CHARACTERISATION OF *AGROBACTERIUM* VIRD2 INTERACTING PROTEIN DIP AND ITS HOMOLOGUES

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LIST OF PUBLICATIONS RELATED TO THIS STUDY

Chang, L., **Tang, H.C.** and Pan, S.Q. (2005). *Agrobacterium* VirD2 protein interacts with plant host DIP (manuscript in preparation)

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LIST OF ABBREVIATIONS

4-MUG	4-methylumbelliferyl β-D-glucuronide
A	adenosine
aa	amino acid(s)
AD	activation domain
Amp	ampicillin
AS	acetosyringone
A/S	Antisense
bp	base pair(s)
BSA	bovine serum albumin
C- terminal	carboxyl terminal
C	cytidine
Cb	Carbenicillin
DBD or BD	DNA binding domain
DIP	VirD2 interacting protein
DMF	N, N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deooxyribonucleoside triphosphate
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DTT	dithiothreitol
EDTA	Ethylenediaminetetra acetic acid
EtBr	ethidium bromide
EtOH	ethanol
Fig	Figure
g	grams or gravitational force, according to the intended meaning
G	guanosine
GUS	ß-glucuronidase
h or hr	hour
hrs	hours
HRP	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase(s) or 1000 bp
kD	kilodalton(s)
Km	kanamycin
lacZ	β-galactosidase gene
LB	Luria-Bertani medium
M	molar
MCS	multiple cloning site(s)
MES	2-[N-morpholino] ethanesulfonic acid
mg	milligram(s)
μ	micro-
μg	microgram(s)
μl	microliter(s)
μm	micrometre
min	minute(s)
ml	milliliter(s)
mM	millimole
mw	molecular weight
N	any nucleoside
n	nano-
nm	manometer
nt	nucleotide(s)
N- terminal	amino terminal
NLS	Nuclear localization sequence
OD	Optical density

Oligo	oligodeoxyribonucleotide
ORF	open reading frame
p	pico-
PAGE	polyacrylamide gel electrophoresis
ppt	phosphinothricin
^{R}	resistant/resistance
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
^{S}	sensitive/sensitivity
SDS	sodium dodecyl sulfate
sec	second(s)
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
T	thymidine
T4SS	Type IV secretion system
1× TAE	40 mM Tris-acetate, 1 mM EDTA
TBS	Tris-buffered saline
UV	ultraviolet
V/V	volume per volume
w/v	weight per volume
wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl β-D glucuronide

Summary

Agrobacterium tumefaciens is a soil-borne plant pathogen that can transfer part of its DNA (T-DNA) into plant cells and integrate the DNA into the plant genome. It has been widely used as a vector for plant transformation to create transgenic plants. However, the host range of *A. tumefaciens* is not limited to plant species as it has been shown to be capable of transferring its DNA into yeasts, fungi as well as some mammalian cells, such as human cells. While the virulence proteins of *A. tumefaciens* have been well characterized, the studies on the host factors are still emerging.

In this study, it was shown that when the plant factor – *A. tumefaciens* VirD2-Interacting Protein, DIP, was "knocked down" transiently in tobacco BY-2 cells or tobacco leaf tissues by RNA interference (RNAi), the plant cells and tissues were shown to become less receptive to transformation by *A. tumefaciens*. When the DIP "knock down" genotype was selected on the selective medium, the resultant stable transgenic BY-2 cells were found to possess a slower rate of growth as well as a similarly reduced efficiency of transformation by *A. tumefaciens.* Subsequently, it was found that homozygous *DIP* $^{-/-}$ "knock out" *Arabidopsis* plants from heterozygous seed line could not be generated. Taken together, these results demonstrate that DIP plays a critical role in the basic biological process(es) and it is important for *Agrobacterium*-mediated transformation of plant cells.

Furthermore, the delineation of DIP-interacting domain of VirD2 via yeast two-hybrid analysis has indicated that the nuclear localization sequences (NLSs) of VirD2 are not required for its interaction with DIP. This sets DIP apart from those plant factors that bind to the NLSs of VirD2 to localize the T-DNA to the nucleus. Based on

its identity as a homologue of the evolutionarily conserved exocyst complex subunit and its conserved Vps52 domain, DIP may receive the T-DNA from host factors interacting with the *A. tumefaciens* T-DNA export machinery during the early phase of *Agrobacterium*-mediated transformation of plant cells and subsequently direct the T-DNA to the endocytotic pathway.

Subsequent study of DIP homologues has shown that the mammalian homologues are homologous to one another, especially between the human and the mouse that share over 95 % amino acid sequence identity. This is reflected in the fact that antibodies against the human homologue, hDIP, could not be raised in both rabbits and mice. Such findings imply that the conserved exocyst complex function in the secretion and/or endocytotic pathway is likely to be 'hijacked' and manipulated for its own cause when *A. tumefaciens* transforms its host cell.

Chapter 1. Literature Review

Agrobacterium tumefaciens is a Gram-negative, soil-borne phytopathogen that causes crown gall disease on a wide range of plant species, particularly the dicotyledonous plants (van Larebeke *et al*, 1974; Waston *et al*, 1975). Initial research in *Agrobacterium*-plant interaction was aimed to understand the molecular mechanism of *Agrobacterium*-mediated tumor formation and to shed light on animal tumors. Although no relationship was found between animal and plant tumors, the research effort has culminated in the possible revolution in plant genetic engineering and transgenic technology. *Agrobacterium*-mediated transformation of plant cells has since become the mainstay in plant molecular biology and a useful tool for scientists to create transgenic plants possessing various desirable characteristics, such as herbicide resistance.

An overview on the mechanism of *Agrobacterium*-plant cell interaction is illustrated in Fig. 1.1. In brief, when *A. tumefaciens* encounters and is attracted to the wounded plant cell by chemotaxis, part of its DNA (the transferred DNA or T-DNA) is processed from the large tumor-inducing (Ti) plasmid to give rise to a T-strand. This T-strand is made up of the single stranded T-DNA with the *A. tumefaciens* virulence (Vir) protein VirD2 bound to its 5' end. *A. tumefaciens* VirE2 proteins, which bind single stranded DNA non-specifically, will then associate with the T-strand to form the T-complex. Whether this T-complex is formed within the bacterial cell or assembled within the plant cell cytoplasm still remains controversial. However, it is clear that the T-DNA is eventually transferred into the plant cell via the VirB/D4 channel, a transfer apparatus formed by 11 different VirB proteins and a single VirD4 protein. After its successful passage through the plant cell cytoplasm, possibly by interacting with

various plant factors, the T-complex is targeted to the plant cell nucleus, where the T-DNA is integrated into the plant genome. In nature, the subsequent expression of the genes carried on the T-DNA, which encodes plant hormone genes, will result in the formation of neoplastic growths, known as crown gall tumors that secrete opines. These opines, which are major sources of carbon and nitrogen, can only be catalyzed by the infecting *A. tumefaciens* strain. In this manner, *A. tumefaciens* can effectively transform plant cells and manipulate the plant cell metabolism to create a favorable niche for itself. It is for this reason that *A. tumefaciens* has been dubbed the natural genetic engineer, a prokaryotic organism that can genetically modify its eukaryotic host for its own benefit. (Kado, 1991; Sheng and Citovsky, 1996; Zupan and Zambryski, 1997; Stafford, 2000; Zhu *et al,* 2000; Gelvin, 2003).

Besides its natural hosts, which are dicotyledonous plants such as fruit trees and grape vines, *A. tumefaciens* has also been successfully used to transform monocotyledonous plants like rice (Komari *et al*, 1998; Hiei *et al*, 1994; 1997) and wheat (Cheng *et al,* 1997). Furthermore, the accumulated knowledge of *Agrobacterium*-mediated transformation has been applied to fungus, yeast and mammalian cells as well (Bundock *et al*, 1995; Relic *et al*, 1998). Undoubtedly, the development of *A. tumefaciens* as a plant genetic vector has been one of the most important technical developments in the past two decades.

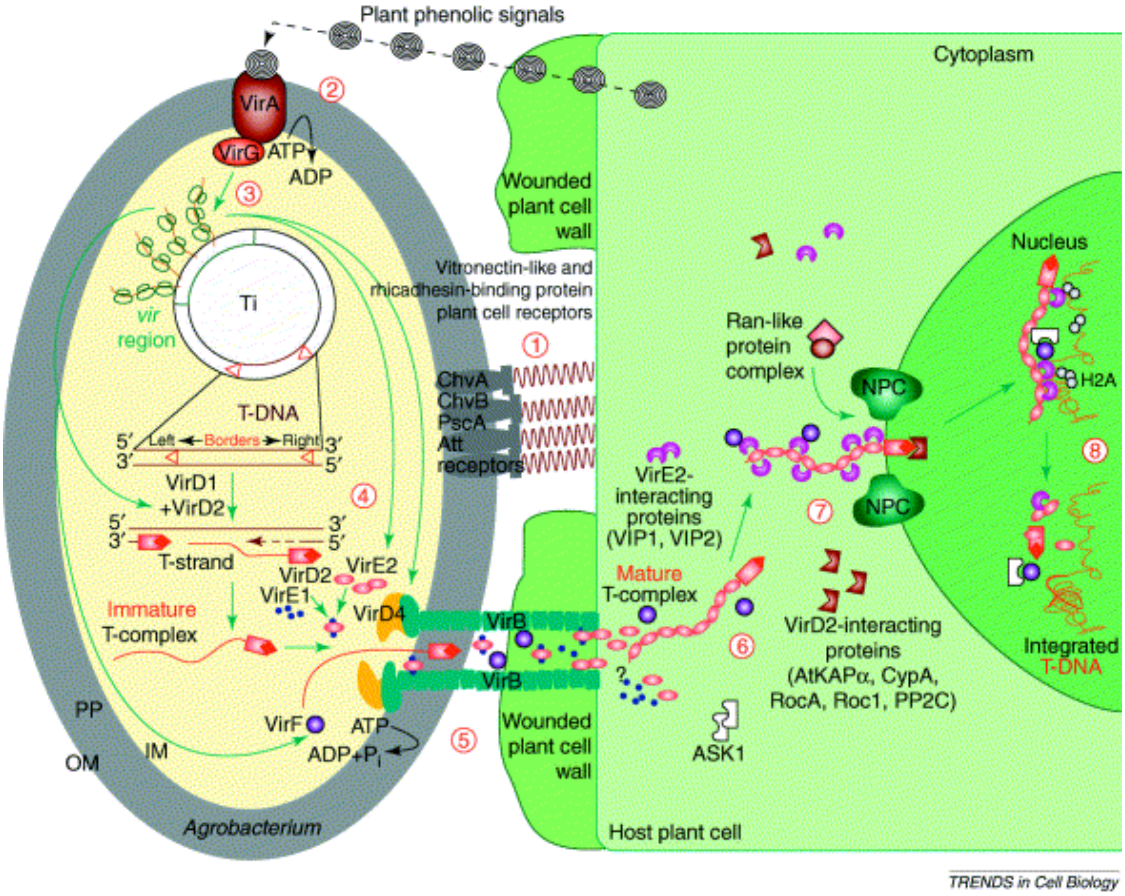


Fig. 1.1. *Agrobacterium*-plant cell interaction. Critical steps that occur to or within the bacterium and those within the plant cell are highlighted, along with genes and/or proteins known to mediate these events:

1. Attachment of *A. tumefaciens* to host cell surface receptors;
2. Recognition of plant signals by bacterial VirA/VirG sensor-transducer system;
3. Activation of bacterial *vir* genes;
4. Processing and production of transferable T-strand;
5. Export of T-DNA into plant cell via VirB/D4 channel;
6. Intracytoplasmic transport of T-complex;
7. Nuclear import of T-complex;
8. T-DNA integration.

IM, bacterial inner membrane; NPC, nuclear pore complex; OM, bacterial outer membrane; PP, bacterial periplasm.

(Cited from Tzfira and Citovsky, 2002)

1.1. Overview of *Agrobacterium*-mediated transformation of plant cells

Agrobacterium-mediated transformation of plant cells is the only well studied example of natural interkingdom gene transfer. This process of T-DNA transfer involves several critical steps: bacterial chemotaxis and attachment to plant cell surface receptors, signal perception and transduction by the highly conserved two-component regulatory system, *vir* gene induction, T-DNA processing, T-DNA transfer into plant cells, nuclear localization of T-complex into plant cell nucleus, T-DNA integration into the plant genome and the expression of transferred gene, as illustrated in Fig. 1.1.

This T-DNA transfer is initiated when *A. tumefaciens* perceives and responds to certain phenolic compounds, sugar, acidic pH and low phosphate level, which are present at plant wound sites. The signal perception is mediated by the VirA/VirG two-component transduction system. Autophosphorylation of VirA protein and the ensuing transphosphorylation of VirG protein results in the activation and transcription of virulence (*vir)* genes. These *vir* gene products or Vir proteins are directly involved in the processing of T-DNA from the Ti-plasmid and the transfer of T-DNA from the bacterium into the plant cell nucleus (reviewed in Tzfira *et al,* 2000; Kado, 2000; Gelvin**,** 2000).

The T-DNA transfer process from *A. tumefaciens* into a plant cell involves many factors from both the bacterium and the host. There are at least three genetic components of *A. tumefaciens* that are essential for plant cell transformation. The first component is the T-DNA, the transferred segment, which is transported from the bacterium into the plant cell (Wang *et al,* 1984; 1987). The T-DNA is located on the 200-kb Ti-plasmid of *A. tumefaciens* and is delimited by two flanking 25-bp imperfect direct repeats known as the T-DNA borders or T-borders. Since border sequences of

the T-DNA are the only *cis* elements necessary for effective transformation of the plant cell, any foreign DNA placed between the T-borders will be transferred into the host plant cell (Miranda *et al,* 1992). The second component is the aforementioned *vir* genes that are also located on the Ti-plasmid. This 35-kb region of DNA, which is not transferred to the plant cells, codes for proteins that are required for the sensing of plant wound metabolites as well as the processing, transfer, nuclear targeting and integration of T-DNA. There are eight major loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, *virJ* and *virH*) in this region. All of the *vir* operons are induced as a regulon via the VirA/VirG two-component system by plant phenolic compounds, such as acetosyringone (AS) and specific monosaccharides. The third component is a set of chromosomal virulence (*chv*) genes, which have been identified as necessary for tumorigenesis. Some of the *chv* genes are involved in bacterial chemotaxis and attachment to wounded plant cells, while others might be involved in the regulation of *vir* gene expression (Uttaro *et al,* 1990; Thomashow *et al,* 1987; O'Connell and Handelsman, 1989; Kamoun *et al,* 1989; Sheng and Citovsky, 1996).

1.2. *A. tumefaciens* genes involved in plant transformation

Both *vir* genes and *chv* genes play important roles in the processing and transfer of the T-DNA from *A. tumefaciens* into the plant cell nucleus, as described briefly above. In the following sections and subsections, the characteristics and functions of these Vir proteins, Chv proteins and other *A. tumefaciens* gene products that are involved in the transformation of plant cells are described in detail.

1.2.1. VirA/VirG, a conserved two-component regulatory system

Perception of signal molecules released by wounded plant cells is the first step of signal transduction that will lead to the expression of *vir* genes in *A. tumefaciens.* The

vir operons constitute a regulon which is strongly and coordinately induced in bacterial cells growing under acidic pH conditions by two classes of plant signal molecules: phenolic compounds, such as acetosyringone, and sugars such as glucose and glucuronic acid. The expression of these virulence genes is under the control of a highly conserved two-component regulatory, which is comprised of VirA and VirG (Winans, 1992; Olson, 1993).

Based on protein sequence similarities, VirA and VirG have been assigned to a large group of His-Asp two-component regulatory systems, involving a sensor and a response regulator. Functioning as an inner membrane histidine kinase, when VirA senses the phenolic compounds released from the wounded plant cells, it will get autophosphorylated at His-474 (Lee *et al,* 1995; 1996; Ninfa *et al*, 1988; 1991; 1993). This phosphorylated VirA will then transfer the phosphate moiety to the response regulator, VirG, at Asp-52 before the phosphorylated VirG activates the transcription of the *vir* genes.

Both physical and genetic evidences have indicated that VirA protein exists as a homodimer in its native conformation and the homodimer is the functional state in the plant-bacterium signal transduction (Pan *et al,* 1993). The VirA protein can be divided into four functional domains, which include periplasmic, linker, kinase and receiver domains. The periplasmic domain has been found to sense a variety of monosaccharides required for *vir* gene induction and also to interact with a periplasmic sugar-binding protein, ChvE (Cangelosi *et al,* 1990; 1991). Though its interaction with ChvE alone does not induce *vir* gene expression, this periplasmic domain sensitizes the VirA molecule to the phenolic inducers. The fact that VirA protein has variable efficiency in different strains of *A. tumefaciens* suggests that different chromosomal

backgrounds, especially the difference in ChvE, may give rise to differential degrees of VirA function.

Found on the same protein, the VirA linker domain has been reported to be necessary for perceiving phenolic compounds and acidity while the kinase domain that contains the conserved phosphorylatable His-474 is found to be required for signal transduction in all sensor molecules. Single-base mutations that cause the change of this residue from His-474 to Gln-474 have resulted in a VirA protein with abolished or attenuated functions. VirA with such mutations could no longer be phosphorylated at this residue and a mutant carrying this modification has been shown to be avirulent and unable to induce *vir* gene expression in the presence of plant signal molecules (Huang *et al*, 1990; Jin *et al,* 1990a; 1990b; 1990c). Despite its similarity to the region of VirG that is phosphorylated by VirA, the function of VirA receiver domain still remains unclear and it has been proposed to play an inhibitory role in signal transduction. This stems from the observation that once the receiver domain was deleted, monosaccharides alone could induce *vir* gene expression even in the absence of phenolic compounds (Jin *et al,* 1990a; 1990b; 1990c).

Unlike VirA, VirG is a cytoplasmic protein that can bind specifically to a 12-bp conserved consensus, termed the *vir*-box. This *vir*-box is present in the upstream region of most of the *vir* genes. By binding to this *vir*-box, VirG acts as a transcriptional activator of these *vir* genes. While the C-terminus region of VirG is responsible for this DNA binding activity, its N-terminal is the phosphorylation domain that shows high homology to the VirA receiver (sensor) domain. Regardless of the mutagenesis approach chosen, mutants with non-phosphorylatable VirA or VirG

protein have not been able to induce *vir* gene expression (Jin *et al,* 1990a; 1990b; 1990c).

In addition, the number of copies and the types of *virG* gene can influence some biological properties of *A. tumefaciens*. For instance, multiple copies of VirG in *A. tumefaciens* can greatly enhance *vir* gene expression and thus the transient transformation frequency of some plants tissues (Liu *et al,* 1992). Having multiple copies of VirG also allow a higher level of *vir* gene induction by acetosyringone (AS) even at alkaline pH (Liu *et al,* 1993).

Recently, studies have revealed that quantitative differences exist in the interactions between VirG and *vir* boxes of different Ti-plasmids, suggesting that efficient *vir* gene induction in octopine and nopaline strains requires *virA*, *virG,* and *vir* boxes from the respective Ti-plasmids for maximal induction efficiency.

1.2.2. VirC, VirD and VirE

1.2.2.1. Formation of T-complex

A. tumefaciens virulence proteins responsible for the production of T-complex are encoded by *virD* and *virE* operons (Grimsley *et al,* 1989; Toro *et al,* 1989; Citovsky *et al,* 1988; 1989; Gietl *et al,* 1987; Sen *et al,* 1989). The T-complex is made up of the T-strand that is coated with a large number of VirE2 proteins along its entire length. This T-strand is the end product after the single-stranded T-DNA is processed from the Ti-plasmid with a molecule of VirD2 covalently bound to its 5' end.

The T-DNA is delimited by two 25-bp imperfect direct repeats, also known as the T-border, at its ends. Since any DNA between the T-borders can be transferred into the plant cell as a single-strand DNA and integrated into the plant genome,

transformation vectors harboring T-borders have been used widely to facilitate the creation of transgenic plants.

In vivo, VirD2, together with VirD1, is sufficient for T-DNA processing in both *E. coli* and *A. tumefaciens*. VirD2 is an endonuclease, which cleaves the bottom strand of the T-DNA at the T-borders and remains covalently bound to the 5' end of the nicked DNA (Pansegrau *et al*, 1993; Jasper *et al*, 1994; Zupan *et al,* 2000; Gelvin, 2000). This endonuclease domain lies in the N-terminal 228 aa of VirD2 and is the only known highly conserved domain in VirD2 protein besides the two short NLS regions near the C-terminus.

VirD1 might assist the endonuclease activity of VirD2 through its interaction with the T-borders, where ssDNA is originated. This interaction can induce local double helix DNA destabilization and provide a single-stranded loop substrate for VirD2. *In vitro* studies have shown that VirD2 alone is enough for mediating the precise cleavage of T-border sequence carried by ssDNA templates even in absence of VirD1 protein. However, VirD1 is essential for the cleavage of T-borders on plasmid or supercoiled DNA substrate by VirD2.

Another factor, VirC1, has been found to increase the efficiency of T-strand production when VirD1 and VirD2 proteins were limited (De Vos and Zambryski**,** 1989). It can specifically recognize and bind to an enhancer or overdrive sequence next to the right T-border, found on many Ti-plasmids. For optimal T-DNA formation, this additional VirC1-mediated function appears to be non-redundant.

After the processing of T-strand from Ti-plasmid, VirE2 subsequently coats the single stranded T-DNA along its entire length, forming the so called T-complex (Citovsky *et al,* 1988; 1989; Gietl *et al,* 1987; Sen *et al,* 1989; Zupan *et al,* 2000). As

a non-sequence-specific ssDNA binding protein, VirE2 can protect the T-DNA from potential nucleolytic attacks. However, recent evidences have suggested that VirE2 protein might function primarily in the plant cell but not necessarily in the bacterium because plants expressing *vir*E2 can be successfully transfected by *A. tumefaciens* lacking *vir*E2 (Citovsky *et al,* 1992).

Currently, it is still unclear whether the association of VirE2 with the T-strand occurs within the bacterial cell soon after the T-strand is formed or VirE2 and T-strand molecules meet each other only inside the host plant cell. Due to the controversial nature with regards to the actual mechanism of VirE2 association with the T-strand, there are two major proposed models for this process and VirE2 transport.

In the first model, VirE2 is thought to bind to the T-strand in the early steps of the infection process since it is one of the most abundant Vir proteins in *A. tumefaciens* and it can bind ssDNA strongly in a cooperative way. In addition, VirE2 and T-strand are believed to be transported from the bacterium into the plant cells through the same VirB/D4 channel, described in a later section. The supporting evidence for this model is based on the finding that T-strand and VirE2 could be coimmunoprecipitated from the extracts of *vir*-induced *A. tumefaciens.*

In the second model, T-strand and VirE2 are proposed to be independently exported into plant cells from the bacterium. This is based on the accumulating evidence and research data which begin to support such notion. Findings from complementation and co-infection studies have indicated that VirE2 is not required for the export of T-strand, while VirE2 export can be inhibited without affecting T-strand export (Citovsky *et al,* 1992),. Furthermore, a recent biophysical observation has suggested that VirE2 itself could form channels on the artificial membranes and this

implies that VirE2 is transported through the VirB/VirD4 channel or an alternative route and subsequently inserts into the plant plasma membrane, allowing the transport of the T-strand (a ss-T-DNA-VirD2 complex) (Dumas *et al*, 2001).

In support of the second model, a specific molecular chaperone for VirE2, VirE1, is found to be essential for the export of VirE2 to plant cells, but not that of the T strands (McBride and Knauf, 1988; Winans *et al,* 1987; Deng *et al,* 1999). VirE1 is a small, acidic protein with an amphipathic α-helix at its C-terminus. Yeast two-hybrid studies and extracellular complementation suggest that VirE1 mediates T-complex formation in several possible ways. First of all, though VirE1 does not influence *virE2* transcription from the native P_{virE} promoter, VirE1 indeed regulates the efficient translation of VirE2. Secondly, VirE1 stabilizes VirE2 via an interaction with the N-terminus of VirE2 and such VirE1-VirE2 complex is composed of one molecule of VirE2 and two molecules of VirE1. Apart from these, the formation of VirE1-VirE2 complex, which inhibits self-interacting of VirE2 to form aggregates, might help to maintain the VirE2 molecule in an export-competent state.

Based on the current research data reported by various groups, it is hard to ascertain which model is the correct model for the actual mechanism of T-complex formation and where this complex is formed. To elucidate this pathway, more investigations coupled with better research tools may be necessary before this mystery can be unraveled.

1.2.2.2. Nuclear localization of T-complex

Despite the controversial nature of T-complex formation, it is certain that T-complex will be targeted to the plant cell nucleus and this nuclear localization is a critical step for tumorigenesis. Since T-DNA itself does not contain any specific

sequence and the fact that any foreign DNA fragment placed between T-DNA borders can be transported into the plant cells and subsequently integrated into the plant genome, this implies that the associated protein components must have played some roles in the nuclear localization of the T-complex. They must have specifically mediated T-complex nuclear localization instead of the nucleic acid molecule itself.

Indeed, both VirD2 and VirE2, which are the integral subunits of T-complex, contain conserved bipartite nuclear localization sequence (NLS) that can direct the T-complex into the plant nucleus through the nuclear pores (Tinland *et al,* 1992; Citovsky *et al,* 1992; 1994). VirD2 mutants with altered or mutated NLS have been shown to possess a reduced capability for tumorigenesis, while the VirE2 mutants were completely avirulent. For the import of short ssDNA, VirD2 alone was sufficient, but the import of long ssDNA required VirE2 additionally (Ziemienowicz *et al*, 2000; 2001). These research data imply that the NLS of these two proteins might play different roles in nuclear localization.

The targeting of T-complex to the nucleus is thought to occur in a polar fashion (Howard *et al,* 1992). VirD2, which is attached to the 5' end of the T-strand, may provide this piloting function. VirD2 molecule contains two NLS sequences, one at each end of the molecule (Herrera-Estrella *et al,* 1990; Howard *et al,* 1992). The N-terminal NLS of VirD2 is a monopartite NLS that resembles the NLS found in the SV40 large T-antigen, whereas the C-terminal NLS is a bipartite NLS which is characterized by two adjacent basic amino acids, a variable-length spacer region and a basic cluster in which any three out of the five contiguous amino acids must be basic (Dingwall and Laskey, 1991, Howard *et al,* 1992).

The N-terminal half of VirD2 required for nicking at the T-border sequences may be involved in T-DNA integration in the plant nucleus, but it is not required for T-DNA transfer because mutations in this domain could not affect T-DNA transfer significantly (Koukolikova-Nicola *et al,* 1993; Shurvinton *et al,* 1992)**.** It has been reported that the N-terminal NLS of VirD2 might be occluded by the covalently bound T-DNA because the tyrosine-29 residue, with which VirD2 is bound to T-DNA, is only a few amino acids away from the N-terminal NLS.

The C-terminal NLS has been found to be involved in the tumorigenesis of *A. tumefaciens* (Rossi *et al,* 1993; Narasimhulu *et al,* 1996). *A. tumefaciens* mutants with genes that code for a VirD2 protein missing its C-terminal part have been found to lose their ability to induce tumors but were efficient in the processing of T-DNA (Young and Nester, 1988). Results from translational fusion protein and coimmunoprecipitation experiments showed that the C-terminal of VirD2 was capable of directing a reporter gene into the plant cell nucleus. Interestingly, the C-terminal NLS of VirD2 protein was found to retain this function even in the mammalian cell systems.

Recent evidences have supported that VirD2 alone is sufficient to transfer short single stranded DNA into the nuclei of tobacco cell and this function is strictly dependent on the presence of the C-terminal NLS of the VirD2 protein. A VirD2 mutant lacking its C-terminal NLS was unable to mediate the plant nuclear targeting of the T-complexes (Rossi *et al,* 1993; Ziemienowicz *et al*, 2000; 2001).

VirE2 protein contains two separate bipartite NLS regions (NLS1 and NLS2) that are located in the central region of the molecule in residues 212-252 and residues 288-317 respectively. Both NLSs might participate in piloting the T-DNA into the plant

cell nucleus (Gietl *et al,* 1987; Christie *et al,* 1988; Citovsky *et al,* 1988; Das, 1988). Nonetheless, the relative importance of VirE2 NLSs for T-strand transfer is difficult to assess because mutations in these sequences might also affect the binding of VirE2 proteins to ssDNA.

Analysis of VirE2 sequence has revealed that the ssDNA binding domain or the cooperativity domain is overlapped with the NLSs of VirE2 (Citovsky *et al,* 1992; Citovsky *et al,* 1994). Based on the results obtained from such analysis, NLS1 and NLS2 might also be involved in binding the single stranded T-DNA. Deletion of NLS1 in VirE2 would reduce its cooperative ssDNA binding activities while deletion of NLS2 or both NLS1 and NLS2 together would completely abolish ssDNA binding and nuclear localization activities. Therefore, the contribution of VirE2 NLSs for T-complex nuclear targeting is still a controversial issue. Some research groups have thus suggested that both VirD2 and VirE2 proteins play important roles in the nuclear targeting of T-complex and both are needed for the optimal nuclear localization activity.

In one experiment, the VirE2-GUS fusion protein was found to localize in the plant cell nuclei due to the nuclear targeting function of VirE2. Meanwhile, another experiment showed that the fluorescently labeled single stranded DNA together with VirE2 proteins were found to accumulate in the plant nuclei after microinjection into plant cells, but the naked single stranded DNA remained exclusively in the cytoplasm. Also, VirE2 mediated nuclear localization was found to be blocked by nuclear import inhibitors (Guralnick *et al,* 1996; Zupan *et al,* 1996).

Unlike that in VirD2 and octopine VirE2, the NLSs of VirE2 derived from the nopaline-specific Ti-plasmids are not functional in the nuclear import of proteins in

Xenopus oocytes, *Drosophila* embryos (Guralnick *et al,* 1996) and yeast cells (Rhee *et al,* 2000). However, the modified VirE2 whose NLS amino acids was altered to resemble more closely to animal NLS sequences could target DNA to animal cell nuclei (Guralnick *et al*, 1996), suggesting that nuclear targeting signals in plant and animal cells might differ slightly (Gelvin, 2000).

On the other hand, recent studies from Ziemienowicz group showed that VirD2 alone could import a small covalently attached oligonucleotide into the plant nucleus without VirE2 NLS function and that this import was absolutely dependent on the C-terminal NLS of VirD2. Additional evidences showed that the presence of VirE2 protein could not functionally compensate for the deletion of the VirD2 NLS (Ziemienowicz *et al,* 1999; 2001). However, when it comes to the nuclear import of large ssDNA above 250nt, VirE2 molecule is required even in the presence of functional VirD2 molecules.

In an attempt to clear up the controversy surrounding VirE2 and its NLS function, a series of nuclear import assays were performed using nopaline VirE2 and octopine VirE2 into both dicotyledonous and monocotyledonous plants, as well as living mammalian and yeast cells by one research group (Tzfira and Citovsky, 2001). Their research findings clearly demonstrate that nuclear import of both nopaline and octopine VirE2 proteins is plant-specific, occurring in plant but not in non-plant systems. Their results also suggest that the nuclear import of VirE2 in a cell-free system (Ziemienowicz *et al*, 1999) may be different from that within living cells and this difference may be the reason why octopine VirE2 alone does not mediate the import of ssDNA into the nuclei of permeabilized plant protoplasts (Ziemienowicz *et al*, 2001). As for the lack of nuclear import of VirE2 in animal and yeast cells, it is

suggested that plant-specific host cellular factors are involved in interacting with VirE2 to facilitate its nuclear uptake and this NLS function of VirE2 in animal and yeast cells may be substituted by unidentified unknown cellular proteins.

Furthermore, it has also been found that RecA, which is an ssDNA binding protein, could be a substitute for VirE2 in the nuclear import of T-DNA but not in the efficient T-DNA transformation of tobacco. This research finding suggests the following implications. Firstly, VirD2 might play a role in directing the T-complex to the nuclei and the NLS in VirE2 is perhaps really not necessary for the nuclear localization because RecA protein contains no motif resembling known NLSs. Secondly, VirE2 may assist nuclear uptake of the T-complex more by keeping the T-strand in an unfolded state to facilitate the traverse through nuclear pore complex rather than by its NLS function.

In order to decipher the relative roles of the VirD2 and VirE2 NLSs in nuclear targeting of the T-strand and to ascertain their respective contributions to nuclear localization, more experiments may have to be performed to dissect and understand the complicated and intertwined pathways in which the recognition and functionality of these NLSs are involved.

Aside from the VirD and VirE elements mentioned above, VirE3 has just recently been shown to be involved in the nuclear targeting of T-complex by facilitating the nuclear import of VirE2 via the karyopherin α-mediated pathway and thus allowing the subsequent T-DNA expression (Lacroix *et al*, 2004). Earlier studies have suggested that VirE3 is exported into the host yeast (Schrammeijer *et al*, 2003) and plant cells (Vergunst *et al*, 2003) during transformation. VirE3 is now eventually

demonstrated to act as an 'adaptor' molecule between VirE2 and karyopherin α and to 'piggy-back' VirE2 into the host cell nucleus (Lacroix *et al*, 2004).

1.2.2.3. Integration of T-DNA

Upon its entry into the plant cell nucleus, the final step of T-DNA transfer is its integration into the plant genome. Due to the lack of suitable systems for detailed investigation, the mechanism of T-DNA integration into the plant genome is still unclear. It has been proposed that this process occurs by illegitimate recombination and most of the T-DNA transferred to the plant cell nucleus does not integrate into the plant genome. It is also perceivable that various host factors of the DNA repair/synthesis machinery are involved in this process and a few models have been proposed for the mechanism of T-DNA integration (reviewed in Tzfira *et al*, 2004). In this process, the bacterial components that can participate in this process are those that make up the T-complex and translocated through the nuclear pore, namely VirD2 and VirE2.

The integration of the 5' end of the T-strand into the plant genomic DNA is generally precise as VirD2 is covalently linked to the 5' end of T-strand. These facts suggest that VirD2 might play an active role in the precise T-DNA integration into the plant chromosome although it does not influence the efficiency of the integration step (Tinland *et al,* 1992). Shurvinton *et al.* (1992) demonstrated that deletion of the conserved omega domain located near the C-terminal end of VirD2 resulted in an approximate two orders of magnitude decrease in tumorigenesis, while the same mutation resulted in only a three to five fold decrease in T-DNA transient expression in tobacco and *Arabidopsis* cells (Mysore *et al,* 1998; Narasimhulu *et al,* 1996). These results indicated that this mutation affected T-DNA integration to a much greater extent

than it affected T-DNA transfer and nuclear targeting. Mysore *et al.* (1998) further proved that an *A. tumefaciens* strain harboring this mutation was deficient in T-DNA integration.

The function of VirE2 protein in integration of the T-DNA into the plant genome is still unclear. Rossi (1996) suggested that, instead of contributing to the efficiency of integration, VirE2 might be involved in maintaining the integrity of the T-DNA during the integration process.

1.2.3. VirB and VirD4, a type IV secretion system (T4SS)

A type IV secretion system (T4SS) that is assembled from 11 VirB proteins and the VirD4 protein is responsible for the transfer of T-DNA from *A. tumefaciens* into plant cells (Zupan *et al,* 1998; Deng and Nester, 1998). This T4SS apparatus has a pilus and a transmembrane complex for translocating the oncogenic T-DNA and effector proteins from the donor to recipient cells during the process of *Agrobacterium*-mediated transformation of host cells.

The 9.5 kb *virB* operon is the largest operon of the *vir* region and it encodes 11 proteins, VirB1 to VirB11, which are thought to be located in or transported to the *A. tumefaciens* inner membrane (Thompson *et al,* 1988; Ward *et al,* 1988; 1990; Kuldau *et al,* 1990; Shirasu *et al,* 1990). The proteins VirB2 through VirB11 are absolutely required for gene transfer and the efficient assembly of extracellular T pili, while VirB1 is an efficiency factor for T-complex transmembrane assembly (Berger and Christie, 1994; Fullner, 1998; Lai and Kado, 1998; Dale *et al,* 1993).

Sequence analysis has revealed that the N-terminus of VirB1 is predicted to contain motifs conserved among lysozymes and lytic transglycosylases, suggesting its

role as a putative lysozyme that can locally lyse the murein cell wall to create channels for transporter assembly (Mushegian *et al,* 1996; Baron *et al,* 1997a). This hypothesis is supported by the findings that mutants with deletion in the putative lysozyme motif were attenuated in virulence (Mushegian *et al,* 1996).

Processed from VirB1, VirB1* is a smaller protein that contains only the C-terminal 73 amino acids of VirB1 protein. This VirB1* protein is found to be secreted and loosely associated with the outer membrane. Coimmunoprecipitation analysis showed that VirB1* and VirB9 form a large complex (Baron *et al,* 1997b). These findings suggest that VirB1* may mediate pilus formation by stabilizing pilus-based contacts between *A. tumefaciens* and plant cells (Zupan *et al*, 1998).

Suggested to be the major structural component, the processed form of VirB2 proteins will form a pilus with VirB5 proteins, which function as essential protein stabilizers. This is the T-pilus which presumably promotes host-recipient interaction (Lai and Kado, 1998; Shirasu and Kado, 1993). Though they are not the structural components, VirB3 and VirB4 might be accessory pilus proteins that are required for pilus assembly (Jones *et al,* 1994; Shirasu *et al,* 1994; Dang and Christie, 1997; Dang *et al*, 1999).

Other than the T-pilus, a putative transmembrane apparatus or complex, possibly assembled from the other five VirB proteins (VirB6 to VirB10) is also an essential feature of T4SS (reviewed in Kado, 2000). Most of these proteins interact with one another and form various protein complexes.

Firmly embedded in the inner membrane with its five transmembrane regions, previous studies have suggested that the presence of VirB6 is required for the stability

of several other VirB proteins. Recently, it has been proven to localize to the cell poles and 5 proteins, VirB7 to VirB11, are required for its polar localization. When a conserved tryptophan residue at position 197 and the extreme C-terminus were altered or deleted respectively, mislocalization of the mutant VirB6 protein was observed, indicating their importance for the subcellular location of VirB6. Subsequent colocalization experiments showed that VirD4 colocalized to the same pole as that of VirB6, demonstrating that the two proteins are in close proximity and VirB6 is probably a component of the transport apparatus (Judd *et al*, 2005).

Aside VirB6, the core of the transfer apparatus is likely to be composed of VirB7-VirB9 heterodimers that are linked by a disulfide bridge and anchored in the outer membrane by lipid modification of VirB7. This VirB7-VirB9 heterodimer interacts, either directly or indirectly, with VirB10 and is shown to be required for the stability of VirB4, VirB8, VirB10 and VirB11 (Christie, 1997; Kado, 2000). Recently, VirB10 has been proposed to function as an energy sensor for the VirB/D4 T4SS, based on the findings that VirD4 and VirB11 ATP-binding subunits induce a structural transition in VirB10 that most probably is necessary for a late stage of machine biogenesis and, in turn, passage of substrate from the inner membrane to the cell surface (Cascales and Christie, 2004a; 2004b).

Purified VirB4 (Shirasu *et al,* 1994; Dang and Christie, 1997; Dang *et al,* 1999) and VirB11 (Christie *et al,* 1989; Rashkova *et al,* 1997) were shown to possess ATPase and this has reaffirmed the notion that export of T-DNA is an energy dependent process. Mutations in VirB4 ATPase have been shown to abolish the biogenesis of T-pilus and this clearly indicates that VirB4 promotes the production of

T-pilus and configures the transfer apparatus as a dedicated export machinery via an energy dependent mechanism.

As for VirB11, it has been postulated to function as chaperones to facilitate the movement of unfolded proteins and DNA substrates across the cytoplasmic membrane, by supplying energy for a possible gated secretion channel (Lai and Kado, 2000). Besides that, VirB11 was found to localize at the inner face of the cytoplasmic membrane independently of interactions with other VirB proteins. Analysis of mutants with defects in the nucleotide triphosphate binding pocket (Walker A motif) suggests that this membrane interaction is modulated by ATP binding or hydrolysis.

The third ATPase, VirD4 that is encoded by the *virD* operon, is also demonstrated to be essential for T-DNA transfer into plant cells because the VirD4 mutants showed complete inactivity in T-DNA transfer (Zupan *et al,* 1998). VirD4 is an inner membrane protein with two membrane spanning domains near its N-terminus, while both its N- and C-termini are cytoplasmic. The large cytoplasmic region of VirD4 contains a nucleotide-binding domain, and both the periplasmic and cytoplasmic domains are essential for substrate transfer. Although VirD4 is not required for T-pilus assembly, it is required for virulence and possibly plays a role as the coupling protein for the transfer of virulence factors (VirD2, VirE2, VirE3, VirF and T-DNA) to the membrane bound components of the type IV transporter by an energy dependent mechanism. It has been recently demonstrated to localize to the cell pole and a polar VirD4 –VirB complex of this kind is likely to function in substrate transfer from the cytoplasm (Pantoja *et al,* 2002; Kumar and Das, 2002).

By using a simple but sensitive and elegant TrIP (transfer-DNA immunoprecipitation) assay and by examining a variety of *vir* mutants, a temporal

order of proteins the T-DNA comes in contact with as it journeys through the T4SS has been established (Cascales and Christie, 2004a; Lybarger and Sandkvist, 2004). Based on this latest research finding and the existing literature, a model of bacterial DNA transfer with unprecedented detail has been proposed. Fig. 1.2 depicts the possible subcellular locations and interactions of various VirB/D4 components involved in T-DNA translocation. In the postulated pathway, the T-DNA first binds the VirD4 receptor and thereafter forms close contacts with the VirB11 ATPase, the VirB6 and VirB8 inner membrane (IM) subunits before its final interactions with VirB2 and VirB9 localized in the periplasm and outer membrane (OM). As for the remaining VirB subunits that do not form detectable contacts with the translocating substrate, VirB4 coordinates substrate transfer to the VirB6 and VirB8 subunits, whereas VirB3, VirB5, and VirB10 promote transfer from VirB6 and VirB8 to the VirB2 and VirB9 subunits (Cascales and Christie, 2004a; 2004b).

Though the assembly and functions of some of the components of VirB/D4 T4SS are better understood now, further investigations are still necessary to elucidate the detailed mechanism of assembly and function of this T4SS, especially on how the interplay of various subunits and other factors involved in these processes can bring about the efficient translocation of T-DNA and/or other substrates from *A. tumefaciens* to its host cells.

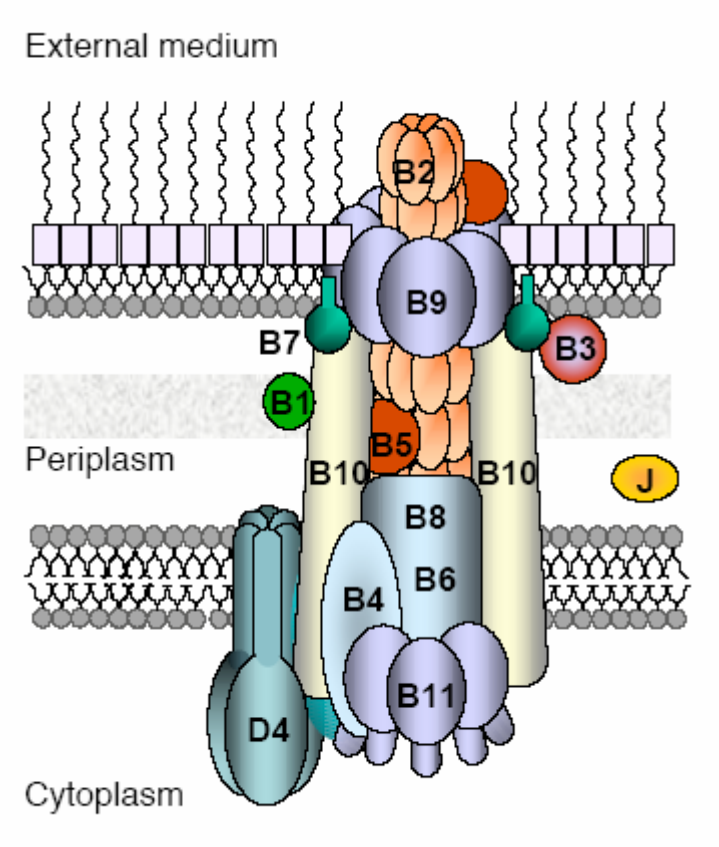


Fig. 1.2. A model depicting the subcellular locations and interactions of the VirB and VirD4 subunits of the *A. tumefaciens* VirB/D4 T4SS. The VirD4 coupling protein assembles as a homohexameric, F1-ATPase-like structure juxtaposed to the VirB channel complex. VirB11, a hexameric ATPase structurally similar to members of the AAA ATPase superfamily, is positioned at the cytoplasmic face of the channel entrance, possibly directing substrate transfer through a VirB6/VirB8 inner membrane (IM) channel. The VirB2 pilin and VirB9 comprise channel subunits to mediate substrate transfer to and across the outer membrane (OM). VirB10 regulates substrate transfer by linking IM and OM VirB subcomplexes.

(Cited from Cascales and Christie, 2004a)

During the interaction with their hosts, many animal pathogens also employ T4SS and these include *Bartonella henselae*, *Bordetella pertussis*, *Brucella abortus*, *Brucella suis*, *Helicobacter pylori*, *Legionella pneumophila* and *Rickettsia prowazekii*. In these pathogens, proteins homologous to the subunits of *A. tumefaciens* VirB/VirD4 system can be found. In these mammalian pathogens, the T4SS is required for the delivery of pathogenesis-related effector proteins and other molecules as well as for intracellular survival. But in the case of *A. tumefaciens*, both the T-strand and its associated proteins are transferred into the plant cells through this T4SS.

In any of the conjugal transfer systems found in the pathogens mentioned above, the precise role of the pilus and the transport complex in substrate transfer still remains elusive. But in the case of *A. tumefaciens* T-pilus, several plausible functions have been assigned to it (Hwang and Gelvin, 2004; Gelvin, 2003). First and most possibly, the T-pilus could serve as a conduit for export of several components needed for virulence, including T-pilin subunits, VirE2, VirE3, VirF proteins, and single stranded T-DNA that is piloted by the covalently linked VirD2 protein.

