LIVE-TRACKING VIRE2 PROTEIN AND MOLECULAR ANALYSIS OF YEAST FACTOR PMP3P DURING *AGROBACTERIUM*-MEDIATED TRANSFORMATION

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NATIONAL UNIVERSITY OF SINGAPORE

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

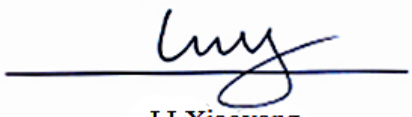

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TABLE OF CONTENTS

SUMMARY

As a natural genetic engineer, *Agrobacterium tumefaciens* is capable of transferring single-stranded DNA molecule (T-DNA) into various recipients. Infection of this bacterium is greatly facilitated by the translocated virulence protein VirE2, which is involved in the entire transformation process inside recipient cells including T-DNA uptake, nucleus import and chromatin integration. However, previous studies of VirE2 lead to conflicting results due to lack of appropriate tagging approaches. In this study, a bipartite split-GFP system was adopted to track the *Agrobacterium* delivered VirE2 inside recipient cells. Using the split-GFP strategy, the VirE2 was visualized for the first time inside host cells after the delivery. This Split-GFP tagging system does not affect VirE2 function, and thus is suitable for VirE2 behavior study *in vivo*. Relatively high VirE2 delivery efficiency has been observed in non-natural host yeast, highlighting the *Agrobacterium* as an excellent protein transporter. Besides, filamentous structures of VirE2 in the absence of T-DNA have also been observed *in vivo* for the first time. Bacteria-delivered VirE2 was actively transported into plant nucleus in a nuclear localization signal (NLS)-dependent manner, while it stayed exclusively inside yeast cytoplasm and no clear movement could be observed. This study helps to further understand the mechanism of VirE2 trafficking inside host cells and also enabled other *in vivo* studies of *Agrobacterium* virulence proteins in the future.

Previous studies of *Agrobacterium*-mediated transformation (AMT) mainly focused on the transformation process inside the bacteria; however, little is known about the host factors that also play important roles. Using yeast as the model, the role of a host membrane protein Pmp3p in AMT process has been identified. Deletion of this protein resulted in decreased efficiencies of virulence protein delivery as well as the transformation, suggesting a role of this membrane protein in bacterial attachment and virulence factor translocation.

Subsequent studies of yeast PMP3 family revealed the potential role of RCI2 family proteins in *Arabidopsis* immunity responses. Active down regulation of these genes was observed upon either *Agrobacterium* infection or flg22 treatment, indicating that these genes might be involved in plant immunity system through interaction with the plasma membrane ion channels. The results from this study help to further understand the host factors in AMT process and also shed light on the complex signaling network of plants in response to both biotic and abiotic stresses.

MANUSCRIPTS RELATED TO THIS STUDY

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABA	abscisic acid	ml	milliliter(s)
Amp	ampicillin	mM	millimole(s)
AMT	*Agrobacterium*-mediated transformation	MS	Murashige & Skoog
AS	acetosyringone	NHEJ	non-homologous end-joining
ATP	adenosine triphosphate	NHR	non-homologous recombination
bp	base pair(s)	NLS	nuclear localization signal
CM	co-cultivation medium	PAMP	pathogen-associated molecular pattern
CRAfT	Cre Recombinase Assay for Translocation	PBS	Phosphate Buffered Saline
DAPI	4',6-diamidino-2-phenylindole	PCR	polymerase chain reaction
DNA	deoxyribonucleic acid	PEG	polyethylene glycol
EF-Tu	elongation factor Tu	PTI	PAMP-triggered immunity
g	gram(s)	RT-PCR	reverse transcription polymerase chain reaction
GUS	β-D-glucuronidase	SDS	sodium dodecyl sulfate
Km	kanamycin	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
LB	Luria-Bertani	T4SS	type IV secretion system
LiAc	lithium-acetate	T-DNA	transfer DNA
LRR	leucine rich repeat	Ti	tumor-inducing
M	mole(s)	µg	microgram(s)
MAPK	mitogen-activated protein kinase	µl	microliter(s)
mg	milligram(s)		

Chapter 1. Literature Review

Agrobacterium tumefaciens, as one of the most commonly studied *Agrobacterium* species, is a soil borne phytopathogen that causes tumor-like growth or gall at the wound parts of host plants during infection. The molecular basis is related to the (~200 kb) tumor-inducing (Ti) plasmid of the bacteria (Hooykaas *et al.* 1992). During infection process, the bacteria can transfer a part of the Ti plasmid (T-DNA) into plant cells, which subsequently enters host nucleus and integrated into the host genome through non-homologous recombination (NHR) (Offringa *et al.* 1990). The integrated T-DNA is responsible for uncontrolled plant cell proliferation by producing enzymes that catalyze the synthesis of plant hormone such as auxin and cytokinin. The transferred T-DNA can also synthesize several kinds of amino acid–sugar conjugates named opines, which could be uniquely used as the carbon and nitrogen resources and thus could provide selective advantages for the pathogen (Dessaux *et al.* 1988).

A. tumafaciens is able to transfer any DNA sequence within the T-DNA region into host cells; thus various efforts have been made to introduce genes of interest into T-DNA region for intended genetic manipulations (Garfinkel *et al.* 1981; Zambryski *et al.* 1983; Fraley *et al.* 1985). However, plenty of difficulties had emerged concerning the relatively large size of the Ti plasmid, which makes it hard to be manipulated in molecular cloning works, such as difficulty in isolation, lack of unique restriction endonuclease sites, low copy number as well as containing oncogenes. To address this, binary vector systems were developed in 1983 to separate the T-DNA region apart from the Ti plasmid onto a new vector (Deframond *et al.* 1983; Hoekema *et al.* 1983). The bacterium with its original T-DNA region deleted was regarded as a *vir* helper strain; the helper strain could recognize and deliver the T-DNA region as long as the T-DNA harboring vector was introduced into the same bacterial cell. The separated binary vector has greatly simplified the genetic manipulation process and

also makes it practical to use multiple copies of T-DNA with different features at the same time. With the binary vector systems, the utility of *Agrobacterium*-mediated transformation (AMT) in plant researches become wide and diverse.

In this section, a brief review will be included concerning the usage of *Agrobacterium* in biotechnology, process of AMT, host factors involved in AMT process, as well as the *Agrobacterium*-induced plant immunity.

1.1. *Agrobacterium tumefaciens* as a genetic tool in biotechnology

1.1.1. Genetic engineering of plants in the era of functional genomics

The natural host range of *A. tumefaciens* spans most of the plant family in the plant kingdom. Early studies in 1970s showed that up to 56% of the gymnosperms and 58% of the angiosperms were able to be transformed by wild type *Agrobacterium* strains (Decleene *et al.* 1976; Decleene *et al.* 1981). Moreover, by using combination of different *Agrobacterium* strains and inoculation approaches, some recalcitrant plants also displayed susceptibility to AMT under laboratory conditions (Ishida *et al.* 1996; Hiei *et al.* 1997; Chen *et al.* 2006); and the number of plant species reported to be transformed by *Agrobacterium* is still increasing. The extremely wide host range of *A. tumefaciens* greatly increases its application in plant genetic manipulations.

With the advancing technology in the field of biological sciences, we have entered the era of functional genomics and more and more genome sequences of various plant species become available. Meanwhile, the need of different tools in large-scale genomic studies is increasing. Using *Agrobacterium* as a vector for efficient horizontal gene transfer becomes convenient in random mutagenesis of plant genome.

Plenty of systemic studies have been carried out by using *Agrobacterium* as the insertional mutagenesis tool in plants, such as *Arabidopsis thaliana*, *Oryza sativa*, and *Nicotiana* species (Koncz *et al.* 1989; Koncz *et al.* 1992; Jeon *et al.* 2000; Radhamony *et al.* 2005). These useful works have established foundations for the

functional genomics studies in various research fields.

1.1.2. *Agrobacterium*-mediated transformation of non-plant species

Except for the natural host species in plant kingdom, the range of *Agrobacterium* host has been extremely expanded under laboratory conditions.

It has been shown that more than 80 non-plant species were able to be transiently or stably transformed by *Agrobacterium*, in the presence of plant wounding-related phenolic compounds such as acetosyringone (AS), including bacteria, algae, fungi and mammalian cells (Michielse *et al.* 2005; Lacroix *et al.* 2006). The “promiscuous” characteristic of *Agrobacterium* suggest that it could also be used as genetic tools in the study of other non-plant organisms.

Interestingly, unlike the non-homologous end joining recombination happens in plant cells, T-DNA mainly relied on homologous recombination in chromosomal integration of non-plant hosts such as *Saccharomyces cerevisiae* (Bundock *et al.* 1995). This enables the targeted genetic manipulation of these organisms. Moreover, the relatively conserved transformation process in these different *Agrobacterium* hosts makes it possible to using simplified and efficient system such as yeast to study the transformation process as well.

1.2. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation is a complex process which begins with plant signal recognition and ends in the expression of T-DNA integrated in the host genome. The transformation is a long-term evolved process which requires the participation of both pathogen as well as various host factors. This section will mainly focus on the bacterial factors involved in this process.

1.2.1. Host recognition and virulence gene expression

A. tumefaciens is an environmental microorganism and can live in the soil

independent of host plants. However, opines production after plant cell transformation serves as a selective advantage thus provides a preferable environment for the bacteria.

Agrobacterium-mediated transformation commonly happens at the wound sites of the host plants, where the plant wound associated phenolic compounds such as acetosyringone serve as the activation signals (Stachel *et al.* 1985). Except the host associated compounds including phenols and aldose monosaccharides, some environmental factors such as low pH and low PO_4 have also been shown to be involved in virulence gene induction (Palmer *et al.* 2004; Brencic *et al.* 2005).

