfection, transgene DNA was found to accumulate more rapidly in histone *HTA1*-overexpressing plants. The authors proposed that enhanced *Agrobacterium*-mediated transformation through histone overexpression is due to the protection of incoming transgene DNA during the initial stages of transformation. The main mechanism by which histones confer susceptibility seems to be conserved, as overexpression of *Arabidopsis HTA1* can not only enhance transformation efficiency in *Arabidopsis* (Yi *et al*, 2002, 2006), but also in rice (Zheng *et al*, 2009).

Mutations in *fas1* and *fas2*, encoding two subunits of the chromatin assembly factor CAF1, show greatly increased frequencies of homologous recombination and T-DNA integration (Endo *et al*, 2006). Studies on the *Arabidopsis* protein KU80 further stress the active participation of the host's repair machinery in T-DNA integration. KU80, an important protein in the non-homologous end-joining complex (Jeggio *et al*, 1999), directly binds to double-stranded T-DNA intermediates (Li *et al*, 2005b), which are rapidly converted from T-strands early in the infection process and are essential intermediates of T-DNA integration (reviewed in Tzfira *et al*, 2004a, b). Ku80 mutants are defective in T-DNA integration, but not in transient T-DNA expression, whereas KU80 overexpression results in increased susceptibility to *Agrobacterium* infection and increased resistance to DNA-damaging agents (Li *et al*, 2005b). The function of KU80 during the transformation of germ-line cells, however, is still debatable. Ku80 has been reported to be both required (Friesner and Britt, 2003) and dispensable (Gallego *et al*, 2003) for T-DNA integration.

## *Agrobacterium* and the plant innate immune response

### Plant factors involved in *Agrobacterium* perception

*Agrobacterium*-attacked plants do not simply come to terms with their fate. Similar to other pathogens, *Agrobacterium* is sensed as an invader and triggers the 'innate immune response', characterised by the expression of defence genes

and accumulation of reactive oxygen species (reviewed in Pitzschke *et al*, 2009a). This reaction is achieved through the perception of pathogen-associated molecular patterns (PAMPs) by specific receptors. Although several PAMPs have been isolated, only few receptors are yet identified. Putative plant receptors for *Agrobacterium* include a vitronectin-like protein (Wagner and Matthyssse, 1992), a rhicadhesin-binding protein (Swart *et al*, 1994), and several VirB2-interacting proteins (Hwang and Gelvin, 2004; reviewed in Citovsky *et al*, 2007). A recent in-depth study by Clauce-Coupel *et al* (2008) on vitronectins strongly indicates that this group of proteins is unlikely to act as receptors for site-specific *Agrobacterium* attachment.

The most intensively investigated PAMP is flagellin, a highly conserved bacterial protein. In *Arabidopsis*, it is perceived by the receptor protein FLS2, a leucine-rich repeat receptor such as kinase (LRR-RLK). On perception of flagellin or its derived highly conserved 22 amino-acid peptide, flg22, FLS2 becomes activated and initiates a phospho-relay-based signal transduction through the MAPK cascade MEKK1-MKK1/2-MPK4 (Qiu *et al*, 2008; Pitzschke *et al*, 2009a, b). Subsequently, the MPK4-activated transcription factor WRKY33 contributes to the defence-related transcriptional re-programming (Petersen *et al*, 2008).

The flagellin proteins of *Agrobacterium* and of symbiotic bacteria (rhizobia) are distinct from those of most other microbes in that they are not recognised and do not trigger a defence response, implying that other PAMP-receptor pairs are responsible for recognition of these organisms. Indeed, a prominent *Agrobacterium* PAMP, the elongation factor EF-Tu, has been identified. Although highly conserved in all prokaryotes, *Agrobacterium* EF-Tu is fully active as an elicitor (Kunze *et al*, 2004). Interestingly, despite their chemical dissimilarity, flg22 and EF-Tu share several characteristics. Both PAMPs inhibit seedling growth and activate a common set of signalling events and defence responses, while acting with no apparent synergy (Zipfel *et al*, 2006). These responses include MAPK activation, alkanisation of the medium, and an oxidative burst. Moreover, a microarray analysis of the response of *Arabidopsis* to flg22 and the EF-Tu-derived peptide elf18 revealed a clear correlation of differential gene expression, whereas no apparent flg22 or elf18-specific subsets of genes were identified. A surprisingly high number of RLK-encoding genes (100 of 610) were found to be rapidly induced by these PAMPs (Zipfel *et al*, 2006).