Secondly, 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 some other transfer apparatus (Lai and Kado, 2000; Kado, 2000). Based on the research findings, the T-pilus has been proposed to retract and subsequently draw the bacterial cell into sufficiently close contact with the host cell to permit the transfer of T-DNA and Vir proteins to the recipient cell.

Thirdly, the T-pilus could also serve as a sensor for potential mating-signal molecules from the host cell as plant cells may have a receptor for the T-pilus or a pore for T-DNA transfer through the plant cell wall and plasma membrane.

In order to firmly assess and ascertain these ascribed functions of T-pilus and that of the VirB/D4 transport complex for efficient *Agrobacterium*-mediated transformation of host cells, further studies are needed.

1.2.4. VirF

VirF is a 23-kDa protein that is encoded by a gene presents in only the *vir* region of the octopine-type Ti-plasmid but absent from the nopaline-type Ti-plasmid (Melchers *et al,* 1990; Schrammeijer *et al,* 1998). It is originally ascribed a role as the host range determinant, because the presence of *virF* gene on the octopine-type Ti-plasmid made *Nicotiana glauca* susceptible to the infection by *A. tumefaciens virF* mutants.

Besides VirD2, VirE2 and VirE3, VirF is another Vir protein that is exported to the plant cells from *A. tumefaciens.* The transport of VirF from *A. tumefaciens* into the plant cells is depended on the VirB/D4 transport system. The C-terminal amino acid motif Arg-Pro-Arg, which is also present on the VirE2 molecule, is thought to be the export signal that can be recognized by the VirB/D4 secretion system.

VirF might function in the plant cells because *virF* mutant strain can be complemented by the expression of the *virF* gene in the plant host cells. The results from yeast two-hybrid experiment suggest that VirF is the first prokaryotic protein with an F box, by which it can interact with the plant homologue of Skp1 protein of the yeast. Since Skp1 proteins are part of the complexes involved in targeted proteolysis and are thought to regulate the cells into S phase, it is suggested that VirF might help in stimulating the plant cells to divide and become more susceptible to transformation by *A. tumefaciens* (Schrammeijer *et al,* 2001).

1.2.5. VirJ

virJ lies between *virA* and *virB* in the *vir* region of an octopine-type Ti-plasmid, but it is not found in the nopaline-type Ti-plasmid, pTiC58 (Pan *et al,* 1995; Kalogeraki and Winans, 1995). VirJ protein shares about 50% identity in its amino acid sequence with the chromosomally encoded protein, AcvB, which could be found in both octopine-type and nopaline-type strains. The homologous region lies in the C-terminal half of AcvB. However, the *virJ* gene contains a putative *vir* box and can be induced in a VirA/VirG dependent fashion by the *vir* gene inducer acetosyringone which has no effect on *acvB*.

Currently, the exact role of VirJ and that of AcvB in tumorigenesis are still not clear. It has been shown that either VirJ or AcvB is required for the transfer of T-DNA from *A. tumefaciens* into the plant cells (Pan *et al,* 1995). The two proteins share at least some degree of functional similarity because *virJ* could heterologously complement an *acvB* mutation in the tumorigenesis of *A. tumefaciens* on plant wound sites. Though both proteins did not affect the attachment of *A. tumefaciens* to plant cells, agroinfection experiments had proven that VirJ or AcvB might be required for the T-DNA transfer (Pan *et al,* 1995).

It has also been reported that AcvB might play a role in virulence by influencing the formation of the pili (Parimal *et al,* 1999). In this report, AcvB is thought to be a single stranded DNA binding protein that could interact with the T-strand and assist in the export of T-DNA from the bacteria to the host cells. If proven correct, this model would explain how the T-strand could be transferred from *A. tumefaciens* as a T-strand/protein complex independent of VirE2.

1.2.6. VirH

With the aid of an electron microscope, a Ti-plasmid converged genetic locus was identified at the left end of known *vir* genes. This locus flanks an operon designated as *virH*. The *virH* operon contains two genes that resemble P-450-type monooxygenases (Kalogeraki and Winans, 1998), which may detoxify plant defense compounds and allow the bacteria to survive in the presence of bacteriocidal or bacteriostatic plant compounds. Since VirH1 and VirH2 are homologous to each other, it seems plausible that they could be functionally redundant. The role of VirH in plant–microbe interaction requires additional studies before their roles can be accurately assessed.

1.2.7. Other genes on Ti plasmid

In addition to the *vir* genes and their protein products described above, there are some other gene loci on the Ti plasmid besides *vir* genes. Some of them confer ancillary functions in tumor formation, such as inter-bacterial conjugation genes and vegetative replication genes. The inter-bacterial conjugation genes, which include *oriT*, *traAFB* and *trbB*, are involved in controlling the conjugative transfer of Ti-plasmid. On the other hand, the vegetative replication genes, which include *rep*AB and *repC*, are involved in controlling the replication and partition of Ti-plasmid.

Some of the T-DNA genes, which direct the production of plant growth hormones in plants, also affect tumor morphology and physiology. Interestingly, the non-transcribed regions of these genes possess many features of plant genes, including typical eukaryotic TATA and CAAT boxes, transcriptional enhancers and poly (A) sites. These genes include *iaaM* (also called *aux1, tms1*), *iaaH* (also called *aux2, tms2*) and *ipt* (also called *cyt, tmr*), which encode enzymes catalyzing the synthesis of

auxin and cytokinin respectively. Added to that, the gene *ons* (or *6a*) controls octopine and nopaline export from plant cells, while *tml* (or *6b*) increases the sensitivity of plant cells to phytohormones (Kado, 1991; Sheng and Citovsky, 1996; Winans *et al,* 1986; 1989).

1.2.8. Chromosomal virulence genes

Apart from *vir* genes encoded by the Ti-plasmid, chromosomal virulence (*chv*) genes have also been shown to play important roles in tumorigenesis (Gelvin, 2000; Zhu *et al,* 2000; Zupan *et al,* 2000; Liu *et al*, 2001). Unlike the virulence genes on the Ti-plasmid, the functions of chromosomal virulence genes have not been well elucidated, as the pleiotropic functions of these genes make it difficult to assess their precise roles in tumorigenesis.

chv genes exert their functions mainly in the events of bacterial attachment to the plant cell wall, the promotion of growth efficiency in wound site on the plant and the regulation of virulence genes on the Ti plasmid during the early stages of infection (Sheng and Citovsky, 1996; Zupan and Zambryski, 1997). In contrast to those *vir* genes on the Ti-plasmid, which are dedicated solely to specific steps in the interaction of *A. tumefaciens* with its host, the chromosomal virulence genes exert their functions by regulating the general physiology of *A. tumefaciens* and have been conscripted to play ancillary but significant roles in the interaction of the bacterium with its host plants.

The best understood chromosomal virulence gene is *chvE.* It was shown to play important roles in the sugar enhancement of *vir* gene induction and in bacterial chemotaxis, as mutants at this locus displayed a strongly attenuated *vir* gene induction and limited host range. *chvE* codes for a periplasmic glucose-galactose binding

protein, which is required in the VirA/VirG two-component regulatory system (Winans *et al,* 1994; Doty *et al,* 1996). This protein can sense monosaccharides in the environment and then interact with the periplasmic domain of VirA, a requirement for maximal activation of VirG and the subsequent activation of all Ti-plasmid encoded *vir* genes.

Characterizing the beginning of infection is chemotaxis and attachment of *A. tumefaciens* to wounded plants (Vande Brock and Vanderleyden, 1995). Genetic studies have shown that non-attaching mutants could not cause tumors on plants and that this attachment to the plant cells is a two-step process. The first step involves a cell-associated acetylated and acidic capsular polysaccharide and this step is reversible because vortexing or washing with a stream of water could dislodge the bacteria. *attR* encodes a transacetylase, which is required for the synthesis of this polysaccharide. *attR* mutants that could not synthesize the acetylated polysaccharide were found to be avirulent and could not attach to carrot suspension cells (Matthysse and McMahan, 1998).

In the second step of attachment, the cellulose fibrils are elaborated by the bacterium, causing a large number of bacteria to colonize at the wound surface (Matthysse and McMahan, 1998). Three chromosomal virulence genes, *chvA, chvB*, and *pscA* (*exoC*), are required for this process and they are involved in either the synthesis (*chvB* and *pscA*) or export (*chvA*) of cyclic ß-1,2-glucans and other sugars into the periplasm (Uttaro *et al,* 1990; Thomashow *et al,* 1987; O'Connell and Handelsman, 1989; Kamoun *et al,* 1989) and may be involved indirectly in bacterial attachment by an unknown mechanism. *chvA* or *chvB* mutants were found to have lost the ability to attach to the host cells and to cause tumor formation under normal

inoculation conditions (Douglas, 1982; 1985). Interestingly, *chvB* mutants could partially regain virulence if the bacteria were grown and inoculated at 19°C, a lower culture and cocultivation temperature.

chvH is a recently characterized chromosomal gene that encodes a homologue of an elongation factor P (*efp*) involved in protein synthesis (Peng *et al,* 2001). This gene is present as a single copy in *A. tumefaciens* and is important but not essential for the growth of *A. tumefaciens*. The *chvH* mutant A6880 is an avirulent, pleiotropic mutant which is more sensitive to detergents such as SDS and acidic pH than its parent strain, suggesting that the integrity of the outer membrane is impaired. In *E. coli*, the elongation factor P can increase the efficiency of formation of peptide bonds involving aminoacyl acceptors that bind poorly to the ribosome in its absence (Glick 1980). Since heterologous complementation of *chvH* mutation in *A. tumefaciens* could be achieved by the expression of the *efp* gene of *E. coli, chvH* and *efp* are probably functionally homologous.

Functioning as an elongation factor protein, ChvH exerts its roles at the posttranscriptional level. And the avirulence of the *chvH* mutant is due to the low level expression of key proteins required for T-DNA transfer such as VirB, VirE2 and VirG, though the possibility that the *chvH* gene product may contribute in other ways to tumorigenesis cannot be ruled out. Other studies showed that wild-type *chvH* locus is essential not only for full expression of *vir* genes encoded by the Ti-plasmid but also for that of some chromosomal genes. These genes might code for particular sequences of amino acids, perhaps near the start of translation, which are exceptionally dependent on elongation factor P for translation.

In addition, some other genomic genes such as c*hvD*, *ros* and *miaA* are also involved in virulence (Gray *et al,* 1992; D'souza-Ault *et al,* 1993). *chvD* encodes an ABC transporter homologue that plays important roles in the virulence regulatory pathway. Findings from Liu *et al.* (2001) have indicated that ChvD controlled the virulence genes by affecting the *virG* expression and *A. tumefaciens* strains carrying a mutant *chvD* gene showed greatly attenuated virulence and *vir* gene expression, while constitutive expression of *virG* in the same strain restored the virulence. Furthermore, even though the interaction between VirB8 and ChvD has been verified by using yeast two-hybrid screening, the biological relevance of this interaction still remains a puzzle.

ros encodes a 15.5-kDa C2H2 zinc finger protein that represses the expression of *virC* and *virD* (Cooley *et al,* 1991) and a plant oncogene *ipt*, whose promoter contains typical TATA boxes and is regulated by eukaryotic transcriptional machinery in the host plant (Chou *et al,* 1998). C2H2 zinc finger proteins are a large superfamily of eukaryotic transcription factors that are originally thought to occur exclusively in eukaryotes. Phylogenetically, *ros* is distantly related to eukaryotic zinc finger regulators.

Some *chv* genes have counterparts in other bacteria that are associated pericelluar or intracellularly with animals and plants, either as pathogens or as endosymbionts. A good example is the *chvG/chvI* genes that have been under extensive studies and are found widely in the chromosomal loci of many organisms such as *A. tumefaciens*, *Brucella abortus* and *Sinorhizobium meliloti* (Sola-Landa *et al,* 1998; Galibert *et al,* 2001). These genes are found to be required for establishing a successful relationship between the bacteria and their hosts and sequence analysis of the 16S rRNA gene showed that these genera all belong to the same α-2 subdivision of the proteobacteria.

Based on the amino acid sequence analysis, the *chvG*/*chvI* genes encode a two-component signal transduction system. Once bacteria are internalized into plant or animal cells, it is quite likely that they will encounter an acidic pH environment within the vesicles containing them. Sensing the acidity appears to be important for *A. tumefaciens* to cope with the environment in plants and to cause tumors on these plants. ChvG is proposed to be a histidine protein kinase that might act as the sensor to directly or indirectly sense extracellular acidity, while ChvI is suggested to be the response regulator. Site-specific insertion mutations in either *chvG* or *chvI* have been shown to make *A. tumefaciens* avirulent (Charles and Nester, 1993).

Recently, our lab has identified two additional chromosomal genes, *katA* and *aop*B, both of which are participants in *A. tumefaciens* tumorigenesis (Xu and Pan, 2000; Jia *et al,* 2002). *katA* encodes a catalase that is involved in the dismutation of hydrogen peroxide to water and oxygen (Xu and Pan, 2000). Mutation in this gene highly attenuates the bacterial ability to cause tumors on plants and tolerate H_2O_2, but not the bacterial viability in the absence of exogenous H_2O_2. Further research showed that mutation at *katA* caused a 10-fold increase of the intracellular H_2O_2 concentration in the bacteria grown on an acidic medium (Xu *et al,* 2001). These suggest that during the *Agrobacterium*-plant interaction, plants might produce an H_2O_2 burst and KatA would serve to detoxify the H_2O_2 released by plant cells. This process will protect the bacterial cells against the damage caused by reactive oxygen species to cellular components, including nucleic acids, proteins and cell membranes (Imlay and Linn, 1988; Storz and Imlay, 1999).

Even though *A. tumefaciens* does not elicit a typical hypersensitive response (HR) on its host plants, unlike other plant pathogens (Staskawicz *et al,* 1995; Deng *et al,*

1995), it might still trigger some other kinds of plant defense response when it interacts with the host cell, such as the H_2O_2 burst. Thus, proteins like KatA are quite likely to be involved in the *Agrobacterium*-plant interaction, in order to ensure a higher chance of successful transformation.

Another chromosomal gene, *aopB,* is homologous to a *Rhizobium* gene encoding an outer membrane protein and when this gene is mutated, the bacterial ability to form tumors on plants is very much attenuated (Jia *et al,* 2002). Interestingly, the expression of this gene required the wild type ChvG/ChvI two-component system. Further research is needed before the roles of AopB and other membrane proteins in *Agrobacterium*-mediated plant transformation can be established.

1.2.8. Summary of roles of *A. tumefaciens* virulence genes

The functions of *A. tumefaciens* virulence proteins, both Ti-plasmid encoded and chromosomally encoded, can be summarized as shown in Table 1-1. Although the roles of these virulence factors in plant cell transformation have been relatively and progressively well understood, the mechanisms of the transport or the pathways some of these factors take to achieve their functional roles still remain unclear. For instance, whether the binding of VirE2 to T-strand occurs in the bacterium (Christie *et al,* 1988) or in the plant cell (Binns *et al,* 1995; Sundberg *et al,* 1996) still remains a much debated topic. What is certain is that mutations in the *virA*, *B*, *D*, *E* and *G* loci result in avirulence, whereas mutations in *virC* causes attenuated virulence (Yanofsky *et al,* 1985; Horsch *et al,* 1986). Some members of *vir* operon, such as *virJ*, *F*, *H* and *E3*, are required for tumorigenesis in specific instead of all hosts or play other roles in pathogenesis.

Results from recent studies showed that the exported virulence proteins, VirD2, VirE2 and VirF, could also be exported from bacterial cells by a specific pathway independent of VirB/D4 (Chen *et al,* 2000). Although the precise biological function of this process is still not clearly addressed, it suggests that the transfer of the T-DNA from *A. tumefaciens* may take place in two steps, with the first step mediated by an unidentified pathway and the second step by the virB/D4 system (Chen *et al,* 2000).

The fact that *A. tumefaciens* possesses genes which are not only typically eukaryotic genes with eukaryotic expression signals but also prokaryotic genes coding for proteins with eukaryotic features, such as the nuclear localization sequences (VirD2, VirE2 and VirE3), the F box (VirF) and eukaryotic promoter (*iaaM* and *iaaH*), infers that *A. tumefaciens* is not a typical pathogenic bacterium but a sophisticated manipulator of its environment (Valentine, 2003). It is also perhaps for this reason and the plasticity of these genes to function in various eukaryotic cells that *A. tumefaciens* can interact with diversely different host cells such as plant cells, yeast and mammalian cells and transform them for its own benefits.

Table 1-1. Functions of *A. tumefaciens* virulence proteins involved in plant transformation.*

Virulence protein	Function(s) in *A. tumefaciens* and/or plant cells [#]
Ti plasmid encoded virulence proteins	
VirA	Phenolic sensor of VirA/VirG two-component regulatory system
VirG	Phenolic response regulator of VirA/VirG two-component regulatory system
VirB1-11	Synthesis and assembly of T-pilus and transmembrane apparatus
VirB1	Transglycosylase
VirB1*	Bacterial-host cell contact
VirB2	Cyclic T-pilin subunit; cell contact
VirB3	Minor component of T-pilus; requires VirB4 for stability
VirB4	ATPase; VirB4–VirB4 self-association; transport activation
VirB5	Probable chaperone; minor component of T-pilus as stabilizer
VirB6	Component of transport apparatus; candidate pore former
VirB7	Lipoprotein; VirB7–VirB7 homodimer; VirB7–VirB9 heterodimer required for stability of VirB4, VirB9, VirB10 and VirB11; probable chaperone
VirB8	VirB8–VirB8, VirB8–VirB9 and VirB8–VirB10 interactions
VirB9	VirB9–VirB9, VirB8–VirB9, and VirB9–VirB10 interactions
VirB10	VirB10–VirB10, VirB10–VirB8, and VirB10–VirB11 interactions; thermostability; energy sensor
VirB11	ATPase; VirB11–VirB11 self association; transport activation
VirC1	Putative "overdrive" binding protein; enhancement of T-strand formation
VirD1	Required for T-DNA processing *in vivo* and for double stranded T-DNA border nicking *in vitro*
VirD2	1. T-border specific endonuclease 2. Putative "pilot protein" that leads T-strand through transfer apparatus into the plant cell 3. Nuclear targeting of the T-strand 4. Protection of T-strand from 5' exonucleolytic degradation 5. T-strand integration into the plant genome
VirD4	ATPase; coupling protein for the transfer of virulence factors to VirB channel

VirE1	Association with VirE2; may be needed for VirE2 export from *A. tumefaciens*
VirE2	1. Binding to T-strand to form T-complex
	2. Protection of T-strand from nucleolytic degradation
	3. Nuclear targeting of the T-strand
	4. Passage of T-strand through the nuclear pore complex
VirE3	'Adaptor' between VirE2 and karyopherin α; facilitation of nuclear import of VirE2 and T-complex
VirF	Host range factor; interaction with Skp1 proteins to regulate plant cell division
VirH (PinF)	Putative cytochrome P450 enzyme; detoxification of toxic plant compounds
VirJ/AcvB	Putative T-strand binding protein; T-strand export from *A. tumefaciens*
Chromosomally encoded virulence factors	
AttR	Transacetylase; required for synthesis of a capsular polysaccharide involved in host cell attachment
ChvA	Export of cyclic ß-1,2-glucans and sugars involved in host cell attachment
ChvB	Synthesis of cyclic ß-1,2-glucans and sugars involved in host cell attachment
PscA (ExoC)	Synthesis of cyclic ß-1,2-glucans and sugars involved in host cell attachment
ChvD	Control of virulence genes by affecting *virG* expression
ChvE	Sensing of monosaccharides and interaction with VirA periplasmic domain
ChvH	A homologue of elongation factor P; pleiotropic effect on tumorigenesis
ChvG	Acidity sensor of ChvG/ChvI two-component regulatory system
ChvI	Acidity response regulator of ChvG/ChvI two-component regulatory system
Ros	Repression of *virC* , *virD* and *ipt* expression
KatA	Detoxification of H_2O_2 released by plant cells
AopB	Outer membrane protein; required for tumorigenesis

*Adapted and modified from Gelvin, 2000

References to these functions can be found in Gelvin, 2000 and in the subsections of Section 1.2 from 1.2.1 to 1.2.7.

1.3. Plant genes involved in *Agrobacterium*-mediated transformation

From the perspective of host-pathogen interaction, *A. tumefaciens* infection represents a major physiological, biochemical and genetic challenge to the host plant. Based on the research findings from the past three decades, the molecular events that occur within the bacterium during this process are partially understood. However, little is known about the plant genes and their encoded factors that are involved in the tumorigenesis. In recent years, some plant factors that are involved in this process are gradually being discovered (reviewed in Tzfira and Citovsky, 2002).

Even though it does not induce the hypersensitive response in plants, *A. tumefaciens* can trigger changes in the gene expression patterns by inducing or repressing specific sets of plant genes. By using cDNA amplified fragment length polymorphism (AFLP) technique, four plant genes whose expression were uniquely regulated by *A. tumefaciens* infection have been identified (Ditt *et al*, 2001). One of them encodes a nodulin-like protein belonging to a class of proteins induced in the root nodules of *Rhizobium*-infected plants and might be involved in cell division and differentiation, while another one encodes a lectin-like protein kinase, which has been proposed to play a role in cell-to-cell recognition. Both genes might play putative roles in plant signal transduction and defense response, indicating that *A. tumefaciens* and *Rhizobium* might elicit similar changes in gene expression in their host cells.

By adopting combinatorial approaches of suppressive subtractive hybridization, macroarray and RNA blot analyses, Veena *et al.* (2003) have identified numerous genes that were differentially expressed during the early stages of *Agrobacterium*-mediated transformation of tobacco BY-2 cells. Genes that were differentially expressed include those involved in defense responses, cell division and growth,

chaperones, as well as primary and secondary metabolism. Their findings indicate that *A. tumefaciens* infection has induced the expression of plant genes necessary for the transformation process while simultaneously repressing host defense response genes. The expression profiles strongly suggest that *A. tumefaciens* is capable of successfully utilizing existing host cellular machinery for its genetic transformation purposes.

The various steps in which plant factors are likely to be involved in the *Agrobacterium*-mediated transformation process include (1) bacterial attachment to the plant cell surface; (2) transfer of T-DNA from the bacteria to plant cells across the plant cell wall and membrane; (3) nuclear localization of T-complex and (4) stable integration of T-DNA into the plant genome. In the following sections, the plant factors involved in these steps are described in details.

1.3.1. Plant factors involved in bacterial attachment to the plant cell surface

For efficient *A. tumefaciens* infection, it generally requires wounding and/or a rapidly dividing cell suspension culture. In the absence of a wound site, the efficiency of such infection is low. Earlier experiments showed that the bacterial attachment to the plant cell was inhibited when the plant cell surface was treated with various proteinases (Wagner and Matthysse, 1992; Swart *et al,* 1994), suggesting that some cell wall materials present on the cell surface might play a role in *A. tumefaciens* attachment.

Research in this direction has given rise to two plant cell wall proteins, a vitronectin-like protein and a rhicadhesin-binding protein, which might mediate such bacterial attachment. Since many pathogenic bacteria utilized vitronectin as a specific receptor in their interactions with their animal hosts, it is quite likely that plant vitronectin-like protein might also be required for *A. tumefaciens* binding.

Indeed, among the 21 identified *Arabidopsis rat* mutants, which are resistant to *A. tumefaciens* transformation, *rat1* and *rat3* have been shown to be blocked at the early steps in *Agrobacterium*-mediated transformation and microscopic analysis revealed that these ecotypes are deficient in the binding of *A. tumefaciens* to their roots under various incubation conditions (Nam *et al*, 1998). DNA sequence analysis has subsequently revealed that *rat1* encodes an arabinogalactan protein (AGP) and *rat3* encodes a small protein that is a potential cell-wall protein. The involvement of AGPs in *Agrobacterium*-mediated transformation was then confirmed by using ß-glucosyl Yariv reagent, which binds AGPs specifically. When *Arabidopsis* root segments were incubated with an active Yariv reagent prior to inoculation with *A. tumefaciens*, transformation was blocked. This result was verified with control experiments which indicated that ß-glucosyl Yariv reagent did not affect the viability of *Arabidopsis* root segments or *A. tumefaciens* cells.

1.3.2. Plant factors involved in the export of T-DNA

For the translocation of T-DNA from *A. tumefaciens* into the plant cell through a type-IV secretion system, the assembly of the virulence pilus and the transporter complex might not be sufficient to initiate such export. Physical contact with the recipient plant cell might be required to activate the transport machinery, suggesting that unidentified host factors are most probably required for the export of T-DNA and Vir proteins into the plant cells.

Recently, three VirB2-interacting proteins (BTI), BTI1, BTI2 and BTI3, and a membrane-associated GTPase, AtRAB8, were identified from an *Arabidopsis thaliana* cDNA library via yeast two-hybrid system (Hwang and Gelvin, 2004). Besides their interaction with VirB2, these three related BTI proteins were found to interact with one

another and with AtRAB8 *in vitro* and when pre-incubated with 100 μg/ml of GST-BTI1 protein, the ability of *A. tumefaciens* to transform *Arabidopsis* suspension cells was decreased by about 25%. This suggests that a competitive binding of GST-BTI1 to VirB2, which is the major component of T-pilus, has decreased the number of available T-pili for interaction with *Arabidopsis* cells.

Apart from this, transgenic *Arabidopsis* plants with disrupted BTI and AtRAB8 expression (via antisense or RNAi constructs) have been shown to be less susceptible to transformation by *A. tumefaciens*, whereas overexpression of BTI1 protein in transgenic *Arabidopsis* has given rise to plants that are hyper-susceptible to *Agrobacterium*-mediated transformation. When *BTI1* was inserted with T-DNA through mutagenesis, reduced levels of *Agrobacterium*-mediated root transformation were observed in the mutant *Arabidopsis* plants. All these results demonstrate the functional significance of these plant factors in transformation.

Further results have shown that the level of BTI1 protein is transiently increased immediately after *A. tumefaciens* infection, and confocal microscopic data have indicated that GFP tagged BTI proteins preferentially localize to the periphery of root cells in transgenic *Arabidopsis* plants. All these suggest that BTI proteins may contact the *A. tumefaciens* T-pilus and it is quite likely that they are essential in assisting the export of T-DNA due to their interaction with VirB2.

Sequence analysis has revealed that the three BTI proteins contain a C-terminal 150 to 201 amino acid reticulon homology domain comprising of two large hydrophobic regions separated by a 66 amino acid loop. Based on the reticulon domain present in their C termini, 15 reticulon-like proteins are found in *Arabidopsis* and BTI1, BTI2, and BTI3 are found to correspond to RTNLB1, RTNLB2, and RTNLB4

(Oertle *et al*, 2003). Though the functions of RTN are unknown, more than 250 reticulon-like (RTNL) genes were identified in divergent eukaryotes, fungi, plants, and animals (Oertle and Schwab, 2003; Oertle *et al*, 2003). It is probable these genes encode protein factors that interact with VirB2 or T-pilus components during *Agrobacterium*-mediated transformation of these eukaryotic cells.

As for AtRAB8, little is known about its functions in plant cells. Previous studies have suggested that AtRAB8 is similar to RAB8 and RAB10 of mammals, to Ypt2 of the fission yeast *Schizosaccharomyces pombe* and to Sec4 of the budding yeast *Saccharomyces cerevisiae* (Haubruck *et al*, 1990; Rutherford and Moore, 2002). Sec4 is essential for post-Golgi events in yeast secretion, while Rab8 regulates transport from the trans-Golgi network to the basolateral plasma membrane in epithelial cells and to the dendritic plasma membrane in cultured hippocampal neurons (Huber *et al*, 1993). Overall, this class of Rab proteins is found to be membrane associated proteins that modulate tubulovesicular trafficking between compartments of the biosynthetic and endocytic pathways (Olkkonen and Stenmark, 1997; Martinez and Goud, 1998; Schimmoller *et al*, 1998; Moyer and Balch, 2001).

It is probable that during *Agrobacterium*-mediated transformation of these eukaryotic cells, AtRAB8 and its homologues in these cells might be utilized by *A. tumefaciens* to interfere with the membrane trafficking pathways for its own purposes. Further characterization of BTI and AtRAB8 proteins and those yet unidentified proteins that potentially interact with T-pilus components will provide information on how T-DNA is transferred from *A. tumefaciens* into plant cells and how the *A. tumefaciens* T-pilus contacts the plant cell surface.

1.3.3. Plant factors necessary for nuclear localization of T-complex

After translocation, the entry of T-complex into the host cell nucleus is the central event in the genetic transformation of plants by *A. tumefaciens*. A number of proteins from plant cells have been identified to bind with VirD2 or VirE2, which are integral subunits of the T-complex. These plant factors are probably intrinsic plant proteins with their own cellular functions that have been 'hijacked' for the delivery or targeting of the T-complex into the plant cell nucleus.

With the use of yeast two-hybrid system (Golemis *et al,* 1994), several plant-encoded proteins have been identified to interact with VirD2 nuclear localization sequence (NLS). This includes an *Arabidopsis* karyopherin α (AtKAPα, importin-α1) protein that specifically binds to the NLS of VirD2 (Ballas and Citovsky, 1997). In other species, importin-α has been shown to bind to NLS regions of karyophilic proteins to assist their nuclear targeting (Gorlich and Mattaj, 1996; Catimel *et al,* 2001). Using a similar approach, a tomato DIG3 cDNA clone that encodes an enzymatically active type 2C serine/threonine protein phosphatase (PP2C) was shown to interact with VirD2 (Tao *et* al, 2004; Gelvin, 2000). Co-electroporation of GUS (β-glucuronidase)-VirD2 NLS gene together with PP2C protein has resulted in the cytoplasmic localization of GUS in the majority of tobacco BY-2 cells and overexpression of PP2C has been shown to enhance nuclear localization.

Apart from AtKAPα and PP2C, Arabidopsis cyclophilins, RocA, Roc4 and CypA, have been shown to interact with VirD2 as well (Deng *et al*, 1998). The findings have shown that when VirD2-cyclophilins interaction was disrupted by an inhibitor, cyclosporin A, *Agrobacterium*-mediated transformations of *Arabidopsis* and tobacco were inhibited. Also, it has been found that these cyclophilins did not interact

with VirD2 NLS domain and the VirD2 domain interacting with these cyclophilins is distinct from the endonuclease, omega and the NLS domains. Since some cyclophilins possess peptidyl-prolyl isomerase activity, it has been hypothesized that this protein might serve as a chaperone for VirD2 during the T-strand trafficking in the plant cell.

Besides those VirD2 interacting proteins, a VirE2 interacting protein from *Arabidopsis*, VIP1, was identified. VIP1 has been implicated to be required for the nuclear import of VirE2 and tumorigenesis of *A. tumefaciens* during the early stages of T-DNA gene expression (Tzfira *et al,* 2001). This plant protein contains a β-ZIP motif made up a long basic domain followed by a leucine zipper, which is composed of seven leucine repeats evenly separated from each other by six amino acid residues. When disrupted by antisense approach, the *VIP1* antisense plants were shown to be resistant to *A. tumefaciens* induced tumor formation, suggesting that this plant factor plays a critical role in this process. By conducting a recently developed genetic assay for nuclear import and export (Rhee *et al,* 2000), VIP1 was shown to facilitate the transport of VirE2 into the nuclei of yeast and mammalian cells and participate in the early stages of T-DNA expression (Tzfira *et al,* 2001).

Other than VIP1, VIP2 was also demonstrated to interact with VirE2 and VIP1 but had no effect on intracellular localization of VirE2 when co-expressed in yeast or mammalian cells. In order to define the precise role of this protein in nuclear localization in plant cells, new nuclear import system using purified plant nuclei and fractionated cellular extracts has to be developed (Gelvin, 2000).

1.3.4. Plant factors involved in T-DNA integration

T-DNA does not have to been integrated into the plant genome before the genes between the T-borders are expressed. As such, transient expression of reporter genes has been used to assay for the efficiency of T-DNA transfer in many experiments. It is perhaps for the same reason that some *A. tumefaciens* cocultivated plant cells or tissues with high transient expression of reporter genes have actual low transformation efficiency or are sometimes recalcitrant to transformation to the extent that transgenic plants cannot be regenerated from these cocultivated samples.

The roles of plant proteins in the T-DNA integration process are only beginning to be defined recently. After inoculation by *A. tumefaciens*, one of the *Arabidopsis rat* mutants (*rat5*) was found to be deficient in T-DNA integration, despite the observation that T-DNA encoded reporter gene was expressed in the plant cells. Subsequent genetic analysis showed that *rat5* contains two tandem copies of T-DNA integrated into the 3' untranslated region of a histone H2A gene and complementation of the *rat5* mutant with histone H2A gene resulted in restored tumorigenesis phenotype. When overexpressed in plants, H2A has been shown to increase the susceptibility of these plants to transformation. The histone H2A genes comprise a small multigene family in *Arabidopsis* and histone H2A might potentially specify the conformation at the T-DNA integration site (Mysore *et al*, 2000). To understand the exact mechanism of the involvement of histone H2A and other still unidentified factors in T-DNA integration, further investigations are necessary to shed light on how this process occurs within the plant cell nucleus.

1.3.5. Summary of roles of plant genes involved in transformation

The roles and possible functions of the various plant genes encoded factors that are involved in *Agrobacterium*-mediated transformation can be summarized in Table 1-2 and in Fig. 1.3. The functions of some of these factors require further studies and characterization before their roles can be ascertained. Unlike the *A. tumefaciens* virulence factors which have been relatively well characterized, the studies of their host cellular partners are just beginning. Identification and characterization of these host factors will lead to a better understanding of basic biological processes such as cell communication, intracellular transport and DNA repair and recombination, because it is highly probable that *A. tumefaciens* has adapted these and other existing cellular processes for its own purposes of genetic transformation (Tzfira and Citovsky, 2002).

As a genetic engineering tool, it is perceivable that the modulation of these host factors may be the next step forward in increasing the transformation efficiency by *A. tumefaciens* and in expanding the host range to those recalcitrant species, especially many of those agronomically important species that still cannot be transformed by *A. tumefaciens*.

Table 1-2. Functions of plant factors involved in various steps of *Agrobacterium*-mediated transformation [†]

Plant factor/mutant	Roles and possible functions [#]
Bacterial recognition and attachment	
Vitronectin	Binding of *A. tumefaciens* to host cells
Rhicadhesin-binding protein	Binding of *A. tumefaciens* adhesion protein
Arabidopsis rat1 mutant (arabinogalactan protein)	Mutant does not bind to *A. tumefaciens*
Arabidopsis rat3 mutant (putative cell-wall protein)	Mutant does not bind to *A. tumefaciens*
Arabidopsis ecotypes BI-1 and Petergof (unknown factors) [*]	Ecotypes do not bind to *A. tumefaciens*
Nodulin-like protein	Might be involved in cell-to-cell recognition
Lectin-like protein kinase	Might be involved in cell-to-cell recognition
Export of T-DNA	
VirB1* interactor (still unknown) [*]	Establishing cell-cell contact
BTI1, BTI2, BTI3, AtRAB8 (VirB2 interacting proteins)	Recognition of T-pilus and activation of transporter complex
VirB5 interactors (still unknown) [*]	Recognition of T-pilus and activation of transporter complex
T-complex nuclear localization	
RocA, Roc4, CypA (cyclophilins)	Chaperones that are possibly involved in maintaining VirD2 conformation
AtKAPα (importin α-1)	Binds to VirD2 NLS; facilitates VirD2 nuclear import
Abi 1 mutant (type 2C serine/threonine protein phosphatase, PP2C)	Mutant has increased susceptibility to *A. tumefaciens* infection; overexpression of PP2C enhances activity of the VirD2 NLS
Putative protein kinase (still unknown) [*]	Downregulates VirD2 nuclear import by phosphorylating its NLS region
Ran [*]	Facilitates nuclear import of VirD2 and VirE2

VIP1	Binds to VirE2 to facilitate VirE2 nuclear import; might assist subsequent intranuclear transport of T-complexes and T-DNA integration
<u>Intranuclear transport of T-complexes and T-DNA integration</u>	
VIP2	Binds to VirE2 and VIP1; might participate in intranuclear transport of VirE2 and T-complexes and/or in T-DNA integration
ASK1 and SCF complex components *	Targeted proteolysis during uncoating of T-complexes and/or exposing the host cell genome DNA prior to or during integration
DNA Ligase *	Ligation of integrating T-DNA into the plant genomic DNA
DNA Polymerase *	T-strand conversion to double-stranded DNA
Arabidopsis rat5 mutant (H2A histone)	Mutant deficient in T-DNA integration; H2A histone might specify chromatin conformation at the integration site
Arabidopsis ecotype UE-1 (unknown factor) *	Ecotype deficient in T-DNA integration

† Adapted and modified from Tzfira and Citovsky, 2002

References to these functions can be found in Tzfira and Citovsky, 2002 and in the subsections of section 1.3 from 1.3.1 to 1.3.4

* The functions of these factors require further studies for verification and are not discussed in section 1.3

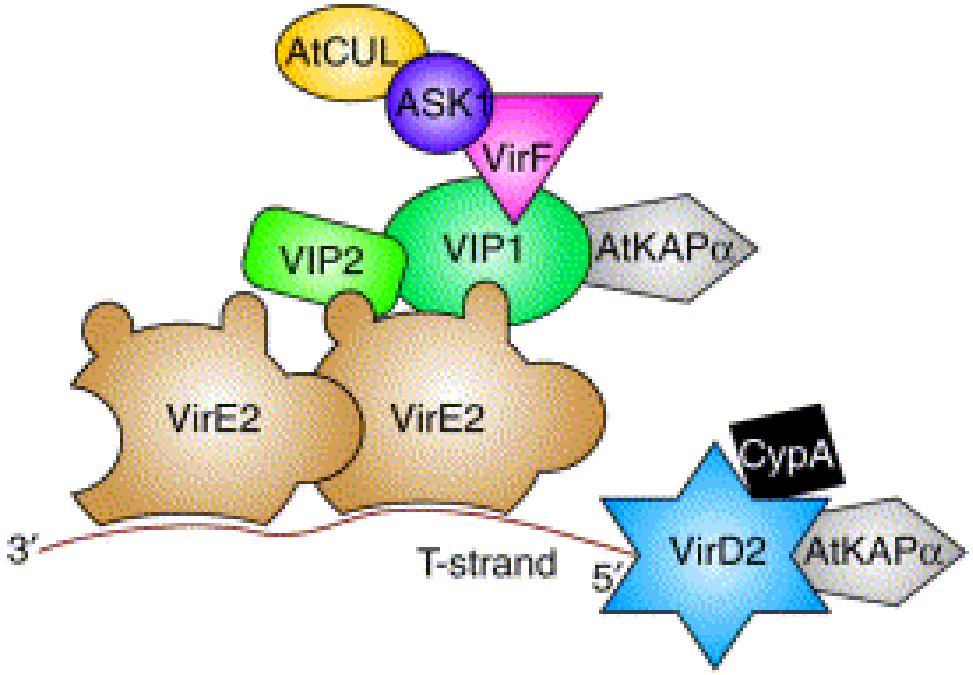


Fig. 1.3. Possible interactions between host cell proteins and the molecular components of the mature *A. tumefaciens* T-complex. The mature T-complex is thought to comprise multiple VirE2 molecules bound along the length of the T-strand and interacting with each other for binding cooperativity, and a single molecule of VirD2 covalently attached to the 5′ end of the T-strand. This T-complex interacts with the following host cell proteins: to preserve its proper conformation within the plant cell, VirD2 might bind to the CypA chaperone; for nuclear import, VirD2 interacts directly with AtKAPα, whereas VirE2 interacts with AtKAPα via VIP1; for intranuclear transport to the integration site, VirE2 might interact with VIP2 (VIP1, which also binds to VIP2, might also play a role in this process); for uncoating of the T-complex and/or removal of its cellular interactors, VirF might bind to VIP1 and bridge it with ASK1 and AtCUL components of the targeted proteolysis machinery.

(Cited from Tzfira and Citovsky, 2002)

1.4. Environmental factors affecting *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation of plant cells is a complex process that involves both bacterial and host factors. Aside from these factors, many parameters may potentially influence the final outcome as to whether a successful and efficient transformation is achieved. Growth conditions, such as pH, temperature and ionic composition of the external medium, have been demonstrated to affect the virulence functions of many pathogenic bacteria including *A. tumefaciens*. When the growth conditions are altered, virulence gene expression in plant and animal pathogenic bacteria will shift in concert with incubated conditions, reflecting their adaptation to the host environment. In many cases, regulation occurs at the level of gene expression by modulating the activity of specific two-component regulatory systems, commonly found in bacteria.

Research findings from previous studies have indicated that a high level of *vir* gene induction could be obtained at a pH below 6.0 and a temperature below 28°C. It has been reported that environmental acidity plays an important role in inducing the virulence gene expression in *A. tumefaciens* (Olson, 1993; Foster, 1999), as acidic pH in the minimal medium resembles the plant environment that *A. tumefaciens* usually encounters during infection. At least two independent regulatory pathways are required for *vir* gene induction by acidic pH (Winans *et al,* 1988; Winans, 1990; Chen and Winans, 1991; Mantis and Winans, 1992). The first one is the pH-inducible promoter of *virG*. Transcription of *virG* is initiated at two promoters, called P1 and P2. While the upstream promoter P1 is inducible by phenolic compounds in the usual VirA/VirG dependent manner and by phosphate starvation, the P2 promoter is primarily induced by low pH and secondarily responsive to certain stress stimuli

(Mantis and Winans, 1992). The other low pH regulatory pathway is the maintenance of an active conformation of VirA in acidic media that affects the VirA periplasmic domain.

Other than acidity or low pH, the activation of *vir* system is also dependent on external temperature. The ability of *A. tumefaciens* to cause tumor on plant wound site was strongly reduced at temperatures above 29°C when compared to that at 22°C. This could attribute to the inefficient expression of some *vir* genes and the denaturation of a protein complex at these elevated temperatures. Studies have shown that the expression of some virulence genes is specifically inhibited at temperature above 32°C, even when the *virA* and *virG* are expressed under a constitutive promoter instead of their native ones. This suggests that the signal transduction mediated by VirA and the subsequent transfer of phosphate to VirG might be sensitive to ambient temperature above 32°C. It has been proposed that the conformational change of VirA protein at high temperature was responsible for the thermal sensitivity of *vir* gene expression (Jin *et al*, 1993).

Besides the expression of virulence genes, the VirB/D4 secretory machinery of *A. tumefaciens* was also affected by high temperature (Fullner and Nester, 1996). At 19 °C, pili could be readily observed on the surface of cultured *A. tumefaciens* cells but not at 28°C. The reasonable explanation for this phenomenon is that the degradation of a limited set of virulence proteins prevents the assembly of the type IV transporter at elevated temperatures. Interestingly, a low temperature also enhances the *virB*-independent secretion of VirE2 and VirD2 and at least fivefold more of VirE2 and VirD2 proteins were shown to be present in the supernatant fraction of cells grown at 19°C when compared to that at 28°C.

1.5. *Agrobacterium*-mediated transformation of other eukaryotic cells

The host range of *A. tumefaciens* has expanded from its native dicotyledonous host plants to include monocotyledonous plants and the number of transformable plant species is increasing. When appropriate conditions and modified parameters are used, *A. tumefaciens* has been proven to be capable of transferring its T-DNA into yeasts, fungi such as *Kluyveromyces lactis,* as well as some mammalian cells.

The transfer of T-DNA from *A. tumefaciens* into *S. cerevisiae* is very similar to that into the plant cells in that the Ti-plasmid encoded *vir* genes required for T-DNA transfer into plant cells were also found to be required for T-DNA transfer into *S. cerevisiae* and that *vir* gene induction is also necessary (Bundock *et al,* 1995; Piers *et al,* 1996). The frequency of *A. tumefaciens* mediated transformation of *S. cerevisiae* is approximately 10^{-3}–10^{-6} transformants per recovered recipient, expectably lower than the transformation of its native host plants.

Despite the aforementioned similarities, the mechanisms of transformation are not entirely conserved. Studies have shown that when chromosomal virulence genes of *A. tumefaciens* involved in attachment and subsequent transformation of plant cells were mutated, no effect was observed on the efficiency of T-DNA transfer into *S. cerevisiae.* This suggests that the yeast transformation system does not emulate plant cell transformation in the attachment step.

Apart from this, the T-DNA integration step is also slight different in the case of T-DNA transfer into yeast. If the T-DNA shares homology with the genome of *S. cerevisiae*, it is able to efficiently integrate into the host genomic DNA via homologous recombination, while T-DNAs lacking homology with *S. cerevisiae* genome could still integrate via illegitimate recombination, albeit at a very low

frequency. However, this is not the case in plants, where gene targeting is difficult to achieve and T-DNA that shares extensive homology with the plant genome integrates primarily via illegitimate recombination.

Besides yeast, it has been demonstrated that the expanded host range of *A. tumefaciens* also includes the filamentous fungi (de Groot *et al,* 1998). Although the mechanism of this transfer is not fully understood, it has been proven that this transfer is dependent on the induction of the bacterial virulence genes, which will lead to the processing of the T-strand and the establishment of VirB pilus that can mediate the transfer of T-strand into these fungal cells. In nature, *A. tumefaciens* and certain species of filamentous fungi share the same habitat and if T-DNA transfer from *A. tumefaciens* to filamentous fungi indeed occurs in nature when these organisms encounter each other, then the interkingdom horizontal DNA transfer may be more extensive than expected.

In 2001, the exciting possibility that the host range of *A. tumefaciens* could be expanded to include human cells was first reported (Kunik *et al,* 2001). Research findings have confirmed that *A. tumefaciens* could transfer its T-DNA into human cells and integrate the T-DNA into the human cell genomes, with an efficiency of stable transfection of about $1.6 \pm 3 \times 10^{-5}$ cells. In stably transformed HeLa cells, the integration event was found to occur at the right border of T-DNA. Such T-DNA transfer supports the notion that *A. tumefaciens* transforms human cells by a mechanism similar to that which it uses to transform the plant cells. Indeed, mutant strains with mutations in the *vir* or *chv* genes (*virA*, *virB*, *virG*, *virD*, *virE, chvA* and *chvB*) were found to have lost their transformation ability, producing no geneticin-resistant cells under the same experimental conditions.