Perception of plant wound signals is achieved through a two-component VirA/VirG system (Stachel *et al.* 1986). Although inducible as the other virulence genes, *virA* and *virG* are constantly expressed at a basic level under normal growth condition (Winans *et al.* 1988). The *virA* gene encodes a dimeric protein containing two transmembrane domains (Brencic *et al.* 2005). It is responsible for sensing phenolic compounds and sugars with the help of a chromosomally encoded protein ChvE (Cangelosi *et al.* 1990; Chang *et al.* 1992; Turk *et al.* 1994; Tzfira *et al.* 2004). VirA contains a cytoplasmic kinase domain which is responsible for VirG phosphorylation (Jin *et al.* 1990; Chang *et al.* 1992). This kinase domain is repressed by the VirA periplasmic domain and a receiver region under normal circumstances, while the suppression could be relieved by interaction of ChvE and signal compounds (Melchers *et al.* 1989; Chang *et al.* 1992; Banta *et al.* 1994). Once the kinase domain of a VirA protein is derepressed, it binds to an ATP molecule followed by phosphorylation of the neighboring VirA molecule in the dimeric state (Brencic *et al.* 2004). The phosphorylation of VirA dimer will then results in accumulation of VirG-PO_4 in a phenol dependent manner (Brencic *et al.* 2004). VirG serves as the transcriptional factor after phosphorylation; it binds to specific promoter region of different *vir* genes and initiates downstream transcription (Brencic *et al.* 2005).

1.2.2. Bacteria attachment and translocation of virulence factors

Agrobacterium-mediated transformation is achieved by a serial of virulence proteins activated by VirA/VirG system; several of them could also be translocated into host cells to facilitate infection, including VirD2, VirD5, VirE2, VirE3 and VirF (Citovsky *et al.* 1992; Howard *et al.* 1992; Vergunst *et al.* 2000; Schrammeijer *et al.* 2003; Tzfira *et al.* 2004).

Physical interaction and attachment of *Agrobacterium* to the host cell surface is required prior to substrates transfer. The physical association between *Agrobacterium* and host cells involves both a nonspecific, aggregation-like interaction and a specific, surface-receptor-required interaction (Neff *et al.* 1985; Gurlitz *et al.* 1987). The specific attachment is Ti plasmid independent, which requires periplasmic β1-2 glucan synthesis and the participation of at least three chromosomally encoded genes including *chvA*, *chvB*, and *pscA* (*exoC*) (Douglas *et al.* 1985; Cangelosi *et al.* 1987; Thomashow *et al.* 1987).

After host recognition and surface attachment, several virulence molecules are delivered into host cells through a VirB/VirD4 type IV secretion system (T4SS) (Cascales *et al.* 2003). The secretion apparatus is comprised of 12 different *Agrobacterium* virulence proteins including VirB1-11 and VirD4; these proteins interact with each other and form a complex pilus-like structure. Among these T4SS components, 3 inner membrane associated proteins, VirD4, VirB4, and VirB11, form the base of the secretion structure. All of these proteins contain NTP-binding domain and are supposed to provide energy for the secretion apparatus biogenesis and substrates secretion through ATP hydrolysis (Berger *et al.* 1993; Stephens *et al.* 1995; Kumar *et al.* 2002). Besides, another inner membrane protein VirB6 was also shown to be able to interact with the base components while its function is not quite clear (Jakubowski *et al.* 2004; Judd *et al.* 2005). The core component of the secretion apparatus is composed of 4 virulence proteins, VirB7, VirB8, VirB9 and VirB10, which spans the bacterial inner and outer membranes (Kado 2000; Christie 2001). The

third part of the T4SS apparatus is comprised of VirB2 and VirB5 which form a pilus-like structure outside the bacterial membrane (Lai *et al.* 1998; Schmidt-Eisenlohr *et al.* 1999; Lai *et al.* 2002). These three components (base structure, core structure and pilus structure) interact with each other to form a cell envelope–spanning structure for T4SS substrates translocation. The VirB/VirD4 complex was shown to be localized around the bacteria cells in a helical pattern thus was supposed to facilitate host cell attachment and substrates transfer (Aguilar *et al.* 2010).

During *Agrobacterium*-mediated transformation, at least 5 *Agrobacterium* virulence proteins have been shown to be able to transfer into host cells, including VirD2, VirD5, VirE2, VirE3 and VirF (Citovsky *et al.* 1992; Howard *et al.* 1992; Vergunst *et al.* 2000; Schrammeijer *et al.* 2003; Tzfira *et al.* 2004). Different from the other T4SS, *Agrobacterium* is also able to transfer the T-DNA fragment into host cells through the VirB/VirD4 channel. The translocation of T-DNA is facilitated by the VirD2 protein (Wang *et al.* 1984). VirD2 nicks the Ti plasmid at the T-DNA border region in the form of VirD1-VirD2 complex; it then stays covalently attached to the 5’ prime end of the T-strand and leads its way into host cells through the T4SS channel as a nucleoprotein complex (Scheiffele *et al.* 1995).

Similarly as the other T4SS systems (Luo *et al.* 2004; Nagai *et al.* 2005; Schulein *et al.* 2005; Hohlfeld *et al.* 2006), the translocation of *Agrobacterium* T4SS substrates is dependent on their C-terminal regions, which share a conserved domain R-X(7)-R-X-R-X-R within their protein sequences (Vergunst *et al.* 2005). This conserved C-terminal domain is necessary for interaction with the T4SS apparatus to facilitate translocation. Protein translocation process is initiated through the interaction with the coupling protein VirD4, which plays an important role in recruiting the T4SS substrates to the secretion apparatus followed by transportation (Hamilton *et al.* 2000; Atmakuri *et al.* 2003; Cascales *et al.* 2004). The virulence effectors are then transferred into host cells through the interaction with the other

T4SS components and finally get into the host cytoplasm, where they could further facilitate the transformation process through various aspects.

1.2.3. Nuclear targeting and T-DNA integration

Although it is still not very clear how the secreted virulence proteins pass through the host cell membrane, the process was hypothesized to be mediated by the VirB pilus structure and is mechanically similar to a typical conjugation process (Schroder *et al.* 2005).

The secreted virulence factors are separately translocated into host cytoplasm through *Agrobacterium* VirB/VirD4 apparatus. Upon delivery into host cells, the VirE2 might be able to form channels on plant cell membrane and "pull" the T-strand in through covalent binding (Dumas *et al.* 2001; Duckely *et al.* 2005). VirE1 binds to VirE2 inside *Agrobacterium* cells to prevent it from self aggregation and binding to T-DNA (Deng *et al.* 1999; Zhao *et al.* 2001; Dym *et al.* 2008), while the translocated VirE2 could interacted with each other in the absence of VirE1 and coat the T-strand to form a putative T-complex (Citovsky *et al.* 1989; Sen *et al.* 1989; Yusibov *et al.* 1994; Dym *et al.* 2008). The T-complex is then delivered into host nucleus through cytoplasm by an active process, which probably involves the participation of plant microtubules (Salman *et al.* 2005; Tzfira 2006).

Various approaches are adopted by *Agrobacterium* in T-complex targeting into host nucleus. The nucleus targeting of T-complex is mainly dependent on VirD2; it has been shown to be able to interact with the plant importin α family protein AtKAPα with its C-terminal bipartite NLS to facilitate the nucleus import (Ballas *et al.* 1997). The T-DNA coating protein VirE2 also contains two putative nuclear localization signals and could localize to the plant nucleus independent of VirD2, indicating that it might also could help T-complex nucleus targeting as well (Citovsky *et al.* 1992; Citovsky *et al.* 1994). Different from the nucleus import of VirD2, VirE2 interacts with *Arabidopsis* transcription factor VIP1, which undergoes nuclear import

after phosphorylation by mitogen-activated protein kinase MPK3 (Tzfira *et al.* 2001; Djamei *et al.* 2007). Recent studies also showed that the VirE2 was able to directly interact with *Arabidopsis* importin α isoform IMPa-4 to get into the plant nucleus (Bhattacharjee *et al.* 2008). Besides, the translocated virulence protein VirE3 might also mimic the function of VIP1 in plant cell to facilitate the nucleus uptake of T-complex (Lacroix *et al.* 2005).

Once getting into the host nucleus, the T-complex is recruited to the host chromatin through interaction with host VIP1 and VIP2 (Li *et al.* 2005; Loyter *et al.* 2005; Anand *et al.* 2007). Uncoating of T-complex is required prior to integration into host genome. Uncoupling of VirE2 from T-complex is mediated by the *Agrobcaterium* effector VirF, which contains an F-box domain and initiate the proteasomal degradation of VIP1 together with VirE2 (Vergunst *et al.* 2000; Tzfira *et al.* 2004).

After T-complex uncoating, the T-strand is integrated into host genome, which mainly occurs as non-homologous recombination while homologous recombination also occurs in some non-natural host species (van Attikum *et al.* 2003; Tzfira *et al.* 2004).

1.3. Host proteins involved in AMT process

Agrobacterium-mediated transformation is a complex process which requires the participation of both bacterial and host factors. A variety of host proteins involved in the AMT process have been identified through different approaches, including forward genetic screening, protein two-hybrid interaction assay, transcriptional profiling and reverse genetic experiments. In this section, the host factors related to the *Agrobcaterium*-mediated transformation will be reviewed.

1.3.1. *Agrobacterium* attachment and virulence factors transfer

Agrobacterium attachment to the plant cell surface represents one of the earliest

events in the AMT process and is critical for successful transformation. Different *Agrobacterium* attachment deficient mutant displayed attenuated virulence or even avirulent in transformation of plants (Douglas *et al.* 1982; Douglas *et al.* 1985; Matthysse 1987; Thomashow *et al.* 1987; Cangelosi *et al.* 1989; Deiannino *et al.* 1989).

Previous studies have shown the involvement of two *Arabidopsis* proteins in the *Agrobacterium* attachment, an arabinogalactan protein AtAGP17 and a cellulose synthase-like protein CslA-09; and the T-DNA insertional mutants of these genes displayed decreased susceptibility to *Agrobacterium*-mediated transformation (Zhu *et al.* 2003; Zhu *et al.* 2003; Gaspar *et al.* 2004). Besides, some other plant proteins including a rhicadhesin binding protein and an avitronectin-like protein have been shown to be important in bacterial attachment; however, further confirmation is still needed for these observations (Wagner *et al.* 1992; Swart *et al.* 1994).