A targeted reverse genetics approach has led to the identification of a receptor kinase essential for EF-Tu perception, EFR1. EFR1, similar to FLS2, is a member of the LRR-RLK protein family. The important function of LRR-RLKs in plant-microbe sensing has already become evident for root nodule symbiosis. *Lotus japonicus* mutants affected in the LRR-RLK SYMRK (symbiosis receptor kinase) fail to engage in symbiosis (Stracke *et al*, 2002). Likewise, *Arabidopsis* mutants lacking *fls2* show no response to flg22 treatment (Asai *et al*, 2002). The *efr* mutants are insensitive to elf18, while showing a normal flg22 response (Zipfel *et al*, 2006) and an otherwise normal phenotype. The striking feature of *efr* mutants is their high susceptibility to *Agrobacterium* infection, as shown by the enhanced expression of a T-DNA-harboured reporter gene (GUS) on transient transformation of seedlings. On pre-treatment with flg22, an increase of receptor-binding sites for EF-Tu was observed. Co-injection

of flg22 resulted in abolishment of GUS expression in wild type and *efr* mutants, whereas co-injection of elf18 only abolished GUS expression in wild type, but not in *efr*. *N. benthamiana*, an easily transformed plant species, does not have an EF-Tu recognition system. However, transgenic *N. benthamiana* plants expressing *Arabidopsis* EFR are capable of inducing elf18-triggered defence responses. Together, these observations lead to the conclusion that EFR-mediated EF-Tu perception restricts *Agrobacterium*-mediated transformation (Zipfel *et al*, 2006).

A recent study sheds light on the molecular mechanism that links EFR receptor activation to intracellular signal transduction and stresses differences between flg22- and elf18-triggered limitation of *Agrobacterium* transformation (Ishikawa, 2009). *Arabidopsis* mutants affected in the G-protein b-subunit (*agb1-2*) show significantly reduced ROS production on flg22 or elf18 treatment, whereas stress-triggered MAPK activation (analysed by immunoblotting with an antibody recognising active MPK3 and MPK6) is apparently not affected. Moreover, these mutants are impaired in the elf18- but not in flg22-triggered immunity against *Agrobacterium*. Therefore, a function of AGB1 as positive regulator integrating flagellin and EF-Tu perception into ROS production, and specifically in EF-Tu signalling to limit *Agrobacterium* transformation, has been proposed.

## Gene expression re-programming in response to *Agrobacterium*

*Agrobacterium* attack leads to a major re-programming of gene expression in plants. Already in the pre-microarray-era, large-scale expression analyses (cDNA-AFLP) have revealed that many *Agrobacterium*-induced genes are related to plant defence and to general stress responses (Ditt *et al*, 2001). Using different *Agrobacterium* strains, Veena *et al* (2003) could show that the transfer of T-DNA and Vir proteins can modulate the expression of plant genes in tobacco cell culture. The authors concluded that T-DNA and Vir protein transfer acts as suppressors of the defence response. Later, it was found that an attachment-deficient *Agrobacterium* mutant hyper-induced defence-related genes in *Ageratum conyzoides* cell culture. Interestingly, also non-pathogenic *Escherichia coli* triggered such hyper-induction (Ditt *et al*, 2006), and it was concluded that *Agrobacterium* can dampen plant defence in an attachment-dependent manner (Ditt *et al*, 2006). The authors also observed a strong variability in transformation efficiency between individual experimental series, and further analyses revealed that enhanced basal defence gene expression correlates with resistance to *Agrobacterium* transformation. These findings correlate with the observation that co-injection of plants with *Agrobacterium* plus flg22 or elf18 elicitors abolishes transformation (Zipfel *et al*, 2006). One factor that may explain the reported negative correlation between transformation efficiency and stress status may be salicylic acid (SA). This plant hormone is an important signal in regulating the plant response to pathogens. It accumulates in local and systemic tissues of stress-exposed plants and induces expression of pathogenesis-related genes. A study by Yuan *et al* (2007) now implicates SA in the repression of the Vir regulon, the attenuation of the function of the VirA kinase as well as in the degradation of an *Agrobacterium* quorumone. Accordingly,



plant mutants overproducing SA were recalcitrant to tumour formation. These findings are further supported by Anand *et al* (2008). In summary, from the bacterial point of view, for maximal transformation efficiency, a minimally stressed state of host cells seems desirable. Reciprocally, plants may evade *Agrobacterium* infection if their defence system is in an alerted state.

A kinetic study on the response of tobacco BY2 cell cultures to various *Agrobacterium* strains, including T-DNA and Vir protein transfer-incompetent strains, helped to dissect the stress versus transformation efficiency ambivalence and allowed to distinguish general transcriptional responses resulting from attachment or proximity of *Agrobacterium* near BY2 cells from transformation-specific responses induced by the transfer of T-DNA and/or Vir proteins into plant cells (Veena *et al*, 2003). *Agrobacterium* triggers an early (3–12 h) induction of stress genes, whose expression is subsequently repressed by transfer-competent strains. Concomitant with the decline of this general defence, T-DNA and Vir protein transfer occurs. In contrast, Vir transfer-deficient strains trigger a second wave (after 24 h) of defence gene expression and fail to transfer T-DNA. Through their cDNA macroarray, Veena Jiang *et al* (2003) also revealed that several histone genes were more strongly induced by transfer-competent strains than transfer-deficient strains. Together with the observed constitutive repression of numerous histone genes in the *vip2* mutant, which are affected in stable, but not in transient transformation (Anand *et al*, 2007), these findings emphasise the importance of histones in *Agrobacterium* transformation and indicate that an elevated pool of histones is required for facilitating T-DNA integration into the host genome.