However, *Agrobacterium*-mediated transformation of mammalian cell does not always agree with that of plant transformation because mammalian cell transformation could occur at 37°C and uninduced *A. tumefaciens* could still transform HeLa cells. Under these conditions, the expression of *virA*, which is involved in perceiving the *vir*-inducing plant signals and other components of the T-DNA transfer machinery, is inhibited (Winans *et al,* 1994). Thus, additional experiments have to be performed to elucidate the exact mechanism by which *A. tumefaciens* transforms mammalian cells.

In addition to human cells, recent research data from our lab have indicated that *A. tumefaciens* is capable of transfecting cultured fish cells (Lin and Pan, unpublished) and mice cells (Hou and Pan, unpublished) under similar conditions in which the human cells were transformed. Despite the establishment of these new findings, further investigations are required to determine if the T-DNA transfer into these cells also emulate that of the plant or human cells.

Regardless of the difference in species, cell types and mechanisms of T-DNA transfer, it is certain that host factors play important roles in *Agrobacterium*-mediated transformation of these diverse types of cells from various organisms. Also, it is perceivable that *A. tumefaciens* may 'hijack' evolutionary conserved cellular machineries and/or pathways to achieve this incredible feast of gene transfer, when they interact with these cells. Further research in this direction may shed some light on how this remarkable and unusual pathogenic bacterium performs such gene transfer 'trick'.

1.6. DIP, a novel *Arabidopsis* VirD2 interacting protein

By using a yeast 2-hybrid system, our lab identified a novel plant gene product designated as DIP (VirD2 Interacting Protein) from an *Arabidopsis* cDNA library that can interact with *A. tumefaciens* VirD2 protein (Chang, 2002). This VirD2-DIP interaction was confirmed by an independent *in vitro* immunoprecipitation assay and sequence analysis of DIP protein revealed that it is homologous to yeast Sec3p protein, a subunit of the yeast exocyst complex involved in secretion (Finger *et al*, 1997; 1998; Wiederkehr *et al*, 2003). Since exocyst complex is evolutionary conserved, DIP or Sec3p homologues are also found in various organisms, including human (Matern *et al*, 2001) and mice (Zhang *et al*, 2001).

Subsequent immunohistology and confocal microscopy experiments have revealed that DIP colocalizes with GUS reporter protein and T-DNA molecules in the cytoplasm of the same transformed plant cells but not in those untransformed cells or cell clusters. All the data indicate that DIP proteins are usually randomly located in the cytoplasm of plant cells, but become coexisted with T-DNA in the *A. tumefaciens* cocultivated cells. This suggests that DIP not only could interact with VirD2 *in vivo* and *in vitro*, but also is involved in *Agrobacterium*-mediated transformation of plant cells, by assisting the T-complex movement within the plant cell cytoplasm.

1.7. Objectives of this study

Nuclear localization of T-complex is a central event in *Agrobacterium*-mediated transformation of its host cells and several host factors have been shown to be necessary or rather utilized in assisting such intra-cytoplasmic transport (Section 1.3.3). The newly identified DIP protein has been shown to be involved in *Agrobacterium*-mediated transformation of plant cells by probably aiding in the nuclear localization of T-complex through its interaction with VirD2.

This study is aimed to establish the functional significance of DIP in *Agrobacterium*-mediated transformation of plant cells and to characterize DIP and its evolutionarily conserved homologues in other host cells, especially the homologues from human cells, in order to ascertain whether the T-DNA transfer into these cells may involve exocyst complex or secretion pathway.

Chapter 2. General Materials and Methods

2.1. Bacterial strains, yeast strains, plant species and human cell lines

Bacterial strains, yeast strains, plant species and human cell lines used in this study are listed in Table 2-1. *Escherichia coli* strains were grown at 37 °C in LB (Sambrook *et al*, 1989) and *Agrobacterium tumefaciens* strains were grown at 28 °C in MG/L, AB or IB media (Cangelosi *et al,* 1991) supplemented with the appropriate antibiotic when necessary. For long-term storage, the bacteria were kept in LB with 50 % glycerol at –80 °C. Yeast strains were grown at 30 °C in either YPD or SD medium containing the appropriate drop-out formulation. For long-term storage, the yeast cells were kept in YPD or the appropriate SD medium with 50 % glycerol at –80 °C.

Plant cell cultures were grown at room temperature (RT) in MS medium. For selection and subsequent maintenance of transformants, the medium was supplemented with the appropriate selective agent. For long-term storage, the cell cultures were cryopreserved with 5 % DMSO under liquid nitrogen (Menges and Murray, 2004). The human cell lines used in this study were grown at 37 °C in DMEM in a 5 % (v/v) CO_2 incubator. For long-term storage, the cell lines were cryopreserved with DMSO under liquid nitrogen following the instructions of the suppliers.

2.2. Media, stock solutions, plasmids and primers

The media used to culture the bacteria, yeast, plants and human cell lines are listed in Table 2-2. The preparation and concentration of antibiotics and other solutions used in this study are listed in Table 2-3, while the plasmids and primers used are listed in Table 2-4 and Table 2-5 respectively.

Table 2-1. Bacterial strains, yeast strains, plant species and human cell lines

Strains or cell lines	Relevant characteristics	Source or reference
E. coli		
DH5α	*EndA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ*(argF-lacZYA)*U169 ϕ80d*lacZ* ΔM15	Bethesda Research Laboratories
BL21(DE3)	*B F⁻ dcm ompT hsdS(r_B-m_B-) gal (DE3)*	Stratagene
A. tumefaciens		
LBA4404	Ach5, pTiAch5 Sm/Sp®	Ooms *et al*, 1982
MX243	*virB* mutant strain, derived from A348	Stachel and Nester, 1986
WR1715	*virD2* mutant strain, 70 % of *virD2* deleted	Shurvinton *et al,* 1992
Yeast (*Saccharomyces cerevisiae*)		
CG-1945	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, cyh^{r}2, LYS2 :: $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, URA3 :: GAL4 $_{17\text{-}mers(x3)}$-$CYC1_{TATA}$⁻lacZ*	Clontech, Feilotter *et al,* 1994
Y187	*MATα, ura3-52, his3-200, ade 2-101, trp 1- 901, leu 2-3, 112, gal4Δ, met⁻, gal80Δ, URA3 :: $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ, MEL1*	Clontech, Harper *et al,* 1993
Plant species		
BY2	*Nicotiana tabacuum* L. cv. Bright Yellow 2	Laboratory Collection
Col-0	*Arabidopsis thaliana* Columbia wild-type	LEHLE seeds
SALK-140590	*Arabidopsis thaliana* Columbia T-DNA inserted $DIP^{+/-}$ mutant	SALK Institute seeds
Xanthi	*Nicotiana tabacuum* L. cv. Xanthi	Laboratory Collection
Human cell lines		
EcoPack2-293	An ecotropic, HEK 293-based packaging cell line used for transiently or stably producing virus capable of infecting mouse and rat cells	Clontech
NT2	An embryonal carcinoma cell line derived from a human teratocarcinoma that can differentiate into neuron-like NT2N cells *in vitro* upon retinoic acid treatment; also known as NT-era2 cells	American Tissue Culture Collection (ATCC)

Table 2-2. Media preparation

Media or solutions	Preparation [a, b]	Reference
E. coli		
LB (Luria broth)	Tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; pH 7.5	Sambrook *et al*, 1989
SOB	Tryptone, 20 g; yeast extract, 5 g; NaCl, 0.5 g; 10 ml of 250 mM KCl; pH 7.0; sterilize by autoclaving and add 5ml of filter-sterilized 2 M $MgCl_2$ before use	Sambrook *et al*, 1989
TB	10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl	Sambrook *et al*, 1989
A. tumefaciens		
MG/L	LB, 500 ml; mannitol, 10 g; sodium glutamate, 2.32 g; KH_2PO_4, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; biotin, 2 μg; pH 7.0	Cangelosi *et al*, 1991
AB (Minimal medium)	20 × AB salts, 50 ml; 20 × AB buffer, 50 ml; 0.5 % glucose, 900 ml; autoclave each constituent separately before mix together	Cangelosi *et al*, 1991
IB (Induction Medium)	20 × AB salts, 50 ml; 20 × AB buffer, 1 ml; 0.5 M MES (pH 5.5), 8 ml; 30% glucose, 60 ml; autoclave each constituent separately before mix together	Cangelosi *et al*, 1991
20 × AB salts	NH_4Cl, 20 g; $MgSO_4 \cdot 7H_2O$, 6 g; KCl, 3 g; $CaCl_2$, 0.2 g; Fe $SO_4 \cdot 7H_2O$, 50 mg	Cangelosi *et al*, 1991
20 × AB buffer	K_2HPO_4, 60 g; NaH_2PO_4, 23 g; pH7.0	Cangelosi *et al*, 1991
0.5 M MES	MES, 97.6 g; pH5.5	Cangelosi *et al*, 1991
1000 × AS [c]	14.6 mg/ml AS in DMSO	Sambrook *et al*, 1989
Yeast		
YPD	Difco peptone, 20 g; yeast extract, 10 g; glucose, 20 g	Clontech user manual
SD medium	Minimal SD base, 26.7 g; appropriate drop-out	Clontech user manual

Plant		
MS medium	Murashige and Skoog salts and vitamin mixture, 4.42 g; sucrose, 30 g; 2,4-D (0.1 mg/ml), 2 ml	Murashige and Skoog, 1962
Human cell lines		
DMEM	25 mM HEPES; 4 mM L-glutamine; 4.5 g/l glucose; 10% (v/v) heat inactivated fetal bovine serum; 100 units/ml penicillin; 100 μg/ml streptomycin	Sigma

[a] Preparation for 1 liter and sterilized by autoclaving
[b] For solid media, 1.5 % agar was added
[c] No autoclaving is necessary

Table 2-3. Antibiotics and other stock solutions used in this study

Names	Preparations	Stock con.* (mg/ml)	Working con. in *E. coli* (μg/ml)	Working con. in *A. tumefaciens* (μg/ml)	Working con. in Plant (μg/ml)
Ampicillin (Ap)	Dissolved in dH_2O, filter sterilized	100	100	–	–
Kanamycin (Km)	Same as above	100	100	100	100
Carbenicillin (Cb)	Same as above	100	100	100	300
Cefotaxime (Cef)	Same as above	200	–	–	200
Acetosyringone (AS)	Dissolved in dimethyl sulfoxide	100 mM	–	100 μM	–
IPTG	Dissolved in dH_2O, filter sterilized	24	24	24	–
X-Gal	Dissolved in dimethyl sulfoxide	20	20	20	–
X-Gluc	Dissolved in dH_2O	100	–	–	0.5
Proteinase K	Dissolved in dH_2O	20	50	50	–
RNase	Dissolved in dH_2O	10	20	20	–
Phosphinothricin (ppt)	None	150	–	–	5

* 'con.' is the abbreviation for 'concentration'

Table 2-4. Plasmids used in this study

Plasmid	Characteristics	Source or reference
pUCA19	pUC19 (US Biochemical) harboring *repA* for efficient replication in both *E. coli* and *A. tumefaciens*, Amp^R	Lab collection
pIG121-Hm	Vector for plant transformation containing a 35S:intron:GUS reporter gene, Km^R	Ohta *et al*, 1990
pCB302-1	Vector for plant transformation containing 35S:*bar* herbicide (phosphinothricin) resistance gene, Km^R	Xiang *et al*, 1999
pHC19	pCB302-1 harboring the C-terminal 588 bp fragment (from nucleotides no. 1899 to 2486) of the 2664-bp coding sequence of *DIP*, Km^R	This study
pHC20	pCB302-1 harboring the antisense sequence to the first 498 bp (from nucleotide no. 1899 to 2396) of the fragment from pHC19, Km^R	This study
pHC18	pCB302-1 harboring the adjoining sequences of the 588 bp fragment and the 498 bp fragment from pHC19 and pHC20 respectively, Km^R	This study
pRSET	Vector for overexpression of proteins, Amp^R	Invitrogen
pDual GC	High-level dual mammalian and bacterial protein expression vector containing human ORF coding for hypothetical protein FLJ10893 (Accession: AF208854), corresponding to the C-terminus of hDIP, the human homologue of DIP, Km^R	Stratagene
pHC2	pRSET-A containing the C-terminal 621 bp *EcoR* I subtending fragment of pDual GC, Amp^R	This study
pAS2-1	Vector for expressing bait:GAL4 DNA-BD fusion protein, Amp^R	Clontech
pACT2	Vector for expressing prey:GAL4 AD fusion protein, Amp^R	Clontech
pCL1	Positive control plasmid, encoding the full-length wild-type GAL4 protein, Amp^R	Clontech
pVA3-1	Positive control plasmid used with pTD1-1, encoding a DNA-BD/murine p53 fusion protein in pAS2-1, Amp^R	Clontech
pTD1-1	Positive control plasmid used with pVA3-1, encoding an AD/SV40 large T-antigen fusion protein in pACT2, Amp^R	Clontech

pLAM5'-1	False positive detection plasmid, encoding a DNA-BD/human lamin C fusion protein in pAS2-1, Amp^R	Clontech
pGAD10-DIP	pGAD vector (Clontech) encoding GAL4 AD fused to VirD2-interacting protein (DIP) that was fished out from *Arabidopsis* cDNA library	Lab collection
pAS-D2	pAS2-1 harboring VirD2:GAL4 DNA-BD fusion, Amp^R	Lab collection
pAS-D2 (74)	pAS-D2 harboring a fusion construct with VirD2 lacking the N-terminal 73 amino acids	This study
pAS-D2 (174)	pAS-D2 harboring a fusion construct with VirD2 lacking the N-terminal 173 amino acids	This study
pAS-D2 (274)	pAS-D2 harboring a fusion construct with VirD2 lacking the N-terminal 273 amino acids	This study
pAS-D2 (354)	pAS-D2 harboring a fusion construct with VirD2 lacking the N-terminal 353 amino acids	This study
pAS-D2 (-NLS)	pAS-D2 harboring a fusion construct with VirD2 lacking the C-terminal NLS sequence	This study
pAS-D2 (N)	pAS-D2 harboring a fusion construct with VirD2 containing only the N-terminal 212 amino acids	This study
pAS-D2 (C)	pAS-D2 harboring a fusion construct with VirD2 containing only the C-terminal 212 amino acids	This study

Table 2-5. Primers used in this study

Primer	Relevant sequence
S1(C)	5’-AACTGCAGGATTTGTTCGCCTTTTGCTTGG-3’
S2	5’-CGGGATCCTTGCTGCAATCGATTTGTCC-3’
AS1	5’-TCCCCCGGGTATCTCTTCAGGGGTGATAG-3’
AS2	5’-CGGAATTCCTGCAGGATTTGTTCGCC-3’
D2 (1)	5’-CATGCCATGGATGCCCGATCGCGCTC-3’
D2 (74)	5’-CATGCCATGGGACGATGATAGGCAA C-3’
D2 (174)	5’-CATGCCATGGCACGGCATAGTCCTG G-3’
D2 (274)	5’-CATGCCATGGCGGATCCGCGTATCATTG-3’
D2 (354)	5’-CATGCCATGGGGATTGAAGGCTGCGC-3’
D2 (-NLS)	5’-CGGAATTCTGATCGCTGCTGGCGC-3’
D2 (N)	5’-CGGAATTCTTCGAATTGAATCTTTTGAG-3’
D2 (C)	5’-CATGCCATGGGATACAGATTTTGATG-3’
D2 (end)	5’-CGGAATTCGGTCCTTCCTTCCTGTC-3’
Dip-ex1	5’-ATGGCGAAATCAAGCGCCGAC-3’
Dip-ex2	5’-AAGAAATGCTTTCTTTCGTGGACCCTTTG-3’
RT-PCRF	5’-CGGGATCCATGACAGCAATCAAGCATGCA-3’
RT-PCRR	5’-CCCAAGCTTTTAGTGGGACTGTGCAATGCTG-3’
Mid-Up	5’-GCTCAGTGTTCAGAGTTCAGGG-3’
Mid-Down	5’-GCTCATTCGCGAACCCTGAACTCTGAAC-3’
FWD	5’-GTACCACTGGCATCGTGATG-3’
BCK	5’-GCTTGCTGATCCACATCTGC-3’
BM012F	5’-CGGGATCCATGCCTGGAACTATG-3’
BM012R	5’-CGGGATCCTTAGTGGGACTGTGC-3’

2.3. Cell and tissue cultures

2.3.1. Plant cell culture and subculture

Tobacco BY2 (*Nicotiana tabacuum* L. cv. Bright Yellow 2) calli were maintained on solid Murashige and Skoog medium (MS; Murashige and Skoog, 1962) supplemented with 3 % sucrose and 0.2 mg/ml 2,4-D. These calli were subcultured or transferred to fresh MS plates every 3 to 4 weeks before they turned black or brownish, an indication of dead tissues. For use in *A. tumefaciens* mediated transformation, BY2 cells were grown in liquid MS medium at RT with shaking at 100 rpm and were subcultured every week with a 5 % inoculum.

2.3.2. Human cell culture and subculture

DMEM was used to culture the human embryonic kidney (HEK) EcoPack2-293 cells (Clontech) and NT2 cells (ATCC). For optimal growth, DMEM was supplemented with 1nM sodium pyruvate for the culture of HEK-293 cells, while DMEM was supplemented with 1.5 mg/ml of sodium bicarbonate ($NaHCO_3$) for the culture of NT2 cells. Both EcoPack2-293 and NT2 cells were grown in the recommended media following the instruction of suppliers at 37 °C in a 5 % (v/v) CO_2 incubator. EcoPack2-293 cells were grown as a monolayer in 75 cm^2 flasks and subcultured at least once every 5 days by trypsin/EDTA treatment and at a dilution of 1:4 in fresh medium. NT2 cells were subcultured similarly, except that a sterile cell scrapper was used to scrap down the cells from the flask surface instead of using the trypsin/EDTA treatment to dislodge the cells from the flask surface.

2.4. DNA manipulations

2.4.1. Plasmid DNA preparation from *E. coli*

Plasmid DNA was prepared following the method described previously with some modifications (Sambrook *et al*, 1989). Briefly, *E. coli* cells from 2 ml of overnight culture were collected by centrifugation at 10, 000 rpm (Eppendorf 5417C) for 1 min. The cell pellet was resuspended in 100 µl of ice-cold solution I (50mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0) thoroughly by vigorous vortex. Then, 200 µl of freshly prepared solution II (0.2 N NaOH, 1 % SDS) was added and the contents were mixed by inverting gently for 4 to 6 times. After the addition of 150 µl of Solution III (3 M potassium, 5 M acetate), the mixture was inverted for 4 to 6 times to disperse Solution III through the viscous bacterial lysate. The lysate was extracted with equal volume of chloroform once by centrifuging at 14, 000 rpm (Eppendorf 5417C) for 5 min. The supernatant was then transferred to a clean Eppendorf tube. To precipitate the plasmid DNA, 2 volumes of ethanol was added and the mixture were centrifuged as above. The DNA pellet was washed once with 70 % ethanol and dried in a vacuum concentrator. The extracted plasmid DNA was dissolved in 20 µl of sterile water and stored at -20 °C, ready for subsequent use after thawing.

2.4.2. Plasmid DNA preparation from *A. tumefaciens*

Plasmid DNA was isolated from *A. tumefaciens* cultures using the QIAprep Spin Miniprep Kit (QIAGEN) following the user-developed protocol (Weber *et al*, 1998) with some modifications. Briefly, 10 to 15 ml of overnight MG/L culture (Cangelosi *et al*, 1991) supplemented with antibiotics was harvested by centrifugation at 10, 000 rpm (Eppendorf 5417C) for 1 min. The resultant combined cell pellet was

resuspended in 500 µl of buffer P1 before 500 µl of lysis buffer P2 was added to the suspension. After gentle mixing by inverting 4 to 6 times, 1 ml of neutralization buffer N3 was added to the mixture. Another gentle inversion of 4 to 6 times was performed and the mixture was then subjected to centrifugation at 14, 000 rpm (Eppendorf 5417C) for 10 min. The cell lysate was applied into a QIAprep column and centrifuged at 14, 000 rpm for 30 sec to1 min. After discarding the flow through, the spin column was washed with PB buffer and then PE buffer, following the standard procedure for plasmid isolation using this kit. The plasmid DNA was finally eluted in 30 to 50 µl sterile water and subjected to further analysis or manipulation.

2.4.3. DNA digestion and ligation

DNA digestion and ligation were conducted following the instructions of the manufacturers supplying the enzymes (Fermentas). The digestion reaction system used in this study is comprised of buffer, enzyme, DNA and sterile deionized water, with incubation at 37 °C or other recommended temperature for 1 hr to overnight. For vectors digested with a single restriction enzyme, dephosphorylation was carried out by adding 0.5 µl (1 unit) of shrimp alkaline phosphatase into the digestion mixture. Digested vectors and gene fragments used for ligation were cleaned or purified by using the Gel Extraction Kit (QIAGEN) before the ligation reaction was carried out by incubating the mixture of T4 DNA ligase (Fermentas), vector DNA, insert DNA, ligase buffer and sterile water at RT for 4 hrs or overnight.

2.4.4. Polymerase chain reaction (PCR)

Polymerase chain reaction was carried out using a PCR machine in a thin-walled PCR tube with a volume of 200 µl to amplify any target DNA fragment. The reaction mixture for PCR was made up of the following components in a final volume of 50 µl:

10 × PCR buffer (without $MgCl_2$)	5 µl
25 mM $MgCl_2$	3 µl
Primer 1 (10 pmol/µl)	1 µl
Primer 2 (10 pmol/µl)	1 µl
dNTPs (10 mM each)	1 µl
Template DNA	20 to 100 ng
Taq DNA polymerase (Fermentas)	1 µl (1 unit)
Add sterile distilled water to a final volume of	50 µl

The PCR was run using the following program:

1 cycle	95 °C for 1 min
25 to 30 cycles	95 °C for 30 sec
	Annealing at (Tm-5) °C or lower for 30 sec
	Extension at 72 °C for 1 min per kb
1 cycle	72 °C for 10 min

2.4.5. DNA gel electrophoresis and purification

DNA fragments or PCR products were electrophoresized in a 1 × TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) agarose gel along with a standard DNA marker (Fermentas). Digested DNA vectors and fragments or PCR products to be used for ligation and subsequent transformation reaction were purified with QIAquick Gel Extraction Kit (QIAGEN) following the instructions provided by the manufacturer. Briefly, DNA was separated in a 1 % agarose gel before the gel slice containing the desired DNA band was excised and transferred to a pre-weighted Eppendorf tube. Then, 3 gel volumes (100 mg gel ≈ 100 µl) of buffer QG were added and the tube was incubated in a 55 °C waterbath for 5 to 10 min to dissolve the gel completely. For DNA fragments larger than 4 kb or smaller than 500 bp, 1 gel volume of isopropanol was added prior to transferring the mixture into a QIAquick spin column in a 2-ml collection tube. The binding of DNA to the column was achieved by centrifugation for 1 min at 14, 000 rpm (Eppendorf 5417C). After discarding the flow through, the column was then washed once by applying 750 µl of buffer PE to the column and subjecting the column to centrifugation at 14, 000 rpm for 30 sec to 1 min. A second centrifugation at 14, 000 rpm for 1 min was then performed, after the removal of the flow through, to eliminate any residual ethanol. The column was placed into a clean 1.5-ml centrifuge tube before 30 to 50 µl of sterile water was applied to the center of the column membrane. To elute the DNA, the column was centrifuged at 14, 000 rpm for 1 min.

2.4.6. DNA sequencing

Adapting from the instructions of Big Dye™ automated sequencing protocol, the PCR reaction mixture, the PCR program and the subsequent post-PCR precipitation of PCR products for sequencing reaction were carried out as outlined below.

PCR reaction mixture:

Big Dye™ Ready Mix (Version 3.0 or 3.1)	2 μl
Primer (10 pmol/μl)	1 μl
Plasmid or DNA	100 to 500 ng
Add distilled water to a final volume of	10 μl

PCR program:

1 cycle	96 °C for 15 sec
25 cycles	50 °C for 5 sec
	60 °C for 4 min
1 cycle	4 °C for ∞

Post-PCR precipitation mixture:

PCR product	10 μl
3 M sodium acetate (pH 4.6)	1.5 μl
Non-denatured 95 % Ethanol	31.25 μl
Sterile distilled water	7.25 μl

After adding the PCR product, the precipitation mixture in a sterile 1.5-ml microcentrifuge tube was vortexed and kept at RT for 30 min before the tube was spun

in a microcentrifuge (Eppendorf 5417C) at 14, 000 rpm for 30 min. Thereafter, the supernatant was carefully aspirated without disturbing the pellet. The pellet was washed with 500 µl of 70 % ethanol, followed by centrifugation at 14, 000 rpm for 10 min before the 70 % ethanol was removed. After a repeat wash at the same conditions, the pellet was dried in a vacuum centrifuge or concentrator and then kept at -20 °C, ready to be sent to a DNA sequencer.

2.4.7. Introduction of plasmid DNA into *E. coli*

2.4.7.1. "Heat shock" transformation

E. coli DH5α was routinely used as the host for cloning experiments unless or otherwise specified. High efficient competent cells were prepared as described previously (Inoue *et al*, 1990). *E. coli* cells were streaked from frozen stock and cultured overnight on a LB plate at 37 °C. Then, several colonies were picked and inoculated into 100 ml of SOB medium in a 1-liter conical flask. The cells were cultured at RT (around 20 °C) with vigorous shaking (250 rpm) to an OD_{600} of 0.5 to 0.7. The cells were chilled on ice for 10 min before they were collected by centrifugation at 2600 rpm (Eppendorf 5810R) for 5 min at 4 °C. The cell pellets were resuspended in 30 ml of ice-cold TB buffer (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250mM KCl, pH 6.7; all components except $MnCl_2$ were dissolved and autoclaved; 1 M $MnCl_2$ solution was filter-sterilized and added to make TB buffer; stored at 4 °C) and then incubated on ice for 10 min. Cells were collected by centrifugation as above and resuspended in 5 ml of ice-cold TB buffer. Thereafter, DMSO was added to a final concentration of 7 % and the cell suspension was

aliquoted into pre-cooled sterile Eppendorf tubes at 100 µl each. The competent cells were kept at -80 °C until needed.

To introduce a plasmid or a ligation reaction product into *E. coli* by transformation for amplification or screening (Sambrook *et al*, 1989), a frozen vial of competent cells (100 µl) was thawed on ice. Plasmid DNA (50 to 100 ng in 10µl or less sterile water) or ligation product (10 to 20 µl) was added and the contents of the tube were mixed by gently tapping the tube a few times. The tube was then incubated on ice for 30 min before the mixture of cells and DNA was heat shocked at 42 °C for 90 sec. After chilling the cells on ice for 2 min, 900 µl of fresh LB medium was added and the culture was incubated at 37 °C for 45 min to 1 hr with agitation. The cells were then collected by centrifugation at 10, 000 rpm for 1 min and resuspended in 50 to 100 µl of LB before the cell suspension was spread onto a LB agar plate containing the appropriate antibiotic(s) or substrate(s). Colonies would usually appear after 12 to 16 hrs of incubation at 37 °C.

2.4.7.2. Electrotransformation

The electrocompetent *E. coli* cells were prepared following the method described by Dower *et al.* (1988). *E. coli* cells were grown overnight on LB plate supplemented with the appropriate antibiotics at 37°C, before suitable amount of the cells was scraped off the plate and resuspended in 1 ml of cold 1 mM HEPES (pH 7.0). The cells were spun down at 10,000 rpm in for 1 min at RT. The resultant pellet was washed twice with 1 ml of cold 1 mM HEPES (pH 7.0) and spun down as above. The cells were resuspended in 50 µl cold 10 % glycerol after which the cells may be used immediately or frozen in liquid nitrogen and stored at -80 °C.

The electrotransformation of *E. coli* was carried out following the protocol described previously (Dower *et al*, 1988). In brief, the electrocompetent *E. coli* cells were gently thawed on ice before 1 to 2 μl of plasmid DNA was added and mixed well with the cells. After incubating the mixture on ice for 1 min, the mixture was transferred into a cold, 0.2 cm electroporation cuvette (BioRad). One pulse was applied to the electroporation cuvette on the pulse generator at the settings of 25 μF capacitor, 2.5 kV, and 200 Ω in parallel with the sample chamber. This should result in a pulse of 12.5 kV/cm with a time constant of 4.5 to 5 sec. Immediately after the pulse, 800 μl of LB medium was added to the cuvette and the cells were gently resuspended with pipette. The cell suspension was transferred to a 1.5 ml centrifuge tube and incubated at 37°C for 45 min to 1 hr with shaking before appropriate aliquots were plated on selective LB plates. Colonies would usually appear after overnight incubation of these plates at 37°C.

2.4.8. Introduction of plasmid DNA into *A. tumefaciens* by electroporation

Electrocompetent *A. tumefaciens* cells were prepared as follows. Cells cultured overnight at 28 °C were scraped from the plate with a sterile wooden stick and then transferred into a sterile Eppendorf tube. The cells were washed once with ice-cold water and once with ice-cold 15 % glycerol. The cell pellet was resuspended in 50 to 100 μl of ice-cold 15 % glycerol and then plasmid DNA (50 to 100 ng in 10 μl or less sterile water) was added. The mixture of cells and DNA was transferred into a chilled BioRad electroporation cuvette and kept on ice for 10 min. Gene Pulser II Electroporation System (BioRad) was set to the 25 μF capacitor, voltage of 2.5 kV and a controller unit of 400 Ω. The outside of the cuvette was wiped with tissue paper to get rid of moisture before the cuvette was slide into the shocking chamber base. The

cells were usually pulsed once with a time constant of 8 to 10 msec. Then, 1 ml of MG/L medium was immediately added and the mixture was transferred into a 15-ml culture tube. After culturing at 28 °C for 45 min to 1 hr, the cells were collected and spread onto an MG/L plate containing the selectable antibiotics. Colonies would usually appear 2 to 3 days later.

2.5. RNA manipulations

2.5.1. RNA isolation from human cells

Total RNA of mammalian cells was prepared using TRIZOL Reagent (GIBCO/Life Technologies, Grand Island, NY) according to the manufacturer's instructions. In brief, mammalian cells from one 75 cm^2 flask were washed once with 10 ml of PBS before 2 ml of TRIZOL Reagent was added onto the flash surface. The homogenized sample was then vortexed for 30 sec and incubated at RT for 5 min. Residual protein was removed after the addition of 400 μl of chloroform, mixing for 30 sec, incubation at RT for 3 min and centrifugation for 15 min at 12000 × *g* and 4 °C. The RNA in the colorless aqueous phase was precipitated in 1 ml of isopropanol by mixing for 15 sec, incubation for 10 min at RT and centrifugation for 10 min at 12000×*g* and 4 °C. The resulting RNA pellet was washed with 1 ml of 75 % ethanol and centrifuged for 5 min at 7500 × *g* and 4 °C. The RNA pellet was air dried, resuspended in DEPC treated water and stored at -80 °C. The extracted RNA was treated with RNase-free DNase before RT-PCR was conducted.

2.5.2. RNA isolation from *Arabidopsis* tissues

Total RNA was isolated from *Arabidopsis* plant tissues using the RNeasy® Plant Mini Kit (QIAGEN), following the instructions of the manufacturer. First of all,

leaves or other tissues were collected and weighed to ensure that the weight of each sample was less than 100 mg. After weighing, the tissues from each sample were flash frozen in liquid nitrogen before they were ground in an appropriate volume of liquid nitrogen by using a mortar and pestle. Thereafter, the powder derived from each sample was decanted into a pre-chilled Eppendorf tube. Upon the evaporation of liquid nitrogen but before the tissues started to thaw, 450 µl of buffer RLT was added to the sample and then vortexed vigorously. The resultant lysate was pipetted directly into a QIAShredder spin column placed in a 2-ml collection tube and centrifuged for 2 min at 14,000 rpm (Eppendorf 5417C). The supernatant of the flow-through fraction was then transferred carefully into a new 1.5-ml tube, without disturbing any pellet that might have formed. Then, 0.5 volume of absolute ethanol was added and mixed by pipetting. The mixture was subsequently applied into an RNeasy minicolumn placed in a 2-ml collection tube and subjected to centrifugation for 15 sec at 8000 g. After discarding the flow through, 350 µl of buffer RW1 was added into the column and the sample was centrifuged for 15 sec at 8000 g. Following that, the second flow through was discarded and an incubation mixture of 10 µl of DNase I stock solution and 70 µl of buffer RDD was added directly onto the membrane of the column and incubated at RT for 15 min. After the on-column DNase digestion, the ensuing washes with buffer RW1 and buffer RPE were carried out as recommended in the protocol before the RNA was eluted with RNase-free sterile water in a sterile microcentrifuge tube provided in the kit.

2.5.3. RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was carried out using QIAGEN One-step RT-PCR kit (QIAGEN). The 50-μl RT-PCR reaction mixture was comprised of the following components:

5 × RT-PCR buffer	10 μl
RNase-free water	19.7 μl
Primer 1 (10 pmol/μl)	3 μl
Primer 2 (10 pmol/μl)	3 μl
dNTP Mix	2 μl
RNase inhibitor	0.3 μl
RNA template	10 μl
QIAGEN One-step RT-PCR Enzyme Mix	2 μl

The RT-PCR was run using the following program:

1 cycle	50 °C for 30 min
1 cycle	95 °C for 15 min
40 cycles	94 °C for 1 min
	Annealing at (Tm-5) °C for 30 sec
	Extension at 72 °C for 1 min per kb
1 cycle	72 °C for 10 min

2.6. Protein techniques

2.6.1. Buffers for protein manipulations

Buffers used in protein manipulations are listed in Table 2-6.

2.6.2. SDS-PAGE gel electrophoresis

Protein profiles were analyzed using SDS-PAGE (Laemmli, 1970) based on molecular weight. The electrophoresis apparatus used was the Mini-Protean III Electrophoresis Cell (BioRad). The apparatus was assembled according to the instructions provided by the manufacturer. The monomer stock solution of acrylamide/bis-acrylamide (30.8 % T : 2.7 % C) was prepared as described in *Molecular Cloning* (Sambrook *et al*, 1989) and stored in dark at 4 °C. Ammonium Persulfate (APS) (10 %) solution was freshly prepared before each use. Separating gel buffer (4 ×, 1.5 M Tris-HCl, pH 8.8) and stacking gel buffer (4 ×, 0.5 M Tris-HCl, pH 6.8) were stored at RT. Tank buffer was prepared as a 10 × stock solution (0.25 M Tris-HCl, 1.92 M glycine, 1 % SDS, pH 8.3) and stored at RT. Gel loading buffer (2 ×, 100 mM Tris-HCl, pH 6.8, 4 % SDS, 0.2 % bromophenol blue and 20 % glycerol, 0.2 M DTT) was prepared without DTT and stored at RT. DTT was added from a 2 M stock solution that was stored at -20°C before each use. The preparation of polyacrylamide gel and the separation of protein were performed following the instructions of Hoefer Scientific Instruments (Protein electrophoresis-applications guide, 1994). In this study, 10 or 12 % PAGE gel was used for the analysis of proteins, unless or otherwise specified.

Table 2-6. Buffers used in protein manipulations

Name	Components (for 1 L)	pH adjustment
10 × Tris-buffered saline (10 × TBS)	0.2 M Tris base 1.37 M Sodium chloride 38 ml 1M Hydrochloric acid	Adjust pH to 7.6
1 × TBST	0.1 % Tween-20 (v/v) in 1 × TBS	
10 × Tank buffer	0.25 M Tris 1.92 M Glycine 0.1 % SDS	No need to check pH
10 × Transfer buffer	48 mM Tris 38 mM Glycine 0.37 g SDS 20 % Methanol	Adjust pH to 8.3
4 × Separating gel buffer	1.5 M Tris-HCl	Adjust pH to 8.8
4 × Stacking gel buffer	0.5 M Tris-HCl	Adjust pH to 6.8
1 × SDS gel-loading buffer	50 mM Tris-HCl (pH 6.8) 100 mM Dithiothreitol 2 % SDS 0.1 % Bromophenol blue 20 % Glycerol	
Staining solution	0.25 g Coomassie Brilliant blue R (Gibco) 400 ml Methanol 70 ml Acetic acid	
Destaining solution I	400 ml Methanol 70 ml Acetic acid	
Destaining solution II	70 ml Acetic acid 50 ml Methanol	

2.6.3. Staining of SDS-PAGE separated proteins with standard Coomassie blue

SDS-PAGE separated proteins were stained according to the instructions of Hoefer Scientific Instruments (Protein electrophoresis-applications guide, 1994). The gel was placed in the staining solution and shaked at low speed for 1 h. The staining solution was then discarded and replaced with destaining solution I. After the gel had been destained for 30 min, destaining solution I was removed and replaced with destaining solution II. The destaining solution II was changed twice a day until the gel background was clear.

2.6.4. Western blot analysis

The sample was mixed with equal volume of 2 × loading dye buffer (Laemmli, 1970), and boiled in a water bath for 5 to 10 min. After cooling down, the sample was loaded into a 10 or 12 % SDS polyacrylamide gel and separated at a constant voltage of 100 V. The protein was transferred to an Immun-Blot^{TM} PVDF membrane (BioRad) from the gel in Mini Gel Transfer System for 4 hr to overnight at 200 mA, before the non-specific binding sites on the membrane were blocked by immersing the membrane in 10 % non-fat milk (Nestle) in TBST for 2 h at RT on an orbital shaker. The membrane was then washed in TBST buffer for 3 times, with each wash that lasted 10 min. The membrane was then incubated in the diluted primary antibody for 1 h at RT and washed three times as above before incubation in the diluted secondary antibody for 1 h at RT. After washing thoroughly as above, the membrane was processed for signal detection according to the recommendations of the manufacturer (Amersham).

Chapter 3. Functional Characterization of DIP by RNA Interference

3.1. Introduction

As mentioned in sections 1.6 and 1.7 of Chapter 1, *Arabidopsis* DIP (VirD2 Interacting Protein; At1g47550) was found to interact with *A. tumefaciens* virulence protein, VirD2, both *in vitro* and *in vivo* via independent immunoprecipitation, immunohistological and confocal microscopy assays. The series of analyses have indicated that DIP is localized in the cytoplasm of the plant cells and the exclusive colocalization of DIP with T-DNA in *A. tumefaciens* transformed plant cells but not in those untransformed cells has strongly suggested DIP is involved in *Agrobacterium*-mediated transformation of plant cells, by probably assisting the T-complex movement within the plant cell cytoplasm (Chang, 2002).

The proposed role of DIP as the facilitator of T-complex trafficking within the plant cell cytoplasm can be attributed to the fact that DIP is homologous to yeast Sec3p protein, which is a subunit of the yeast exocyst complex involved in secretion (Finger *et al*, 1997; 1998; Wiederkehr *et al*, 2003). Sequence analysis has revealed that the subunits of the exocyst complex are evolutionarily conserved and homologues can be found in various organisms, including human and mice (Matern *et al*, 2001; Zhang *et al*, 2001).

Despite a strong correlation between the colocalization of DIP with T-DNA and the involvement of DIP in *Agrobacterium*-mediated transformation of plant cells, a direct functional assay of the involvement of DIP in such process has not been demonstrated. To verify such a correlation, a RNA interference approach was used in

our study to directly establish the functional role or significance of DIP in *Agrobacterium*-mediated transformation of plant cells.

3.1.1. General overview of RNA interference

3.1.1.1. Definition and assay of RNA interference

Cropping up again and again in biology research these days, RNA interference (RNAi) is a powerful laboratory tool, partly because it is a widespread natural phenomenon (Novina and Sharp, 2004). Hailed as the "Scientific Breakthrough of the Year" for 2002 by the journal *Science* (Couzin, 2002), RNAi is a recently discovered and evolutionarily conserved gene silencing phenomenon in which small pieces of double-stranded RNA (dsRNA) suppress the expression of genes with sequence homology (Fire *et al*, 1998; Dykxhoorn and Lieberman, 2005). Together with quelling in fungi and posttranscriptional gene silencing (PTGS) in plants, RNAi in animals and basal eukaryotes are examples of a broad family of phenomenon collectively called RNA silencing (Kooter *et al*, 1999; Li and Ding, 2001; Matzke *et al*, 2001; Vaucheret *et al*, 2001; Waterhouse *et al*, 2001; Hannon, 2002; Plasterk, 2002).

In a recent attempt to advocate the standardized use of terms for RNAi experimentation, RNAi has been defined as 'the inhibition of gene expression requiring a dsRNA or dsRNA domain-containing molecule processed by a RNase III-like endonuclease and/or the generation of a ribonucleoprotein (RNP) complex containing a small RNA molecule and member(s) of the Argonaute (Ago) family of proteins' (Huppi *et al*, 2005).

As a terminology in the newly standardized usage, functional analysis using RNAi will thus be referring to an assessment of protein function through the use of

RNAi-based methods, resources, and technologies. The assumed aim is that silencing is mediated through transcript cleavage using most of the approaches developed to date and that downstream phenotypes are as consequence of a reduction in the level of the target protein (Huppi *et al*, 2005). Such an approach is thought to produce a "knock down" phenotype as a result of the decrease in the protein level, but not total elimination as in the case of the "knock out" approach.

3.1.1.2. Mechanism of RNA interference

Although the exact and detailed mechanism of RNAi is not fully understood, biochemical and genetic studies have begun to unravel the mystery surrounding the once puzzling natural phenomenon that contributes to a wide range of developmental, cellular-defensive and regulatory processes (reviewed in Novina and Sharp, 2004). As illustrated in Fig. 3.1, RNAi is a cellular process in which small or short interfering RNAs (siRNAs) of about 21 to 25 nucleotides induce sequence-specific degradation of cognate mRNAs.

As shown in Fig. 3.1, when dsRNAs that are produced from an introduced transgene, a viral intruder or a parasitic genetic element such as transposon are cleaved by the ribonuclease III enzyme – Dicer, into siRNAs, RNA-induced silencing complex (RISC) will incorporate a single-stranded RNA (ssRNA) into this complex. This ssRNA is usually, though not always, the antisense strand of the siRNAs after the unwinding of siRNAs by a yet unidentified unwinding enzyme. The incorporated ssRNA is also known as the 'guide' strand and upon its incorporation into RISC, it will serve as the guide to target those mRNAs with sequence complementarity for destruction (Novina and Sharp, 2004; Sontheimer, 2005). Besides participating in the initiation phase of RNAi, where dsRNAs are cleaved to produce siRNAs, Dicer

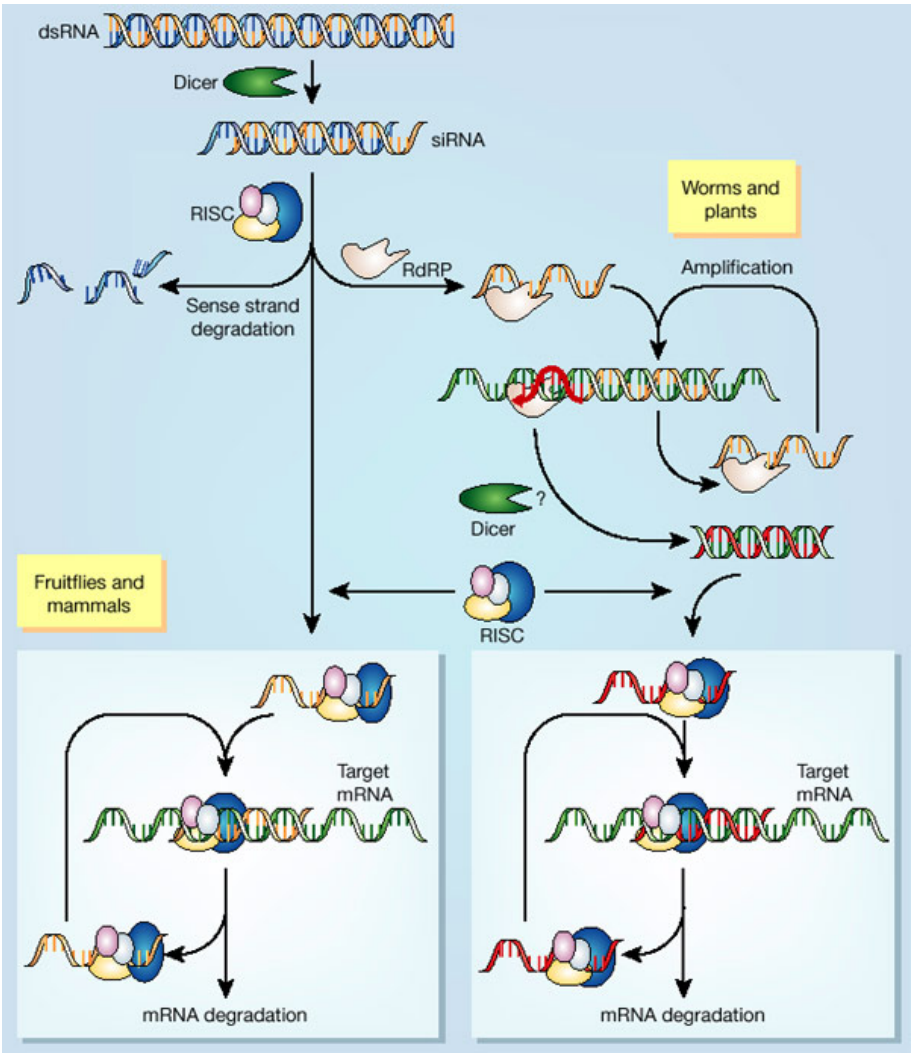


Fig. 3.1. The mechanism of RNA interference (RNAi). RNAi is triggered when a cell encounters a long double-stranded RNA (dsRNA), which might be produced from an introduced transgene, a viral intruder or a rogue genetic element, e.g. transposon. An enzyme called Dicer cleaves the long dsRNA into small or short interfering RNAs (siRNAs). An RNA-induced silencing complex (RISC) then distinguishes between the different strands of the siRNA. The sense strand (blue) is degraded. The antisense strand (yellow) is used to target genes for silencing, and has one of several fates depending upon the organism. In fruitflies and mammals, the antisense strand is incorporated directly into RISC to target a complementary mRNA (green) for destruction. In the absence of siRNAs, the RISC lacks sequence-specific mRNA-binding properties. But when bound to the antisense strand, the now activated RISC can participate in repeated cycles of degradation of specific mRNAs, such that no protein is made — effectively silencing the gene from which the mRNAs are produced. In worms and plants, the antisense strand of the siRNA might first be used in an amplification process. The antisense strand, bound by an RNA-dependent RNA polymerase (RdRP) enzyme, can pair up with a complementary mRNA (green) and act as a start point for the synthesis of a new long dsRNA. Dicer is then required to generate new siRNAs (red), which are specific to different sequences on the same mRNA. Again, the target mRNA is destroyed.