After *Agrobacterium* attachment, physical interaction between the T4SS pilus structure and host cell surface proteins is required for the subsequent delivery of virulence factors. The T-pilus is comprised of two virulence proteins, the major component VirB2 which forms the body of the structure and the minor component VirB5 which localizes to the pilus tip (Lai *et al.* 1998; Eisenbrandt *et al.* 1999; Aly *et al.* 2007). Both of these two virulence proteins might be involved in the interaction with host surface proteins, while little is known about the host cell receptors for the T-pilus contact and the substrates transfer. A yeast two hybrid screening experiment identified several *Arabidopsis* interaction partners for VirB2, including AtRTNLB1, AtRTNLB2, AtRTNLB4 and a Rab8 GTPase; these proteins might form protein complex with T-pilus components at the host cell membrane to facilitate the virulence translocation (Hwang *et al.* 2004; Marmagne *et al.* 2004; Nziengui *et al.* 2007).

1.3.2. Cytoplasmic trafficking and Nucleus targeting

Once assembled inside host cell, the T-complex has to move across the

cytoplasm to enter the host nucleus for successful integration and T-DNA expression.

Proteins containing nuclear localization signal sequences are supposed to be imported into nucleus though interaction with importin α proteins. Both of the two T-complex components, VirD2 and VirE2, contain NLS sequences and are supposed to co-operatively help T-complex in nucleus targeting. It has been shown that both VirD2 and VirE2 can interact with several *Arabidopsis* importin α isoforms (KAPα, IMPa-2, IMPa-3, and IMPa-4) in yeast cells and two additional importin α isoforms (IMPa-7 and IMPa-9) in plants (Bhattacharjee *et al.* 2008); thus these plant importin proteins are supposed to be responsible for T-complex nuclear targeting through interaction with VirD2 or/and VirE2. Besides, the VirE2 might also abuses the *Arabidopsis* VIP1 defense signaling pathway, which could be activated by the mitogen-activated protein kinase (MAPK) MPK3 upon *Agrobacterium* infection, to facilitate its nucleus import (Djamei *et al.* 2007).

In addition to these host proteins directly involved in nucleus import, some other host factors might also be indispensible for the cytoplasmic trafficking of T-complex. Several studies have implicated the involvement of plant cytoskeleton structures in T-complex transport inside host cytoplasm, including the microtubules and actin microfilaments (Zhu *et al.* 2003; Salman *et al.* 2005); however, the role of these host factors is still not conclusive enough and requires further investigations.

1.3.3. Chromatin targeting and T-DNA integration

Once inside the host nucleus, the T-DNA will be recruited to the host chromatin followed by integration.

Several host proteins might be involved in the chromatin targeting of T-strand, including a kinase CAK2Ms, which indirectly help target VirD2 to the transcriptionally active regions through phosphorylation of the largest subunit of RNA polymerase II (Bako *et al.* 2003). Besides, the VirE2 interaction protein VIP1 acts as a transcription factor; its association with VirE2 might also help in T-DNA chromatin

targeting (Li *et al.* 2005; Loyter *et al.* 2005; Lacroix *et al.* 2008).

Once arriving at the host chromatin region, the coating protein VirE2 will be removed from the T-complex through the VirF mediated proteosome degradation pathway (Regensburgtuink *et al.* 1993; Schrammeijer *et al.* 2001; Tzfira *et al.* 2004; Lacroix *et al.* 2008). Some plant species such as *A. thaliana* also encode F-box proteins that function similarly as the VirF to mediate the degradation of VIP1-VirE2 protein complex (Zaltsman *et al.* 2010).

The integration of T-DNA into plant genome requires double-strand break at the insertion site of host DNA. The prevailing model for this process has suggested the association between T-DNA integration and host double-strand break repair mechanism (Tzfira *et al.* 2004). In this model, T-DNA inside host nucleus could replicate to a double-strand form and subsequently insert into the genome double-strand breaks through the non-homologous end-joining (NHEJ) process. Thus those host proteins required for NHEJ might also help in the T-DNA integration, including Ku70, Ku80, XRCC4, and DNA ligase IV (Pansegrau *et al.* 1993; Friesner *et al.* 2003; Watt *et al.* 2009). Using yeast as a model to study AMT process also revealed the involvement of several NHEJ proteins (Ku70, Mre11, Sir4, Rad50, and Xrs2) in T-DNA integration (van Attikum *et al.* 2001).

As a potential transcriptional regulator, the VirE2 interaction protein VIP2 might also be involved in T-DNA integration by recruiting T-strand to the transcription active regions (Anand *et al.* 2007).

Besides, a variety of histones and the related proteins are shown to play an important role in T-DNA integration, including various histones (H2A, H2B, H3, H4), histone chaperones (CAF-1, SGA1), nucleosome assembly factors, histone deacetylases and acetyltransferases (Nam *et al.* 1999; Mysore *et al.* 2000; Zhu *et al.* 2003; Endo *et al.* 2006; Crane *et al.* 2007). Although how these host proteins affect the T-DNA integration is not quite clear, they are supposed be related to the T-DNA access to the host genome thus affect the AMT process.

1.4. *Agrobacterium* and plant immunity response

Agrobacterium cause uncontrolled cell proliferation in plants to create a preferable microenvironment to facilitate the bacterial reproduction. On the other hand, perception of the bacteria triggers the plant cell immunity responses, which in turn also affects the *Agrobacterium*-mediated transformation.

1.4.1. *Agrobacterium* perception by plant cells

Different from the mammalian cells, plant cells mainly depend on the innate immune system instead of adaptive immune system for pathogen defense (Dangl *et al.* 2001; Ausubel 2005; Chisholm *et al.* 2006).

Plant cell surface receptors could recognize the pathogen-associated molecular patterns (PAMPs) and result in PAMP-triggered immunity (PTI) to repel further colonization of pathogenic microorganisms (Nurnberger *et al.* 2004; Jones *et al.* 2006). Two well established archetypal PAMPs are bacterial flagellin and elongation factor Tu (EF-Tu) (Gomez-Gomez *et al.* 2002; Zipfel *et al.* 2006). Although with different chemical characteristics, treatment with flagellin or EF-Tu resulted in almost identical downstream transcriptional changes in plant cells, indicating that the perception of different PAMPs might converge on similar signaling pathways to induce the common immune response in plants (Zipfel *et al.* 2006).

Unlike most of the other microorganisms, *Agrobacterium* flagellin proteins do not contain the conserved 22 amino-acid peptide, flg22, thus is insufficient to elicit PTI in plant cells (Felix *et al.* 1999). Instead, other PAMPs of *Agrobacterium* including the EF-Tu could actively act as the PTI elicitors (Kunze *et al.* 2004). Perception of *Agrobacterium* EF-Tu by the LRR-kinase receptor EFR will lead to the activation of innate immune system as well as downstream defense response (Zipfel *et al.* 2006).

1.4.2. Host cell transcriptional re-programming

Perception of PAMPs by host cell receptors will subsequently initiate the downstream response, including ion fluxes, oxidative burst, signaling pathway activation, receptor endocytosis and transcriptional re-programming (Boller *et al.* 2009).

PAMP receptors are usually membrane associated kinases with leucine rich repeat (LRR) domains; these receptors recognize the PAMPs from the pathogen and activate MAPK cascade. Two complete MAPK cascades, MEKK1/MKKKs-MKK4/5/9-MPK3/6 and MEKK1-MKK1/2-MPK4, are involved in signaling transduction of MAMP induced primary response (Tena *et al.* 2011). Activation of MAPK signaling cascades will lead to modulation of the downstream transcription factor activity and result in massive gene re-programming in plant cells.

Large scale microarray analysis revealed that *Agrobacterium* attack triggered the modulated expression of a variety of genes related to the plant immunity response (Ditt *et al.* 2001). And the enhanced defense response also has been shown to be correlated with the resistance to *Agrobacterium*-mediated transformation (Zipfel *et al.* 2006). Further analyses implied the important role of salicylic acid (SA) in regulation of *Agrobacterium vir* genes expression (Yuan *et al.* 2007). However, the mechanisms involved in *Agrobacterium*-induced host cell transcriptional re-programming are still mostly unknown.

Interestingly, the *Agrobacterium* effector VirE3 has also been shown as a potential transcription factor and could be delivered into plant nucleus, where it might functions as a transcriptional activator to regulate immunity-related specific genes expression (Garcia-Rodriguez *et al.* 2006).

1.4.3. Evading of *Agrobacterium* from the host defense response

In the presence of host defense responses, the bacterium itself could also develop diverse approaches to interfere with the host immunity systems to facilitate its

proliferation (Jones *et al.* 2006).

Early studies showed that the attachment-deficient *Agrobacterium* mutant triggered enhanced defense gene expression in *Ageratum conyzoides* cells, indicating that *Agrobacterium* might suppress the plant immunity system in an attachment-dependent pattern (Veena *et al.* 2003). They also showed that the translocated T4SS substrates, including T-DNA and *vir* proteins, could regulate the host genes expression in tobacco cells (Veena *et al.* 2003). All these observations have implied the important role of translocated virulence factors in plant defense response modulation.

1.5. Objectives

Although *Agrobacterium*-mediated transformation has been well studied inside the bacteria, the process in the host cells is still not clear and requires further investigations. Studies in this thesis mainly focus on the host part and aimed in the following aspects including bacterial virulence factors trafficking and host factors involved in the AMT process.

As a crucial virulence factor, *Agrobacterium* VirE2 is involved in various aspects of the transformation process inside recipient cells including T-DNA uptake, nucleus import and chromatin integration. However, *in vivo* studies of VirE2 in recipient cells remain difficult due to lack of appropriate methods and resulted in controversies. This study aims to develop a new approach for study of the VirE2 trafficking in host cells.

Successful transformation process requires the participation of both bacterial and host factors, however, little is known for the host part that also plays important roles in the AMT. This study adopted *Saccharomyces cerevisiae* and *Arabidopsis thaliana* as the model organisms and aimed to find out and study the host factors that are potentially involved in the transformation process.