The mechanisms of transcriptional re-programming of *Agrobacterium*-infected tissue and the extent to which bacterial factors contribute to this re-programming are still mostly unknown. On the basis of the findings that VirE3 has transactivating activity in yeast, localises to plant nuclei, and that VirE3 can bind pBp, a general plant-specific transcription factor, a function of VirE3 as potential plant transcriptional activator mediating the expression of tumour development-specific genes was proposed (Garcia-Rodriguez *et al*, 2006).

## Defence versus transformation—how to evade the innate immune response

From the above sections, it becomes apparent that a major barrier for *Agrobacterium* infection is the defence response triggered in the host cell. Once plant defence can be blocked, reduced, or circumvented, much higher transformation efficiencies can be achieved. Even more, in at least one aspect, *Agrobacterium* have learnt to turn the tables in that they can even benefit from being recognised as a pathogen. Similar to numerous other microbes, *Agrobacterium* triggers the activation of MAPKs, primarily MPK3, MPK4, and MPK6 (Djamei *et al*, 2007). Y2H experiments, *in vivo* interaction, and kinase assays have shown that VIP1, the VirE2-interacting protein 1, interacts with and becomes specifically phosphorylated by MPK3 (Djamei *et al*, 2007). Stress-triggered phosphorylation of VIP1 results in the rapid translocation of this protein from the cytoplasm to the nucleus. In contrast to VirD2, which passes into the host nucleus on its own, VirE2 needs the assistance of both importin  $\alpha$  (Bhattacharjee *et al*, 2008, see

above) and the host protein VIP1. *Agrobacterium* thus does not only abuse VIP1 as such for delivering the VirE2/T-DNA into the host cell nucleus, but also actively manipulates the subcellular localisation of this plant protein. As this was not enough, once nuclear transfer of the T-DNA complex has been accomplished, T-DNA integration into the plant genome is likely to be achieved through abuse of yet another manipulation of the host cell machinery. Tzfira *et al* (2004a,b) suggest that nuclear VirF protein may mediate the degradation of the T-DNA complex through the plant proteasome, resulting in the release of the T-DNA, which subsequently can integrate into the host genome, the final step of stable transformation.

As not only *Agrobacterium*, but also flg22, triggers MPK3 activation and rapid nuclear accumulation of VIP1 (Djamei *et al*, 2007), the question arises which function the stress-dependent cytoplasmic-nuclear translocation of VIP1 has in the plant response to other stresses. We have recently shown that VIP1 is a functional bZIP transcription factor, which binds to a novel DNA regulatory motif, VIP1 response element (VRE) (Pitzschke *et al*, 2009c). VREs are overrepresented in the promoter regions of stress-responsive genes. VIP1 binds *in vivo* to the promoter of a stress-responsive transcription factor, MYB44, under conditions that activate the MPK3 pathway. The *mpk3* mutants are impaired in stress-triggered induction of VIP1 target genes, whereas VIP1 over-expressing plants show constitutively elevated transcript levels. From these observations, a function of VIP1 as a mediator of MPK3-mediated stress gene modulation was derived. How does the function of VIP1 as activator of the defence responses correlate with the finding that VIP1-over-expression results in enhanced transformation efficiency (Tzfira *et al*, 2002)? A plausible explanation is that the higher transformation rate is achieved through a more efficient nuclear transfer of the T-DNA complex, as more VirE2-T-DNA molecules can be 'piggy-packed' by VIP1. The degradation of the T-DNA complex and VIP1 through the action of VirF and the plant proteasome serves both the release of the T-DNA and the prevention of VIP1-induced defence gene expression. According to this model, not VirF, VirD2, or the fidelity of the plant proteasomal machinery, but VIP1 phosphorylation/localisation and the presumably directly correlated nuclear import of VirE2/T-DNA might be the limiting factors in transformation.

Owing to its dual function as a stress-responsive transcription factor on the one hand and as T-DNA/VirE2 shuttle on the other hand, VIP1 may be one of the factors that 'tips the scales', that is it decides between successful transformation and failure of transformation because of elevated basal stress levels. As MPK3 is very sensitive to numerous stresses, its target protein, VIP1, can potentially be phosphorylated, translocate to the nucleus and induce stress gene expression equally easily. The battle between *Agrobacterium* and plant, therefore, is to 'compete' for one of the two VIP1 functions.

## Conclusions/summary

The transformation of plants by *Agrobacterium* is a complex process that involves multiple steps and the concerted action of both microbial and host factors. As evidenced by a still increasing number of reports on novel plant-*Agrobacterium* protein interactions, much is still to be learnt to understand

the entire transformation process in detail. The main recent progress includes the characterisation of Vir-interacting proteins *in vivo*, the function of histones in stable integration and the elucidation of surprisingly smart strategies used by *Agrobacterium* to circumvent or even abuse the plant defence system. Clearly, the in-depth study and molecular analysis of the plant-*Agrobacterium* interaction will not only add to our understanding of pathogen strategies for host infection, but harbour also the potential to render plants transformable that are otherwise recalcitrant to *Agrobacterium* transformation

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and to speed up the progress in generating stress-resistant plants.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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