(Cited from Novina and Sharp, 2004)

might also be involved in the effector phase of RNAi, where siRNA programmed RISC degrades target mRNAs (Sontheimer, 2005).

Despite the widespread occurrence of RNAi in virtually every eukaryotic system apart from *Saccharomyces cerevisiae* and some trypanosomes (reviewed in Ullu *et al*, 2004) and its associated cellular functions in transcriptional regulation of gene expression, heterochromatin formation, centromere maintenance and more recently in DNA elimination and silencing of unpaired DNA during meiosis (Matzke and Birchler, 2005), this evolutionarily conserved pathway runs differently in different organisms and a few important distinctions are apparent among the RNAi pathways in different species (reviewed in Tian *et al*, 2004).

First of all, research findings have indicated that different proteins are involved in different RNAi pathways in different organisms. For example, no R2D2/RDE-4 homologue has been found in human, though this protein was well characterized and shown to function in the fruitfly, *Drosophila.* Other than that, the double-stranded-RNA-binding motif (dsRBM) containing proteins from different organisms were found to possess different domain structures (Tian *et al*, 2004).

Similarly, biochemical analyses with various techniques which include X-ray crystallography and nuclear magnetic resonance (NMR) have revealed that different homologues of Argonaute (Ago) protein and Dicer or Dicer-like (DCL) proteins were found in different organisms (Lingel and Sattler, 2005). While these Ago and DCL proteins all contain the conserved PAZ domain, these crucial components of RISC are not found in equal number in different organisms. For instance, 4 Dicer homologues and 10 Ago proteins were found in *Arabidopsis* while *C. elegans* harbors only 1 Dicer but 27 Ago proteins (Matzke and Birchler, 2005).

Therefore, it is perceivable that RISC is probably differentially assembled in various organisms and such assembly is likely to involve different subunits or associated proteins, some of which could exclusively be found in certain organisms but not in others (reviewed in Sontheimer, 2005). This demonstrates that RNAi is an ancient mechanism which might have been adopted to achieve a similar regulatory function, but was then adapted to suit the need or customized design in different organisms.

In addition to the involvement of different proteins or conserved proteins with different structures and numbers, RNAi in some species, such as plants and worms, entails an amplification step by an RNA-dependent RNA polymerase (abbreviated as RDR or as RdRP in Fig. 3.1) (Tian *et al*, 2004). Besides this difference, systemic RNAi effects were also observed in these species where siRNAs could be used by RDR for amplification and the RNAi signal could be transported across the cells. In the case of plants, the channel of such translocation of signal was the plasmodemata, the pore channels linking the plant cells (Voinnet, 2005).

3.1.1.3. Relation of microRNAs and other short RNAs to siRNAs

MicroRNAs (miRNAs) have been dubbed the 'cousins' of siRNAs (Tomari and Zamore, 2005). They are a class of 19 to 25 nucleotides ssRNAs that are encoded in the genomes of most multicellular organisms studied. Some are evolutionarily conserved and are developmentally regulated (Novina and Sharp, 2004). They silence certain cellular genes at the stage of protein synthesis by repressing the expression of target genes (reviewed in He and Hannon, 2004). As shown in Fig. 3.2, miRNAs and siRNAs have a shared central biogenesis, which involved the processing by both Dicer and RISC. Therefore, as two separate classes of silencing RNAs, miRNAs and

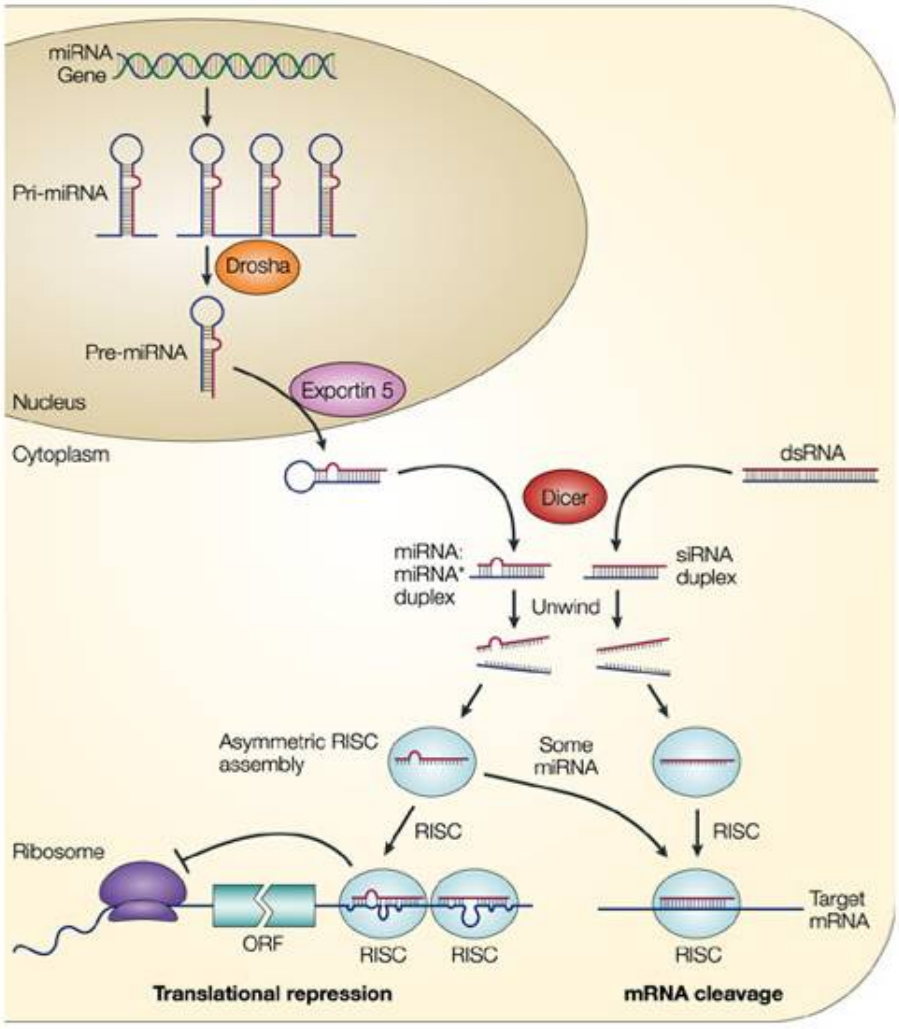


Fig. 3.2. Biogenesis of miRNAs and siRNAs and post-transcriptional suppression. The nascent primary-microRNA (pri-miRNA) transcripts are first processed into ~70-nucleotide pre-miRNAs by the ribonuclease Drosha inside the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into miRNA:miRNA* duplexes by Dicer. Dicer also processes long dsRNA molecules into siRNA duplexes. Only one strand of the miRNA:miRNA* duplex or the siRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC) , which subsequently acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target. ORF, open reading frame.

(Cited from He and Hannon, 2004)

siRNAs cannot be distinguished by their chemical composition or mechanism of action. However, important distinctions can still be made in regard to their origin, evolutionary conservation and the types of genes they silence (reviewed in Bartel, 2004).

Although the discovery of miRNAs has added a new dimension to the understanding of complex gene regulatory networks, the question as to 'whether miRNAs are truly different from siRNAs or the current understanding fails to functionally distinguish these two species of small RNAs under physiological conditions' still remains to be addressed (He and Hannon, 2004). What is better elucidated from various studies is that like siRNAs, plant and animal miRNAs can direct cleavage of their mRNA targets when the two are extensively complementary (Hutvágner and Zamore, 2002; Llave *et al*, 2002; Tang *et al*, 2003; Xie *et al*, 2003; Zeng and Cullen, 2003; Mallory *et al*, 2004; Okamura *et al*, 2004; Yekta *et al*, 2004), but repress mRNA translation when they are not (Olsen and Ambros, 1999; Chen, 2004; Doench *et al*, 2003; Saxena *et al*, 2003; Zeng *et al*, 2003; Doench and Sharp, 2004).

Aside from miRNAs, tiny non-coding RNAs (tncRNAs) as well as small modulatory RNA (smRNA) have been discovered. But their precise functions are still not very well understood or are localized to neuron-specific genes only, respectively (Novina and Sharp). It is probable that other species of small RNAs are yet to be discovered. Thus, it is important to consider the potential effect, if any, of all these small RNAs when performing RNAi experiments.

3.1.1.4. Relation of cosuppression and antisense inhibition to RNAi

The discovery of RNAi in *C. elegans* (Fire *et al*, 1998) had precedents that dated back to the late 1980s and early 1990s. At those times, plant biologists working with petunias were surprised to find that when they introduced numerous copies of a gene that codes for deep purple flowers into the plants, the resultant transgenic petunias produced white or patchy flowers instead of flowers with darker purple hue associated with overexpression of the transgene, chalcone synthease (Napoli *et al*, 1990; van der Krol *et al*, 1990). The transgenes had somehow silenced both themselves and the plants' endogenous 'purple flower' genes. Similarly, when plants were infected an RNA virus that had been genetically engineered to contain fragments of a plant gene, the plant's gene itself became silenced (Wassenegger *et al*, 1994). These phenomena have led to the coining of the term 'cosuppression'.

On the other hand, antisense sequences or transgenes have also been found to be capable of silencing genes, albeit with a relatively low efficiency with weak suppression (Bruening, 1998; Murfett *et al*, 1995). This is a seeming paradox when the potential of antisense transgene to form duplex RNA with the target mRNA is taken into account. This phenomenon can be termed as antisense inhibition.

Though the molecular bases of sense and antisense transgene-mediated silencing still remain unclear, it is believed that the 'copying' of these transgene-encoded ssRNAs into dsRNAs is responsible for converting them into silencing triggers and such 'copying' is mediated by RNA-dependent RNA Polymerase 6 (RDR6 in Fig. 3.4) (Baulcomble, 1996; 2004; Jorgensen, 2003; Tomari and Zamore, 2005; Gendrel and Colot, 2005). Based on this, a threshold sensing model has been proposed, in which

"aberrant" single-stranded RNA accumulates *in vivo* beyond some critical level before triggering its 'copying' into dsRNA (Zamore 2001; Jorgensen, 2003).

In an alternative nuclear model, nuclear accumulated sense and/or antisense transcripts have been proposed to form pre-miRNA-like structure that upon cleavage by Dicer or Drosha-like proteins will give rise to miRNA-like small RNAs, which can then be amplified or enter the RNAi pathway as outlined in Fig. 3.3 (Wang and Metzlaff, 2005; Voinnet, 2003).

Even though the molecular bases of sense and antisense transgene-mediated silencing are still not well characterized, these transgenes have the potential to cause gene silencing, possibly through the RNAi pathway. Therefore, in addition to expressing the inverted repeat transgene that will give rise to hairpin RNAs (hpRNAs) responsible for siRNA generation, over-expression of sense and antisense sequences of the introduced transgene should also be included as controls when designing an RNAi experiment.

3.1.1.5. Advantages and applications of RNAi

As a simple, cheap and powerful tool, RNAi has been used extensively in the laboratory to generate cells, tissues, or even animals with reduced expression of specific genes, allowing scientists to probe the functional significance of the genes of interest (Dillon *et al*, 2005). It has also facilitated the study of physiological processes by offering a quicker way to generate transgenic "knock down" animals when comparing to the conventional approaches, such as "knock out" mice (Leung and Whittaker, 2005). The RNAi approach has perceivably the added advantage of being able to generate transgenic cell lines or animals even in the event of a lethal mutation.

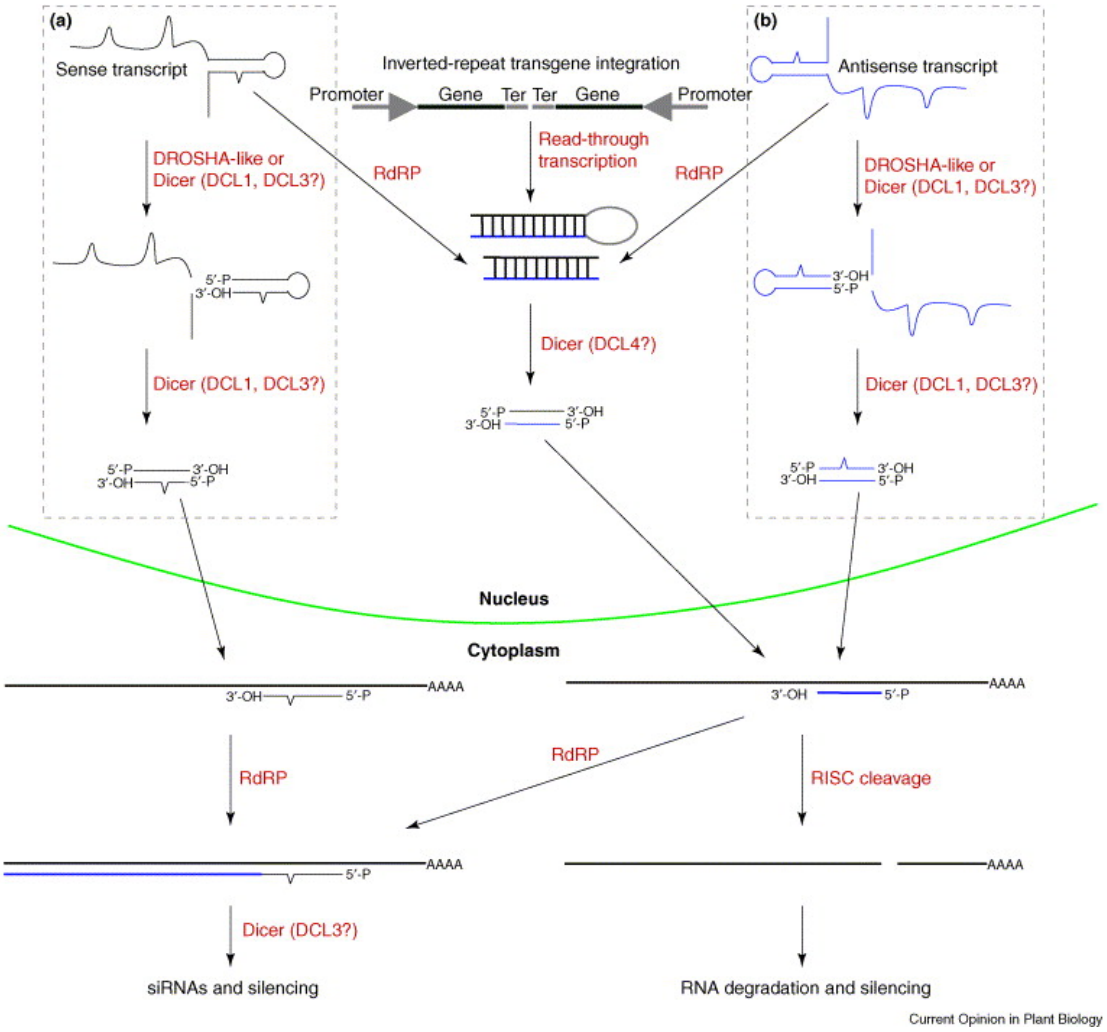


Fig. 3.3. A nuclear model for sense and antisense transgene-mediated silencing. (a) Nuclear-accumulated sense transcript forms a pre-miRNA-like structure and, upon cleavage by Dicer or Drosha-like proteins, gives rise to miRNA-like small RNAs. These small RNAs are then used as primers by RNA-dependent RNA polymerase (RdRP) to synthesize secondary dsRNA, resulting in gene silencing (cosuppression). (b) Similarly, nuclear-localized antisense transcript can also form pre-miRNA-like structures and hence miRNA-like small RNAs. These small RNAs have perfect complementarity with the target mRNA. They guide RISC to cleave target mRNA or are used as primers for RdRP or both. Alternatively, nuclear sense or antisense transcript is the preferred template for RdRP to synthesize secondary dsRNA in either a primer-dependent or a primer-independent manner. Another possibility is that read-through transcription occurs in a tail-to-tail inverted transgene repeat, generating long hairpin RNA (hpRNA) and triggering silencing. Ter, transcriptional terminator; DCL, Dicer-like.

(Cited from Wang and Metzlaff, 2005)

This is because the varying degrees of gene silencing or "knock down" by RNAi will ensure at least that in some transgenic lines, residual expression of the targeted endogenous gene is still feasible for the survival of the transgenic lines, a feast which is not quite possible in the "knock out" scenario.

As a powerful reverse genetic tool for research, RNAi has also been applied to plant science in the study of plant cytoskeleton (Klink and Wolniak, 2000), root biology (Limpens *et al*, 2004), oncogene silencing in crown gall tumors caused by *A. tumefaciens* (Escobar *et al*, 2001) as well as other studies that aim to understand plant biology or for crop improvement (Pattanayak *et al*, 2005).

Likewise, RNAi is also applied to research studies based in animals, worm, fly and other organisms. However, in the mammalian and some non-plant systems, research findings have shown that long dsRNA more than 30 bp will give rise to non-specific effects, which are characterized by degradation of all mRNAs and the inhibition of all protein synthesis. These non-specific effects were found to arise as a consequence of activation of two enzymes, PKR and 2,'5' oligoadenylate synthetase (2', 5'-AS), and could be circumvented by using duplexes of siRNAs of about 21- to 22-nt instead of the inverted repeat sequence used in plant studies (Bass, 2001; Elbashir *et al*, 2001; Zamore, 2001). This difference in non-specific effects reflects the divergent evolutions of various organisms while the ancient cellular pathway of RNAi is evolutionarily conserved. And such difference should be taken into consideration when using RNAi as an experimental approach in different systems originated from different organisms

Besides its usefulness as an experimental tool, RNAi is also a potent therapeutic approach to silence the expression of exogenous disease causing genes, such as those

from pathogens, as well as endogenous genes that play a role in the disease process (Dillon *et al*, 2005). Its use in therapeutic approaches for cancer, neurological diseases, infectious diseases, respiratory diseases and its potential use in treatment for human immunodeficiency virus (HIV-1) induced diseases has been explored (Shankar *et al*, 2005; Leung and Whittaker, 2005; Forte *et al*, 2005; Cullen, 2005; Dillon *et al*, 2005). In addition, its use as a therapeutic strategy against viral infections both in plants and animals has been well examined too (Voinnet, 2005; Tan and Yin, 2004). Despite several obstacles or limitations to its use that include aspects of delivery, vector system, safety, efficacy and "off target" effect, phase I clinical studies of RNAi are on the horizon (reviewed in Dillon *et al*, 2005; Shankar *et al*, 2005; Leung and Whittaker, 2005).

Apart from all the aforementioned applications, RNAi has also revolutionized the functional analysis of genomes, as genome-scale RNAi analyses have provided new approaches for probing the inner workings of the cell. With the myriad of phenotypic data collected from these studies at hand, a new era of bioinformatics related to the phenome has emerged (reviewed in Gunsalus and Piano, 2005; Bengert and Dandekar, 2005). Even though it is still a budding technology, RNAi cell microarrays is a promising approach and tool that can increase the efficiency, economy and ease of genome-wide RNAi screens in metazoan cells (reviewed in Wheeler *et al*, 2005).

As a natural occurring and endogenous cellular pathway, the applications of RNAi in many aspects are still being studied, discovered and refined for its usage. Just like any newly emerging technology, RNAi has also some limitations and drawbacks that have to be considered and, if possible, overcome before its current and future

applications will yield maximum benefits to whichever arena it is applied (Campbell and Choy, 2005).

3.1.2. RNAi-mediated silencing pathways in plants

RNAi in plants is also known as posttranscriptional gene silencing (PTGS) and it is related to or at least shares similar, if not identical, silencing pathway with virus induced gene silencing (VIGS) (Voinnet, 2005). Research findings have indicated that PTGS or RNAi in plants is likely to involve a number of RNA-dependent RNA polymerases (RdRP or RDR) and Dicer or Dicer-like (DCL) factors (Xie *et al*, 2004).

As illustrated in Fig. 3.4, studies of *Arabidopsis* insertion mutants have revealed that unlike many animals, plants encode multiple DCL and RDR proteins. DCL1 was found to be involved in miRNA processing, DCL3 for that of endogenous siRNAs and DCL2 for viral siRNAs. Though *Arabidopsis* contains 4 DCL factors, function for DCL4 is yet to be determined. As for RDR proteins, RDR2 was found to be required for all endogenous siRNAs analyzed, while RDR6 was necessary for sense transgene mediated RNAi (Gendrel and Colot, 2005; Xie *et al*, 2004; Tang *et al*, 2003; Beclin *et al*, 2002; Dalmay *et al*, 2000; Mourrain *et al*, 2000).

While there is only one Dicer in mammals and *C. elegans*, the flowering plant *Arabidopsis* has 4 DCL factors. At the same time, while RDR was not found to be involved in RNAi in human and fly, at least 2 such polymerases (RDR2 and RDR6) were found to be working in plants. Together with other silencing pathways that are operating simultaneously, such as RNA-dependent DNA methylation (RdDM), it is apparent that transgene induced RNAi is only just a small part of the complicated regulatory circuit found in the plant systems. Therefore, when designing

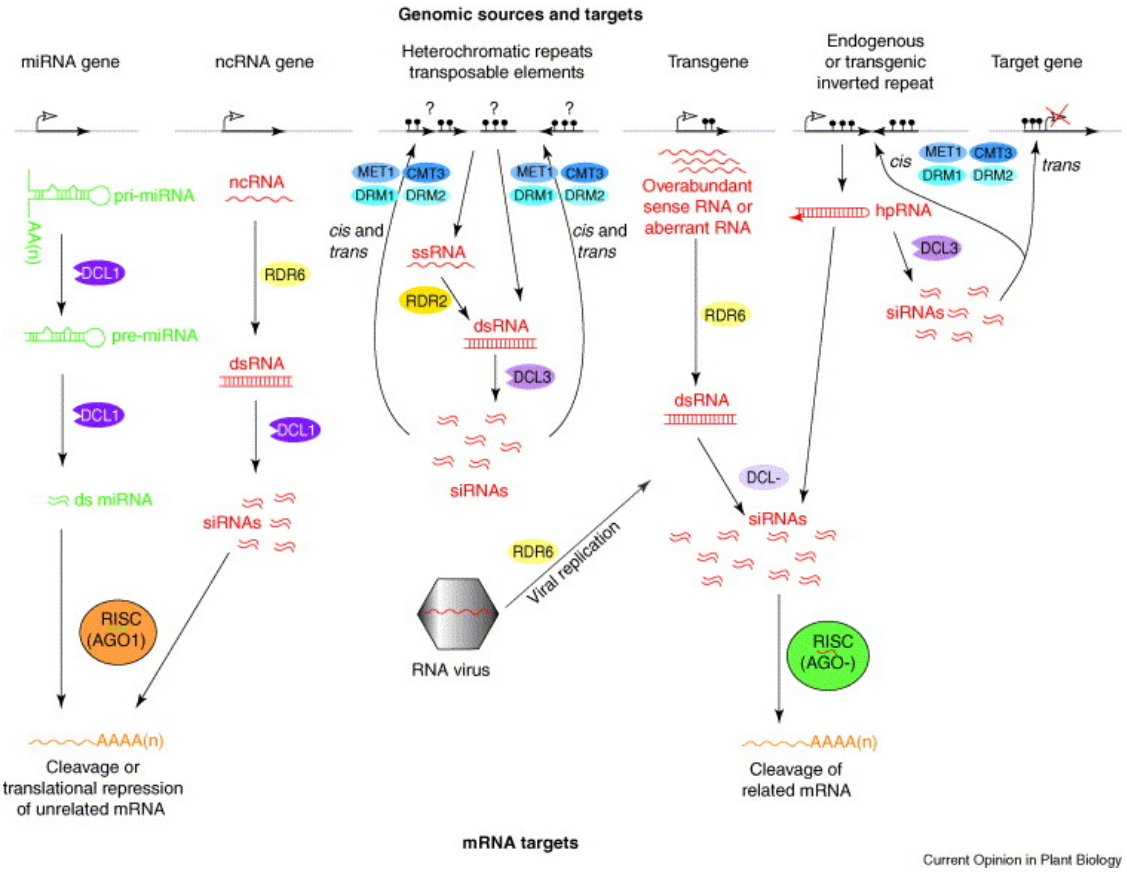


Fig. 3.4. RNAi-mediated silencing pathways in plants. Genomic sources of RNAs that are processed into small RNAs are illustrated at the top. Open arrows and question marks indicate transcription start sites and uncharacterized transcription, respectively. Filled circles indicate DNA methylation; the open arrow with a red cross indicates promoter silencing by RNA-dependent DNA methylation (RdDM). For simplicity, the RdDM pathway that is associated with posttranscriptional gene silencing is not depicted and neither is the distinction between nucleus and cytoplasm. DCL, Dicer-like; RDR, RNA-dependent RNA polymerase; ncRNA, non-coding RNA.

(Cited from Gendrel and Colot, 2005)

an RNAi experiment using the plant systems, it is crucial to consider a number of factors such as the genomic locus of the endogenous gene to be silenced, the sequence of the siRNA used or whether to use a viral vector to deliver the transgene.

3.1.3. RNAi in suspension cultured plant cells

Cultured *Drosophila* cells (Caplen *et al*, 2000) and mammalian cells such as human embryonic kidney 293 (HEK-293) and HeLa cells (Elbashir *et al*, 2001) have been used for the studies of RNAi. Suppression of gene expression by RNAi was subsequently shown in suspension cultured tobacco BY-2 cells (Akashi *et al*, 2001). As mentioned earlier, *Arabidopsis* DIP is a protein that has been shown to interact with *A. tumefaciens* VirD2 protein. Western blot analysis of DIP has shown that this protein, or rather its conserved homologue, could also be detected in tobacco BY-2 cells, as detection has yielded a protein of the correct and expected size in both species of plants. Subsequent investigations in BY-2 cells have further verified the cytoplasmic location of such DIP homologue and its colocalization with T-DNA and involvement in *Agrobacterium*-mediated transformation of plant cells (Chang, 2002).

Despite the absence of the actual or exact sequence of tobacco DIP homologue in the current tobacco sequence database, the lack of PKR and 2', 5'-AS mediated non-specific effects observed in mammalian RNAi experiments means that a larger inverted repeat of DIP sequence can be utilized for RNAi experiment. This dsRNA-forming transgene of sense and antisense sequences joined in a head-to-head manner with a loop between them, i.e. sense-loop-antisense, will generate a number of siRNAs, some of which, if not all, are capable of inducing RNAi in tobacco BY-2 cells. For example, if the sense and antisense DIP sequences are 500 bp each, then the endogenous cellular machinery involving DCL factor will generate about 23 to 24 of

21-nt siRNAs from the hairpin dsRNA that is formed after the transcription of the sense-loop-antisense transgene. Depending on the sequence homology between DIP and the tobacco DIP homologue, some of the siRNAs with high sequence homology will target the mRNAs of tobacco DIP homologue in BY-2 cells for degradation, or for translational repression of the homologue if the homology is low. For the intended aim of achieving RNAi in tobacco cells and tissues in this study, all that is needed is just one single siRNA which can induce the degradation or cleavage of the mRNAs of tobacco DIP homologue.

3.1.4. Novel approach of sequential *Agrobacterium*-mediated transformations of suspension cultured plant cells

As one of the important objectives of this study is to verify and establish the functional significance of DIP in the process of *Agrobacterium*-mediated transformation of plant cells, the use of an RNAi approach will lead inevitably to the circumstances where the plant cells will have to be transformed twice. This is because the first round of transformation used to induce RNAi is designed to study the phenomenon of *Agrobacterium*-mediated transformation of plant cells, which itself is a transformation process.

In other words, while the first round of transformation is used to introduce a transgene to induce the "knock down" of DIP via an RNAi pathway, the second round of transformation is to assay whether the "knock down" of DIP will have any effect on the *Agrobacterium*-mediated transformation of plant cells. Therefore, the approach of co-transformation cannot be used in this study. Neither is any harsh or inefficient approach of transformation. For the first round of transformation, the chosen approach must be efficient and does not require extensive manipulations or disturbance to the

cells. This is to ensure that the viability of the cells is retained or not compromised before they are transformed again by *A. tumefaciens.* For this purpose, *Agrobacterium*-mediated transformation was chosen to bring about RNAi in our study.

Unlike the approach adopted by Akashi *et al* (2001), which requires the manipulation of protoplasts and the subsequent electroporation of protoplasts, the tobacco cells or tissues were subjected to two rounds of *Agrobacterium*-mediated transformations in our study, the first round for RNAi and the second round for examining the effect of DIP "knock down" on the efficiency of *Agrobacterium*-mediated transformation. This sequential Agrotransformation approach has been chosen for our study because *Agrobacterium*-mediated transformation of plant cells is efficient, mild to the plant cells that are to be subjected to further analysis and it usually inserts only a copy of the T-DNA into the plant genome, while affecting the viability of the cells the least.

3.2. Materials and methods

3.2.1. Construction of plasmids and strains

pGAD10-81 harboring DIP (also named as pGAD-DIP) was isolated from *A. thaliana* cDNA library via the GAL4-based two-hybrid system. DIP (VirD2 Interacting Protein) encoded by this plasmid was found to interact with VirD2 of pTiA6 (encoded by pAS-D2) from *A. tumefaciens* strain A348 (Chang, 2002). To construct a plant transformation plasmid for RNAi experiments, the C-terminal 588-bp fragment (from nucleotides no. 1899 to 2486) of the coding sequence of *DIP* , which is 2664 bp, was amplified from pGAD10-DIP using primers S1(C) and S2. This 588-bp fragment contains a 90-bp spacer or loop sequence downstream of the 498-bp region chosen for RNAi analysis. After a double digestion with *Pst* I and *Bam*H I, the PCR fragment was cloned into *Pst* I and *Bam*H I digested pUCA19 (lab collection) to obtain pHC9. After verification by DNA sequencing, the *Pst* I and *Bam*H I fragment was subcloned into the MCS2 of the low-copy pCB302-1 binary vector (Xiang *et al*, 1999) to obtain pHC19 (Fig. 3.5).

Likewise, the antisence sequence to the 498-bp region was also amplified from pGAD10-DIP by using primers AS1 and AS2. After a sequential digestion by *Sma* I at 30 ºC and *Eco*R I at 37 ºC, the PCR fragment was ligated into *Sma* I and *Eco*R I digested pUCA19 to obtain pHC10. After verification by DNA sequencing, the *Sma* I and *Eco*R I fragment was subcloned into the MCS2 of pCB302-1 to create pHC20 (Fig. 3.6). Excision and ligation of the antisense fragment from pHC10 into *Sma* I and *Eco*R I digested pHC9 was subsequently performed to obtain pHC12. After verification by DNA sequencing, the *Pst* I RNAi fragment (sense-loop-antisense) was subcloned from pHC12 into *Pst* I digested pCB302-1 to give rise to pHC18. Double

digestion by *Pst* I and *EcoR* to create pHC18 was not necessary since the antisense sequence also contains a *Pst* I site (Fig. 3.7). The expression of the RNAi *DIP* fragment (sense-loop-antisense) encoded by pHC18 in plant cells will produce dsRNAs which has been shown to be responsible for the RNAi phenomenon in cultured plant cells (Akashi *et al*, 2001).

The constructed plasmids mentioned above were then transformed into *A. tumefaciens* strain LBA4404 (Ooms *et al*, 1982) via electroporation (Cangelosi *et al*, 1991), as described in Chapter 2. Serving as a negative control, the empty binary vector, pCB302-1, was also transformed into LBA4404. As a summary, the T-DNA regions, between the left border (LB) and right border (RB), of all these plasmids can be outlined as shown in Fig. 3.8. Plasmid DNA from the LBA4404 based strains harboring these plasmids were then isolated by a modified Miniprep procedure (Weber *et al*, 1998) and verified by restriction digestion analysis, as outlined in Chapter 2 (data not shown). These strains were used in all the ensuing RNAi experiments.

To serve as a positive transformation control, LBA4404 harboring pIG121-Hm (Ohta *et al*, 1990) was used in all transformation and RNAi experiments (Fig. 3.9). pIG121-Hm contains a GUS reporter gene inserted with a modified intron of the castor bean catalase. This intron inserted GUS reporter gene that is fused to the 35S cauliflower mosaic virus promoter and can only be expressed in plant cells but not in *A. tumefaciens* cells, due to inability of the prokaryotic cellular machinery to excise this intron. After a histochemical GUS assay, LBA4404(pIG121-Hm) transformed plant cells will be stained blue. If fluorogenic GUS assay was performed, the transformed plant cells will emit a fluorescence that can be measured by using a fluorometer.

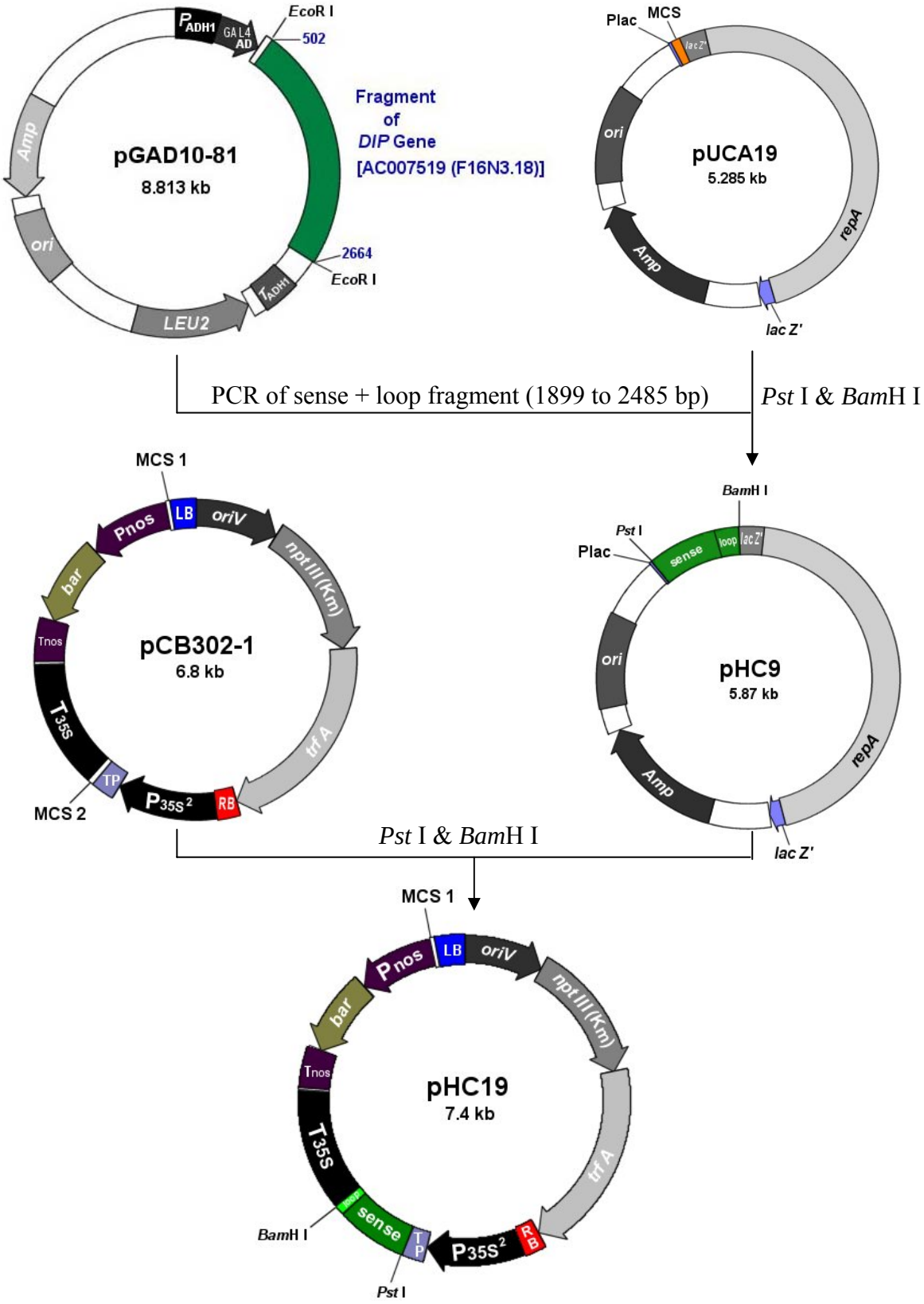


Fig. 3.5. Construction of pHC19. A 588-bp *DIP* fragment was amplified from pGAD10-81 (also renamed as pGAD10-DIP), digested with *Pst* I & *Bam*H I and then ligated into the *Pst* I & *Bam*H I digested pUCA19 to obtain pHC9. pHC19 was derived by subcloning the *Pst* I & *Bam*H I fragment (verified by DNA sequencing) from pHC9 into pCB302-1.

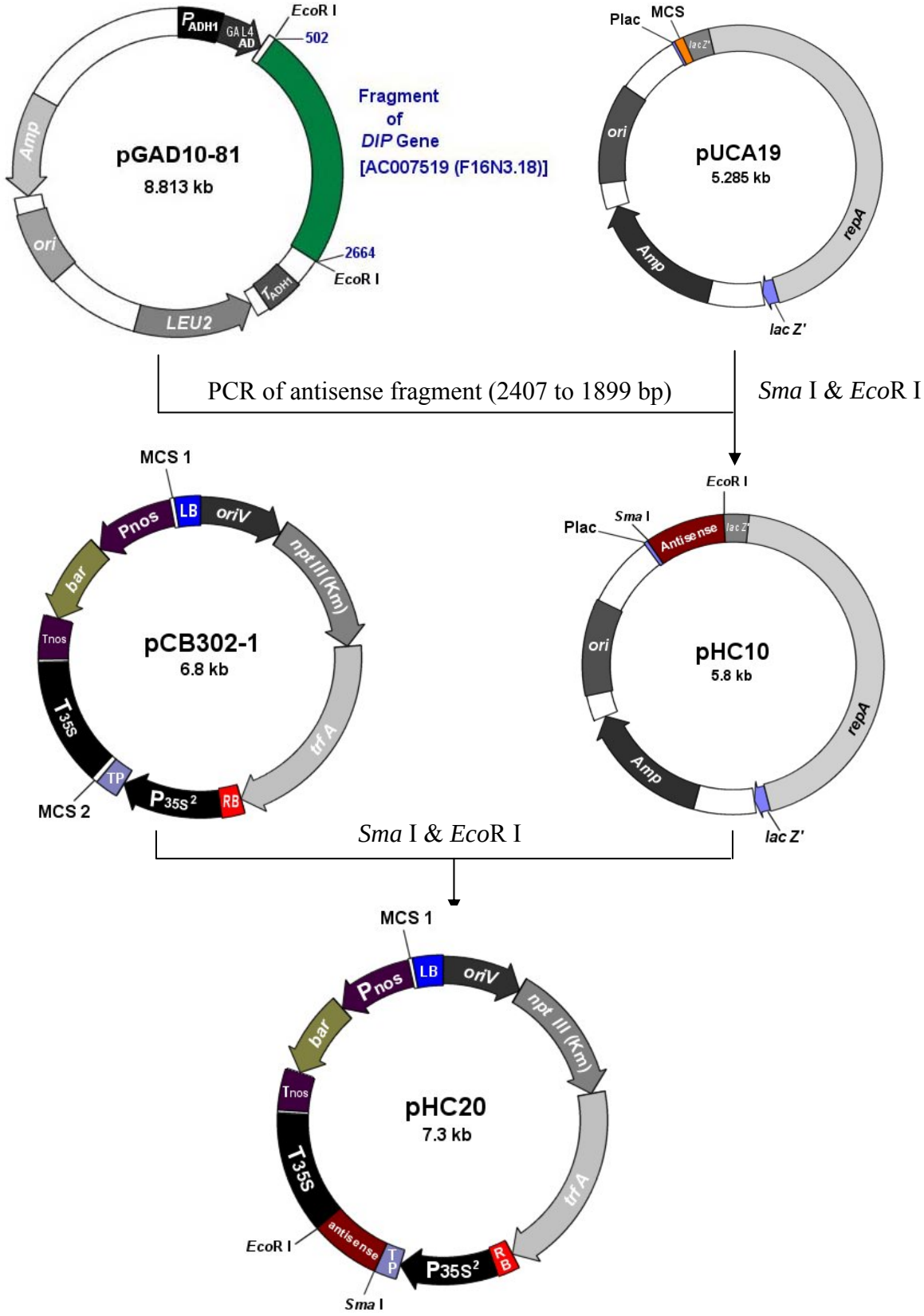


Fig. 3.6. Construction of pHC20. A 498-bp antisense *DIP* fragment was amplified from pGAD10-81 (also renamed as pGAD10-DIP), digested with *Sma* I & *Eco*R I and then ligated into the *Sma* I & *Eco*R I digested pUCA19 to obtain pHC10. pHC20 was derived by subcloning the *Sma* I & *Eco*R I fragment (verified by DNA sequencing) from pHC10 into pCB302-1.

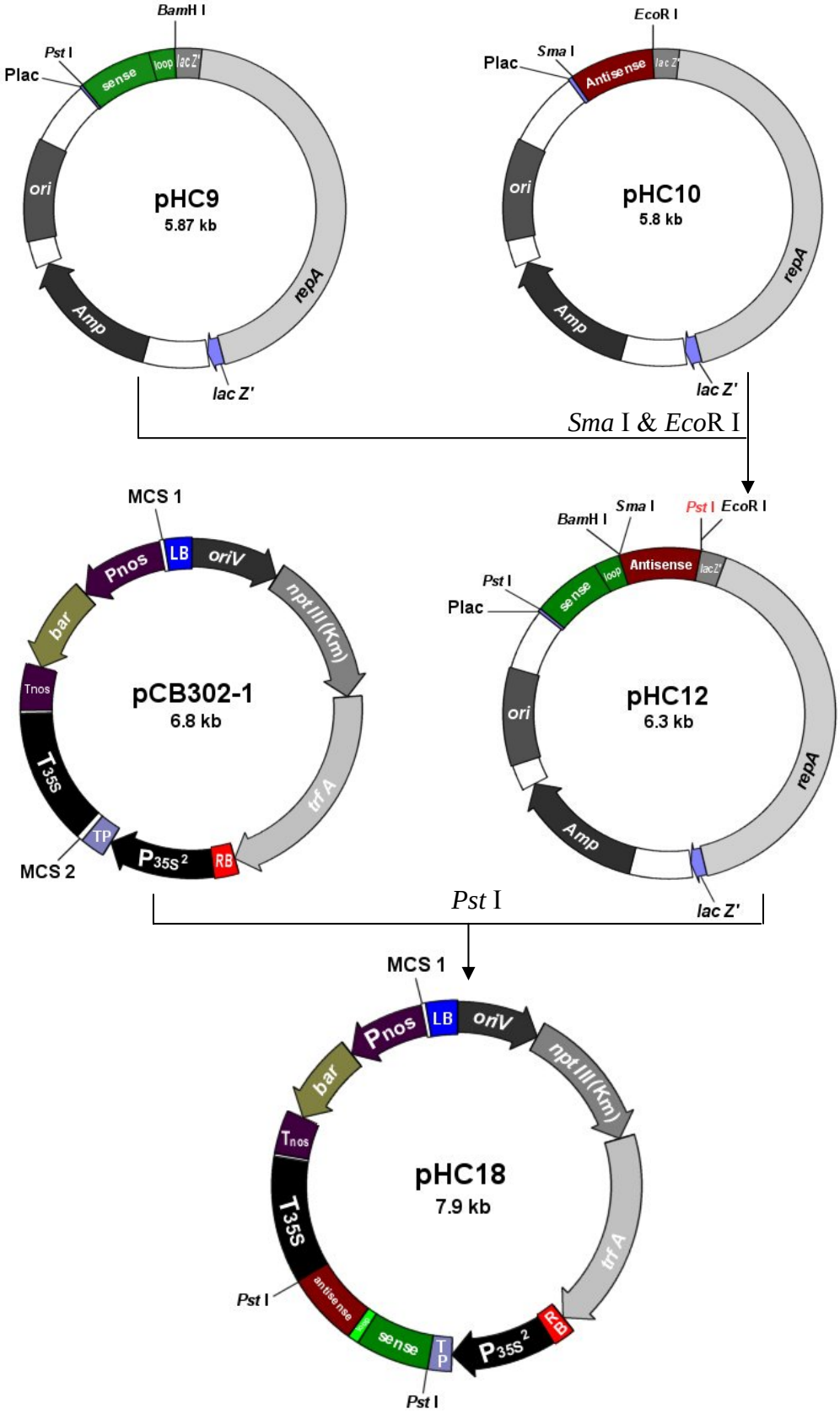


Fig. 3.7. Construction of pHC18. The *Sma* I & *Eco*R I fragment from pHC10 was subcloned into *Sma* I & *Eco*R I digested pHC9 to obtain pHC12, as both pHC9 and pHC10 possess the same pUCA19 vector backbone. Since the antisense sequence also contains a *Pst* I site, the *Pst* I RNAi fragment (sense-loop-antisense; verified by DNA sequencing) was subcloned from pHC12 into *Pst* I digested pCB302-1 to give rise to pHC18.