Chapter 2. Materials and Methods

2.1. Strains, plasmids and Culture

Yeast and bacterial strains used in this study are listed in Table 2.1. Plasmids used in this study together with their features are listed in Table 2.2. *E. coli* DH5α strain was used for cloning experiments.

Media for yeast and bacterial culturing were prepared as described in Table 2.3. *E. coli* strains were grown in Luria-Bertani (LB) liquid or agar medium at 37 °C. *A. tumefaciens* strains were grown in MG/L liquid or agar medium at 28 °C. 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin were supplemented when necessary.

2.2. DNA manipulations

2.2.1. Molecular cloning

Competent cell preparation, polymerase chain reaction (PCR), DNA digestion, ligation, and bacterial cells transformation were carried out following standard protocols as described (Gannon *et al.* 1988). *E. coli* DH5α strain was used for cloning experiments.

2.2.2. Preparation of yeast genomic DNA

Total genomic DNA of yeast was prepared as described with a few modifications (Gannon *et al.* 1988). Yeast cells from 3 ml of overnight culture were harvested by centrifugation. Cells were washed once with PBS and re-suspended in 450 μl TES (10 mM Tris-HCl, 25mM EDTA, 150mM NaCl, pH 8.0).

Table 2.1. Yeast and bacterial strains used in this study

Strains	Relevant characteristics	Source
Escherichia coli		
DH5α	*EndA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 (argF-lacZYA)* U169 φ80d*lacZ*	Bethesda Research Laboratories
Saccharomyces cerevisiae		
BY4741	**MAT***a his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Open Biosystem
pmp3Δ	**MAT***a his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *pmp3Δ::KanMX*	Open Biosystem
Agrobacterium tumefaciens		
A348	Wild-type, A136 (pTiA6NC) (octopine-type)	(Knauf *et al.* 1982)
A348*ΔvirE2*	A348 derivative, with *virE2* gene deleted	This study
A348-*105virE2*	A348 derivative, with original *virE2* gene replaced by EHA105 *virE2*	This study
A348-*105virE2::GFP11*	A348 derivative, with original *virE2* gene replaced by *virE2::GFP11* from EHA105	This study
EHA105	C58 strain containing pTiBo542 harboring a T-DNA deletion	(Hood *et al.* 1993)
EHA105*ΔvirE2*	EHA105 derivative, with *virE2* gene deleted	This study

EHA105*ΔvirD4*	EHA105 derivative, with *virD4* gene deleted	This study
EHA105*GFP11-virE2*	EHA105 derivative, with *virE2* replaced by *GFP11-virE2*	This study
EHA105*virE2-GFP11*	EHA105 derivative, with *virE2* replaced by *virE2-GFP11*	This study
EHA105*virE2::GFP11*	EHA105 derivative, with *virE2* replaced by *virE2::GFP11*	This study
EHA105*virE2::GFP11nls1*	EHA105*virE2::GFP11* derivative, with NLS1 coding sequences mutated	This study
EHA105*virE2::GFP11nls2*	EHA105*virE2::GFP11* derivative, with NLS2 coding sequences mutated	This study
EHA105*virE2::GFP11nls*	EHA105*virE2::GFP11* derivative, with both two NLS coding sequences mutated	This study
EHA105*GFP-virE2*	EHA105 derivative, with *virE2* replaced by *GFP-virE2*	This study
EHA105*virE2::GFP11ΔvirD4*	EHA105*virE2::GFP11* derivative, with *virD4* gene deleted	This study
EHA105*virE2::GFP*	EHA105 derivative, with *virE2* replaced by *virE2::GFP*	This study

Table 2.2. Plasmids used in this study

Plasmids	Relevant characteristics	Source
pCB301	A mini binary vector, *nptIII*	(Xiang *et al.* 1999)
pEX18Tc	Counter-selectable plasmid carrying *sacB*, *oriT*, Tc^R	(Hoang *et al.* 1998)
pEX18TcKm	pEX18Tc derivative, with a *nptIII* insertion, Km^R	This study
pEX18TcKm-A348VE2KO	pEX18TcKm derivative, with insertion of A348 *virE2* upstream and downstream sequences, Km^R	This study
pEX18TcKm-EHA105VE2KO	pEX18TcKm derivative, with insertion of EHA105 *virE2* upstream and downstream sequences, Km^R	This study
pEX18TcKm-GFP11-VirE2	pEX18TcKm derivative, with insertion of GFP11-VirE2 coding sequence and relative flanking sequence, Km^R	This study
pEX18TcKm-VirE2-GFP11	pEX18TcKm derivative, with insertion of VirE2-GFP11 coding sequence and relative flanking sequence, Km^R	This study
pEX18TcKm-VirE2::GFP11	pEX18TcKm derivative, with insertion of VirE2::GFP11 coding sequence and relative flanking sequence, Km^R	This study

pEX18TcKm-GFP-VirE2	pEX18TcKm derivative, with insertion of GFP-VirE2 coding sequence and relative flanking sequence, Km^R	This study
pEX18TcKm-EHA105VD4KO	pEX18TcKm derivative, with insertion of EHA105 *virD4* upstream and downstream sequences, Km^R	This study
pEX18TcKm-105VirE2	pEX18TcKm-A348VE2KO derivative, with EHA105 VirE2 coding sequence inserted between A348 *virE2* upstream and downstream sequences, Km^R	This study
pEX18TcKm-105VirE2::GFP11	pEX18TcKm-A348VE2KO derivative, with EHA105 VirE2::GFP11 coding sequence inserted between A348 *virE2* upstream and downstream sequences, Km^R	This study
pEX18TcKm-VirE2::GFP11NLS1M	pEX18TcKm-VirE2::GFP11 derivative, with NLS1 coding sequences mutated, Km^R	This study
pEX18TcKm-VirE2::GFP11NLS2M	pEX18TcKm-VirE2::GFP11 derivative, with NLS2 coding sequences mutated, Km^R	This study
pEX18TcKm-VirE2::GFP11NLSM	pEX18TcKm-VirE2::GFP11 derivative, with both two NLS coding sequences mutated, Km^R	This study

pCMV-mGFP1-10 Hyg Amp	A vector containing GFP1-10 coding sequence	American Peptide Company
pmGFP Cterm S11	A vector containing GFP11 coding sequence	American Peptide Company
pACT2	Yeast expression vector, 2μ origin, *ADH1* promoter, *ADH1* terminator, *LEU2*, Amp^R	Clontech Laboratories
pACT2A	pACT2 derivative, with the GAL4AD domain deleted, 2μ origin, *LEU2*, Amp^R	This study
pACT2A-GFP1-10	pACT2A derivative, expressing GFP1-10, 2μ origin, *LEU2*, Amp^R	This study
pQH04-GFP1-10	pACT2A-GFP1-10 derivative, with *LEU2* replaced by *HIS3*, Amp^R	This study
pHT101	A derivative of the binary vector pCB301, ligated at SalI site with pACT2, in which the GAL4AD gene is replaced by EGFP reporter, 2μ origin, *LEU2*, Km^R, Amp^R	Lab collection
pHT101-2A	pHT101 derivative, with EGFP reporter deleted, *LEU2*, Km^R, Amp^R	This study
pYES2	Yeast expression vector, 2 μ origin, *GAL1* promoter, *CYC1* terminator, *URA3*, Amp^R	Invitrogen

pYES2-DsRed-GFP11	pYES2 derivative, expressing DsRed-GFP11 fusion protein, *URA3*, Amp^R	This study
pYES2-GFP-VirD2	pYES2 derivative, expressing GFP-VirD2 fusion protein, *URA3*, Amp^R	Lab collection
pYES2-GFP	pYES2 derivative, expressing GFP, *URA3*, Amp^R	Lab collection
pHT105	Yeast expression vector, 2 μ origin, *ADH1* promoter, *ADH1* terminator, *URA3*, Amp^R	Lab collection
pHT105-GFP11-VirE2	pHT105 derivative, expressing GFP11-VirE2 fusion protein, *URA3*, Amp^R	This study
pHT105-VirE2	pHT105 derivative, expressing VirE2 protein, *URA3*, Amp^R	This study
pHT105-VirE2-GFP11	pHT105 derivative, expressing VirE2-GFP11 fusion protein, *URA3*, Amp^R	This study
pHT105-VirE2::GFP11	pHT105 derivative, expressing VirE2::GFP11 fusion protein, *URA3*, Amp^R	This study
pHT105-GFP-VirE2	pHT105 derivative, expressing GFP-VirE2 fusion protein, *URA3*, Amp^R	This study

pQH05	Yeast expression vector, 2 μ origin, *ADH1* promoter, *ADH1* terminator, *HIS3*, Amp^R	Lab collection
pQH05-VIP1	pQH05 derivative, expressing *A. thaliana* VIP1 protein, *HIS3*, Amp^R	This study
pQH05-VIP1-DsRed	pQH05 derivative, expressing VIP1-DsRed fusion protein, *HIS3*, Amp^R	This study
pST203	Yeast expression vector, 2 μ origin, *ADH1* promoter, *ADH1* terminator; *URA3*, Amp^R	Lab collection
pST203-PMP3	pST203 derivative, expressing PMP3 from its natural promoter and terminator, *URA3*, Amp^R	This study
pST203-GFP	pST203 derivative, expressing GFP, *URA3*, Amp^R	This study
pST203-PMP3-GFP	pST203 derivative, expressing PMP3-GFP fusion protein from yeast *PMP3* promoter and terminator, *URA3*, Amp^R	This study

Table 2.3. Media and solutions used in this study

Medium/Solution	Preparation*	Reference
LB	Tryptone, 10 g ; yeast extract, 5 g; NaCl, 10 g	(Chong 2001)
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)
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)
$IBPO_4$	20 × AB salts, 50 ml; 20 × AB buffer, 1 ml; 62.5 mM KH_2PO_4 (pH 5.5), 8 ml; 30% glucose, 18g; autoclave separately	(Piers *et al.* 1996)
YPD	Difco peptone, 20 g; yeast extract, 10 g; glucose, 20 g	(Gannon *et al.* 1988)
SD (Yeast Minimal Media)	Yeast nitrogen base without amino acids, 6.7g; pH 5.8	Clontech Laboratories
SD Gal/Raf	SD media with raffinose and galactose instead of glucose	Clontech Laboratories
1/2 x Murashige & Skoog (MS)	Murashige and Skoog basal medium lacking phytohormones, 2.2g; Sucrose, 10g; MES, 0.5g; pH5.8 (0.8% phyto agar for solid media)	(Murashige *et al.* 1962)

*Recipe for 1 liter; 1.5% agar was added for solid media.

50 µl of 10 × lyticase was added into cell suspension and followed by incubation at 37 °C for 30 minutes. The mixture was then incubated at 95 °C for 5 minutes to lyse the cell and then transferred to 4 °C for another 5 minutes. The cell lysate was subsequently extracted once with 1 volume of phenol (pH 8.0) followed by 1 volume of chloroform respectively. The aqueous phase was then transfer to a clean 1.5 ml tube. Genomic DNA was precipitated with 2 volume of cold 100% ethanol supplemented with 1/10 volume of 3M NaOAc (pH 5.2) at 4 °C for at least 1 hour. Precipitated genomic DNA was washed twice with 70% ethanol and dissolved in distilled H_2O.

2.2.3. Preparation of *A. tumefaciens* genomic DNA

Agrobacterium genomic DNA was prepared as described with a few modifications (Charles *et al.* 1993). Bacterial cells from 4 ml of overnight culture were collected by centrifugation. The cells were washed once with TES and re-suspended in 500 µl of TE (10 mM Tris-HCl, 25mM EDTA, pH 8.0) supplemented with 75 µl of 3M NaCl, 62.5 µl of proteinase K (5 mg/ml) and 62.5 µl of 10% SDS. The mixture was then incubated at 68 °C for 30 minutes to lyse the cells. The cell lysate was subsequently extracted once with 1 volume of phenol (pH 8.0) and 1 volume of chloroform respectively. The aqueous phase was then transfer to a clean 1.5 ml tube. Precipitation of genomic DNA was carried out the same as described for yeast genomic DNA extraction. Genomic DNA was dissolved in distilled H_2O after wash with 70% ethanol.

2.2.4. Transformation of *A. tumefaciens* by electroporation

Plasmids were introduced into *A. tumefaciens* using electroporation as described (Cangelosi *et al.* 1991). *Agrobacterium* was grown in MG/L medium till early log phase (OD_{600}=1.0), cells were collected by centrifugation at 4 °C and routinely 1 × 10^9 cells were used in each experiment. Prior to electroporation, cells were washed

twice with ice-cold H_2O followed by washing once with ice-cold 15% glycerol. The cell pellet was then re-suspended in 50 µl of ice-cold 15% glycerol and incubated on ice for 2 minutes. The cells suspension was transferred into a pre-chilled 0.2-cm electroporation cuvette (Bio-Rad). The Gene Pulser Ⅱ Electroporation System (Bio-Rad) was used for electroporation (Capacitance 25 µF, Voltage 2.5 kV, Pulse controller set to 400 Ω). After electroporation, 1 ml of MG/L broth was quickly added into the cuvette and the cell suspension was transferred into a culture tube for recovery. After 1 hour recovery at 28 °C, cells were collected and plated onto MG/L agar plate with appropriate antibiotics for selection.

2.2.5. Lithium acetate transformation of yeast

Lithium acetate transformation of yeast was performed as described (Gietz et al. 1995). Overnight culture of yeast cells were harvested by centrifugation and sub-cultured into fresh medium to reach log phase. 3×10^7 cells were then routinely used for each transformation. Yeast cells were washed once with H_2O and 100 mM lithium acetate sequentially. After washing, the cells were re-suspended in transformation buffer (H_2O, 80 µl; carrier DNA, 5 µl ; 1 M lithium acetate, 36 µl; 50% PEG, 240 µl) supplemented with the plasmid and mix by vortex. The mixture was then incubated at 42 °C for 30 minutes. Thansformants were selected using SD agar plates with appropriate amino acids supplements.

2.3. RNA manipulations

2.3.1. Total RNA extraction from yeast cells.

Yeast total RNA extraction was carried out using TRIzol(R)-based method (Hummon *et al.* 2007). Yeast cells were harvested and re-suspended in 450 µl TES supplemented with 50 µl 10 × lyticase. The mixture was then incubated at 37 °C for 30 minutes. Cells were harvested by centrifuging at 6000 rmp for 3 minutes at 4 °C. 800 ml of TRIzol reagent was added to cell pellet and re-suspended. The mixture was

incubated at room temperature for 5 minutes, 160 µl of chloroform was added to the mixture and mixed by vortex. After centrifuging at 12000 g for 15 minutes at 4 °C, the upper aqueous phase was transferred into a clean 1.5 ml tube. 1 volume of isopropanol was then added for RNA precipitation and incubated at room temperature for 10 minutes. After precipitation, the RNA pellet was washed twice with 75% ethanol and subsequently dissolved in double distilled water.

2.3.2. Total RNA extraction from *A. thaliana* cells.

Total RNA extraction of *A. thaliana* cells were carried similarly as described for yeast. Plant tissues were frozen in liquid nitrogen and grinded into powder prior to re-suspension in 800 ml of TRIzol reagent. The total RNA was then extracted using the same procedure as described for yeast RNA extraction.

2.3.3. Real time RT-PCR analysis

Total RNA was extracted as described above; Reverse transcription polymerase chain reaction (RT-PCR) was then carried out using iScript cDNA SynthesisKit (Bio-Rad). Real-time PCR was performed using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech) and the amplification was performed using CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad).

The $2^{-\Delta\Delta CT}$ method was adopted for data analysis from qRT-PCR experiments (Livak *et al.* 2001). Primers used in real-time PCR are listed in Table 2.4. Amplification efficiencies were determined using standard curve method.

2.4. Protein analytical Techniques

2.4.1. SDS-PAGE gel electrophoresis

Standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein separation as described (Laemmli 1970).

Table 2.4. Primers used for real-time PCR in this study

Primers	Sequence (5’ → 3’)	Purpose	Efficiency
ACT1F	CCACCACTGCTGAAAGAGAA	Amplify *S. cerevisiae ACT1*	96.03%
ACT1R	AGAAGATTGAGCAGCGGTTT		
PMP3F	ATTATCCCTTTTCTTACCACCAGT	Amplify *S. cerevisiae PMP3*	103.53%
PMP3R	ATCTTGTAGGACAATGTACAAGGC		
UBQ10F	GGCCTTGTATAATCCCTGATGAATAAG	Amplify *A. thaliana* UBQ10	93.67%
UBQ10R	AAAGAGATAACAGGAACGGAAACATAGT		
RCI2AF	CTTTCTCAGATTTGGTTGCG	Amplify *A. thaliana RCI2A*	100.68%
RCI2AR	GGTGAGGACATAAATGGCGTA		
RCI2BF	TGCTATCATCTTGCCTCCTCT	Amplify *A. thaliana RCI2B*	100.06%
RCI2BR	TTCCGGGAAGATAACCAAAC		
RCI2DF	CGAGATCTTCATCGCAATTC	Amplify *A. thaliana RCI2D*	95.27%
RCI2DR	CTCAACAGTGCAACAGCCAT		
RCI2EF	GCGAGCAACATGGAAGTTT	Amplify *A. thaliana RCI2E*	96.85%
RCI2ER	AACAGCCACGTTTGAGACAA		
RCI2FF	GCCGAGCAACTGTGAGATT	Amplify *A. thaliana RCI2F*	90.33%
RCI2FR	CAAACTCCGAGAGGAGGAAG		

Table 2.5. Buffers and solutions used in SDS-PAGE gel electrophoresis

Buffer/Solution	Recipe
Acrylamide/bis-acrylamide solution	30% acrylamide, 0.8% bis-acrylamide
Separating gel buffer	3 M Tris-HCl, pH 8.8
Stacking gel buffer	0.5 M Tris-HCl, pH 6.8
Ammonium persulfate (APS) solution	10% (W/V) ammonium persulfate
SDS stock solution	10% SDS
2 × Sample-loading buffer	100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.002% bromophenol blue
DTT stock solution	1 M DTT
10 × Electrophoresis buffer	0.25 M Tris, 2.5 M Glycine, 1% SDS, pH 8.3
Staining buffer	0.5 g Coomassie Brilliant Blue R250 in 180 ml methanol : H_2O (1:1, V/V) and 20 ml glacial acetic acid
Destaining solution	Methanol : H_2O : glacial acetic acid (V/V) = 9:9:1

The buffers and solutions used are listed in Table 2.5. 10% PAGE gel (1.9 ml Separating gel buffer, 5 ml Acrylamide/bis-acrylamide solution, 150 µl 10% SDS, 150 µl 10% APS, 9 µl TEMED 7.71 ml H_2O) was used for protein analysis in this study. Routinely 40 µl of protein sample was mixed with 1 M DTT solution and 50 µl loading buffer followed by incubating at 95 °C for 5 minutes prior to loading.

2.4.2. Western blot analysis

Proteins separated by SDS-PAGE gel electrophoresis were transferred to an Immun-Blot™ PVDF membrane (Bio-Rad) by electrophoresis using an Electro-Blot Unit (Scie-Plas, EB10). After transfer, the membrane was soaked in methanol for 1 minute prior to covering onto the protein gel. Transfer was performed at 200 mA constant current for 2-3 hours. 3% BSA in TBST buffer (20 mM Tris, 154 mM NaCl, 0.1% Tween-20, pH 7.5) was then used for blocking at room temperature for 1 hour. The PVDF membrane was washed at least three times with TBST buffer, followed by incubation with primary antibody (1:5000) for 1 hour with mild shaking. The membrane was then washed in TBST buffer for three times. Secondary antibody was then applied on to the membrane and incubated for 1 hour with mild shaking followed by washing with TBST buffer for three times. After treated with Supersignal® West Pico Chemiluminescent Substrate, the membrane was developed using BioMax XAR films with an X-ray developer.