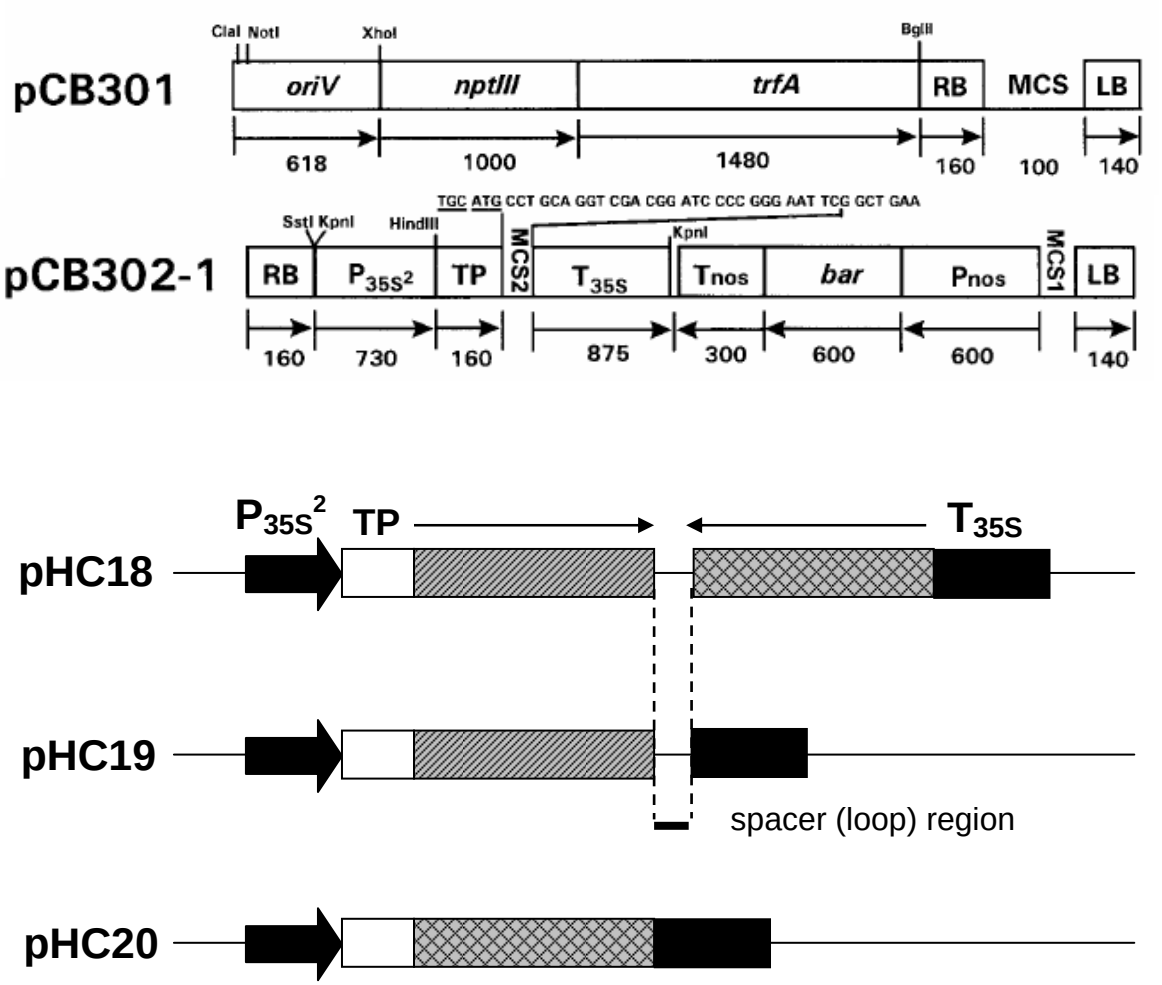


Fig. 3.8. T-DNA regions of the *DIP* RNAi, sense and antisense expression plasmids. The circular mini binary vector pCB301 that is commonly used for the construction of plant transformation plasmids is linearized in this schematic representation. pCB302-1 is identical to pCB301 except for the sequences contained between the RB and LB of T-DNA and that is the only region illustrated (Xiang *et al*, 1999). The numbers under each DNA region indicate the approximate size of that region in base pairs and the arrow indicates the orientation. The plasmids used for RNAi experiments, pHC18, pHC19 and pHC20, are constructed with pCB302-1 as the vector backbone containing a *DIP* fragment in MCS2. pHC19 contains a fragment of the C-terminal region of the *DIP* coding sequence (2664 bp) that ranges from nucleotides no. 1899 to 2486. This region is inclusive of a spacer or loop region that is made up of 90 nucleotides downstream of the chosen sequence (the hatched box). pHC20 contains the antisense sequence (the cross-hatched box) to the chosen *DIP* region without the spacer or loop. Ligation of the sense-loop-antisense RNAi sequence into pCB302-1 gives rise to pHC18. The expression of the *DIP* fragment encoded by pHC18 in plant cells will produce dsRNAs which has been shown to be responsible for the RNAi phenomenon in cultured plant cells (Akashi *et al*, 2001). *bar*, gene for phosphinothricin acetyltransferase; LB, left border of T-DNA; MCS, multiple cloning site (from pBluescript II); *nptIII*, gene for neomycin phosphotransferase for kanamycin resistance (from pBIN19); *oriV*, part of RK2 origin of replication (from pBIN19); P_{35S}^2, 35S cauliflower mosaic virus promoter with double enhancers; Pnos, promoter of *nos* (nopaline synthase) gene; RB, right border of T-DNA; Tnos, terminator of *nos* (nopaline synthase) gene; TP, plastid targeting sequence of Rubisco small subunit; *trfA*, part of RK2 origin of replication.

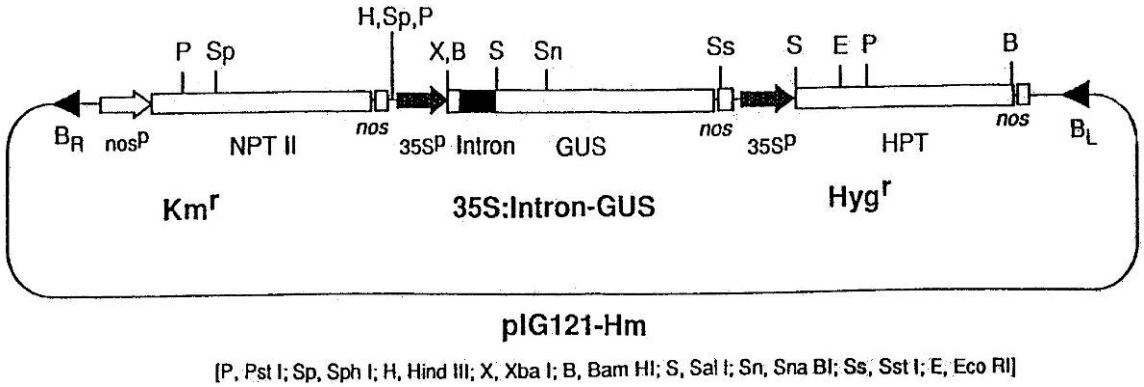


Fig. 3.9. GUS reporter plasmid, pIG121-Hm. This plasmid contains a CaMV 35S promoter-a modified intron of the castor bean catalase-GUS chimeric gene (CaMV 35S::Intron-GUS in pIG121-HM). It was constructed from pBI101 (vector; Jefferson *et al*, 1987), pIG221 (35S:Intron-GUS; Ohta *et al*, 1990) and pLAN101MHYG (Hyg^r; Dr. Ko Shimamoto) (Akama *et al*, 1992; Hiei *et al*, 1994).

3.2.2. *Agrobacterium*-mediated transformation of tobacco BY-2 cells

Nicotiana tabacuum BY-2 suspension cultured cells were maintained in Murashige and Skoog (MS) liquid medium supplemented with 0.2 mg/L of 2,4-D (Linsmaier and Skoog, 1965). The cultures were incubated at room temperature (RT) with shaking at 100 rpm and subcultured every week with a 4 % inoculum.
A. tumefaciens strain was grown overnight in AB minimal medium, supplemented with the appropriate antibiotics, in a 28 °C incubator before the bacterial cells were collected and then resuspended in IB medium supplemented with 100 μM acetosyringone (AS) as well as the appropriate antibiotics and further incubated at 28 °C for 16 to 18 hrs for the induction of virulence gene expression (Cangelosi *et al*, 1991). After washing with MS medium, 100 μl of the bacterial cell suspension (5 X 10^8 cells/ml) was added to 4 ml of BY-2 cell suspension that was 3 to 5 days old after the weekly subculture, in a small petri dish with a diameter of 4 cm. After incubation at RT for a certain period of time (ranging from 1 to 4 days), the bacterial cells were washed away from the plant cells as described previously (Lee *et al*, 1999) before the cocultivated plant cells were then subjected to GUS assays (Jefferson *et al*, 1987; 1991; Cao *et al*, 1998), induction of transformed or transgenic calli on selective medium or another round of cocultivation with the GUS reporter strain, LBA4404(pIG121-Hm).

3.2.3. Sequential Agrotransformations of tobacco BY-2 cells

To investigate the effect of transient "knock down" of *DIP* on *Agrobacterium*-mediated transformation (Agrotransformation) of BY-2 cells, these cells were subjected to 2 rounds of cocultivation with *A. tumefaciens*. BY-2 suspension cultured cells that was 5 days old after the weekly subculture were cocultivated with pre-

induced *A. tumefaciens* LBA4404 harboring pHC18, pHC19, pHC20, pCB302-1 or pIG121-Hm during the first round of cocultivation, following the conditions outlined in the previous section.

After cocultivation for 1, 2 or 3 days, the cocultivated cells were collected in 50-ml sterile tubes and the volume of each tube was made up to 50 ml with fresh MS medium before the samples were centrifuged at 1000 rpm for 4 min at RT with soft start/stop (Eppendorf 5810R). This washing step was repeated once or twice with additional 50-ml volumes of fresh MS medium and centrifugations under the same conditions. The washed cells from each sample were then cocultivated with the pre-induced GUS reporter strain, LBA4404(pIG121-Hm), for 3 to 4 days.

After the second cocultivation with the GUS reporter strain, the BY-2 cells were collected, washed and then subjected to histochemical GUS assay (Jefferson *et al*, 1987; Cao *et al*, 1998) with some modifications. In brief, the cocultivation mixture in a petri dish was pelleted down by centrifugation at 660 rcf for 1 min and the supernatant was removed before the cells were incubated in the GUS staining solution containing 100 mM sodium phosphate buffer (pH 7), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na_2EDTA, 0.5 % (v/v) Triton X-100 and 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) at 0.5 mg/l at 37 °C for 4 hrs to overnight to obtain the final staining results. As the controls, (i) un-cocultivated BY-2 cells; (ii) BY-2 cells cocultivated with the GUS reporter strain only once during the first round of cocultivation; and (iii) BY-2 cells cocultivated with the GUS reporter strain only once during the second round of cocultivation, were also assayed for GUS activity.

3.2.4. Selection and subsequent Agrotransformation of stably transformed tobacco BY-2 cell lines

To investigate the effect of stable "knock down" of *DIP* on *Agrobacterium*-mediated transformation of BY-2 cells, 5 days old BY-2 cells were cocultivated with pre-induced *A. tumefaciens* LBA4404 harboring pHC18, pHC19, pHC20, pCB302-1 or pIG121-Hm for 3 to 4 days. According to the results of previous cytotoxicity assays, the cells were washed with fresh MS medium as described in the previous section before they were plated on MS agar plates supplemented with 5 μg/ml of phosphinothricin (ppt), 200 μg/ml of cefotaxime and 300 μg/ml of carbenicilin. For LBA4404(pIG121-Hm) cocultivated BY-2 cells, they were plated on MS agar plates containing 100 μg/ml of kanamycin instead of ppt. The plates were sealed with Parafilm and incubated in the dark at RT for 3 to 4 weeks until transformed calli were observed on the plates. These calli were then transferred onto fresh plates containing the appropriate antibiotics or selective agents as before and allowed to grow for a further 3 to 4 weeks under the same conditions. Subsequent subcultures were carried out every 3 to 4 weeks after the calli had reached a suitably large size and before they turned brown. After a few such transfers, the stable transformants or calli were initiated into liquid suspension cultures.

The suspension cultures of the transformed BY-2 cell lines were subcultured weekly with a 15 to 20 % inoculum into fresh MS medium supplemented with 100 μg/ml of kanamycin [for LBA4404(pIG121-Hm) transformed line] or 5 μg/ml of ppt (for all other transformed lines). Five days after such subculture, cells from each line were cocultivated with pre-induced *A. tumefaciens* LBA4404(pIG121-Hm), the GUS reporter strain, for 3 to 4 days. Following that, the cells from each line were collected

in Eppendorf tubes by centrifugation at 660 rcf for 1 min, resuspended in 1 to 1.5 ml of GUS extraction buffer (10 mM EDTA, 0.1% Triton X-100, 10mM β-mercaptoethanol in 50 mM phosphate buffer at pH 7.0) and vortexed vigorously for 1 min before the supernatants were collected for fluorogenic GUS assay by using 4-methylumbelliferyl β-D-glucuronide (4-MUG) as the substrate and the fluorescence readings were measured by using a fluorometer (Martin *et al*, 1992; Jefferson *et al*, 1991). To compare the relative transformation efficiency of these cell lines by the GUS reporter strain, the fluorescence readings were normalized to the protein content of each sample, which was measured by using the Bradford assay (Coomassie Plus™ Protein Assay Reagent, Pierce).

3.2.5. Agroinfiltration of tobacco plants

To investigate the effect of transient "knock down" of *DIP* on the efficiency of *Agrobacterium*-mediated transformation of tobacco plants, infiltration of *Nicotiana tabacuum* cv. Xanthi and *Nicotiana benthamiana* by *A. tumefaciens* was performed according to the protocol used in Baulcombe's lab (Voinnet *et al*, 2000) with some modifications. *A. tumefaciens* strain LBA4404 harboring pHC18, pHC19, pHC20, pCB302-1 or pIG121-Hm was first streaked on MG/L plate containing 100 μg/ml of kanamycin and incubated at 28 ºC for 1 to 2 days (Cangelosi *et al*, 1991). A single colony was then picked for each strain and inoculated into 50 ml of MG/L supplemented with 100 μg/ml of kanamycin. After overnight culture with vigorous shaking at 28ºC, the bacterial suspensions ($OD_{600} \approx 1.0$) were spun down at 4000 g for 10 min and the pellets were resuspended each in 50 ml of 10 mM $MgCl_2$ before 75 μl of 100 mM acetosyringone was added. The bacteria were then kept at RT for at least 3 hrs without shaking.

Infiltration was performed with a 1ml syringe without needle. *Nicotiana benthamiana* or *Nicotiana tabacuum* cv. Xanthi plants with 5 to 7 leaves were used for infiltration. Two to three well expanded leaves were punched with a needle and the syringe was applied to the hole on the leaf with blocking by finger from the other side. The syringe barrel was gently pushed and the bacterial suspension was delivered into the intercellular space of the leaf. Three to five plants were infiltrated with each strain of *A. tumefaciens* as described above. As a control, 3 to 5 uninfiltrated plants were maintained together with the infiltrated plants. Three to four days after infiltration, the infiltrated leaves were subjected to a second round of infiltration with the pre-induced GUS reporter strain, LBA4404(pIG121-Hm), following the same infiltration procedure just described. Three days after the second round of infiltration, the leaves from each plant were excised and homogenized in GUS extraction buffer (10 times w/v) and the resultant leaf extract was subjected to fluorogenic GUS assay (Martin *et al*, 1992; Jefferson *et al*, 1991), as described in the previous section.

3.2.6. Analysis of *DIP* $^{+/-}$ heterozygous mutant plants

Both wild-type Col-0 (LEHLE seeds) and T-DNA inserted *DIP* $^{+/-}$ heterozygous mutant (SALK institute seeds) *Arabidopsis* plants were grown in soil. *Arabidopsis* seeds were allowed to imbibe on water wetted filter paper at 4 ºC for 7 days and then planted on *Arabidopsis* mix (three parts Florobella potting compost per 1 part sand). The plants were grown in a growth room with a photoperiod of 16 hrs light and 8 hrs darkness at 20 to 23 ºC. After flowering and seed collection, the seeds from *DIP* $^{+/-}$ mutant plants were germinated and the plants were grown again as above. The progenies were then propagated for a few generations following the same procedure in an attempt to obtain homozygous *DIP* $^{-/-}$ mutant plants through self fertilization or

selfing. Total RNAs were then isolated from the tissues of both the mutant plants (after several generations of selfing) and the control wild-type plants, following the procedure outline in Chapter 2. By using Dip-ex1 and Dip-ex2 primers, the RNAs isolated from these plants were used as the templates for one-step RT-PCR analysis following the instructions of the manufacturer (QIAGEN), also outlined in Chapter 2.

3.3. Results

3.3.1. Transient *DIP* "knock down" and antisense inhibition decrease the efficiency of *Agrobacterium*-mediated transformation of BY-2 cells

To establish a direct functional link of DIP with *Agrobacterium*-mediated transformation of plant cells, RNA interference (RNAi) approach was used to study the effect of *DIP* "knock down" on this process. Fig. 3.8 shows the derivation of plant transformation plasmids that were introduced into *A. tumefaciens* LBA4404 for subsequent RNAi experiments. As shown in Fig. 3.10 panel D, after the first round of cocultivation with LBA4404(pHC18) for 1 day, BY-2 cells became subsequently recalcitrant to transformation by the GUS reporter strain, LBA4404(pIG121-Hm), as evident by the lack of visible blue GUS staining. The expression the *DIP* fragment encoded by pHC18 in plant cells will produce dsRNAs, which have been shown to be responsible for the RNAi phenomenon in cultured plant cells (Akashi *et al*, 2001). Therefore, it is possible that when the expression of tobacco *DIP* homologue is "knocked down" in BY-2 cells, the subsequent T-complex's passage within the plant cells' cytoplasm is disrupted and thus prevented T-DNA from entering the nuclei of the cells for the intron inserted GUS gene to get expressed.

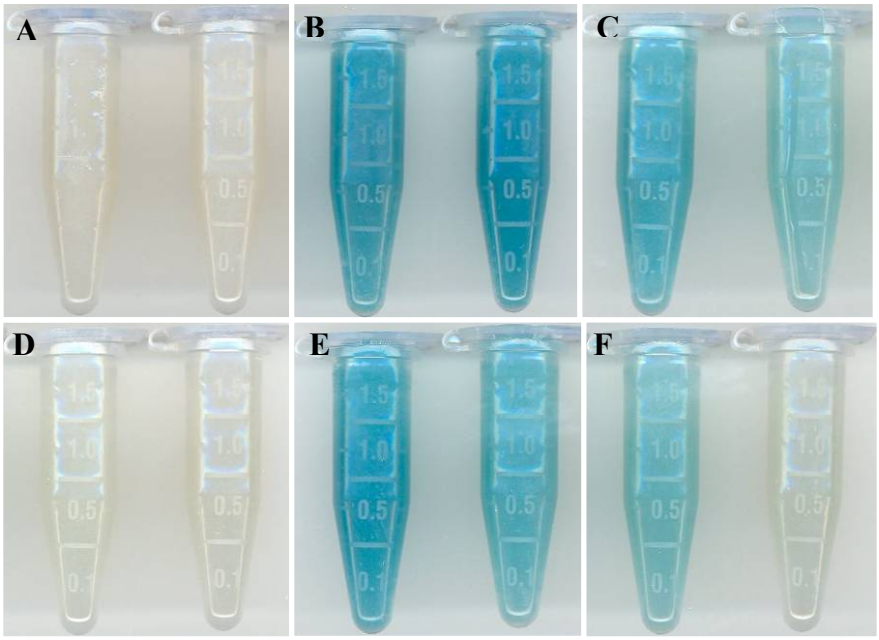


Fig. 3.10. Transient "knock down" of *DIP* decreases the efficiency of *Agrobacterium*-mediated transformation of BY-2 cells. Five days after subculture, tobacco BY-2 cells were cocultivated with pre-induced *A. tumefaciens* LBA4404 harboring pIG121-Hm (panel B), pCB302-1 (panel C), pHC18 (panel D), pHC19 (panel E) or pHC20 (panel F) for 1 day respectively. After washing with fresh MS medium, the cells (panel B to F) were subjected to a second round of cocultivation with pre-induced LBA4404(pIG121-Hm). 3 to 4 days after the second cocultivation, the cells were subjected to histochemical GUS assay. As a control, 5 days old BY-2 cells that were not cocultivated with *A. tumefaciens* (panel A) during the first round of cocultivation were washed with fresh MS medium and subjected to GUS assay 3 to 4 days later together with other samples .

However, when pre-cocultivated with LBA4404 harboring the *DIP* fragment overexpression plasmid, pHC19 (panel E), or the empty binary vector, pCB302-1 (panel C), a positive GUS staining pattern was observed after cocultivation by the GUS reporter strain. This staining pattern is similar to the positive control, in which the BY-2 cells were cocultivated with the GUS reporter strain twice (panel B). When BY-2 cells were pre-cocultivated with LBA4404 harboring pHC20, which expresses an antisense *DIP* fragment (panel F), a partial GUS staining pattern was observed after cocultivation with the GUS reporter strain. This partial stain is characterized by a light blue to faint blue or almost white (but still with a faint streak of light blue) coloration. Although this partial stain was observed in the repeat experiments, the degree of blue coloration varied from one experiment to the other (data not shown). It is possible that the degree of antisense inhibition on the expression of *DIP* might differ from one experiment to the other. Nonetheless, it is apparent that this antisense inhibition of *DIP* has resulted in a decreased efficiency of *Agrobacterium*-mediated transformation of BY-2 cells, confirming a functional role for DIP in assisting the T-complex to traverse across the plant cell cytoplasm.

When assayed for GUS activity, the other two controls, (i) BY-2 cells cocultivated with the GUS reporter strain only once during the first round of cocultivation and (ii) BY-2 cells cocultivated with the GUS reporter strain only once during the second round of cocultivation, also showed a blue stain similar to that of the positive control (panel B) (data not shown). This indicates that both rounds of transformations were successful and the T-DNAs were delivered into the plant cells. Additional transformation controls, both MX243 (an A348 based *virB*$^-$ mutant) and WR1715 (*virD2* mutant; 70 % of *virD2* deleted) strains harboring pIG121-Hm were

used as the negative controls. Negative GUS staining similar to that shown in panel A of Fig. 3.10 have been observed when these strains were used (data not shown).

Apart from the lag time of 1 day between the two rounds of cocultivations, GUS assays were also performed for samples in which the lag time was 2 or 3 days between the two rounds of cocultivations. This lag time is equivalent to how long the bacterial and the plant cells were cocultivated during the first round of cocultivation. Unlike the reproducible results associated with the lag time of 1 day between the two rounds of cocultivations, a few GUS staining patterns were observed for some or all of the experimental samples after many repeat experiments, in which 2 to 3 independent samples were processed for each treatment group. Despite such unpredictability of GUS staining pattern, the GUS assays revealed a few major trends in the staining patterns after cocultivation with the experimental strains for 2 or 3 days at RT and then another cocultivation with the GUS reporter strain for 3 to 4 days at RT

In the first pattern, most or all of the 3 to 5 tubes of the experimental treatment groups showed a negative GUS staining such as that shown in Fig. 3.11, except the positive controls that were cocultivated with the GUS reporter strain twice (panel B of both Fig. 3.10 and Fig. 3.11). This is probably due to the decreased cell viability after prolonged incubation with *A. tumefaciens* after the two rounds of cocultivations, as the experimental conditions are markedly different from the routine culture conditions in which fresh MS medium is supplied to the cells and the culture is gyrated at 100 rpm to supply the cells with maximal nutrients. In addition, disruption of *DIP* expression by RNAi, overexpression or antisense inhibition for a longer period of time may also be detrimental to the overall physiological conditions of the cocultivated cells, since

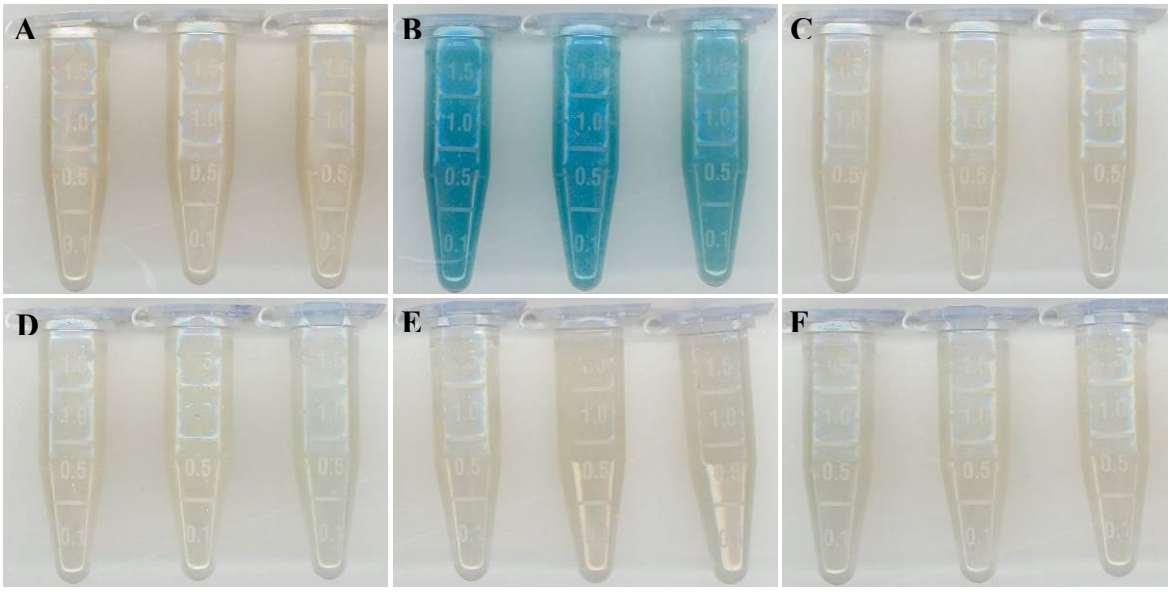


Fig. 3.11. Predominantly negative GUS staining after two rounds of *Agrobacterium*-mediated transformations of BY-2 cells. Five days after subculture, tobacco BY-2 cells were cocultivated with pre-induced *A. tumefaciens* LBA4404 harboring pIG121-Hm (panel B), pCB302-1 (panel C), pHC18 (panel D), pHC19 (panel E) or pHC20 (panel F) for 2 days respectively. After washing with fresh MS medium, the cells (panel B to F) were subjected to a second round of cocultivation with pre-induced LBA4404(pIG121-Hm). 3 to 4 days after the second cocultivation, the cells were subjected to histochemical GUS assay. As a control, 5 days old BY-2 cells that were not cocultivated with *A. tumefaciens* (panel A) during the first round of cocultivation were washed with fresh MS medium and subjected to GUS assay 3 to 4 days later together with other samples.

DIP is found to be a crucial subunit of the evolutionarily conserved exocyst complex involved in secretion via the sequence analysis BLAST programs.

In contrast to the first pattern, all or almost all the samples in the experimental groups were stained blue after GUS assays in the second major staining pattern, such as that shown in Fig. 3.12. It is apparent from Fig. 3.12 that the decrease in the efficiency of Agrotransformation of BY-2 cells could still be reflected in the partial stain or a light blue coloration for the RNAi (panel D) and antisense inhibition (panel F) samples. However, this partial stain is not observed in all the repeated experiments. In some repeated experiments, similar or same degree of blue staining was observed for all the experimental samples, except the negative control (data not shown).

As for this second staining pattern, it is probable that the overall competency of the BY-2 cells to being transformed by the GUS reporter strain was quite high under the experimental circumstances. The cells might still be in relatively better physiological conditions after the first round of transformation and thus might possess better overall cell viability for the second round of transformation by the GUS reporter strain. Alternatively, the efficiency of first round of transformation might have been too low to achieve a "knock down" effect associated with RNAi. This observation is in congruence with the fact that the cells used in the experiments were unsynchronized BY-2 cells (Nagata *et al*, 1992) and that the competency of the suspension cultured cells to transformation by *A. tumefaciens* may differ from one experiment to the next.

In other words, the difference between the first staining pattern and that of the second one may be attributed to the fact that the majority of the cells used for Agrotransformations could reside in different cell cycle stages and that most of the cells used were in a more competent stage to give rise to the second staining pattern.

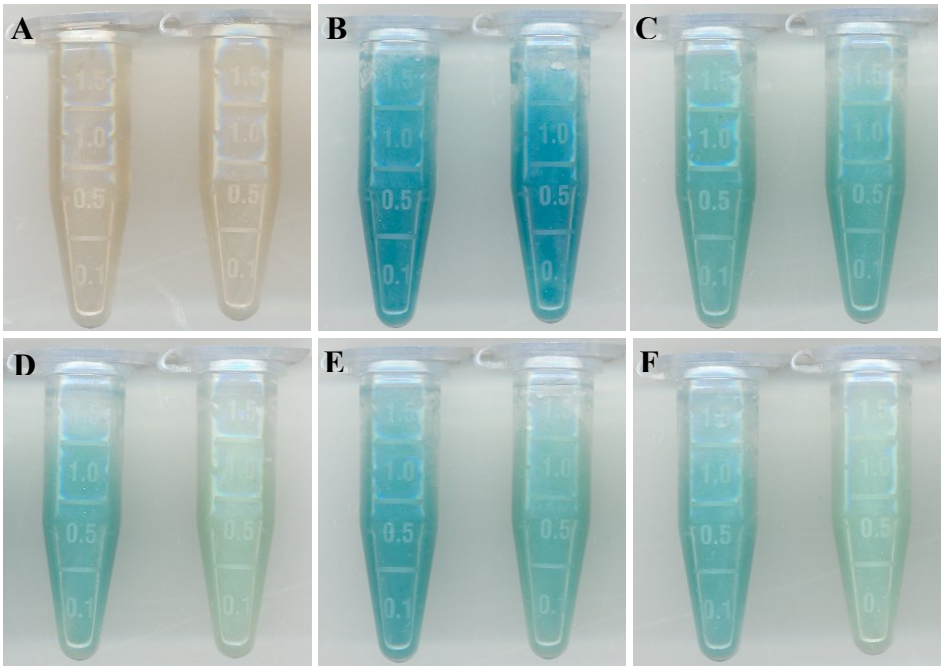


Fig. 3.12. Predominantly positive GUS staining after two rounds of *Agrobacterium*-mediated transformations of BY-2 cells. Five days after subculture, tobacco BY-2 cells were cocultivated with pre-induced *A. tumefaciens* LBA4404 harboring pIG121-Hm (panel B), pCB302-1 (panel C), pHC18 (panel D), pHC19 (panel E) or pHC20 (panel F) for 3 days respectively. After washing with fresh MS medium, the cells (panel B to F) were subjected to a second round of cocultivation with pre-induced LBA4404(pIG121-Hm). 3 to 4 days after the second cocultivation, the cells were subjected to histochemical GUS assay. As a control, 5 days old BY-2 cells that were not cocultivated with *A. tumefaciens* (panel A) during the first round of cocultivation were washed with fresh MS medium and subjected to GUS assay 3 to 4 days later together with other samples.

Aside from these, it is also probable that the effect caused by the transiently expressed genes carried by the T-DNAs outlined in Fig. 3.8 might have been lost or decreased upon prolonged incubation during or after the first round of cocultivation. The cellular machinery might have been trying to bypass the detrimental effects that arise as a consequence of the disruption of *DIP* expression by RNAi, overexpression or antisense inhibition.

In the less frequently observed third major staining pattern (Fig. 3.13), the RNAi sample (panel D), the overexpression sample (panel E) and the antisense inhibition sample (panel F) showed similar degree of GUS staining. These results indicate that the overexpression of sense or antisense *DIP* fragment were equally efficient in decreasing the amount of blue staining. This goes to show that the efficiency of *Agrobacterium*-mediated transformations of BY-2 cells in these samples was relatively diminished when compared to the empty vector control (pCB302-1; panel C). These results might have arisen due to the sense and antisense transgene-mediated silencing, as outline in section 3.1.1.4.

From the unpredictability of GUS staining profiles that arose when the lag time between the two rounds of cocultivations was set at 2 or 3 days, the resultant major staining patterns have reflected the complex nature of *Agrobacterium*-mediated transformation of plant cells in which numerous parameters are involved. By examining the various staining patterns observed for all the repeat experiments, it is certain that when the lag time between the two rounds of cocultivation was set at 1 day apart, the RNAi and antisense inhibition derived decrease in the efficiency of Agrotransformation of BY-2 cells by the GUS reporter strain was more readily

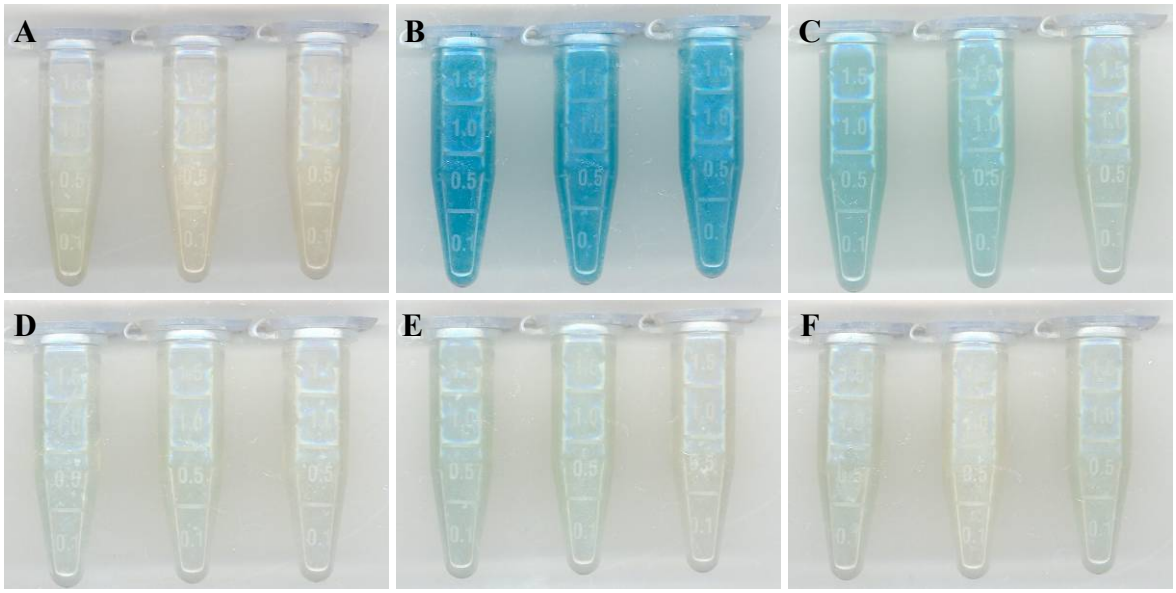


Fig. 3.13. Less frequently observed GUS staining pattern after two rounds of *Agrobacterium*-mediated transformations of BY-2 cells. Five days after subculture, tobacco BY-2 cells were cocultivated with pre-induced *A. tumefaciens* LBA4404 harboring pIG121-Hm (panel B), pCB302-1 (panel C), pHC18 (panel D), pHC19 (panel E) or pHC20 (panel F) for 3 days respectively. After washing with fresh MS medium, the cells (panel B to F) were subjected to a second round of cocultivation with pre-induced LBA4404(pIG121-Hm). 3 to 4 days after the second cocultivation, the cells were subjected to histochemical GUS assay. As a control, 5 days old BY-2 cells that were not cocultivated with *A. tumefaciens* (panel A) during the first round of cocultivation were washed with fresh MS medium and subjected to GUS assay 3 to 4 days later together with other samples.

observed and the results were similar to the staining profile illustrated in Fig. 3.10.

It is quite likely that when the two rounds of cocultivations are 1 day apart, the cells are in a relatively viable and competent stage for the second round of transformation by the GUS reporter strain. Yet at the same time, any detrimental effect due to the disruption of *DIP* expression by RNAi, overexpression or antisense inhibition might not have arisen due to the short cocultivation time of 1 day. Since transient expression of any transferred gene begins at around 2 days after the cocultivation of BY-2 cells with *A. tumefaciens*, this timing is just right for the early examination of the effects of the transient expression of *DIP* dsRNA, overexpression and antisense inhibition gene fragments on the trafficking and targeting of the GUS reporter T-DNAs from the plant cell cytoplasm to the plant cell nucleus, before the viability of the cells are detrimentally affected.

In other words, when the transgenes on the T-DNAs delivered into the nuclei of the plant cells by the first cocultivation are just being transiently expressed, without much detrimental effect on cell viability yet, the T-DNAs harboring the GUS reporter construct delivered into the plant cell cytoplasm by the second round of cocultivation are still being trafficked within the cytoplasm of the plant cells and being targeted to the plant cell nuclei. Therefore, any effect on the trafficking of the GUS-reporter-harboring T-DNAs may be attributed to the transiently expressed transgene. This may serve to explain why a comparatively reproducible negative effect of *DIP* 'knock down" by RNAi or to a certain extent by antisense inhibition was observed, even though the degree and extent of the blue coloration might differ among different experiments.

Last but not least, the more important observation from our data is the occurrence of the highly reproducible negative white GUS staining for almost all the RNAi samples in almost all the experiments performed, except in the predominantly positive GUS staining pattern. Beside this exception, the RNAi sample had always produced the negative stain. This shows that when DIP is "knocked down" by RNAi, the efficiency of *Agrobacterium*-mediated transformation of plant cells is unquestionably and markedly reduced.

Taken together, the results from this series of sequential transient Agrotransformations have demonstrated clearly that DIP is functionally crucial for a successful transformation by *A. tumefaciens.*

3.3.2. Transient *DIP* "knock down" and antisense inhibition decrease the efficiency of *Agrobacterium*-mediated transformation of tobacco plant tissues

To verify if the RNAi disruption of *DIP* expression totally abolishes A*grobacterium*-mediated transformation of plant cells or only decreases its efficiency, a two-round Agroinfiltration approach coupled with a more sensitive fluorogenic GUS assay (Martin *et al*, 1992; Jefferson *et al*, 1991) was adopted. BY-2 cells were not used because when the lag time between the two rounds of A*grobacterium*-mediated transformation increases, the cells (which are usually subcultured weekly) may not be in good physiological conditions for this experiment and that unpredictable GUS profiles may also be observed. Unlike single cells in suspension cultures, such as BY-2 cells, leaves attached to intact plants are nourished by the nutrients transported to them via the plant vascular system and are thus chosen for this experiment. In addition, infiltrated leaves have been shown to be able to recover after Agroinfiltration.

Similar to that of the BY-2 cells based experiment, the *DIP* overexpression and empty vector samples gave a positive GUS result. When viewed under UV, these were the only samples where a weak fluorescence could be observed (data not shown). This fluorescence was unlike the high-intensity and strong fluorescence of the positive controls. For the antisense sample, a very weak to almost null fluorescence was observed. Like the BY-2 cells based experiment, the fluorescence intensity of the antisense sample varied from one experiment to the other. But in the whole plant based experiments, the degree of variation is much smaller.

After normalization to the protein content of each sample, quantitative fluorescence readings (Table 3-1 and Fig. 3.12) reveal that when *DIP* expression was "knocked down" by RNAi, the efficiency of A*grobacterium*-mediated transformation of tobacco leaf tissues was decreased, but not totally abolished, to a level comparable to that of the negative control (the uninfiltrated leaves). Likewise, the antisense inhibition of *DIP* also decreased the transformation efficiency, as indicated by the fluorescence reading. But the reading is higher than that of RNAi sample by approximately 25%.

Surprisingly, the *DIP* overexpression sample gives a higher reading than the empty vector control sample, though the reading is still way below that of the positive controls (Table 3-1, [c]). It is quite probable the overexpression of *DIP* may enhance the interaction between VirD2 and DIP and thus a more efficient intracellular transport within the cytoplasm may give rise to slightly higher fluorescence reading as an indication of a slightly more efficient Agrotransformation.

Table 3-1. The effect of transient *DIP* "knock down" on *Agrobacterium*-mediated transformation of tobacco leaf tissues

Abbreviation [a]	Leaf extracts from infiltrated leaves [d]		Relative fluorescence per mg protein [e]
	1st infiltrated strain	2nd infiltrated strain	
No Agro No Agro	Not infiltrated	Not infiltrated	158 ± 28
RNAi Reporter	LBA4404(pHC18)	LBA4404(pIG121-Hm)	201 ± 23
Sense Reporter	LBA4404(pHC19)	LBA4404(pIG121-Hm)	431 ± 15
A/S [b] Reporter	LBA4404(pHC20)	LBA4404(pIG121-Hm)	266 ± 9
Empty Vector Reporter	LBA4404(pCB302-1)	LBA4404(pIG121-Hm)	381 ± 31
Reporter [c] Reporter	LBA4404(pIG121-Hm)	LBA4404(pIG121-Hm)	5614 ± 424
Reporter [c] No Agro	LBA4404(pIG121-Hm)	Not infiltrated	3687 ± 2
No Agro [c] Reporter	Not infiltrated	LBA4404(pIG121-Hm)	5467 ± 581

[a] These abbreviations are used in Fig. 3.14.

[b] A/S = Antisense *DIP* sequence harbored in pHC20

[C] The relative fluorescence per mg protein of the leaf extracts under these treatment groups are not plotted and included in Fig. 3.14 for clarity reason, as the high fluorescence readings will skew the histogram and depress the bar heights of the other treatment groups markedly. They serve as the positive controls.

[d] 2 to 3 leaves from 3 to 5 different plants were infiltrated for each treatment group

[e] 5 to 6 independent experiments were performed using leaves from both *Nicotiana benthamiana* (shown here) and *Nicotiana tabacuum* cv. Xanthi (data not shown). A similar trend was observed for each experiment despite the difference in absolute readings when leaves from both species of tobacco plants were used.

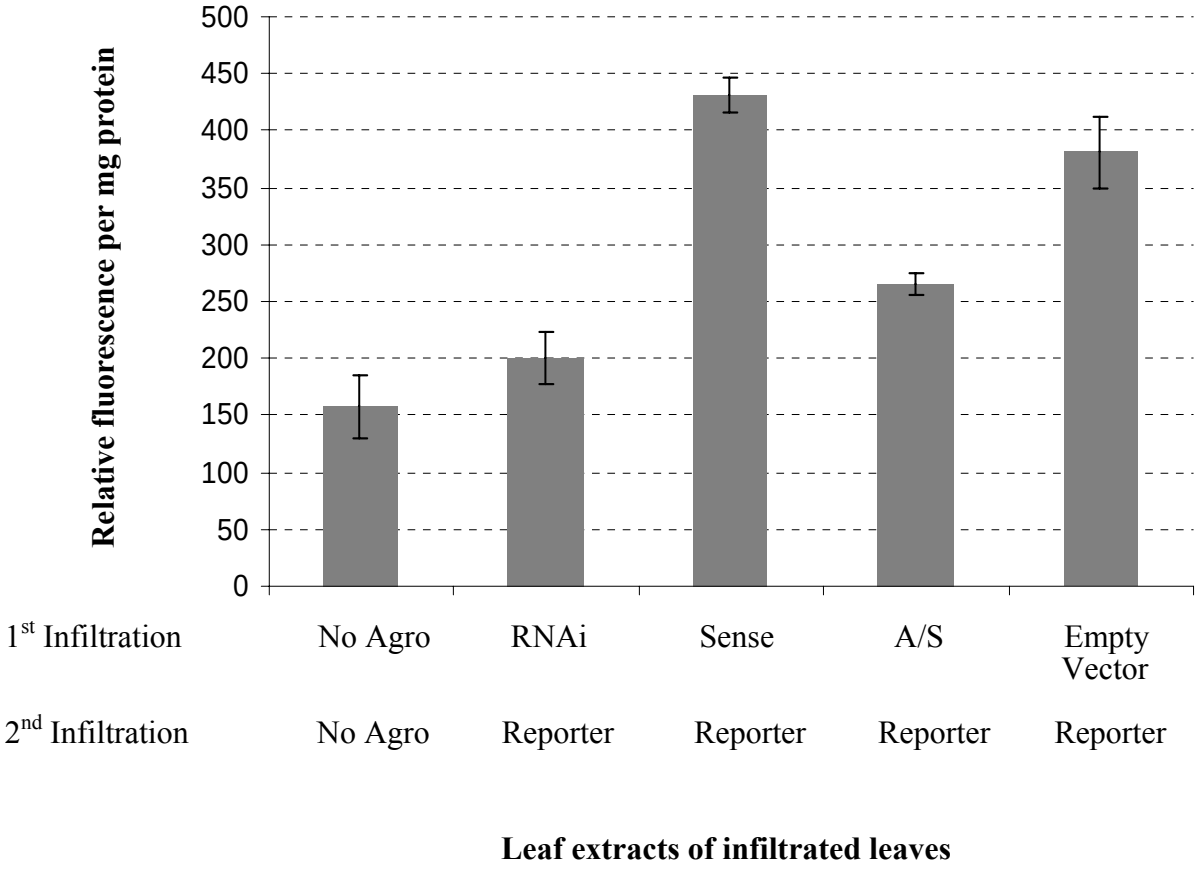


Fig. 3.14. Transient "knock down" of *DIP* decreases the efficiency of *Agrobacterium*-mediated transformation of tobacco leaf tissues. Young expanded leaves of *Nicotiana benthamiana* plants were infiltrated with *A. tumefaciens* LBA4404 harboring pHC18, pHC19, pHC20 or pCB302-1 respectively. After 3 to 4 days, the infiltrated leaves were subjected to a second round of infiltration by the GUS reporter strain, LBA4404(pIG121-Hm). The infiltrated leaves were assayed for GUS protein accumulation by fluorogenic GUS assay. The detailed results of the GUS assays are shown in Table 3-1.

Overall, the Agroinfiltration results support the findings of the BY-2 cell based experiments in that when the *DIP* expression is transiently "knocked down" via RNAi or to a lesser extent by antisense inhibition, a negative effect on the efficiency of *Agrobacterium*-mediated transformation of plant cells or tissues is observed. The perturbations of *DIP* expression do not, however, abolish totally the event of Agrotransformation altogether.

This is in concurrence with the notion that even in the event of a lethal mutation or mutation of a gene with crucial function, varying degree of RNAi in different cell lines or tissues will ensure that at least some transformed cell lines or tissues will harbor residual expression of the targeted endogenous gene for their survival. In tobacco tissues where RNAi did indeed abolish DIP function totally, the leaf tissues would have withered and died, as were observed for some leaves in the experiments. However, more often than not, total abolishment does not take place in most circumstances.

3.3.3. Stable *DIP* "knock down" decreases the efficiency of *Agrobacterium*-mediated transformation of BY-2 cells

As a further verification of the transient *DIP* "knock down" assays, BY-2 cells cocultivated with LBA4404 harboring pHC18, pHC19, pHC20, pCB302-1 or pIG121-Hm were plated on MS agar plates supplemented with antibiotics or selective agents in order to obtain the stably transformed BY-2 cell lines. LBA4404(pIG121-Hm) cocultivated BY-2 cells were selected on MS plates supplemented with 100 μg/ml of kanamycin. As for BY-2 cells cocultivated with other strains harboring pHC18, pHC19, pHC20 or pCB302-1, the *bar* gene encoded by these plasmids codes for phosphinothricin acetyltransferase that confers resistance to phosphinothricin (ppt).

To determine the cytotoxicity effect of phosphinothricin (ppt) on BY-2 cells, various concentrations of ppt were added to untransformed BY-2 cell immediately after the subculture of cell suspension and the subsequent increases in settled cell volume (SCV) of the suspension cultures were monitored. As shown in Fig. 3.15, 1 μg/ml of ppt was already sufficient to suppress the growth of untransformed BY-2 cells not harboring a *bar* gene expressing plasmid, even though higher concentrations of ppt ranging from 2 to 5 μg/ml were also found to inhibit the proliferation of untransformed BY-2 (data not shown). This is in contrast to the control, where the proliferation of BY-2 cells followed the growth curve reported by Nagata *et al* (1992).

Besides assaying the untransformed wild-type BY-2 cells for ppt cytotoxicity, the empty vector strain, LBA4404(pCB302-1), cocultivated BY-2 cells were plated on MS plates supplemented with various concentration of ppt in an attempt to determine a suitable ppt concentration for the selection of transformed BY-2 cells after their cocultivation with LBA4404 strains harboring pHC18, pHC19 and pHC20.

As illustrated in Fig. 3.16, when ppt concentration was lower than 4 μg/ml or when no ppt was added to the MS plates, cocultivated BY-2 cells would proliferate and covered up the whole plate (panel A). This suggests that to select the cocultivated BY-2 transformants on MS plates clearly, a higher ppt concentration is required. However, when un-cocultivated BY-2 cells were spread onto MS plates supplemented with various concentrations of ppt, 1 μg/ml was already sufficient to suppress the growth of untransformed wild-type BY-2 cells, tallying with the result of the cytotoxicity assay described and shown earlier in Fig. 3.15.

On the other hand, when the ppt concentration was 6 μg/ml or higher, it became toxic to even the cocultivated BY-2 transformants, as shown in panel F of Fig. 3.16,

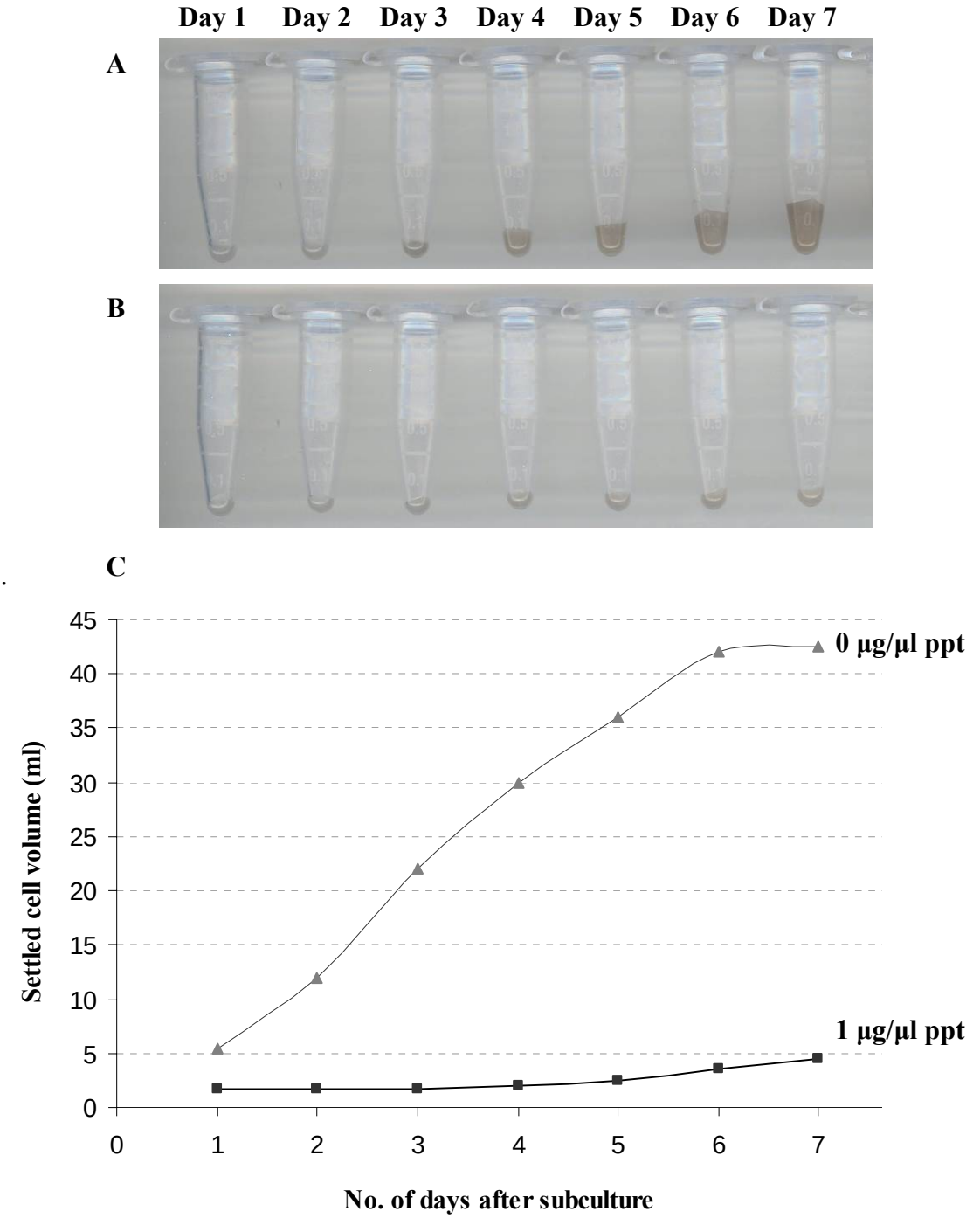


Fig. 3.15. Cytotoxicity effect of phosphinothricin (ppt) on untransformed wild-type BY-2 cells. Immediately after the weekly subculture, 1 µg/ml of ppt was added to the newly diluted BY-2 cell suspension culture, which was then cultured in the usual manner. As a control, no ppt was added to a similar culture. Every 24 hours, 1 ml of each culture was allowed to settle in a microcentrifuge tube before the supernatant was removed. Such procedure was performed for 7 days for the culture added with 1 µg/ml of ppt (panel B) and for the control culture (panel A), where no ppt was added. Similar approach was taken to measure settled cell volume (SCV; Rempel and Nelson, 1995) of 50-ml cultures in 50-ml centrifuge tubes for 7 days for cultures added with 1 µg/ml of ppt or cultures where no ppt was added. The SCV readings were then used to plot the graph illustrated in panel C.

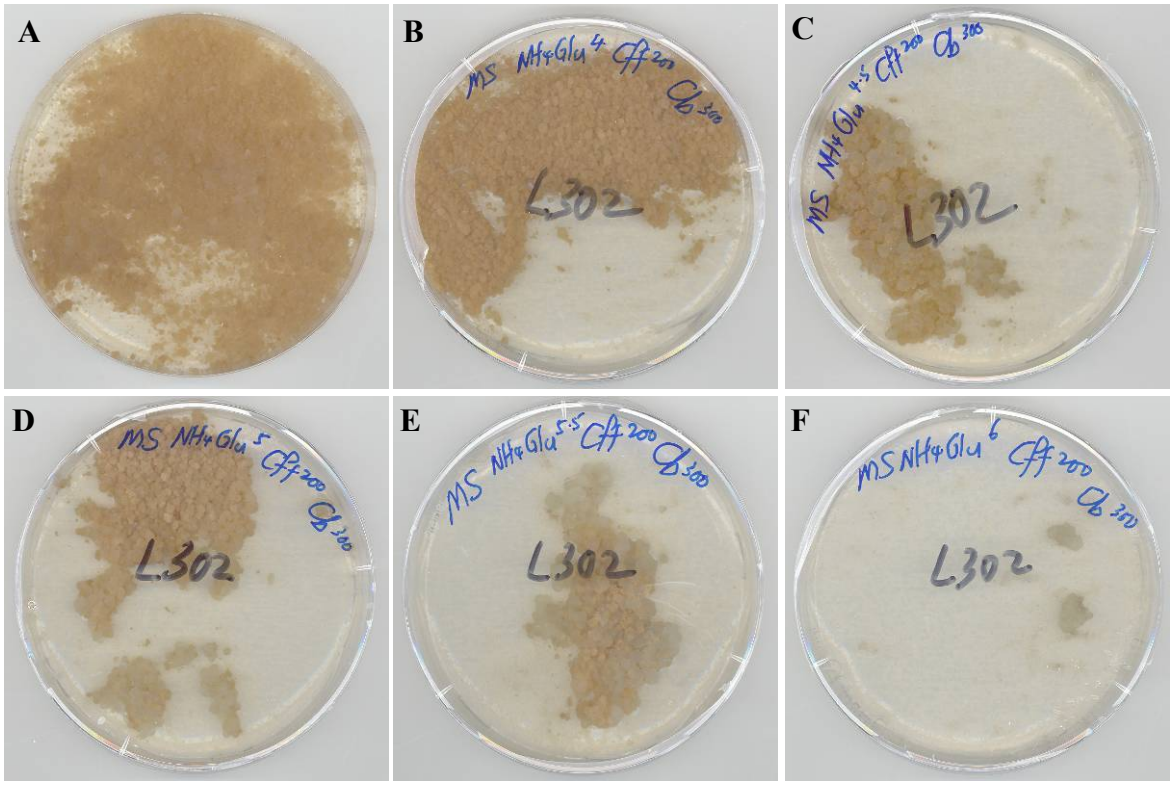


Fig.3.16. Determination of suitable phosphinothricin (ppt) concentration for the selection of transformed BY-2 cells. After cocultivation with the empty vector strain, LBA4404(pCB302-1), BY-2 cells were plated on MS plates supplemented with various concentrations of ppt ranging from 0 µg/ml of ppt to 10 µg/ml of ppt. Photographs of BY-2 cells selected on MS plates supplemented with no ppt (panel A), 4 µg/ml of ppt (panel B), 4.5 µg/ml of ppt (panel C), 5 µg/ml of ppt (panel D), 5.5 µg/ml of ppt (panel E) and 6 µg/ml of ppt (panel F) were taken about 2 months after the initial plating where further proliferation of BY-2 cells was no longer feasible.

where only minute amount of callous tissues was visible. On plates where ppt concentration exceeded 6 μg/ml, ranging from 7 μg/ml to 10 μg/ml, no visible callous tissue could be observed 2 months after the initial plating (data not shown).

Therefore, the effective concentration of ppt for a clearly discernable selection of BY-2 transformants, after cocultivations with the LBA4404 strains harboring pHC18, pHC19, pHC20 and pCB302-1, only ranges from 4 to 5.5 μg/ml (panel B to E). To achieve a balance between the number of available transformants on plate and the selective pressure of ppt on non-transformants, the ppt concentration of 5 μg/ml was used for subsequent selections both on MS plates and in liquid suspension cultures.

After a few subcultures of the transformed calli to fresh selective plates every 3 to 4 weeks, differential grow rates of the transformed BY-2 cell lines were observed (Fig. 3.17). Calli of the stable *DIP* "knock down" line (panel A) grew at a much slower rate than the other stably transformed lines (panel B to E). Though growing at a faster rate than the stable *DIP* "knock down" line, the stable *DIP* antisense line (panel C) still grew comparatively and slightly slower than the other lines. As such, more cell mass or calli from more plates were used to initiate these two cell lines into liquid suspension cultures. These demonstrate that the expression of *DIP* is essential for the proper physiological function of the plant cells.

After initiation into liquid suspension and cocultivation with the GUS reporter strain, the cells from each line were subjected to fluorogenic GUS assay (Fig. 3.18 and Table 3-2). The results confirm the Agroinfiltration data that RNAi "knock down" of *DIP* decreases the efficiency of *Agrobacterium*-mediated transformation of plant cells but does not abolish or prevent the transformation of plant cells. The fluorescence reading of the stable *DIP* "knock down" line was about half that of the *DIP*

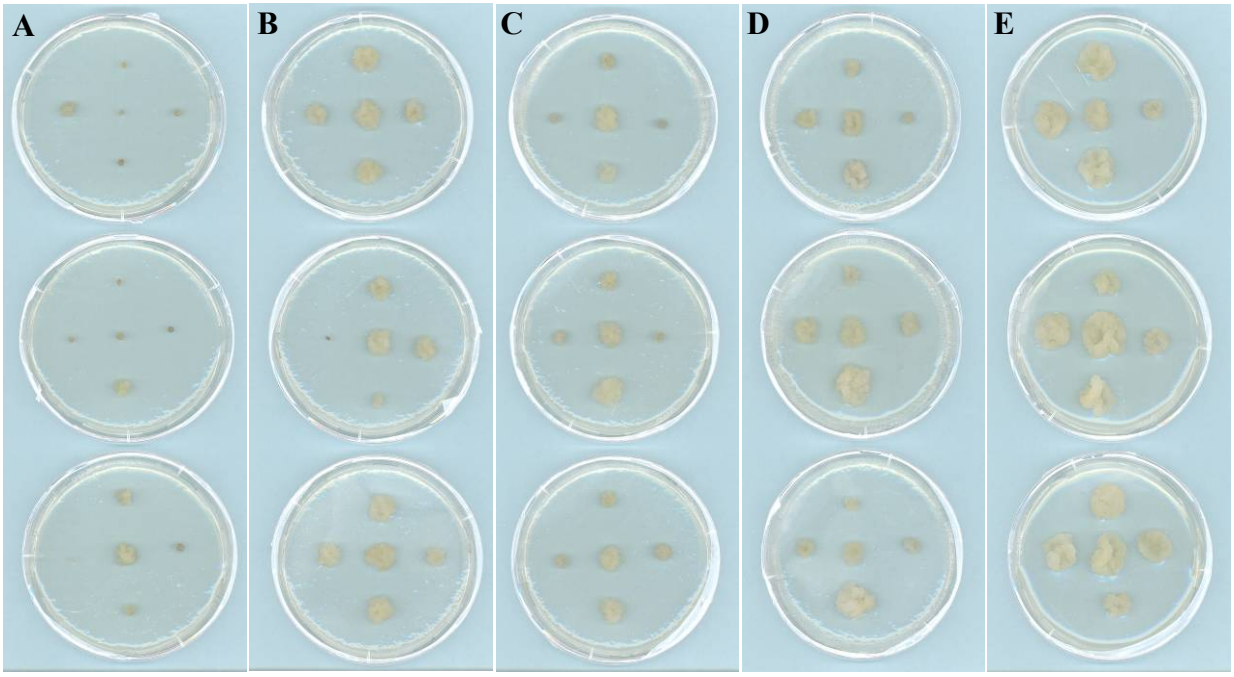


Fig. 3.17. Stable *DIP* "knock down" transformant grows slower than other stably transformed BY-2 cell lines. After cocultivation with *A. tumefaciens* LBA4404 harboring pHC18 (panel A), pHC19 (panel B), pHC20 (panel C), pCB302-1 (panel D) or pIG121-Hm (panel E) for 3 to 4 days, BY-2 cells were washed and selected on MS plates supplemented with 5 μg/ml of ppt (panel A to D) or 100 μg/ml of kanamycin (panel E). The resulting transformed calli were transferred to fresh plates and these transformed cell lines are subcultured every 3 to 4 weeks. The above photographs were taken one month after the previous subculture. The pictures above show that the transformed BY-2 cell lines grow at different rates.

Table 3-2. The effect of stable *DIP* "knock down" on *Agrobacterium*-mediated transformation of BY-2 cells

Abbreviation [a]	BY-2 cell line [b]	Relative fluorescence per mg protein [c]
WT (-)	Uninfected wildtype	504 ± 46
WT	Cocultivated wildtype	3168 ± 49
RNAi	LBA4404(pHC18) transformed	1333 ± 167
Sense	LBA4404(pHC19) transformed	3072 ± 103
Antisense (A/S)	LBA4404(pHC20) transformed	2110 ± 442
Empty Vector	LBA4404(pHC302) transformed	2492 ± 167
Reporter	LBA4404(pIG121-Hm) transformed	10727 ± 522

[a] These abbreviations are used in Fig. 3.13

[b] Except WT (-), all the lines were cocultivated with the GUS reporter strain LBA4404(pIG121-Hm)

[C] 2 to 3 samples from each line were processed for GUS assay in 4 to 5 independent experiments. A similar trend is observed for each experiment despite the difference in absolute readings.

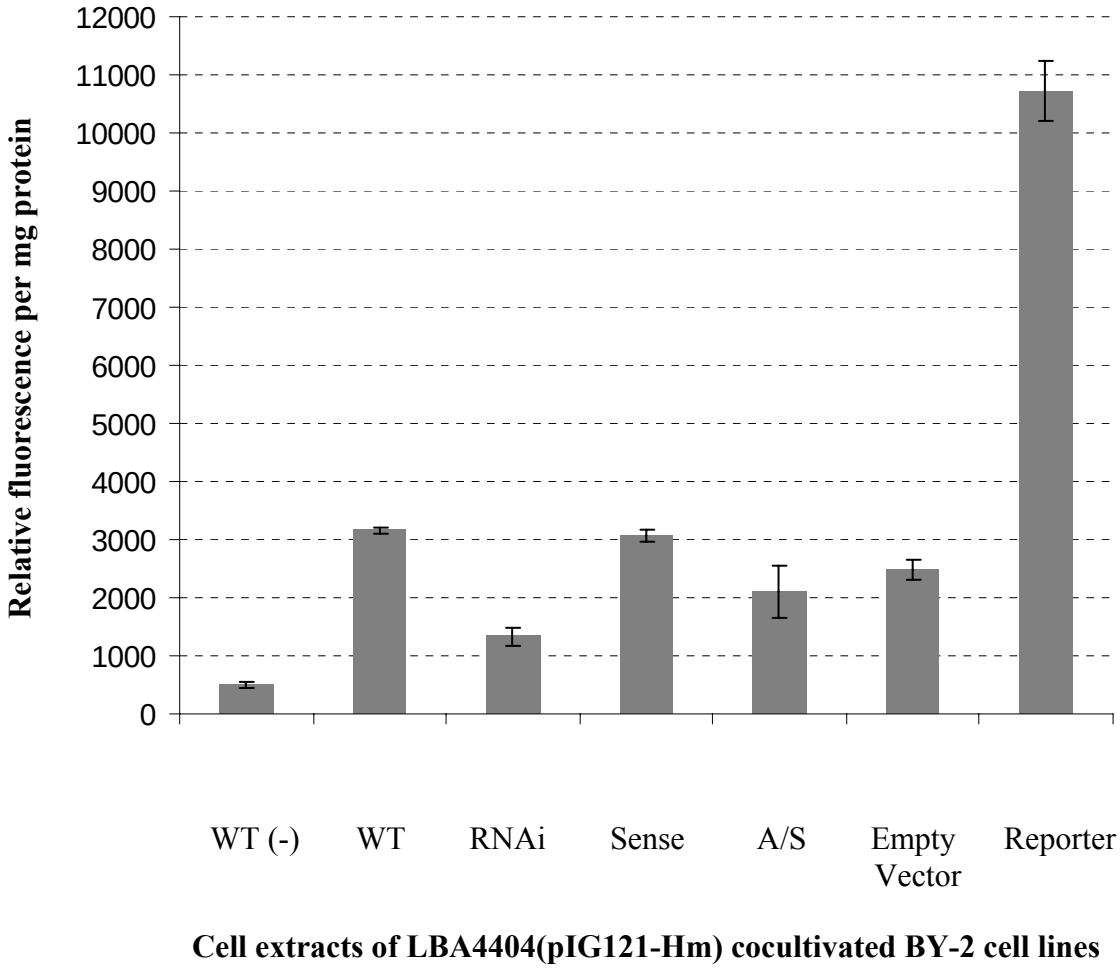


Fig. 3.18. Stable "knock down" of *DIP* decreases the efficiency of *Agrobacterium*-mediated transformation of BY-2 cells. Stably transformed BY-2 cell lines were cocultivated with pre-induced GUS reporter strain, LBA4404(pIG121-Hm) for 3 to 4 days before the cells were collected and subjected to fluorogenic GUS assay. WT (-), uninfected wildtype BY-2 cell line; WT, wildtype BY-2 cell line cocultivated with GUS reporter strain. The detailed results of the GUS assay are shown in Table 3-2.

overexpression line, which gave roughly the same fluorescence reading as that of the wild-type BY-2 cell line cocultivated with the GUS reporter strain. In addition, the empty vector transformed line gave a fluorescence reading that was slightly lower than that of the *DIP* overexpression line. This is in concurrence with the Agroinfiltration data in which the *DIP* overexpression sample is also slightly more susceptible to transformation by the GUS reporter strain, perhaps as a consequence of the increased interaction between DIP and VirD2. As for the stably transformed antisense cell line, the reading was also found to be around 25% higher than the "knock down" line but lower than overexpression cell line, when compared to Agroinfiltration results.

Taken together, these data indicate that *A. tumefaciens* T-complex utilizes DIP and its associated cellular pathway to facilitate its passage through the plant cell cytoplasm on its way to and into the nucleus. And when *DIP* is "knocked down" by RNAi or inhibited by antisense suppression, the efficiency of *Agrobacterium*-mediated transformation is decreased due to the disruption of the intrinsic DIP based transport pathway. Although the overexpression of *DIP* seems to enhance the *Agrobacterium*-mediated transformation of plant cells slightly, it does not increase the susceptibility of these cells to transformation markedly. In another words, the slight increase in fluorescence is most likely not significant and the overexpression may affect the proper functioning of the other subunits or factors participating in the same pathway.

3.3.4. *DIP* is essential for the growth and viability of *Arabidopsis DIP* $^{+/-}$ heterozygous mutant plants

Besides confirming both the effects of transient and stable *DIP* "knock down" and antisense inhibition on Agrotransformations, an attempt was made to characterize

DIP mutant plants and to investigate if Agrotransformation of these plants is also affected.

As shown in Fig. 3.19, intron inserted *Arabidopsis* mutant plants (Alonso *et* al, 2003) are available from the SALK institute at the *Arabidopsis* Biological Resource Center at Ohio State University (http://arabidopsis.org/abrc/). Though 4 independent SALK seed lines are available (Fig.3.19; A and B), sequence alignment has shown that that SALK_140590 line is equivalent to SALK_145185 line. Both these lines are highly homologous to SALK_145187 line from nucleotide no. 66 to 172 and all lines were mapped to the same locus (Fig. 3.19; C). As such, SALK_140590 line was selected for subsequent experiments.

Since the seeds from the SALK institute would give rise to T3 plants that are heterozygous for *DIP* mutation, the T3 plants germinated from SALK_140590 seeds were allowed to self fertilize and the resultant seeds were used to go through a few generations of selfing in an attempt to obtain the homozygous *DIP* $^{-/-}$ mutant line.

Genomically, the approximately 6.6.kb *DIP* or At1g47550 [accession no: AC007519 (F16N3.18)] is made up of 25 exons and 24 introns. After splicing, the final mRNA is around 2.6 kb. As shown in Fig. 3.19 (A and B) and Fig. 3.20 (A), the T-DNA is inserted at intron 1 (the largest intron; 790 bp), between exon 1 (175 bp) and exon 2 (71 bp), of *DIP* gene.

After a few such generations of selfing, the RNAs from 24 selected independent seed lines and the wild-type Col-0 seed line were isolated and used as the templates for RT-PCR using the Dip-ex1 and Dip-ex2 primers. The gel photograph illustrated in Fig. 3.20 (B) clearly demonstrates that all the *DIP* mutant seed lines

Fig. 3.19. *Arabidopsis DIP* $^{+/-}$ heterozygous mutant plant line, SALK_140590. *Arabidopsis* gene At1g47550, named as DIP, was inserted at intron 1 with *A. tumefaciens* T-DNA (Alonso *et al*, 2003) (A). Four independent SALK T-DNA inserted *DIP* mutant lines are available from the *Arabidopsis* Biological Resource Center at Ohio State University (http://arabidopsis.org/abrc/) (B). Sequence alignment of the T-DNAs has shown that SALK_140590 line is equivalent to SALK_145185 line and both lines are highly homologous to SALK_145187 line from nucleotide no. 66 to 172 and all lines were mapped to the same locus (C).

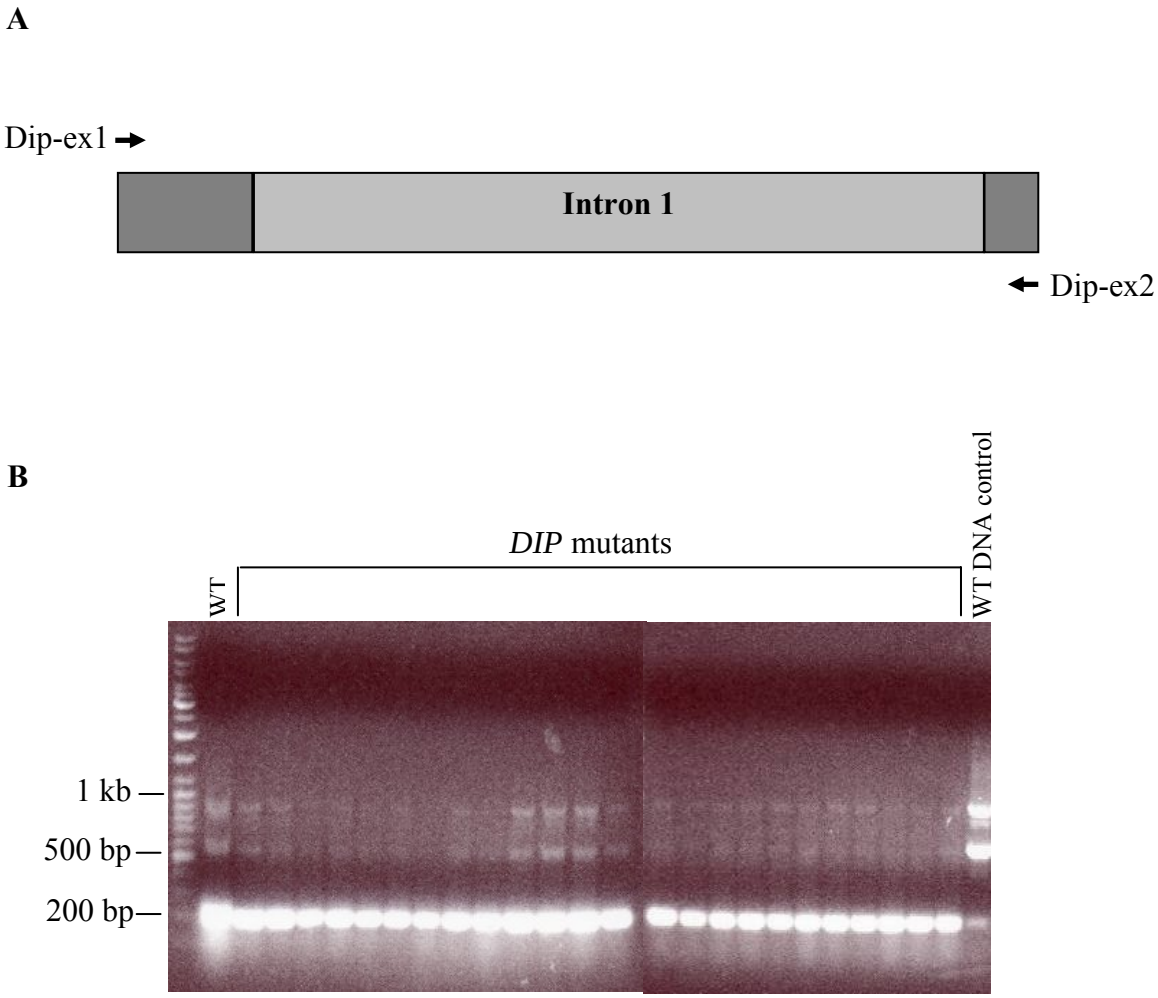


Fig. 3.20. Analysis of *Arabidopsis DIP* insertional mutant plants after several generations of self fertilizations. SALK_149590 *DIP* $^{+/-}$ heterozygous mutant line contains a T-DNA inserted in intron 1 (790 bp), between exon 1 (175 bp) and exon 2 (71 bp), of *DIP* gene (A). After several generations of selfing, total RNAs were isolated from wild-type (WT) and 24 independent SALK_140590 *DIP* mutant seed lines and used as templates for RT-PCR, which was performed by using the Dip-ex1 and Dip-ex2 primers. As a DNA contamination control, total DNA from WT plant was subjected to the same PCR amplification program (WT DNA control) (B).

examined were heterozygous *DIP* $^{+/-}$ seed lines, since all the *DIP* mutant plant samples gave a RT-PCR product of the correct size that is found in the WT plant sample (a joined DNA fragment of exon 1 and exon 2 of 267 bp). The intensity of the 267-bp band in all these ***DIP*** mutant plant samples was roughly halved that of the WT sample, as shown in Fig. 3.20 (B), indicating a heterozygous genotype. No such band would be observed if a homozygous *DIP* $^{-/-}$ mutant was derived after several rounds of selfing, which according to Mendelian genetics has a 25 % chance of obtaining such homozygous mutant.

The failed attempt to generate a homozygous *DIP* $^{-/-}$ mutant may reflect the indispensability of DIP for the proper physiological functions, probably in secretion or intracellular transport based on its identity as an evolutionarily conserved exocyst complex's subunit. If DIP is indeed crucial functionally, this implies that the stably transformed *DIP* "knocked down" BY-2 cell line obtained in the previous studies (Fig. 3.17, Fig, 3.18 and Table 3-2) may have an up-regulated expression of other gene(s) with a similar function to *DIP* to compensate for such "knock down" and that the suppression of DIP function in that particular BY-2 cell line is not absolute and residual DIP function might still be present in those cells.

3.4. Discussion

Although not all plants could be transformed by *A. tumefaciens*, its host range is increasing and has extended to include HeLa cells (Kunik *et al*, 2001), yeast (Bundock *et al*, 1996) and fungi (de Groot *et al*, 1998). Since any foreign DNA placed between the T-DNA borders can be transferred to the host cells (Zambryski, 1992), the protein components of the T-complex, VirD2 and/or VirE2, must have played important roles in targeting the T-complex to the host cell nucleus and integrating the T-DNA into the

host genome. During the course of such events, it is likely that various host factors are involved and are compatible for the pathway or the process which the T-complex or the T-DNA may undertake. Despite the intrinsic differences of the cellular machineries of different host cells, their transformability points towards a notion that these hosts share many factors or components with which the T-complex interacts. These host proteins may be involved in common cellular pathways involved in nucleoprotein uptake, trafficking, nuclear import, and DNA recombination and integration. The lack of some important factors to recognize the T-complex may also explain why some plants or host cells are recalcitrant to transformation by *A. tumefaciens*.

Even though both VirD2 and VirE2 contain nuclear localization signals (NLSs), VirE2 nuclear targeting has been shown to occur in plant but not in animal cells, unlike the more conserved VirD2 nuclear localization mechanism (Guralnick *et al*, 1996; Rhee *et al*, 2000; Relic *et al*, 1998; Ziemienowicz *et al*, 1999). This plant specific nuclear targeting is reported to be facilitated by the cellular VIP1 protein, which interacts with VirE2 and functions as a molecular bridge between VirE2 and karyopherin α (Tzfira *et al*, 2001; 2002; Ward *et al*, 2002; Citovsky *et al*, 2004). Before this, VirE2 has been postulated to localize into the nucleus via a karyopherin independent pathway. Due to this plant cells specificity and the controversial VirE2 contribution to nuclear targeting by contrasting reports, VirD2 instead of VirE2 was chosen for the yeast two-hybrid analysis in previous study conducted by our lab (Chang, 2002). In addition, it is also our intention to correlate any potential plant VirD2 interacting protein identified through this study to the homologues in fish, mouse and human cells which have shown to be transformable by *A. tumefaciens* in our lab (Lin, S. & Pan, S.Q. and Hou, Q. & Pan, S.Q., unpublished).

From the GAL4 based yeast two-hybrid analysis, a previously unidentified VirD2 interacting protein, DIP, was isolated from the *Arabidopsis* cDNA library (Chang, 2002). DIP was found to interact with *A. tumefaciens* VirD2, but subsequent β-galactosidase assay has revealed that the interaction between DIP and VirD2 was weak and could be transient. To ascertain the interaction of DIP and VirD2, an *in vitro* pull down assay using an amylose resin column was performed with a negative control, katA protein, which is a catalase involved in *Agrobacterium*-plant interaction (Xu *et al*, 2001). After BLAST analysis and sequence alignment, the identity of DIP, an unnamed *Arabidopsis* protein, and its homologues in various organisms that include human, mouse, *Drosophila*, *C. elegans* and yeast have been confirmed. These homologous proteins constitute a family of evolutionarily conserved exocyst proteins and DIP is found to be homologous to the yeast Sec3p and the human Sec3 protein.

Sec3p is a subunit the exocyst complex and is involved in polarized secretion by acting as a spatial landmark for secretion in budding yeast (Finger and Novick, 1997; Finger *et al*, 1998; Guo *et al*, 1999; Zhang *et al*, 2001; Wiederkehr *et al*, 2003). The yeast exocyst is composed of 8 subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p. Though the exocyst's role in exocytosis is not understood, it has been proposed to tether secretory vesicles to specialized exocytic sites on the plasma membrane prior to docking and fusion. And Sec3p has been shown to localize to the sites of exocytosis at each stage of the cell cycle and serve as an interface with other subunits prior to exocytosis. Unlike Sec3p, the human Sec3 is cytosolically located and does not appear to function as a spatial landmark for secretion (Brymora *et al*, 2001; Matern *et al*, 2001), while the two homologous *Arabidopsis* genes for *Sec3p* have not been characterized yet (Elias *et al*, 2003). Despite such differences, it is quite probable

that T-complex may interact with and utilize the same Sec3p related cellular pathway for its traverse across the cytoplasm of the various host cells.

To confirm the cytosolic location of DIP, polyclonal antibody against DIP was generated and used in Western blot analysis to assay for the presence of DIP in plant cells (Chang, 2002). The subcellular localization of DIP in plant cells was then verified by *in situ* hybridization and DIP was found to be present in the cytoplasm of *Arabidopsis* and surprisingly also in tobacco BY-2 cells. Since the tobacco homologue of DIP was found to be of the same size as that of DIP and tobacco BY-2 cell suspension has a faster growth and is a better established cell culture system, the ensuing studies have been focused on and conducted with tobacco BY-2 cells (Chang, 2002).

Subsequent immunohistology and confocal microscopy experiments have further confirmed that DIP colocalized with GUS protein and T-DNA molecules in the same transformed BY-2 cells but not in those untransformed cells or cell clusters. Taken together, these results indicate that DIP proteins are usually randomly located in the cytoplasm of BY-2 cells, but become coexisted with T-DNA in the infected BY2 cells, suggesting that DIP may assist the T-complex movement within cytoplasm. After infection or cocultivation for 3 days, the DIP proteins became free again in the cytoplasm of plant cells just like the uninfected cells due to the entry of T-DNA into the nuclei of the cells (Chang, 2002).

From these previous findings (Chang, 2002), it can be deduced that VirD2 interacts with DIP on its way to the nucleus of the plant cells and that DIP is a subunit or a factor that is involved in one of the complicated intracellular transport pathways within the cytoplasm. Indeed, previous studies using endocytotic inhibitors, brefeldin

A and monesin which are Golgi-disrupting agents, have shown that when these inhibitors were added to the plant cells prior to their cocultivation with the GUS reporter strain, LBA4404(pIG121-Hm), a much reduced efficiency of *Agrobacterium*-mediated transformation of BY-2 cells was observed (Chang, 2002). These results show that these inhibitors are effective in blocking the T-DNA movement inside the plant cell cytoplasm, suggesting that the *A. tumefaciens* T-DNA transport inside the plant cells most likely occurs through an endocytotic vesicular protein trafficking pathway. Whether DIP is part of this pathway or that of the still unconfirmed plant exocyst based secretion pathway still remains to be elucidated.

To establish a direct functional link of DIP with *Agrobacterium*-mediated transformation of plant cells, an RNAi approach (Akashi *et al*, 2001; Limpens *et al*, 2004) adapted for novel use with two rounds of *Agrobacterium*-mediated transformation was adopted in this study. After the first round of cocultivation with LBA4404 harboring the plasmids as shown in Fig. 3.8 and Fig. 3.9, the transient *DIP* "knock down" BY-2 cells (Fig. 3.10 panel D) and tobacco leaves (Fig. 3.14 and Table 3-1) have become less susceptible to transformation by the GUS reporter strain, LBA4404(pIG121-Hm), in the second round of cocultivation. These results imply the direct involvement and the important significance of DIP in the T-complex trafficking within the plant cell cytoplasm. When stably transformed BY-2 cell lines were assayed for GUS activity after cocultivation with the GUS reporter strain, the results confirmed the important requirement of DIP for the T-complex movement in the cytoplasm (Fig. 3.17, Fig. 3.18 and Table 3-2).

A vast array of host factors have been reported to be involved in the *Agrobacterium*-plant interaction (reviewed in Tzfira and Citovsky, 2002). Among

these are the VirD2 interacting proteins, including cyclophilin A, RocA and Roc4 identified by Deng *et al* (1998), AtKAPα (now known as importin-α1) by Ballas and Citovsky (1997) and the type 2C serine/threonine protein phosphatase, PP2C, characterized by Tao *et al* (2004), Ran protein (Goldfarb, 1994) and the VirE2 interactor, VIP1 (Tzfira *et al*, 2001; 2002; Ward *et al*, 2002; Citovsky *et al*, 2004). These are the host factors that are involved in localizing the T-complex to the nucleus by interacting with VirD2, VirE2 or both VirD2 and VirE2. It is still unknown whether all these factors function synergistically in targeting the T-complex to the nucleus. Much less is known about the order and sequence they are acting on the T-complex during its traverse through the cytoplasm. It is currently still not clear as to where the functionally indispensable DIP (Fig. 3.20) fits into the whole scenario of trafficking, in conjunction with these factors. It is probable that the T-complex may just interact and use the intrinsic DIP as part of the endocytotic pathway machinery in getting towards the nucleus. To resolve the mystery, further investigations are certainly needed to address the various questions outlined above.

Although DIP is not the only plant factor interacting with T-complex within the plant cell cytoplasm, this study has shown that it is a functionally critical "stepping stone" for the translocation of T-complex within the plant cell cytoplasm and from the cytoplasm to the nucleus.

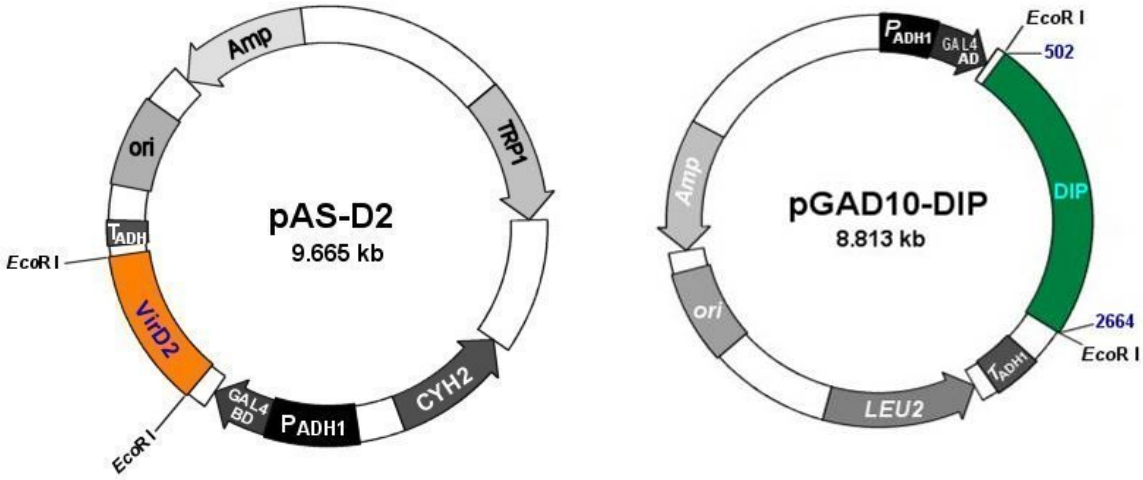
Chapter 4. Nuclear Localization Sequence of VirD2 is not Required for DIP Interaction

4.1. Introduction

Prior studies from our lab have confirmed that *A. tumefaciens* VirD2 protein interacts with *Arabidopsis* factor DIP (VirD2 Interacting Protein), both *in vitro* and *in vivo* (Chang, 2002). As shown in Fig. 4.1, our lab's GAL4 based yeast two-hybrid screen of the *Arabidopsis* cDNA library has identified a previously unidentified protein, DIP, in addition to cyclophilin A that was identified by Deng *et al* (1998) using the LexA based yeast two-hybrid system.

As illustrated clearly in Fig. 4.2, the yeast two-hybrid analysis showed that VirD2 interacted with the plant factor DIP (A), but not with DNA binding domain only (C) or a random protein, lamin C (D). Sector B of the filter shown in Fig. 4.2 was the positive control where the SV40 large T-antigen, which was fused to the GAL4 AD in pTD1-1, interacted with the p53 murine protein that was encoded as a fusion to GAL4 DNA-BD in pVA3-1. When SV40 large T-antigen interacted with p53, they brought the GAL 4 AD and GAL 4 DNA-BD together to activate the transcription of the *LacZ* reporter gene, thus giving rise to a positive β-galactosidase assay that was visible as a blue color. These results demonstrate that VirD2 protein could truly interact with DIP in the two-hybrid system. By using the same GAL4 based yeast two-hybrid system, one of the objectives of this study is to delineate the DIP-interacting domain of VirD2.

A



B

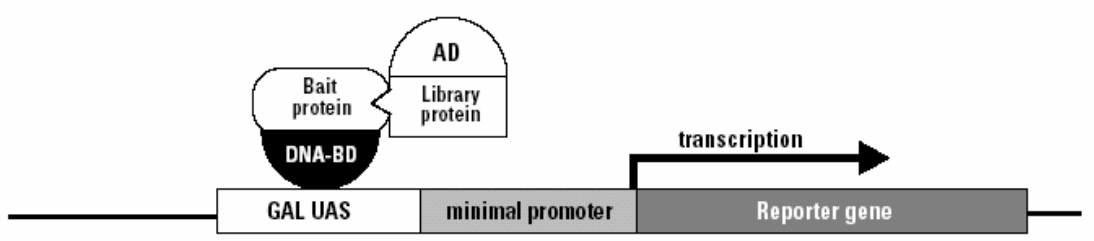
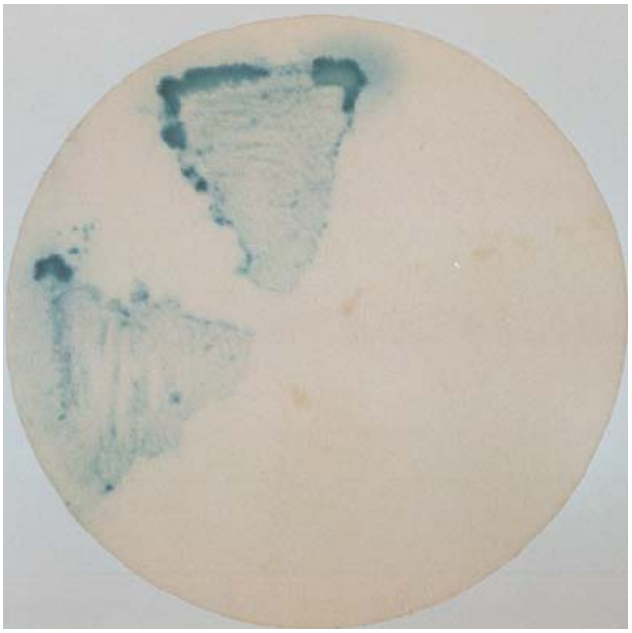


Fig. 4.1. Isolation of VirD2-interacting proteins using the GAL4 based yeast two-hybrid system. (A) VirD2 fused to the GAL4 DNA binding domain (GAL4 BD) was used as a bait protein in the GAL4 based yeast two-hybrid system to isolate GAL4 activation domain (GAL4 AD) containing prey proteins that interact with VirD2. One such previously unidentified protein was isolated and named as DIP (VirD2 Interacting Protein). (B) Such interaction between the bait and the prey proteins will bring the GAL4 AD and GAL4 BD together to effect the transcription of reporter genes, HIS3 or *lacZ*, which will confer the His^+ and blue coloration phenotype to the host cells. By assaying for the presence of the reporter gene activity, the specific interaction between the bait and the prey proteins can be confirmed.

B

A  C

D

Fig. 4.2. Interaction of *Arabidopsis* DIP with VirD2 in the yeast two-hybrid assay. The indicated combinations of bait and prey proteins were achieved by introducing into the yeast host strain, CG-1945, the following plasmids:

A: pAS-D2 and pGAD10-DIP, expressing VirD2 and DIP, respectively;
B: pTD1-1 and pVA3-1, expressing T40 large antigen and p53, respectively;
C: pAS2-1 and pGAD10-DIP; expressing DNA-BD and DIP, respectively;
D: pLAM5'-1 and pGAD10-DIP, expressing Lamin C and DIP, respectively.

Protein-protein interaction was determined by the β-galactosidase assay on a Whatman filter following the recommended protocol (CLONTECH).

(Adapted from Chang, 2002)

4.2. Materials and methods

Unless or otherwise stated, all materials and methods used in this chapter are as described in Chapter 2.