2.5. *Agrobacterium*-mediated transformation of yeast

Agrobacterium-mediated transformation of yeast was performed similarly as described with a few modifications (Piers *et al.* 1996).

Single colony of yeast strain was inoculated into YPD/SD broth followed by growing overnight at 30 °C. The overnight culture was then sub-cultured into fresh medium and grown for additional 5 hours to reach early log phase. Cells were then harvested by centrifugation and washed once with $IBPO_4$; and resuspended to a final

concentration of 1×10^7 cells/ml. *Agrobacterium* cultures were prepared as follows. First, *Agrobacterium* strain was inoculated into MG/L broth and grown overnight at 28 °C, the cells were then sub-cultured into fresh MG/L medium and were grown for additional 8 hours to reach log phase. *Agrobacterium* cells were then collected by centrifugation and subsequently resuspended in $IBPO_4$ supplemented with with acetosyringone (200 μM). Cells were grown at 28 °C for additional 20 hours for induction perpuse. The *Agrobacterium* cells were harvested after induction and resuspended in $IBPO_4$ to a final concentration of 1.2×10^{10} cells/ml. Co-cultivation of *Agrobacterium* and yeast was carried out by mixing 50 μl of both resuspended yeast and bacteria cells and dropping onto $IBPO_4$ agar plates supplemented with acetosyringone (200 μM) and appropriate amino acids supplements. Co-cultivation was carried out at 20 °C for 24 hours, the spots were then washed off and plated onto SD agar plates with full amino acids for recipients recovery or ommiting the marker amino acid for transformants selection.

2.6. Tumorigenesis

2.6.1. Tumorigenesis of Kalanchoe daigremontiana

A. tumefaciens strain was inoculated in MG/L medium for overnight culture at 28 °C, the cells were then sub-cultured into fresh MG/L medium for additional 7-8 hour culturing to reach log phase. Cells were collected by centrifugation and resuspended in fresh MG/L medium. *Kalanchoe* plants were wounded first with a clean tip, and 2-3 μl of *Agrobacterium* cell suspension was inoculated onto the wounded sites of the leaves. The inoculated *Kalanchoe* plants were then kept at room temperature for leaf tumor formation.

2.6.2. Root transformation assay of *Arabidopsis thaliana*

Arabidopsis seeds were surface-sterilized with 15% bleach for 15 minutes and washed 4-5 times with H_2O. The seeds were then incubated at 4 °C for at least 2 days

before germination on 1/2 × MS agar plates. The germination plates were then incubated at 25 °C under a 16-hour photoperiod for 12 more days. Roots from individual seedlings were cut into 3-5 mm segments and mixed with appropriate amount of *Agrobacterium* strains. The mixture were spread onto 1/2 × MS agar plate and incubated at 20 °C in the dark for another 48 hours. After co-cultivation, individual root segments from the same plate were transferred onto a new 1/2 × MS agar plate containing 100 μg mL^{-1} cefotaxime to kill the *Agrobacterium*. The plates were then kept at 25 °C for root tumor formation.

2.7. Agroinfiltration

Agroinfiltration was used for visualization of *Agrobacterium*-delivered VirE2 in *Nicotiana benthamiana* and plant immunity study in *Arabidopsis thaliana*.

For visualization of *Agrobacterium*-delivered VirE2 in *N. benthamiana*, agroinfiltration was performed similarly as described (Lee *et al.* 2006). *Agrobacterium* strain was inoculated into MG/L medium, after overnight culture, the cells were sub-cultured into fresh MG/L medium to reach log phase. The bacteria were then collected and re-suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH5.5) to a final concentration of 1×10^9 cells/ml. Cell suspension was then infiltrated into the *N. benthamiana* leaves using a syringe. The infiltrated plant was kept at 22 °C under a 16-hour photoperiod.

For plant immunity study in *A. thaliana*, agroinfiltration was carried out similarly as described above. The bacteria cells at log phase were collected and re-suspended in infiltration buffer to a final concentration of 5×10^8 cells/ml. Cell suspension was then infiltrated into the underside of *A. thaliana* leaves using a syringe. The infiltrated plant was then kept at 22 °C under a 16-hour photoperiod.

Chapter 3. Live tracking of *Agrobacterium* VirE2 protein in host cells

3.1. Introduction

Successful transformation mediated by *Agrobacterium* requires proper co-operation between various virulence proteins. Among them VirE2 plays an important role by cooperative binding to single-strand DNA and is hypothesized to protect the DNA from nucleolytic disruption (Citovsky *et al.* 1989; Sen *et al.* 1989; Yusibov *et al.* 1994).

It has been shown previously that VirE2 could interact with several importin α isoforms in yeast (KAPα and IMPa-2, IMPa-3, and IMPa-4) and plants (IMPa-7 and IMPa-9) (Bhattacharjee *et al.* 2008); it also interacts with plant protein VIP1, a nucleus localized transcription factor regulated by MAPK-dependent phosphorylation (Tzfira *et al.* 2001; Djamei *et al.* 2007). All these indicate that VirE2 might play a role in helping nucleus transport of T-complex. The close association of VirE2 with T-DNA makes the sub-cellular localization of VirE2 important to understand T-complex movement in host cells. Figure 3.1 shows the possible roles of VirE2 in AMT process.

Various approaches have been adopted to study the localization of *Agrobacterium* VirE2 protein; however, inconsistent results were obtained. When fluorescently labeled single-strand DNA were co-cultivated with VirE2 and microinjected into *Tradescantia* stamen hair cells, the *in vitro* constructed complex was predominantly localized in the nucleus (Zupan *et al.* 1996). However, another similar experiment using permeabilized tobacco cells displayed only cytoplasmic localization (Ziemienowicz *et al.* 2001).

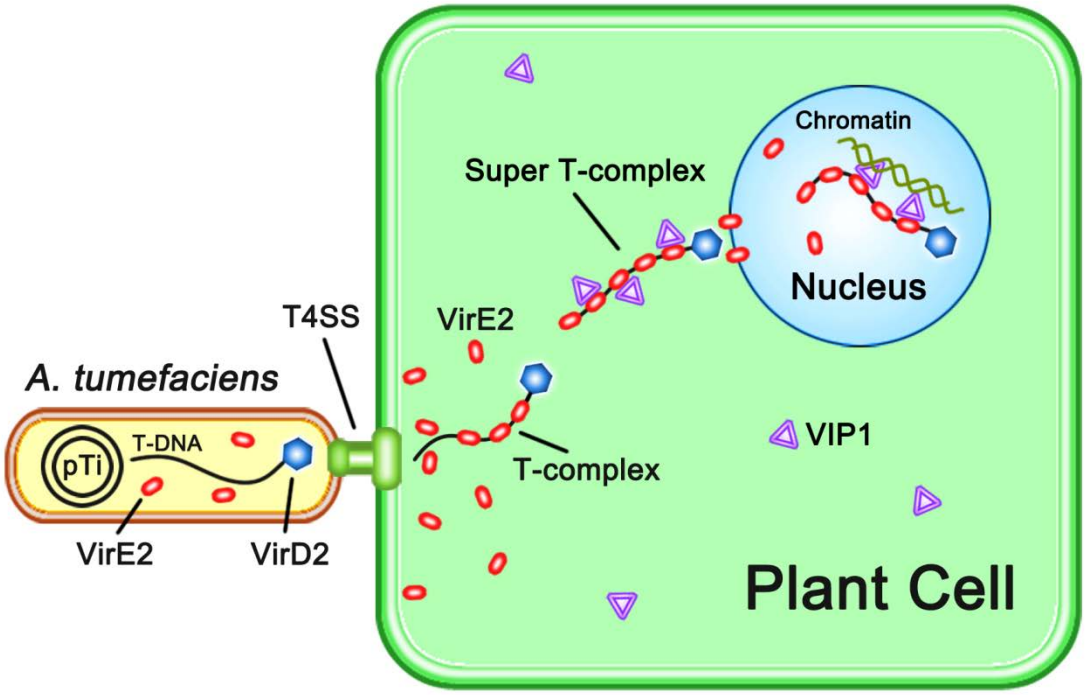


Figure 3.1. Possible roles of VirE2 in *Agrobacterium*-mediated transformation.

(A). After crossing the host cell membrane, VirE2 stays in the membrane and "pull" the T-strand into host cytoplasm (Duckely *et al.* 2003). (B). VirE2 interacts with T-strand and host VIP1 protein inside the plant cytoplasm to form the super T-complex thus protecting the T-strand from degradation and helping its nucleus targeting (Tzfira *et al.* 2001). (C). VirE2 forms pores on the plant nuclear membrane and facilitates T-strands entering into the nucleus (Ream 2009).

Several experiments used microprojectile bombardment to introduce GUS–VirE2 fusion protein expression cassette into plant cells, however, both nucleus localization in maize leaves and cytoplasmic localization in tobacco roots were reported (Citovsky *et al.* 1994). Recent studies also localized the HA tagged VirE2 or YFP tagged VirE2 exclusively inside cytoplasm of tobacco BY-2 cells and *Arabidopsis* root cells respectively (Bhattacharjee *et al.* 2008; Grange *et al.* 2008). The conflicting results from different experiment groups make the role of VirE2 in T-DNA nucleus targeting still unclear.

Different observations described above might result from different experimental environment of these research groups. A major difficulty of such studies lies in the traditional protein labeling approaches as well as the techniques used to introduce VirE2 into host cells. As a single stranded DNA binding protein, VirE2 is vulnerable to traditional protein tagging technique. When tagged at C-terminus using full length GFP, the VirE2 failed to be secreted into plant cells through T4SS, possibly because the structure of GFP blocked its C-terminal secretion signal (Simone *et al.* 2001). On the other hand, the N-terminal labeled VirE2 using YFP failed to gain its original function when trangenicly expressed inside plant cells (Bhattacharjee *et al.* 2008). Considering these difficulties, the published studies usually introduced VirE2 into plant cells either by direct uptake or transgenic expression, which dramatically differ from the natural translocation process. Under natural conditions, the VirE2 might be delivered into host cells by *Agrobacterium* in a much smaller amount and act differently compared to those “introduced” proteins. Besides, the natural translocation of VirE2 also requires T4SS and a trans-membrane process. Some experiment showed that VirE2 molecules were able to form a voltage-gated channel on plasma membrane and help T-DNA translocation (Dumas *et al.* 2001); thus the previous studies ignoring this important process might result in observations different from the natural situation.

In this study, a newly developed protein tagging strategy was adopted. Compared to the traditional way of protein tagging, it introduces less perturbation to the tagged

protein and tends to be more suitable for studying VirE2 in natural AMT process.

3.2. General study of *Agrobacterium* VirE2 in AMT process

3.2.1. Generation of VirE2 deletion mutants in *Agrobacterium* strains

VirE2 plays a crucial role in T-DNA transfer by coating and protecting the T-DNA in host cytoplasm, and possibly participates in nucleus targeting of T-DNA as well. The important role of *Agrobacterium* VirE2 during AMT was confirmed in this study.

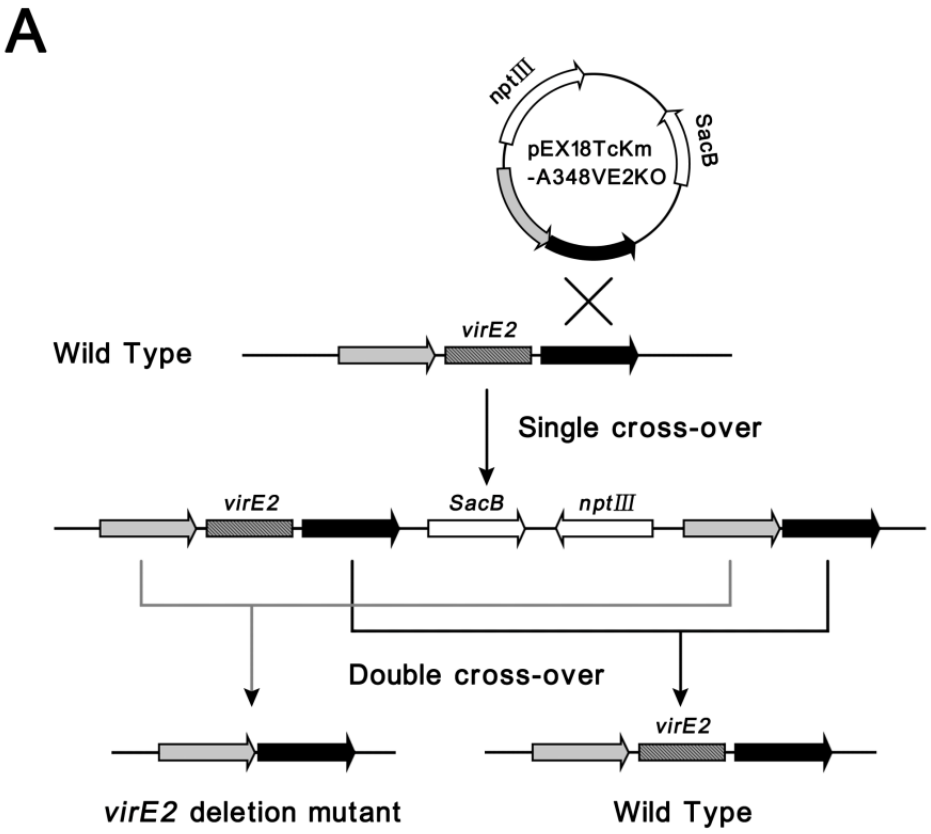
To generate a *virE2* deletion mutant in the tumor-inducing *Agrobacterium* strain A348, a *SacB*-based gene replacement strategy as shown in Figure 3.2 was adopted (Hoang *et al*. 1998).

Firstly, to obtain a suitable vector for *Agrobacterium* gene replacement, a 1029 bp *npt* Ⅲ cassette was amplified from pCB301 and inserted into pEX18Tc to generate pEX18TcKm.

A 834 bp fragment of *virE2* upstream sequence and a 985 bp fragment of *virE2* downstream sequence from A348 Ti plasmid were amplified and inserted into pEX18TcKm to generate pEX18TcKm-A348VE2KO, the resulting vector was then introduced into *A. tumefaciens* A348 by electroporation.

The first round homologous recombination strain was selected using MG/L agar plate containing kanamycin. Single colony was then picked into MG/L broth and cultured overnight before spread onto MG/L agar plate containing 10% sucrose. The deletion mutant A348*ΔvirE2* was subsequently verified using PCR based methods.

To construct a *A. tumefaciens* EHA105 *virE2* deletion vector, a 1500 bp *virE2* upstream fragment and a 1502 bp *virE2* downstream fragment were amplified from pTiBo542 and inserted into pEX18TcKm to produce pEX18TcKm-EHA105VE2KO. The *virE2* deletion mutant of in *A. tumefaciens* EHA105 (EHA105*ΔvirE2*) was then generated similarly as described above. Both of the *A. tumefaciens* deletion mutants were confirmed using PCR and sequencing methods.


A
nptIII
SacB
pEX18TcKm
-A348VE2KO
Wild Type
virE2
Single cross-over
virE2
SacB
nptIII
Double cross-over
virE2
virE2 deletion mutant
Wild Type

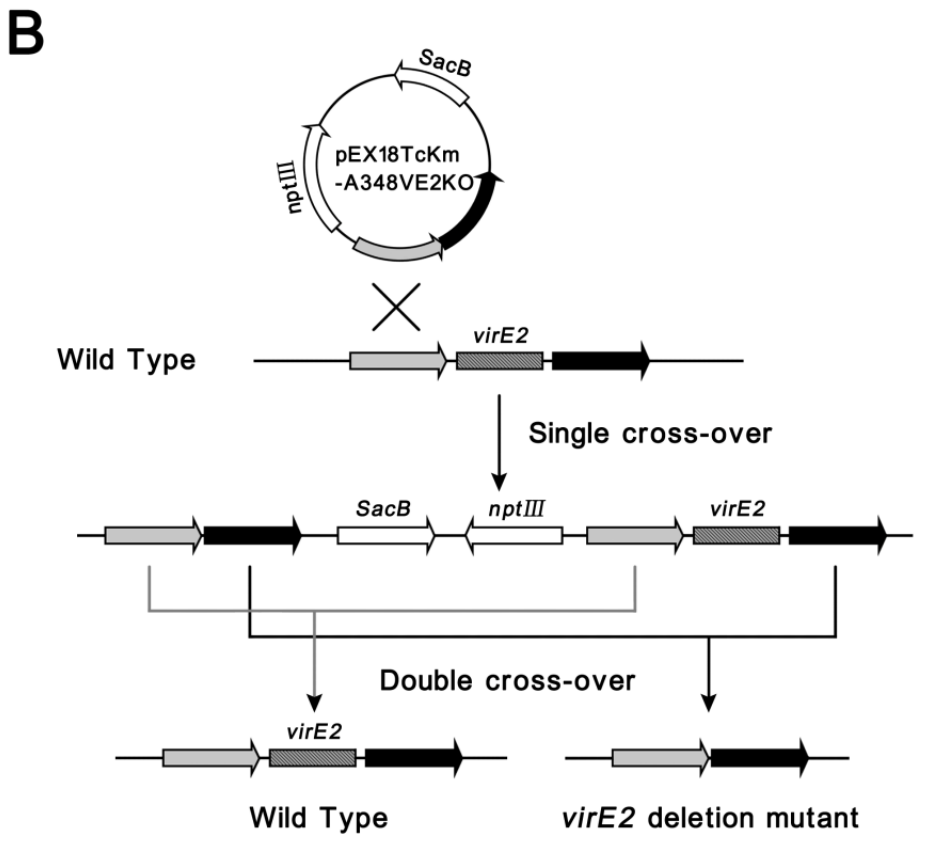

B
SacB
pEX18TcKm
-A348VE2KO
nptIII
Wild Type
virE2
Single cross-over
SacB
nptIII
virE2
Double cross-over
virE2
Wild Type
virE2 deletion mutant

Figure 3.2. Schematic diagram of *virE2* deletion strategy.

The plasmid pEX18TcKm-A348VE2KO containing A348 *virE2* upstream and downstream sequence was used in generation of A348 *virE2* deletion mutant. The first round homologous recombination happens between *virE2* downstream (A) or upstream (B) region, resulting in the integration of pEX18TcKm-A348VE2KO into Ti plasmid. *npt* II cassette on the plasmid was used to select the positive transformation after single cross-over. The second round homologous recombination happens between the homologous regions on the Ti plasmid and the sucrose suicide gene *SacB* was used for selection after double cross-over. The second round homologous recombination results in both wild type and *virE2* deletion strains as shown; *virE2* deletion mutants were then selected using PCR based methods.

3.2.2. *Agrobacterium* VirE2 is indispensable in transformation of plants

To examine the role of *Agrobacterium* VirE2 in plant transformation, the virulence of both *Agrobacterium* strain A348 and *virE2* deletion mutant A348*ΔvirE2* were compared in transformation assay of *Kalanchoe daigremontiana.*

Agrobacterium strains were inoculated into MG/L broth and cultured at 28 °C. Bacteria were harvested after overnight culturing, cells were washed twice and re-suspended in water; the cell concentration was then adjusted to 1×10^9 cells/ml or 1×10^8 cells/ml respectively. 2 µl of the cells suspension were inoculated onto the wound parts of *K. daigremontiana* leaves.

As shown in Figure 3.3, A348 *virE2* deletion mutant failed to induce the formation of crown tumors on *Kalanchoe* leaves as the wide type bacteria, indicating that the VirE2 plays an indispensable role in AMT of plants.

Figure 3.3. Virulence study of *Agrobacterium virE2* mutant in plant.

Agrobaterium strains were inoculated onto the wound parts of *K. daigremontiana* leaves in different concentration as indicated. After inoculation, the plant was grown at room temperature and picture was taken after 4-5 weeks. Experiment was repeated at least two times on different plants.