4.2.1. Construction of VirD2 deletion plasmids and strains

By using pAS-D2 as the template, a series of deletions of the *virD2* gene were generated by PCR amplification by using the primer pairs shown in Table 4-1. The gene fragments were then subjected to double restriction digestion by *Nco* I and *Eco*R I before they were ligated into *Nco* I and *Eco*R I digested pAS2-1 to obtain the resultant VirD2 deletion plasmids, shown in Fig. 4.3. These plasmids were then introduced into the yeast strain CG-1945 harboring pGAD10-DIP by using the high-efficiency transformation approach adapted from Gietz and Schiestl (1995). The latest version of this approach can be found at http://www.umanitoba.ca/faculties/medicine/biochem/gietz/method.html.

4.2.2. Yeast two-hybrid analysis

The yeast two-hybrid analysis was performed following the instructions of the manufacturer, using the strains, which harbor the pDAD10-DIP plasmid and a VirD2 deletion plasmid, in accordance to the protocols of MATCHMAKER GAL4 two-hybrid system (CLONTECH). Protein-protein interaction was determined by both the β-galactosidase assay on a Whatman filter or by assaying for the His^+ phenotype of the transformed yeast cells.

Table 4-1. VirD2 deletion plasmids

Plasmid	Characteristics	PCR Primers
pAS-D2	pAS2-1 harboring GAL4 BD fused to full length VirD2, Amp^R	PCR template
pAS-D2 (74)	pAS2-1 harboring GAL4 BD fused to VirD2 lacking the N-terminal 73 amino acids	D2 (74) & D2 (end)
pAS-D2 (174)	pAS2-1 harboring GAL4 BD fused to VirD2 lacking the N-terminal 173 amino acids	D2 (174) & D2 (end)
pAS-D2 (274)	pAS2-1 harboring GAL4 BD fused to VirD2 lacking the N-terminal 273 amino acids	D2 (274) & D2 (end)
pAS-D2 (354)	pAS2-1 harboring GAL4 BD fused to VirD2 lacking the N-terminal 353 amino acids	D2 (354) & D2 (end)
pAS-D2 (-NLS)	pAS2-1 harboring GAL4 BD fused to VirD2 lacking the C-terminal NLSs and omega sequences	D2 (1) & D2 (-NLS)
pAS-D2 (N)	pAS2-1 harboring GAL4 BD fused to VirD2 containing only the N-terminal 212 amino acids	D2 (1) & D2 (N)
pAS-D2 (C)	pAS2-1 harboring GAL4 BD fused to VirD2 containing only the C-terminal 212 amino acids	D2 (C) & D2 (end)

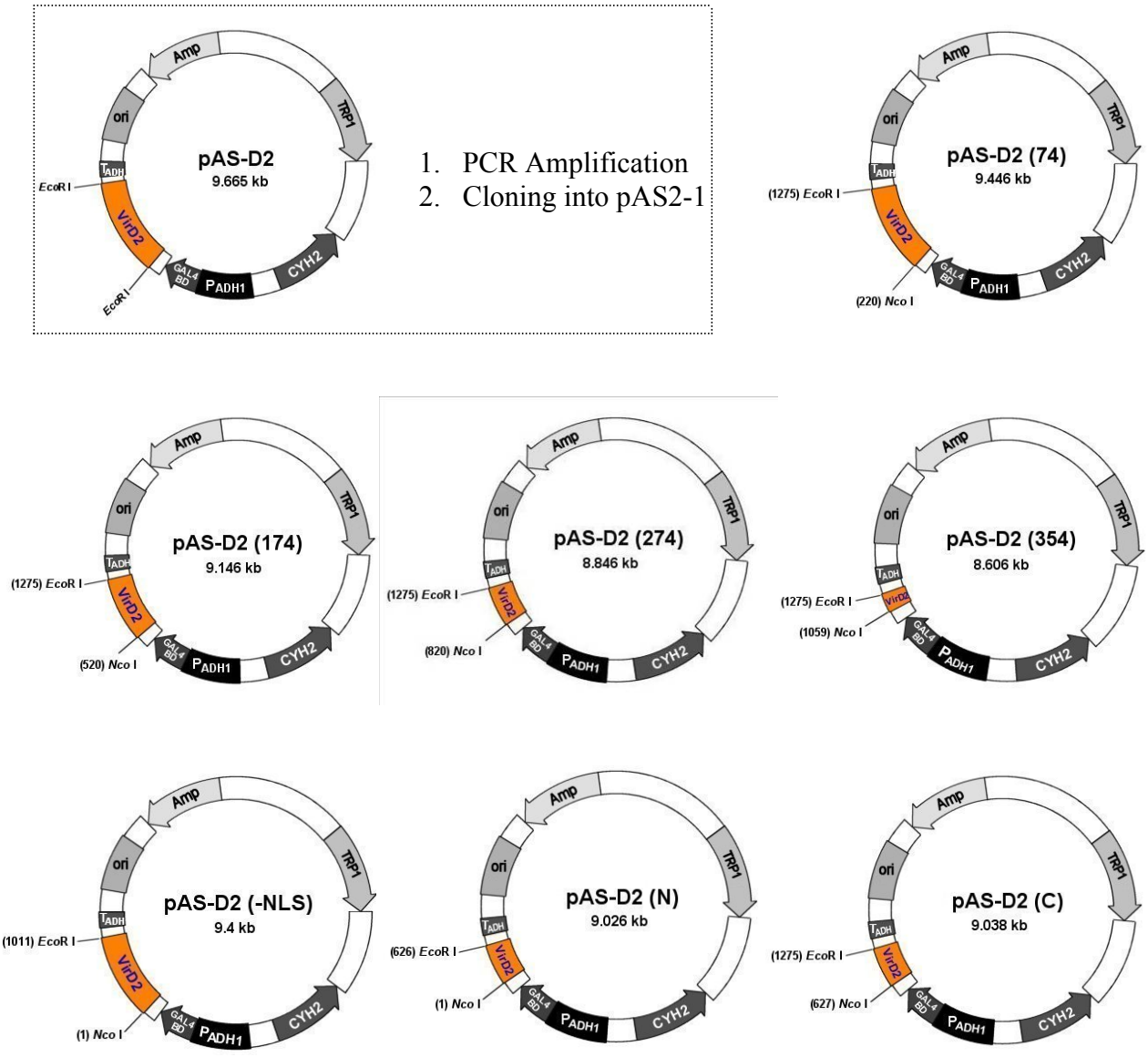


Fig. 4.3. Construction of VirD2 deletion plasmids. These plasmids were constructed by PCR amplification of various *virD2* fragments from pAS-D2 and the subsequent subcloning of these fragments into pAS2-1.

4.3. Results

By assaying for the β-galactosidase activity of the transformed CG-1945 harboring pGAD10-DIP and a VirD2 deletion plasmid, it was found that the C-terminal bipartite nuclear localization sequences (NLSs) of VirD2 are not required for interaction with DIP (Fig. 4.4 and Fig. 4.5). The NLSs have been reported to be essential for the translocation of T-complex from the cytoplasm to the nucleus (Sheng and Citovsky, 1996). Similarly, the C-terminal end omega sequence that was thought to be important for tumorigenesis (Shurvintion *et al*, 1992) is also not needed for the interaction of VirD2 with DIP.

The aforementioned deductions are made on the basis of the β-galactosidase staining observed for sector I of Fig. 4.4, where a positive blue coloration could be observed for CG-1945[pGAD10-DIP & pAS-D2 (-NLS)] cells. Such blue coloration was similar to that produced by the positive control (sector D of Fig. 4.4), but was absent from other control or experimental samples, where the yeast cells harbored different combination of the bait and prey proteins fused to the GAL4 BD and the GAL4 AD domains respectively.

As indicated in Fig. 4.5, the endonuclease domain of VirD2 as well as the domain between the endonuclease domain and the NLSs domain are both crucial for the interaction of VirD2 with DIP. The β-galactosidase assay results were subsequently reaffirmed by similar results from the histidine (His^-) selection assay shown in Fig. 4.6. When selected on SD His^- Leu^- Trp^- plates, only CG-1945[pGAD10-DIP & pAS-D2 (-NLS)] cells and those of the positive controls could grow on SD plates lacking histidine.

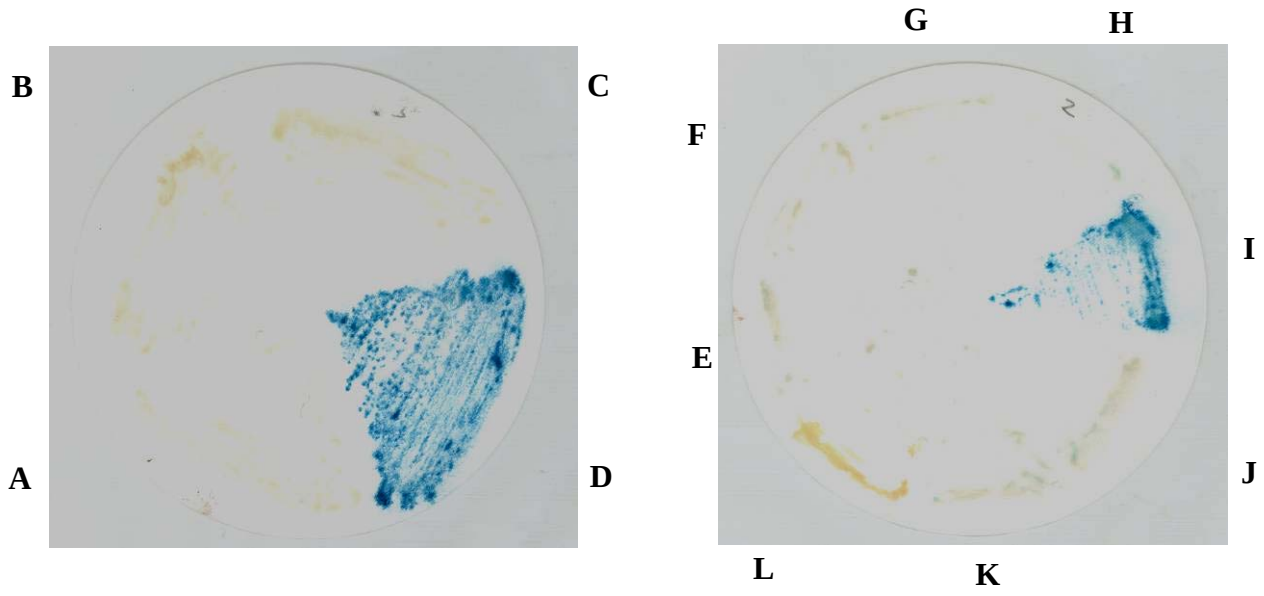


Fig. 4.4. Interaction of *Arabidopsis* DIP with VirD2 deletion fragments in the yeast two-hybrid assay. The indicated combinations of bait and prey proteins were achieved by introducing into the yeast strain, CG-1945, the following plasmids:

A: pAS-D2, expressing VirD2 only;
B: pGAD10-DIP, expressing DIP only;
C: pLAM5'-1 and pGAD10-DIP, expressing Lamin C and DIP, respectively;
D: pAS-D2 and pGAD10-DIP, expressing VirD2 and DIP, respectively;
E: pAS-D2 (74) and pGAD10-DIP, expressing VirD2 $_{(74\text{-}424)}$ and DIP, respectively;
F: pAS-D2 (174) and pGAD10-DIP, expressing VirD2 $_{(174\text{-}424)}$ and DIP, respectively;
G: pAS-D2 (274) and pGAD10-DIP, expressing VirD2 $_{(274\text{-}424)}$ and DIP, respectively;
H: pAS-D2 (354) and pGAD10-DIP, expressing VirD2 $_{(354\text{-}424)}$ and DIP, respectively;
I: pAS-D2 (-NLS) and pGAD10-DIP, expressing VirD2 $_{(1\text{-}337)}$ and DIP, respectively;
J: pAS-D2 (N) and pGAD10-DIP, expressing VirD2 $_{(1\text{-}212)}$ and DIP, respectively;
K: pAS-D2 (C) and pGAD10-DIP, expressing VirD2 $_{(213\text{-}424)}$ and DIP, respectively;
L: pAS2-1 and pGAD10-DIP, expressing GAL4 BD and DIP, respectively.

Protein-protein interaction was determined by the β-galactosidase assay on a Whatman filter following the recommended protocol (CLONTECH).

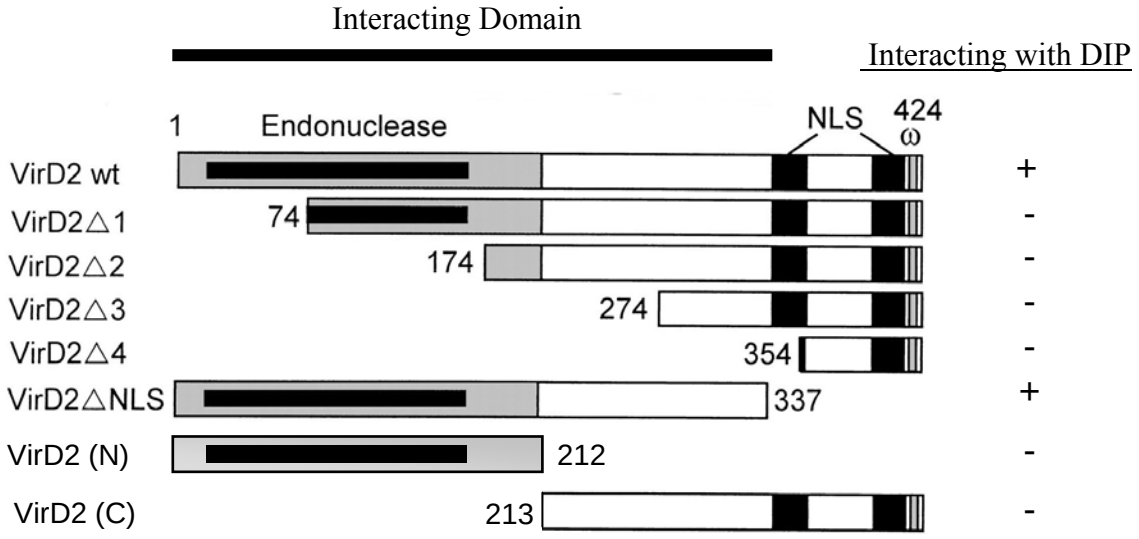


Fig. 4.5. Delineating the DIP-interacting domain of VirD2. A series of deletions of the *virD2* gene were generated by PCR amplification, subcloned into pAS2-1 and then subjected to the GAL4 based yeast two-hybrid analysis that used the β-galactosidase assay on a Whatman filter following the recommended protocol (CLONTECH). A positive interaction was scored if a positive blue coloration was observed after the β-galactosidase assay. Boxes represent the VirD2 region present in pAS-D2. The DIP-interacting domain is indicated by the bar. The endonuclease (Yanofsky *et al*, 1986), NLSs (Sheng and Citovsky, 1996) and omega domains (Shurvintion *et al*, 1992) are indicated. The rectangle within the endonuclease domain denotes critical residues essential to endonuclease activity (Tinland *et al*, 1995; Vogel *et al*, 1995). Results of the yeast two-hybrid analysis are shown to the right of the figure.

(Adapted and modified for illustration from Deng *et al*, 1998)

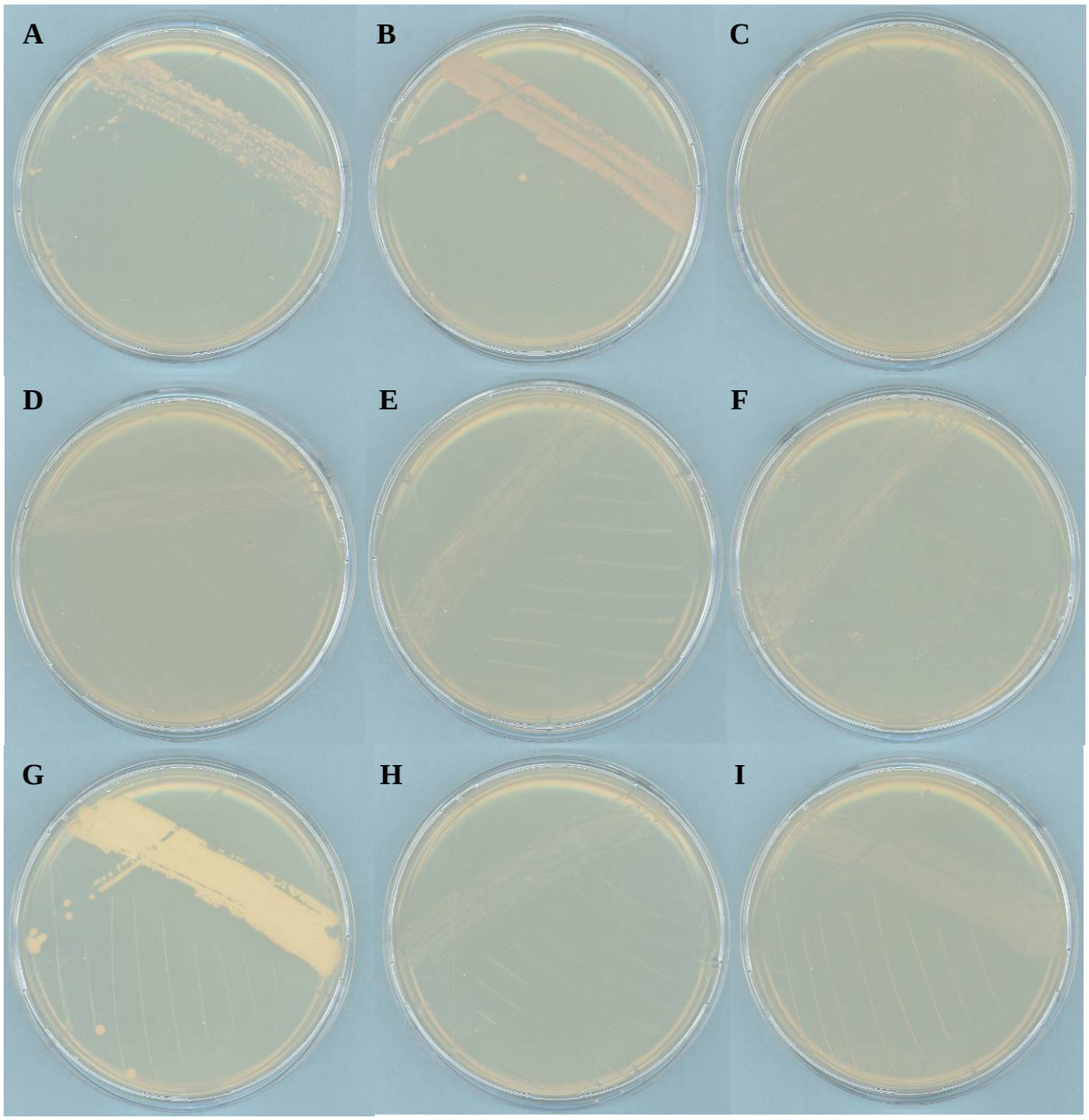


Fig. 4.6. Selection of CG-1945 transformants on His^- plates. Together with a positive control, CG-1945 harboring pVA3-1 and pTD1-1 (panel A), CG-1945 strains harboring pGAD10 and a VirD2 deletion plasmid were plated on SD His^- Leu^- Trp^- plates to assay for protein-protein interaction: (B) CG-1945 harboring pGAD10-DIP and pAS-D2; (C) CG-1945 harboring pGAD10-DIP and pAS-D2 (74); (D) CG-1945 harboring pGAD10-DIP and pAS-D2 (174); (E) CG-1945 harboring pGAD10-DIP and pAS-D2 (274); (F) CG-1945 harboring pGAD10-DIP and pAS-D2 (354); (G) CG-1945 harboring pGAD10-DIP and pAS-D2 (-NLS); (H) CG-1945 harboring pGAD10-DIP and pAS-D2 (N); and (I) CG-1945 harboring pGAD10-DIP and pAS-D2 (C.)

4.4. Discussion

Recent characterization of VirB2-interacting proteins (BTI), BTI1, BTI2 and BTI3, from *Arabidopsis thaliana* has suggested that these plant factors may contact the *A. tumefaciens* T-pilus and it is quite likely that they are essential in assisting the export of T-DNA due to their interaction with VirB2 (Hwang and Gelvin, 2004). These BTI proteins were also found to interact with membrane associated AtRAB8, which has been shown to be homologous to Sec4p of the budding yeast *Saccharomyces cerevisiae* (Haubruck *et al*, 1990; Rutherford and Moore, 2002).

Since the evolutionarily conserved yeast exocyst complex, which contains Sec3p (DIP homologue in yeast), is an effector for Sec4p and that both Sec3p and Sec4p are essential for yeast secretion (Finger and Novick, 1997; Finger *et al*, 1998; Guo *et al*, 1999; Zhang *et al*, 2001; Wiederkehr *et al*, 2003), it is probable that during *Agrobacterium*-mediated transformation of host cells, host factors interacting with the *A. tumefaciens* T-pilus or T-DNA export machinery (such as BTI proteins) may subsequently direct the T-DNA or T-complex to the route of endocytosis. This notion is based on the early research findings which showed both AtRAB8 and Sec4p belongs to a class of membrane associated Rab proteins that modulate tubulovesicular trafficking between compartments of the biosynthetic and endocytic pathways (Olkkonen and Stenmark, 1997; Martinez and Goud, 1998; Schimmoller *et al*, 1998; Moyer and Balch, 2001).

Based on the finding from this study in which the NLSs of VirD2 is not required for its interaction with DIP and the aforementioned relations of Sec4p to AtRAB8 and Sec3p to DIP, it is probable that DIP interaction with VirD2 within the plant cell cytoplasm

could have taken place quite early prior to VirD2 association with other VirD2 interacting proteins, such as cyclophilin A, RocA and Roc4 that function as a chaperones (Deng *et al,* 1998) and AtKAPα (Ballas and Citovsky, 1997), PP2C (Tao *et al,* 2004) and Ran protein (Goldfarb, 1994) that bind to VirD2 NLSs and are involved in facilitating or enhancing the nuclear localization of T-complex into the nucleus.

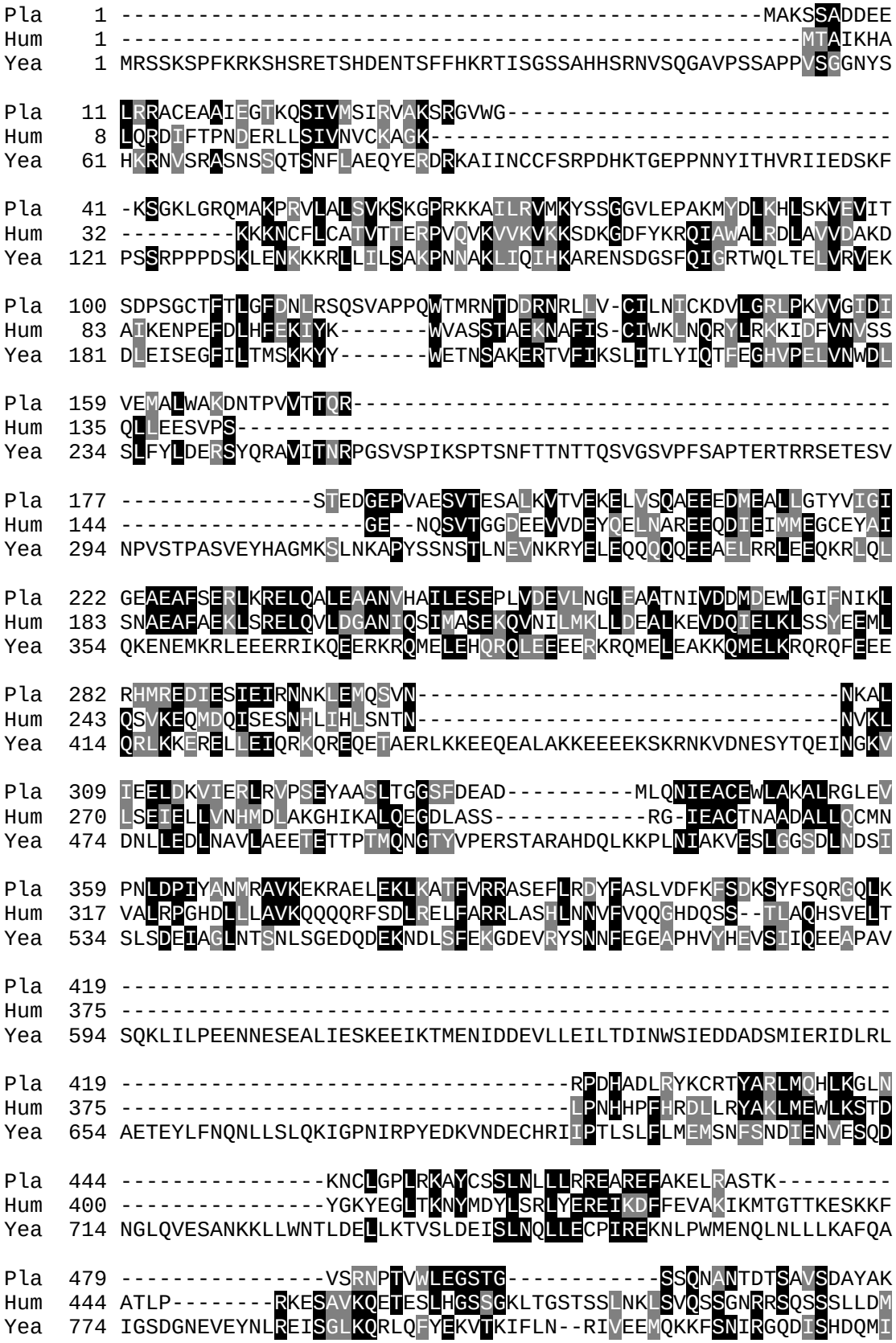
Although the VirD2 interacting proteins in other transformable hosts, e.g. mammalian cells (Kunik *et al*, 2001), still await to be discovered, the presence of AtRAB8 homologues in other organisms such as mammals and fission yeast (Haubruck *et al*, 1990; Rutherford and Moore, 2002) suggests that the evolutionarily conserved DIP homologues in other organisms may also be involved in a similar process during their transformations by *A. tumefaciens.*

Chapter 5. Characterization of DIP Homologues

5.1. Introduction

Earlier work by Chang (2002) has shown that DIP, AC007519 (Gen Bank AAD46030), is homologous to human sec3p like protein FLJ10893 (GenBank NP060731) and yeast sec3p protein. Alignment of the amino acid sequences of DIP and human sec3-like protein by using NCBI BLAST has yielded a significant 27 % identical residues. DIP homologues are also found in other species: *Mus musculus* AK013041 (29% identity), *Drosophila melanogaster* AE003524 (23% identity), *C. elegans* F52E4.7 (22% identity) and yeast sec3p protein (21% identity). Brymora *et al* (2001) has concluded that each of these proteins represents a sec3p homologue in each of these species. The sec3p protein is a subunit of the exocyst complex and is involved in secretion and morphogenesis in the budding yeast *Saccharomyces cerevisiae* (Finger *et al*, 1998). Fig. 5.1 shows the identification of DIP as the plant homologue of yeast Sec3p.

A more recent BLAST analysis has resulted in a greater number of hits – 244 hits in 90 organisms. Additional Sec3 or Sec3-like homologues are found in rice, maize, zebra fish, chicken, dog, rat and orang utan, and 2 to 3 isoforms of Sec3 are reported for some homologues. Other than this, a conserved domain search has also revealed that DIP harbors a Vps52 conserved domain, as shown in Fig. 5.2. *In* vivo, Vps52 complexes with Vps53 and Vps54 to form a multi-subunit complex that is involves in regulating membrane trafficking events. Albeit a low alignment score of 19.3 %, DIP relation to Vps52 further reaffirms its role as an exocyst complex subunit or a subunit of an intra-cytoplasmic transport complex that is functionally essential for *Agrobacterium*-mediated


Pla 1 ----------MAKSSADDEE
Hum 1 ----------MTAIKHA
Yea 1 MRSSKSPFKRKSHSRETSHDENTSFFHKRTISGSSAHHSRNVSQGAVPSSAPPVSGGNYS
Pla 11 LRRACEAAIEGTKQSIVMSIRVAKSRGVWG----------
Hum 8 LQRDIFTPNDERLLSIVNVCKAGK----------
Yea 61 HKRNVSRASNSSQTSNFLAEQYERDRKAIINCCFSRPDHKTGEPPNNYITHVRIIEDSKF
Pla 41 -KSGKLGRQMAKPRVLALSVKSKGPRKKAILRVMKYSSGGVLEPAKMYDLKHLSKVEVIT
Hum 32 ---------KKKNCFLCATVTTERPVQVKVVKVKKSDKGDFYKRQIAWALRDLAVVDAKD
Yea 121 PSSRPPPDSKLENKKKRLLILSAKPNNAKLIQIHKARENSDGSFQIGRTWQLTELVRVEK
Pla 100 SDPSGCTFTLGFDNLRSQSVAPPQWTMRNTDDRNRLLV-CILNICKDVLGRLPKVVGIDI
Hum 83 AIKENPEFDLHFEKIYK-------WVASSTAEKNAFIS-CIWKLNQRYLRKKIDFVNVSS
Yea 181 DLEISEGFILTMSKKYY-------WETNSAKERTVFIKSLITLYIQTFEGHVPELVNWDL
Pla 159 VEMALWAKDNTPVVTTQR----------
Hum 135 QLLEESVPS----------
Yea 234 SLFYLDERSYQRAVITNRPGSVSPIKSPTSNFTTNTTQSVGSVPFSAPTERTRRSETESV
Pla 177 ----------STEDGEPVAESVTESALKVTVEKELVSQAEEEDMEALLGTYVIGI
Hum 144 ----------GE--NQSVTGGDEEVVDEYQELNAREEQDIEIMMEGCEYAI
Yea 294 NPVSTPASVEYHAGMKSLNKAPYSSNSTLNEVNKRYELEQQQQQEEAELRRLEEQKRLQL
Pla 222 GEAEAFSERLKRELQALEAANVHAILESEPLVDEVLNGLEAATNIVDDMDEWLGIFNIKL
Hum 183 SNAEAFAEKLSRELQVLDGANIQSIMASEKQVNILMKLLDEALKEVDQIELKLSSYEEML
Yea 354 QKENEMKRLEEERRIKQEERKRQMELEHQRQLEEEERKRQMELEAKKQMELKRQRQFEEE
Pla 282 RHMREDIESIEIRNNKLEMQSVN----------NKAL
Hum 243 QSVKEQMDQISESNHLIHLSNTN----------NVKL
Yea 414 QRLKKERELLEIQRKQREQETAERLKKEEQEALAKKEEEEKSKRNKVDNESYTQEINGKV
Pla 309 IEELDKVIERLRVPSEYAASLTGGSFDEAD----------MLQNIEACEWLAKALRGLEV
Hum 270 LSEIELLVNHMDLAKGHIKALQEGDLASS----------RG-IEACTNAADALLQCMN
Yea 474 DNLLEDLNAVLAEETETTPTMQNGTYVPERSTARAHDQLKKPLNIAKVESLGGSDLNDSI
Pla 359 PNLDPIYANMRAVKEKRAELEKLKATFVRRASEFLRDYFASLVDFKFSDKSYFSQRGQLK
Hum 317 VALRPGHDLLLAVKQQQQRFSDLRELFARRLASHLNNVFVQQGHDQSS--TLAQHSVELT
Yea 534 SLSDEIAGLNTSNLSGEDQDEKNDLSFEKGDEVRYSNNFEGEAPHVYHEVSIIQEEAPAV
Pla 419 ----------
Hum 375 ----------
Yea 594 SQKLILPEENNESEALIESKEEIKTMENIDDEVLLEILTDINWSIEDDADSMIERIDLRL
Pla 419 ----------RPDHADLRYKCRTYARLMQHLKGLN
Hum 375 ----------LPNHHPFHRDLLRYAKLMEWLKSTD
Yea 654 AETEYLFNQNLLSLQKIGPNIRPYEDKVNDECHRIIPTLSLFLMEMSNFSNDIENVESQD
Pla 444 ----------KNCLGPLRKAYCSSLNLLLRREAREFAKELRASTK---------
Hum 400 ----------YGKYEGLTKNYMDYLSRLYEREIKDFFEVAKIKMTGTTKESKKF
Yea 714 NGLQVESANKKLLWNTLDELLKTVSLDEISLNQLLECPIREKNLPWMENQLNLLLKAFQA
Pla 479 ----------VSRNPTVWLEGSTG----------SSQNANTDTSAVSDAYAK
Hum 444 ATLP--------RKESAVKQETESLHGSSGKLTGSTSSLNKLSVQSSGNRRSQSSSLLDM
Yea 774 IGSDGNEVEYNLREISGLKQRLQFYEKVTKIFLN--RIVEEMQKKFSNIRGQDISHDQMI

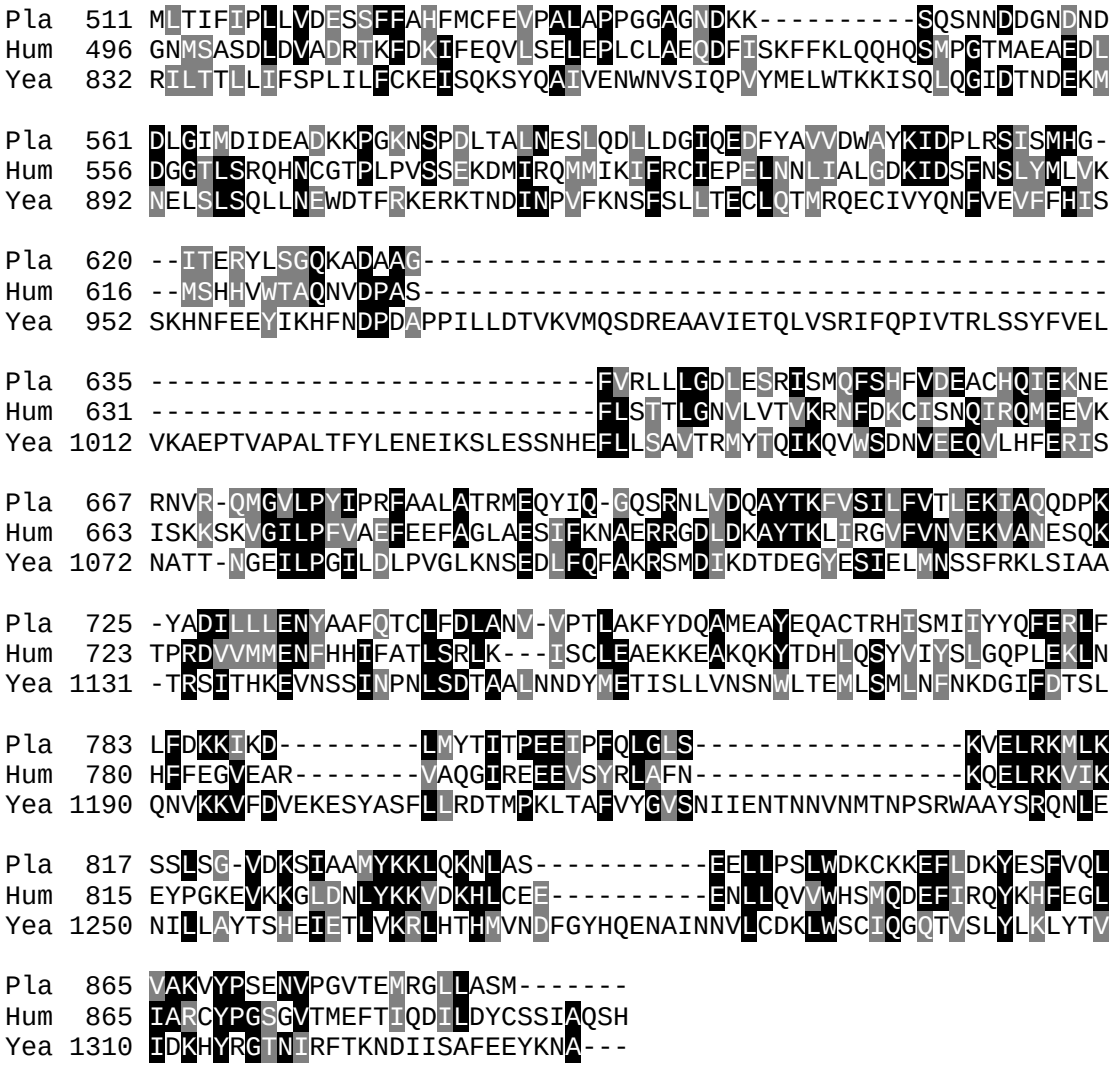


Fig. 5.1. Identification of DIP homologues in yeast and human. The amino acid sequence of DIP (Pla) was aligned with the homologous sequence from human Sec3p-like protein (Hum) and from yeast Sec3p (Yea) using Clustal W. Identical amino acids were shown in black boxes, while similar amino acids were shown in gray boxes.

(Cited from Chang, 2002)

A

gnl|CDD|9688 pfam04129, Vps52, Vps52 / Sac2 family. Vps52 complexes with Vps53 and Vps54 to form a multi-subunit complex involved in regulating membrane trafficking events.

B

```
CD-Length = 509 residues, only 19.3% aligned
Score = 41.5 bits (97), Expect = 4e-04

Query:  246 ILESEPLVDEVLNGLEAATNIVDDMDEWLGIFNIKLRHMREDIESIETRNNKLEMQSVNN 305
Sbjct:  10  IDESENLAS-LHNQIAACDSVLERMEDMLTSFQSDLSSISQDIKFLQEKSNEMQLRLENR  68

Query:  306 KALIEELDKVIERLRVPSEYAASLTGGSFDEADMLQNIE 344
Sbjct:  69  QAVESKLSQFVDDLIVPPELIDTIIDGDVNEPFFLEALE 107
```

Fig. 5.2. Conserved Vps52 domain of DIP. NCBI Conserved Domain Search (Marchler-Bauer and Bryant, 2004) was performed by using the amino acid sequence of DIP [NP_175186; AC007519 (F16N3.18); At1g47550]. (A) The bar represents protein sequence of DIP, while the number on top denotes the amino acid number from 1 to 887. The light blue rectangles depict the masked-out regions with low complexity. The text below the bar provides information about the conserved Vps52 domain, shown in grey open box immediately beneath the bar. (B) Alignment of DIP sequence (Query) to conserved amino acid sequence of Vps52 (Sbjct) from 246 to 344. Identical amino acids were shown in red, while similar amino acids were shown in blue. The above search result was cited from the following URL at NCBI Conserved Domain Search Website: http://www.ncbi.nlm.nih.gov.libproxy1.nus.edu.sg/Structure/cdd/wrpsb.cgi?RID=1123744645-30676-51198934593.BLASTQ3.

transformation of plant cells.

To establish if DIP homologues are also essential for *Agrobacterium*-mediated transformation of other host cells, such as human cells (Kunik *et al*, 2001), human Sec3 protein, which is the human DIP homologue (hDIP), was chosen for our study.

5.2. Materials and methods

Unless or otherwise stated, all materials and methods used in this chapter are as described in Chapter 2.

5.2.1. Cloning of *hDIP*

hDIP (AK027413) was cloned from NT2 cells (ATCC) following the approach outlined in Fig. 5.3. Firstly, total RNA was isolated from NT2 cells by using the TRIZOL Reagent (GIBCO/Life Technologies, Grand Island, NY) according to the manufacturer's instructions. By using two different sets of primers: RT-PCRF & Mid-Down and Mid-Up & RT-PCRR, as shown in Fig. 5.3, the N-terminal half and the C-terminal half of the *hDIP* gene were amplified from total RNA isolated from NT2 cells respectively, following the protocol for One-step RT-PCR kit (QIAGEN). The RT-PCR products were then mixed together and used as the template for the subsequent overlapping PCR to amplify the full length *hDIP* gene. The full length *hDIP* gene was then cloned into pTZ19R (US Biochemical) and subjected to DNA sequencing to verify its sequence with that of the database. As a control, total RNA from HEK-293 cells was also isolated and subjected to the same RT-PCR reactions as that for NT2 cells' total RNA. In addition, another pair of

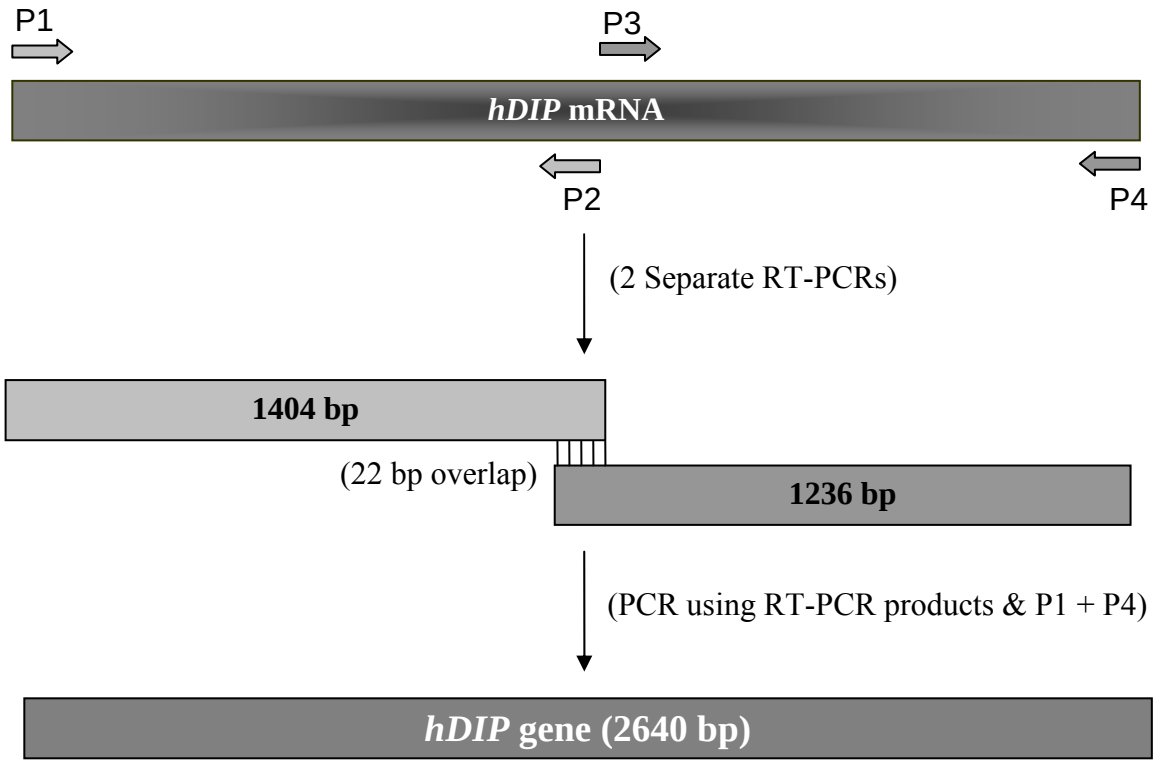


Fig. 5.3. Cloning of *hDIP* from NT2 cells. By using 2 different sets of primers (P1 to P4), the N-terminal half and the C-terminal half of the *hDIP* gene were amplified from total RNA isolated from NT2 cells, following the protocol for One-step RT-PCR kit (QIAGEN). The RT-PCR products were then used as the template for PCR amplification to obtain the full length *hDIP* gene. P1, RT-PCRF; P2, Mid-Down; P3, Mid-Up; P4, RT-PCRR.

primers, FWD and BCK, which would amplify the human ß-actin gene, was also used as a control for both NT2 and HEK-293 RNA samples.

5.2.2. Generation of antibody against hDIP

5.2.2.1. Cloning of *hDIP* gene into the expression vector

As illustrated in Fig. 5.4, a C-terminal 621-bp *Eco*R I digested fragment of the *hDIP* gene was cloned from the GeneConnection™ expression-tested clone (STRATAGENE E05869; UniGene no. Hs.22394; reference accession no. AF208854 or GenBank NP060731), which expresses the hypothetical human protein FLJ10893, into the expression vector pRSET-A (Invitrogen®) to obtain pHC2. The proper construction of pHC2 was confirmed by restriction digestion and the proper in-frame fusion of *hDIP* with the ATG under the control of the T7 promoter in the vector was confirmed by DNA sequencing. After proper verification, pHC2 was transformed into competent cells of BL21(DE3) (Invitrogen®).

5.2.2.2. Pilot expression experiment to monitor the protein expression

In order to determine the optimal time of post isopropyl β-D-thiogalactoside (IPTG) induction, the pilot expression experiment was conducted following the instructions of the manufacturer. In brief, when the cell culture reached a cell density of $OD_{600} = 0.6 – 0.8$, 100 µl samples of BL21(DE3)/pRSET-A and BL21(DE3)/pHC2 were harvested as the uninduced control. The rest of the cells were induced by adding IPTG to a final concentration of 1 mM, followed by incubation with shaking for an additional 2 to 4 hrs.

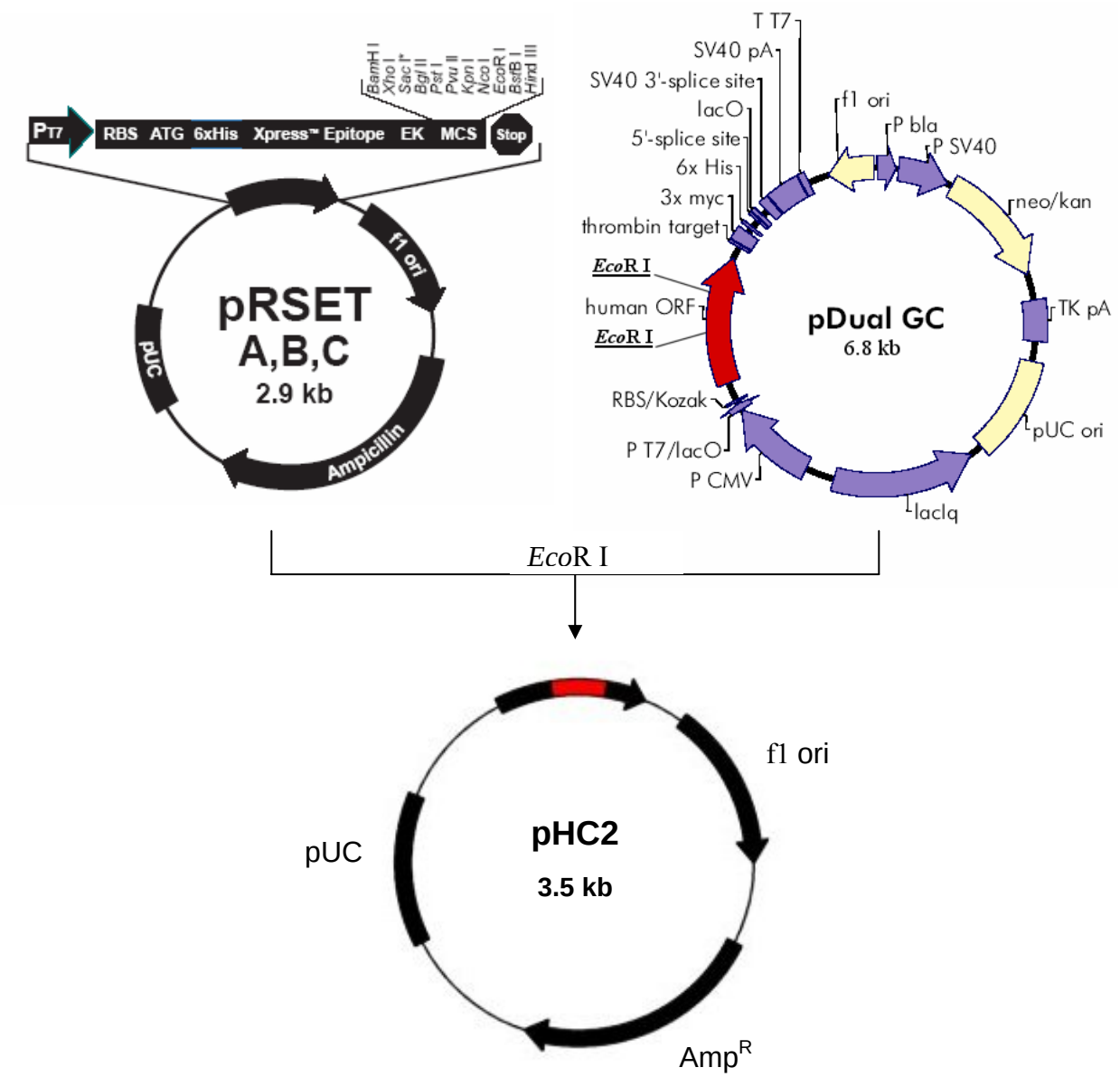


Fig. 5.4. Construction of the expression vector pHC2. pDual®GC vector (STRATAGENE) containing the coding domain for the human hypothetical protein FLJ10893 (reference accession: AF208854) was restriction digested by *Eco*R I to release a 621-bp fragment from the open reading frame (ORF). The fragment was ligated into the *Eco*R I digested pRSET-A expression vector (Invitrogen®), which contained an 6X His-Tag, to create an expression vector, pHC2, that would express the histidine-tagged fusion protein.

100 μl aliquots of the cultures were saved at 30 min intervals following IPTG addition. As an additional control, 100 μl of overnight pre-culture of BL21(DE3)/pRSET-A and BL21(DE3)/pHC2 just prior to the pilot experiment were also harvested. After the cells were collected by centrifugation at each time point, the cell pellet was suspended in 50 μl of 1 × SDS gel sample buffer [50 mM Tris-HCL (pH 6.8), 100 mM dithiothreitol, 2 % SDS, 0.1 % bromophenol blue, 20 % glycerol] and frozen at –20 °C. When the samples at all the time points were collected, they were boiled for 10 min at 95 °C and run on a SDS-PAGE gel. An anti-His monoclonal antibody (CLONTECH) was used as the primary antibody to detect the His-containing proteins by Western Blotting to check if the protein was correctly expressed.

5.2.2.3. Expression of recombinant proteins

A single colony of an *E. coli* strain harboring the fusion protein construct was inoculated into 400 ml of LB (with 100 μg/ml carbenicillin, Cb^{100}) and the culture was grown at 37 °C with shaking for overnight. After that, the cells were harvested by centrifugation at 4,000 rpm for 5 min.

5.2.2.4. Protein Purification

After harvesting the cells, the cell pellet was resuspended in minimal volume of Lysis Buffer (50 mM sodium phosphate, 10 mM Tris-HCl, 8 M urea, 100 mM NaCl, pH = 8.0) as far as possible. Thereafter, the sample was loaded into a Mini Cell and subjected to lysis at 1000 psi by using the FRENCH® Pressure Cell Press, following the instructions

of the manufacturer. The supernatant collected after such cell lysis was used for further purification.

The supernatant collected after the treatment of French Press was the clarified sample containing His_6-fusion proteins. These proteins were purified from the bacterial lysates by affinity chromatography, with the use of TALON resins (CLONTECH) in the batch/gravity-flow column purification approach outlined below.

The TALON resins were thoroughly equilibrated following the protocol supplied by the manufacturer before the sample from bacterial lysates was added. After gently agitating at RT for 1 hr on a platform shaker to allow the polyhistidine-tagged protein to bind the resins, the mixture was then separated by centrifugation at 700 g for 5 min. The supernatant was carefully removed without disturbing the resin pellet. The resins were washed with 10 to 20 bed volumes of 1 × Extraction/Wash Buffer (50 mM sodium phosphate, 8 M urea, 100 mM NaCl, pH = 7.0) and the mixture was gently agitated at RT for 20 min to promote thorough washing. Following that, the mixture was centrifuged as above and the resultant supernatant was discarded. After the washing step was repeated as above, one bed volume of the 1 × Extraction/ Wash Buffer was added to the resins and the pellet was resuspended by vortexing.

After all the aforementioned washing, the mixture was transferred into a 2-ml gravity-flow column with an end cap in place. When the resins had settled out of the suspension, the end cap was removed and the buffer was allowed to drain until it reached the top of the resin bed. After ensuring that no air bubbles were trapped in the resin bed., the column was washed once with 5 bed volumes of 1 × Extraction/Wash Buffer and the

polyhistidine-tagged protein was finally eluted by adding 5 bed volumes of Elution Buffer (50 mM sodium phosphate, 8 M urea, 20 mM MES, 100 mM NaCl, pH = 5.0) to the column. The elution was collected in 0.5 ml fractions, and analyzed by spectrophotometer and SDS-PAGE to determine which fraction(s) contained the majority of the polyhistidine-tagged protein.

5.2.2.5. Gel purification of protein samples

The protein fractions eluted from the TALON resin column were gel purified as described by Hager and Burgess (1980). In brief, the column purified protein was subjected to PAGE and the PAGE gel was stained in 1 M KCl for 10 min at RT with shaking before the subsequent white protein band was excised. The gel slices containing the protein were put into a dialysis tube (PIERCE) and eluted electrophoretically overnight in tank buffer in a transfer apparatus at 200 mA in a cold room. The gel was removed from the tube, and the samples were dialyzed in dH_2O with agitation for at least 18 hr in a cold room, with 3 to 4 changes of dH_2O. The dialyzed sample with a white colloidal appearance was aliquoted and then stored at –20 °C.

5.2.2.6. Antibody production and immunoblot analysis

To obtain polyclonal antibody against hDIP, the partially purified fusion protein was used to raise antibody in a white local female rabbit by intramuscular injection. 2 ml of blood sample was drawn from the rabbit as the negative control before immunization was carried out. A 0.5 ml (100 μg) sample of the purified fusion protein was mixed and

emulsified with an equal volume of Freund's incomplete adjuvant. The preparation was used immediately to inject into one limb of the rabbit.

One month later, 0.5 ml (50 μg) of freshly prepared emulsion was injected into another limb. The limbs were used in rotation every week during each subsequent booster injection. 5 ml of blood was drawn weekly before each booster injection, and the serum was tested for the presence of antibody by Western Blotting (immunoblotting). After the fifth booster, the blood was collected before the rabbit was sacrificed.

Blood from the rabbit was collected in a sterile centrifuge tube, and was allowed to clot at RT for 12 hrs. The supernatant was collected and transferred into a fresh tube, and the antiserum was separated from the clot by centrifugation at 1500 g for 10 min at RT. The antiserum was stored in small aliquots at –20 °C.

5.2.3. Expression profiles of *hDIP* gene and hDIP protein

To examine the accumulation of *hDIP* mRNAs in various human tissues, a Human 12-Lane Multiple Tissue Northern (MTN™) Blot (CLONTECH) was probed with ^{32}P-labeled *hDIP* gene fragment. This *hDIP* probe was amplified from the pDual®GC clone (STRATAGENE) by using the BM012F and BM012R primers and was labeled with ^{32}P by using the *redi*prime™ II random prime labeling system (Amersham). The probe, which is about 1 kb, was produced and hybridized to the MTN Blot by following the instructions of the manufacturers and required the use of the ExpressHyb™ Hybridization Solution (CLONTECH). As a control, the human β-actin control probe that was provided

together with the MTN Blot was also labeled and used to probe the blot, according to the instructions of the manufacturer.

Before the examination of the distribution of hDIP protein in various human tissues, a short and unique peptide of hDIP, KKFGLHGSSGKLTGSTSSLNKL, was commercially synthesized and conjugated to bovine serum albumin (BSA) protein before the conjugated protein was injected into mice to raise antibody against hDIP. This antibody was commercially prepared and obtained from the commercial supplier (Invitrogen^{TM}). The antibody was used as the primary antibody to probe the Human Adult Normal Tissue Western Blot IV (BioChain Institute, Inc; http://www.biochain.com) by following the instructions of the manufacturer.

5.3. Results

5.3.1. Cloned *hDIP* contains several point mutations

The coding domain sequence of *hDIP* is 2640 bp. Repeated effort to clone this gene from the NT2 cells by using one pair of primers for the One-step RT-PCR reaction have failed to amplify this gene from the total RNA of these cells. As such, an approach that used two pairs of primers and a subsequent overlapping PCR was adopted (Fig. 5.3) to clone this gene. As shown in Fig. 5.5, when the N-terminal half (1404 bp; lane 3) and the C-terminal half (1236 bp; lane 4) of this gene were independently amplified from the total RNA of NT-2 cells, one-step RT-PCR products could be obtained. However, these gene fragments could not be amplified from the total RNA samples of HEK-293 cells (lanes 7 and 8). This is in contrast to that of the control human ß-actin gene, which could be

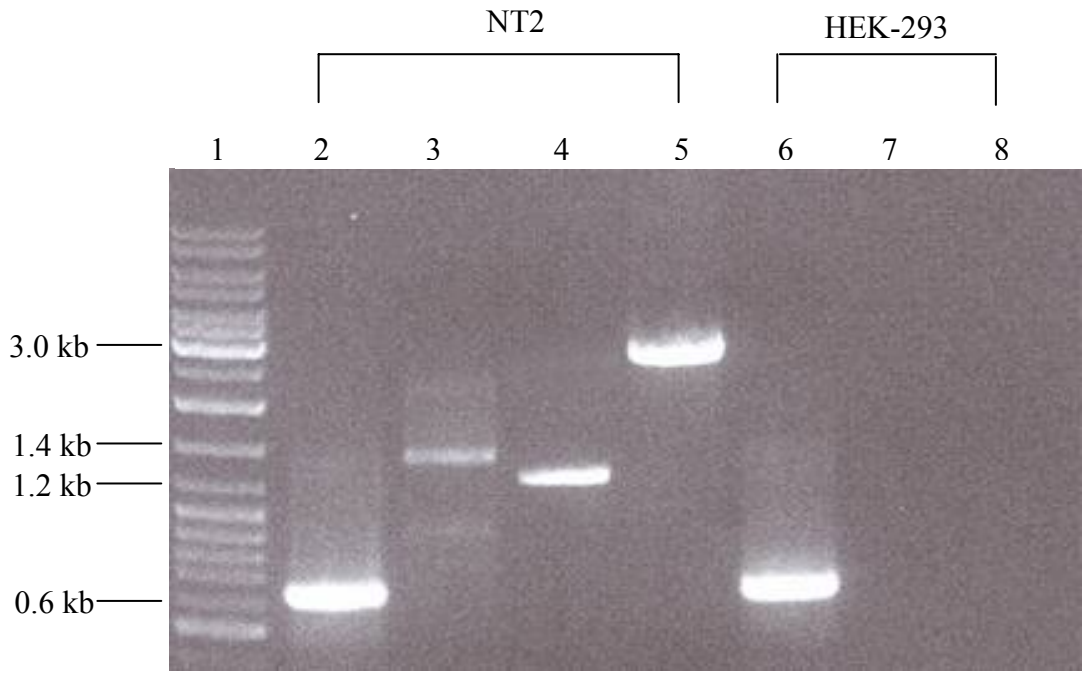


Fig. 5.5. Cloning of *hDIP* from cultured human cells. Primers RT-PCRF and Mid-Down were used to amplify the N-terminal half of *hDIP* gene (lanes 3 and 7) from human RNA samples from both NT2 cells and HEK-293 cells, while primers Mid-Up and RT-PCRR were to use to amplify that of the C-terminal half (lanes 4 and 8). As a control for One-step RT-PCR (QIAGEN), primers FWD and BCK were used to amplify the human ß-actin gene from the RNA samples of both types of cells (lanes 2 and 6). Lane 5 denotes the purified product of overlapping PCR outlined in Fig. 5.3, while lane 1 shows the DNA ladder.

amplified from RNA samples of both types of cells (lanes 2 and 6). The full length *hDIP* gene was finally obtained when purified RT-PCR products from that illustrated in lanes 3 and 4 of Fig. 5.5 were used as the template for overlapping PCR.

After DNA sequencing, it was found that the N-terminal half of the cloned *hDIP* gene contains several point mutations, unlike that published online at the NCBI database. Despite the mutations at the N-terminal half, the nucleotide sequence was not mutated at all for the C-terminal half. As shown in Fig, 5.6, all sequenced clones contain a single base mutation at nucleotides no. 221 and 897. While the mutation at nucleotide no. 897 does not change the ensuing amino acid, the mutation at 221 (from G to A) has resulted in a change of amino acid from glycine to aspartate. Besides the change of bases of the nucleotides at these locations, which were observed for all sequenced clones, there were other mutations that were observed only once or twice for different individual clones at various locations indicated in Fig. 5.6. It is still unclear as to why such a phenomenon is observed.

5.3.2. Antibody against hDIP could not be raised in rabbits and mice

As illustrated in Fig. 5.7, the pilot expression experiment has demonstrated that when the histidine-partial hDIP fusion protein, His_6-$FLJ10893_{(127 - 333)}$, was overexpressed in BL21(DE3) harboring pHC2, a protein slighter larger than 25 kD could be obtained after overnight culture of the bacterial strain. The amount of protein obtained was even more than that after IPTG induction. In both samples, a smaller band was observed after immunoblotting, possibly due to the degradation of the protein into smaller proteins.

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1     ATGACAGCAATCAAGCATGCATTACAAAGAGACATTTTTACACCAAATGATGAACGCCTG   60
61    CTGAGCATTGTGAATGTCTGCAAAGCAGGAAAAAAGAAAAAGAACTGTTTTTTATGTGCC  120
121   ACAGTGACAACTGAACGCCCTGTGCAGGTTAAGGTGGTCAAAGTCAAGAAATCCGATAAG  180
181   GGAGATTTCTACAAAAGGCAGATTGCATGGGCCCTTCGAGGTCTTGCTGTGGTAGATGCC  240
241   AAAGATGCTATCAAAGAAAATCCTGAATTTGATTTACACTTTGAAAAAATATATAAATGG  300
301   GTTGCCAGCAGCACTGCTGAAAAGAATGCATTTATTTCATGCATTTGGAAATTGAATCAG  360
361   CGATATCTCCGGAAGAAAATTGATTTTGTCAATGTTAGCTCACAGCTTTTGGAAGAATCT  420
421   GTTCCAAGTGGAGAAAATCAGAGTGTGACAGGAGGTGATGAAGAAGTAGTAGATGAATAC  480
481   CAAGAGTTAAATGCAAGAGAAGAACAGGATATCGAAATAATGATGGAAGGCTGTGAATAT  540
541   GCAATCTCGAATGCGGAAGCCTTTGCAGAAAAATTGTCCAGAGAGCTGCAGGTGCTAGAT  600
601   GGGGCTAACATCCAGTCAATCATGGCATCTGAAAAACAAGTCAACATCCTGATGAAATTG  660
661   CTAGATGAGGCTCTAAAGGAGGTAGATCAGATTGAATTGAAACTGAGCAGTTATGAGGAA  720
721   ATGCTCCAAAGTGTAAAAGAACAAATGGATCAGATCTCTGAAAGCAACCACCTAATTCAT  780
781   CTTAGTAACACTAATAATGTAAAACTCCTATCTGAGATAGAGTTCCTTGTGAACCACATG  840
841   GACTTGGCCAAAGGTCATATAAAGGCCCTTCAGGAAGGAGATCTTGCTTCTTCCAGGGGC  900
901   ATTGAGGCCTGCACCAATGCTGCTGATGCCCTTCTGCAGTGCATGAATGTAGCTCTTCGA  960
961   CCAGGCCATGACTTGCTTCTGGCAGTCAAACAGCAACAGCAGCGATTCAGTGATTTGCGA 1020
1021  GAGCTTTTTGCCCGGAGACTGGCCAGTCACCTCAACAATGTTTTTGTTCAACAGGGTCAT 1080
1081  GATCAGAGTTCGACTCTTGCCCAACACTCTGTTGAACTGACTTTACCCAATCATCATCCA 1140
1141  TTTCATAGAGATTTGCTCCGATATGCCAAGCTGATGGAGTGGCTAAAGAGTACAGATTAT 1200
1201  GGAAAATATGAAGGACTAACAAAGAATTACATGGATTATTTATCCCGACTATATGAAAGA 1260
1261  GAAATCAAAGATTTCTTTGAAGTTGCAAAGATCAAGATGACTGGCACAACTAAAGAAAGC 1320
1321  AAGAAGTTTGGTCTTCATGGAAGTTCGGGGAAATTAACTGGATCTACTTCTAGTCTAAAT 1380
1381  AAGCTCAGTGTTCAGAGTTCAGGGAATCGCAGATCTCAGTCATCTTCCCTGTTGGATATG 1440
1441  GGAAACATGTCTGCCTCTGATCTCGATGTTGCTGACAGGACCAAATTTGATAAGATCTTT 1500
1501  GAACAGGTACTAAGTGAACTGGAGCCCCTATGTCTGGCAGAACAGGACTTCATAAGTAAA 1560
1561  TTTTTCAAACTACAGCAACATCAAAGTATGCCTGGAACTATGGCTGAAGCAGAGGACCTG 1620
1621  GATGGAGGAACATTATCACGGCAACATAATTGTGGCACACCACTGCCTGTTTCATCTGAG 1680
1681  AAAGATATGATCCGCCAAATGATGATTAAAATATTTCGCTGCATTGAGCCAGAGCTGAAC 1740
1741  AACCTAATTGCATTAGGAGACAAAATTGATAGCTTTAACTCTCTTTATATGTTAGTCAAA 1800
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1801  ATGAGTCATCATGTGTGGACTGCACAAAATGTGGACCCTGCTTCTTTCCTAAGTACTACA  1860
1861  TTGGGAAATGTTTTGGTGACTGTCAAAAGGAACTTTGACAAATGCATTAGTAACCAAATA  1920
1921  AGGCAAATGGAAGAAGTAAAGATCTCAAAAAAGAGTAAAGTTGGAATTCTTCCATTTGTT  1980
1981  GCTGAATTTGAAGAATTTGCTGGACTTGCAGAATCAATCTTCAAAAATGCTGAGCGTCGT  2040
2041  GGAGACCTGGATAAAGCATACACCAAACTTATCAGAGGAGTATTTGTTAATGTGGAGAAA  2100
2101  GTAGCAAATGAAAGCCAGAAGACCCCCAGGGATGTGGTTATGATGGAAAACTTTCACCAT  2160
2161  ATTTTTGCAACTCTTTCTCGATTGAAAATCTCATGTCTAGAAGCAGAAAAAAAAGAAGCC  2220
2221  AAACAAAAATACACAGATCACCTTCAGTCTTATGTCATTTACTCTTTAGGACAACCTCTT  2280
2281  GAAAAACTAAATCATTTCTTTGAAGGTGTTGAAGCTCGCGTGGCACAGGGCATAAGGGAG  2340
2341  GAGGAAGTAAGTTACCAACTTGCATTTAACAAACAAGAACTTCGTAAAGTCATTAAGGAG  2400
2401  TACCCTGGAAAGGAAGTAAAAAAAGGTCTAGATAACCTCTACAAGAAAGTTGATAAACAT  2460
2461  TTATGTGAAGAAGAGAACTTACTTCAGGTGGTGTGGCACTCCATGCAAGATGAATTTATA  2520
2521  CGCCAGTATAAGCACTTTGAAGGTTTGATAGCTCGCTGTTATCCTGGATCTGGTGTTACA  2580
2581  ATGGAATTCACTATTCAGGACATTCTGGATTATTGTTCCAGCATTGCACAGTCCCACTAA  2640
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Fig. 5.6. Cloned *hDIP* contains several point mutations. After DNA sequencing of plasmids isolated from 10 to 20 single bacterial colonies, a number of point mutations were found for nucleotides at various locations (grey shaded boxes). Red letters in shaded boxes refer to mutation which was observed in all clones sequenced, whereas the blue letter denotes mutation which was observed twice. The black letters in shaded boxes refer to mutation which was observed only once.

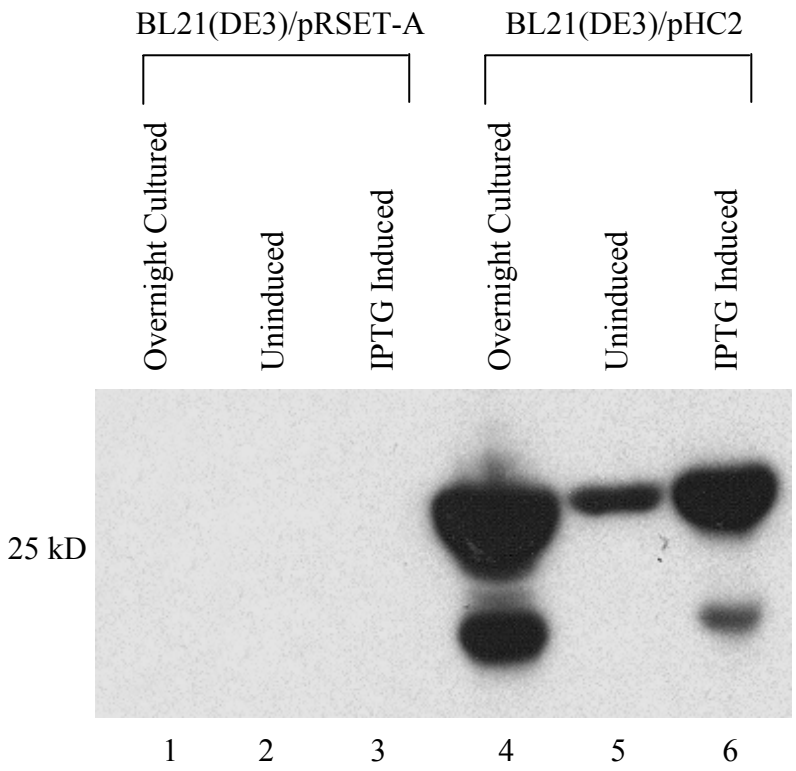

Fig. 5.7. Overexpression of His_6-$FLJ10893_{(127 - 333)}$ partial hDIP fusion protein. *E. coli* strains, BL21(DE3)/pRSET-A and BL21(DE3)/pHC2, grown in LB medium were collected after overnight culture (lanes 1 and 4), before IPTG induction (lanes 2 and 5) and after IPTG induction (lanes 3 and 6). The same amount of bacterial cells was collected for each strain under the three different conditions, and the total protein from each sample was electrophoresed in a 10% SDS-PAGE gel. The fusion proteins were visualized by immunoblotting using the 6×His monoclonal antibody (CLONTECH) as the primary antibody.

As for the uninduced cells, a small amount of the protein could also be detected after Western Blotting. Therefore, this protein appears to be constitutively expressed and the bacterial cells did not require the inducer, IPTG, for maximal expression from the pRSET based vector under the control of the T7 promoter. It is probable that the level of "leaky" expression from uninduced cells was sufficiently high enough to render the addition of IPTG unnecessary.

After French Press mediated cell lysis, purification by affinity chromatography and gel purification, the purified His_6-$FLJ10893_{(127 - 333)}$ fusion protein could be obtained at a relatively high concentration, as shown in Fig. 5.8. However, when the purified fusion protein was used to raise polyclonal antibody in rabbits, the subsequent serum from the rabbits failed to show any immuno-reactivity and specificity against hDIP. When used to probe against hDIP in a Western Blot, a diffused background with numerous bands was observed (data not shown). Thus, it seems that rabbits may possess an hDIP homologue with a very high degree of amino acid sequence similarity to give rise to such results. Such unusual high sequence similarity is a reflective of the highly conserved exocyst function, in which Sec3 plays a role.

Since no sequence data for hDIP homologue(s) in rabbits are available, an attempt was made to raise hDIP antibody in mice. When this experiment was performed in 2001, it was found that the mouse homologue is quite highly homologous to hDIP. But a short stretch of peptide sequence was found to be unique in human and not found in the mouse homologue. This short peptide, KKFGLHGSSGKLTGSTSSLNKL, was synthesized commercially and made to conjugate with the bovine serum albumin (BSA) protein,

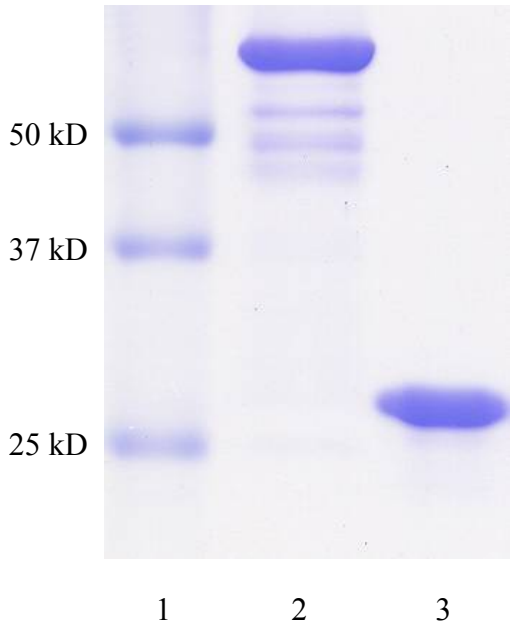

Fig. 5.8. Coomassie blue staining of His_6-FLJ10893$_{(127 - 333)}$ partial hDIP fusion protein after gel purification. The protein samples were electrophoresed on a 10% SDS-PAGE gel and then subjected to Coomassie blue staining, as described in Chapter 2. Lane 1, protein standards; Lane 2, BSA standard; Lane 3, purified His_6-FLJ10893$_{(127 - 333)}$ fusion protein.

before the conjugated protein was sent to a commercial company (Invitrogen^{TM}) for the generation of antibody against hDIP.

When the antibody generated from mice was used to probe the Human Adult Normal Tissue Western Blot IV (BioChain Institute, Inc.), the immunoblot shows that hDIP is expressed in all major tissue types (Fig. 5.9), except a very low level of expression in brain and an additional smaller hDIP protein in the skeletal muscle. But upon closer examination of the immunoblot, it was found that the size of the protein, 62.5 kD, differed markedly from the predicted size of about 100.2 kD (SWISS-PROT/TrEMBL at http://tw.expasy.org/tools/pi_tool.html). The size of 62.5 kD is, however, very similar to that of BSA protein (lane 2 of Fig. 5.8).

It is therefore quite likely that the antibody raised in mice might have been an antibody against BSA protein, which has an expected size of around 62.5 kD. This is quite feasible when considering that BSA was conjugated to the short peptide sent for antibody generation. Indeed, this is subsequently substantiated when the human and mouse Sec3 protein was found to possess at least 2 to 3 isoforms (NCBI database), about 1 year after the initial immunoblot was performed. The multiple sequence alignment of hDIP with its isoforms in human and homologues in mice, shown in Fig. 5.10, has demonstrated very clearly that hDIP is extremely and highly conserved. The homologues in these two species share a sequence identity of over 95 %. This may account for the failure of raising antibody against hDIP in mice and, by in inference, in rabbits.

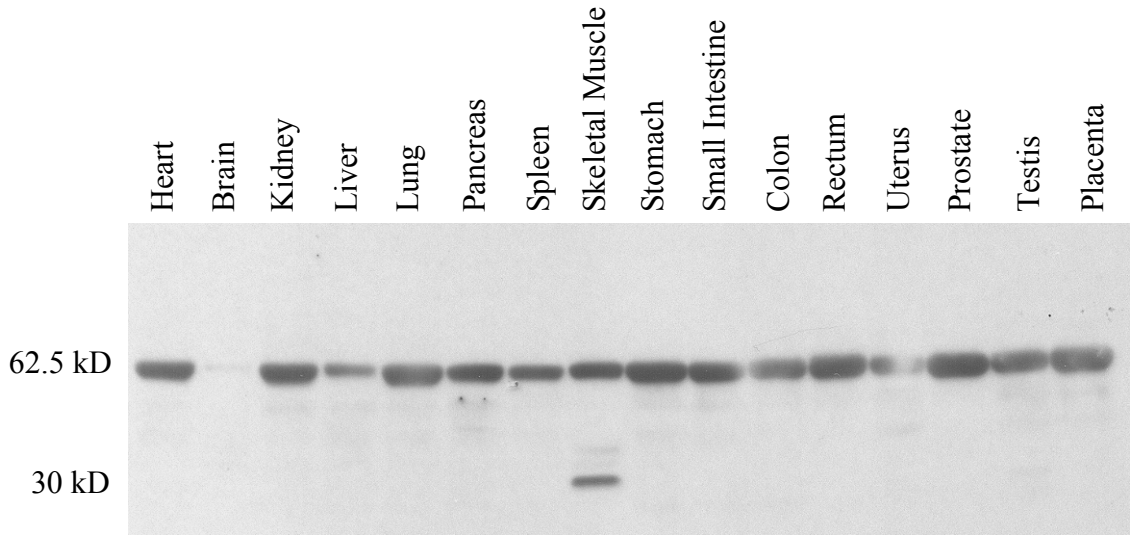

Fig. 5.9. Expression profile of hDIP protein. Human Adult Normal Tissue Western Blot IV (BioChain Institute, Inc.) was subjected to immunoblotting by using the commercially raised antibody (anti BSA: KKFGLHGSSGKLTGSTSSLNKL) generated from mice. The tissue type is indicated on top, while the approximate size range of protein is shown to the left.

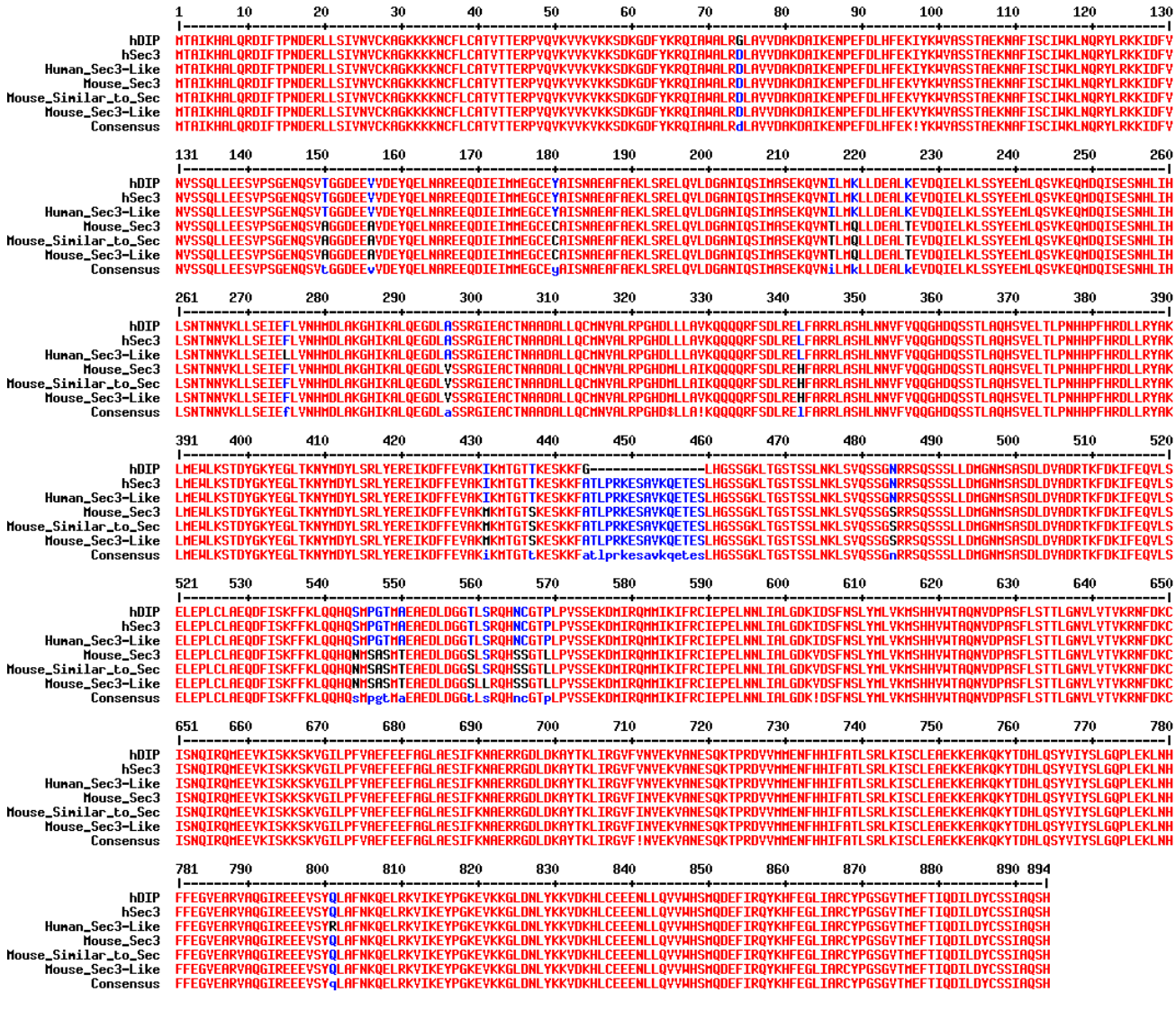

Fig. 5.10. Multiple alignment of hDIP isoforms and mouse homologues. The amino acid sequences of various hDIP isoforms and mouse homologues of hDIP were aligned by using the Multialign program at http://prodes.toulouse.inra.fr/multalin/multalin.html hosted by INRA website. The red letters show identical amino acids while the blue or the black letters show similar amino acids.

5.3.3. *hDIP* is expressed in most human tissues

As shown in Fig. 5.11, *hDIP* gene is expressed in all tissues, except peripheral blood leukocyte, which is not a tissue. The accumulation of *hDIP* mRNAs (~ 3.6 kb) was quite high in brain, heart, skeletal muscle, kidney and placenta, whereas the expression in other tissues was comparatively lower or in some tissues quite low. This differential expression is probably due to the different rates of required polarized secretion in different tissues, where the exocyst function is differentially engaged for cellular functions. On the contrary, such variation in expression level was not observed for the control human β-actin gene, as the immunoblot shows a more or less uniform expression level at around 2 kb.

Although *hDIP* gene could not be amplified via One-step RT-PCR from HEK-293 cells – a cell line derived from embryonic kidney cells, the probe of *hDIP* has managed to detect the mRNA of this gene in the kidney tissue (Fig. 5.11). This discrepancy suggests that even though a certain tissue may be tested positive for the presence of the transcript for *hDIP* gene, the developmental stage and individual cellular function of the various cell types within a tissue may dictate the need for the expression of this gene at various levels and at various time points.

Perceptibly, *hDIP* could also be constitutively expressed in certain cell types or tissues where an ever active exocyst function is engaged at all times. This is probably the case for heart and skeletal muscle tissues, where rhythmic contractions are almost continuous at most time.

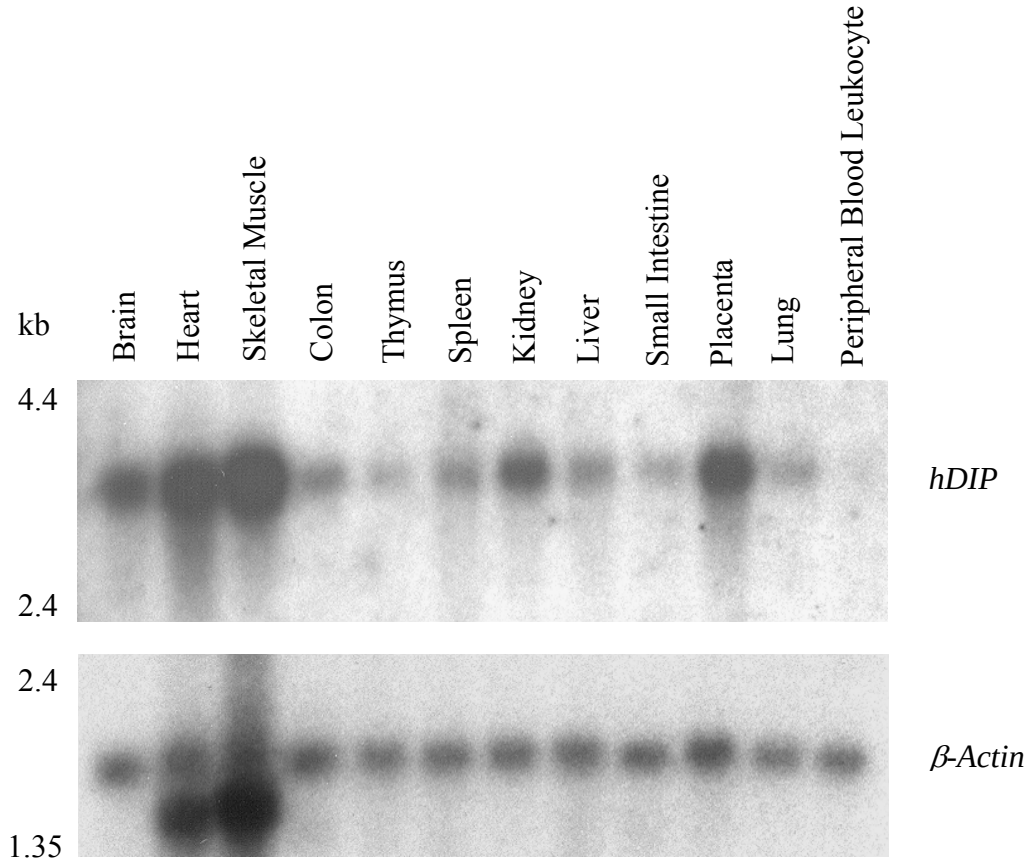

Fig. 5.11. Expression profile of *hDIP* gene. Human 12-Lane Multiple Tissue Northern (MTN^{TM}) Blot (CLONTECH) was probed with a 1-kb *hDIP* gene fragment labeled with ^{32}P. As a control, the same MTN Blot was stripped and reprobed with human β-actin control probe labeled with ^{32}P. The tissue type is indicated on top, while the approximate size range of RNA is shown to the left.

5.4. Discussion

Prior to the availability of all the sequences of the various isoforms of human Sec3 (hDIP) and mice Sec3 proteins, attempts were made in our lab to generate the antibody against hDIP in 2001. The subsequent inability to generate anti-hDIP antibody in both rabbits and mice has suggested that the homologues of the evolutionarily conserved exocyst complex subunit, Sec3, in mammals are probably extremely homologous to one another and an important function is perhaps conserved in these organisms. This high degree of sequence homology is eventually substantiated, when the sequence data of all the isoforms of human and mice DIP homologues became available.

While the identification of the various isoforms of these proteins in different organisms from the database suggests that proteins which are encoded by different genes may play the same function, subsequent sequence analysis has revealed that these isoforms are over 95 % identical to one another, as shown in Fig. 5.10. This is also the case for DIP and its native *Arabidopsis* homologues with very high sequence homology to one another (alignment not shown). All these observations imply that the various isoforms could have been encoded by the same gene and the minor sequence differences among them might have been attributed to different sequencing efforts, which produced slightly different results. The single band of *hDIP* for each tissue type shown in the MTN Blot in Fig. 5.11 seems to support this notion. Besides this, the frequency of mutation of the N-terminal half of *hDIP* shown in Fig. 5.6 could also partly explain the generation of these potentially false “isoforms”.

If the presence of isoforms is disregarded, the sequence homologies of the DIP homologues in various organisms with DIP, as outlined in section 5.1 above, are still relatively and significantly adequate for us to hypothesize that *Agrobacterium*-mediated transformation of cells from these organisms may involve the evolutionarily conserved exocyst complex subunit Sec3. This is based on the indispensability of normal DIP function for an efficient *Agrobacterium*-mediated transformation, shown in Chapter 3. Although not all cells from the organisms with a DIP homologue are transformable by *A. tumefaciens* or shown to be transformable by *A. tumefaciens*, the ever increasing host range of *A. tumefaciens* and the plasticity of this natural occurring "inter-kingdom gene transfer" suggest that *A. tumefaciens* may utilize the endogenous host exocyst function during course of its interaction with the transformable host cells.

Despite the failure to raise antibody against hDIP, which has hindered further immunohistological studies of hDIP and its colocalization with T-DNA, if any, the analysis of our data has implicated an involvement of the exocyst complex in various organisms during *Agrobacterium*-mediated transformation. Besides Sec3, it is perceivable that other exocyst complex component may also be involved during *Agrobacterium*-mediated transformation.

Even though exocyst complex has been largely associated with polarized secretion and exocytosis, a recent study of exocyst complex component Sec5 in *Drosophila* has shown that Sec5 could be found on the endocytic vesicles in the oocytes (Sommer *et al*, 2005). Such finding further supports the feasibility that *Agrobacterium*-mediated transformation of host cells may mediate through the endocytotic pathway, which is

manipulated by *A. tumefaciens* for the traverse of its T-DNA within the cell cytoplasm and towards the nucleus. If that is indeed proven to be the case, further studies and characterization of DIP homologues and other exocyst complex components will give rise to a much clear picture and shed light on the mechanism of *Agrobacterium*-mediated transformation of its host cells.

Chapter 6. General Conclusions and Future Work

6.1. General conclusions

VirD2 Interacting Protein, DIP, isolated from *Arabidopsis* cDNA library has been shown to interact with *A. tumefaciens* virulence protein, VirD2, both *in vitro* and *in vivo* (Chang, 2002). In that study, DIP was shown to co-localize with T-DNA within the plant cell cytoplasm during *Agrobacterium*-mediated transformation of tobacco BY-2 cells, by an immunohistological approach. Even though the aforementioned work on DIP has suggested the involvement of DIP during *Agrobacterium*-mediated transformation of plant cells, the functional requirement and importance of this plant factor was not firmly demonstrated.

In this study, a novel RNAi approach involving sequential rounds of *Agrobacterium*-mediated transformations was adopted to establish the functional significance of DIP in the *Agrobacterium*-mediated transformation of plant cells and tissues. When DIP was "knocked down" transiently in tobacco BY-2 cells or tobacco leaf tissues, the plant cells and tissues were shown to become less susceptible to a second round of transformation by *A. tumefaciens*. This is evident in the decrease of the GUS reporter activity, which indicates a much reduced efficiency of transformation of these cells and tissues by *A. tumefaciens*. When the DIP "knock down" genotype was selected on the selective medium, the resultant stable transgenic BY-2 cells were found to possess a slower rate of growth as well as a similarly reduced efficiency of transformation by *A. tumefaciens.* Apart from these, the failed attempt to generate homozygous *DIP* $^{-/-}$ "knock out" *Arabidopsis* plants from heterozygous seed line by repeated rounds of self fertilization has further shown that DIP is critically important

for plant physiological and cellular functions. Taken together, these results show that DIP is crucial and involved functionally in *Agrobacterium*-mediated transformation of plant cells.

Furthermore, the delineation of the DIP-interacting domain of VirD2 via the GAL4 based yeast two-hybrid analysis has indicated that the C-terminal bipartite NLSs of VirD2 are not required for interaction with DIP. This places DIP in a group of plant factors that do not localize T-complex to the nucleus. In term of the mode of action, DIP is perhaps more akin to chaperone proteins such as cyclophilins, although they are not homologous to each other in term of sequences, (Deng *et al*, 1998).

Since DIP has been identified as a homologue of the evolutionarily conserved exocyst complex subunit (Sec3p) in yeast and partly because it contains a conserved Vps52 domain, it is probable that during *Agrobacterium*-mediated transformation of plant cells, host factors interacting with the *A. tumefaciens* T-pilus or T-DNA export machinery (such as BTI proteins; Hwang and Gelvin, 2004) may subsequently direct the T-DNA or T-complex to the route of endocytotic pathway, in which the exocyst complex, involving DIP, may play a role.

The subsequent effort in characterizing the human homologue of DIP, hDIP, has revealed the surprisingly and extremely homologous amino acid sequences among the various mammalian homologues of Sec3, especially that between hDIP and the mouse homologues and possibly also that between hDIP and the rabbit homologues. This is apparent from the inability of raising antibody against hDIP in both rabbits and mice. Such an observation from this study implies that the conserved exocyst complex function in the secretion and/or endocytotic pathway is likely to be ‘hijacked’ and manipulated for its own cause when *A. tumefaciens* transforms its host cells.

6.2. Future work

Despite the failure to generate antibody against hDIP for immunohistological analyses, further characterization of hDIP and that of the other DIP homologues can still make use of a similar RNAi approach that has been adopted to probe DIP function in this study. After taking into consideration the 'non-specific' effects of RNAi in the mammalian systems as discussed in Chapter 3, RNAi experiments in human cells seem to be the appropriate next step. Depending on the outcomes of such investigation, the role of exocyst complex in *Agrobacterium*-mediated transformation can then be assessed further.

Aside from that, the relative order of interaction and any potentially synergistic relationship of DIP with other plant factors in *Agrobacterium*-mediated transformation of plant cells will also require more studies. Albeit the inherent difficulties that are associated with the studies of such a complex process as *Agrobacterium*-mediated transformation of plant cells, the findings from such studies will certainly and unavoidably yield further insights with great impact.

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