3.2.3. *Agrobacterium* VirE2 is important in AMT of yeast

Besides the plants, *Agrobacterium* is able to transform various non-natural hosts under laboratory conditions. As a simple model organism, the yeast *Saccharomyces cerevisiae* was chosen to study the role of *Agrobacterium* VirE2 in non-plant host transformation.

Similarly as the virulence study in plant, *Agrobacterium* strain EHA105 and *virE2* deletion mutant were used in transformation of yeast BY4741, a commonly used auxotrophic strain. A binary vector pHT101-2A containing leucine synthesis cassette was used for selection of positive transformants. The vector was introduced into EHA105 and EHA105*ΔvirE2* by electroporation prior to transformation assay.

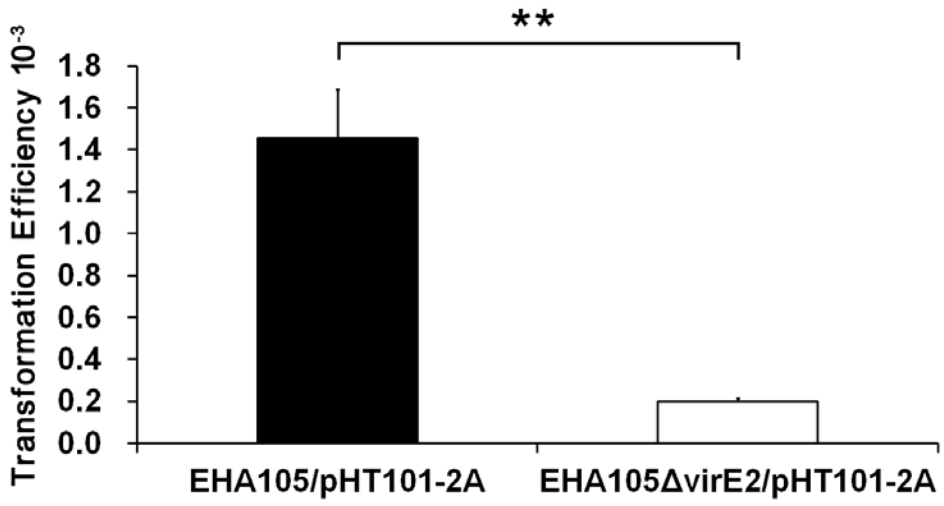


Figure 3.4. Virulence study of *Agrobacterium virE2* deletion mutant in yeast.

Yeast strain BY4741 was co-cultivated with *Agrobacterium* strains as described. Numbers of positive transformants and recipients were counted 3-4 days after transformation. Transformation efficiency is defined as the ratio of the number of transformants to the number of recipients.

Agrobacterium-mediated transformation of yeast was carried out as described in Chapter 2. In brief, *A. tumefaciens* strains were co-cultivated with *S. cerevisiae* on co-cultivation medium (CM) agar plate for 24 hours at 20 °C. Co-cultivation spots were subsequently washed off and spread onto SD Leu$^-$ agar plate and SD plate for transformants selection and recipients recovery respectively. The yeast *LEU2* on the T-DNA sequence was used as the selection marker. Transformation efficiency is defined as the ratio of the number of transformants to the number of recipients. The transformation results were shown in Figure 3.4.

Different from the result observed in plant transformation, EHA105 *virE2* deletion mutant was still able to transform the yeast under experimental condition. However, it showed dramatically decreased virulence compared to the wild type strain, indicating that *Agrobacterium* VirE2 plays a crucial, though not essential, role in transformation of non-natural host.

3.3. Development of Split-GFP detection system in yeast cells

3.3.1. General strategy of Split-GFP system for protein detection

To live track the movement of *Agrobacterium*-delivered VirE2 protein in host cells, a recently developed Split-GFP detection system was adopted in this study (Cabantous *et al.* 2005).

In this Split-GFP detection system, a modified Superfolder GFP molecule was divided into two fragments, a bigger one (GFP1-10) containing Strands 1-10 and a smaller one (GFP11) containing Strand 11 of GFP (Figure 3.5A). Neither component of these two GFP fragments is fluorescent while they could spontaneously bind to each other and restore fluorescence again both *in vitro* and *in vivo* (Figure 3.5B).

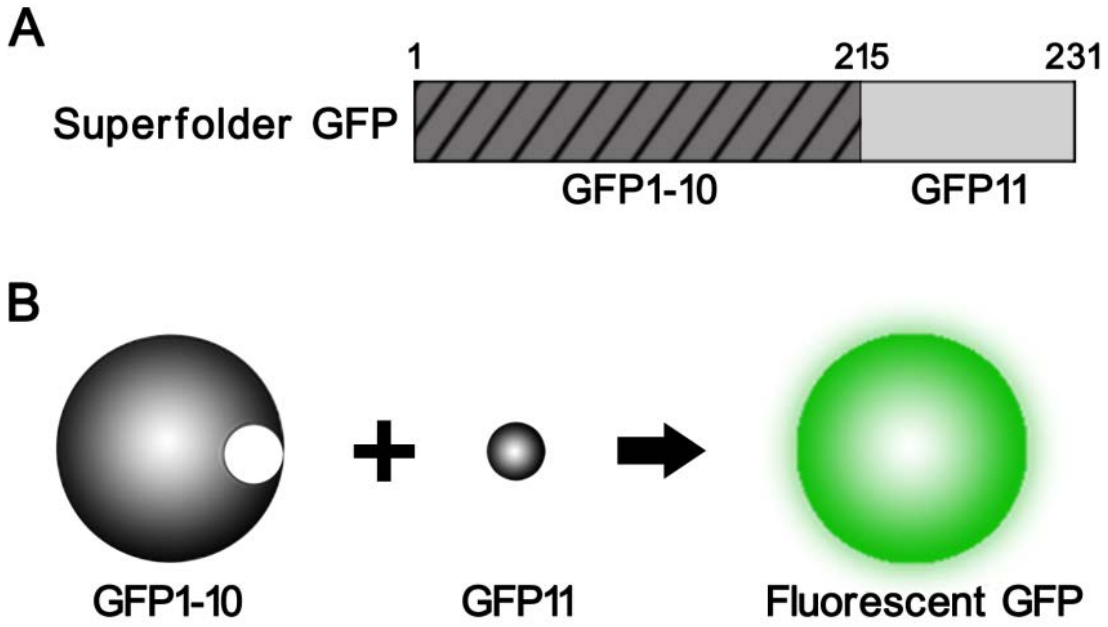


Figure 3.5. Schematic diagram of Split-GFP system.

A Split-GFP strategy was adopted in this study. A superfolder GFP molecule was divided into two fragments, GFP1-10 and GFP11 (A). The two GFP fragment are non-fluorescent while they could bind to each other to restore the green fluorescence (B).

3.3.2. Development of Split-GFP system in yeast cells

Since this protein detection system was commercially developed for either bacteria or mammalian cells, its feasibility in yeast cells was firstly tested.

The vectors pCMV-mGFP1-10 Hyg Amp containing GFP1-10 coding sequence and pmGFP Cterm S11 containing GFP11 coding sequence were purchased from Theranostech.

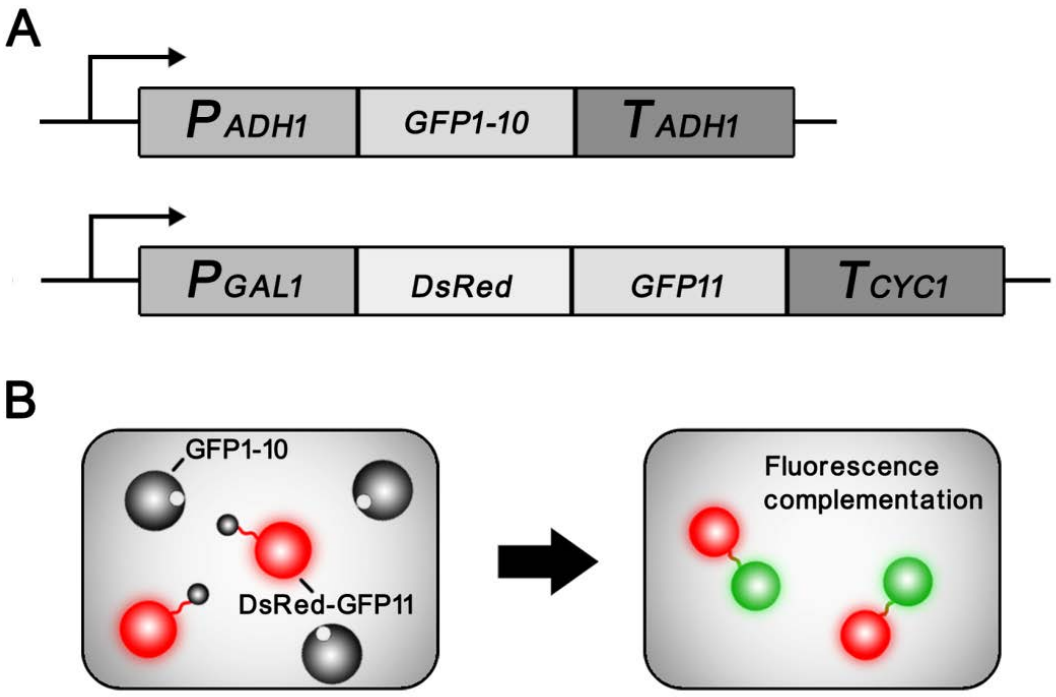


Figure 3.6. Schematic diagram of Split-GFP system testing is yeast cells.

The big fragment GFP1-10 is expressed under the control of yeast *ADH1* promoter while the small fragment GFP11 is fused with DsRed and expressed under the control of yeast *GAL1* promoter (A). GFP fluorescence occurs once the GFP1-10 bind to the DsRed-GFP11 in yeast cells (B). The DsRed is used as the expression control.

Firstly, the GFP1-10 coding sequence was cloned from pCMV-mGFP1-10 Hyg Amp and inserted into a yeast expression vector pACT2A to make pACT2A-GFP1-10; the big fragment GFP1-10 will be controlled by yeast *ADH1* promoter for constant expression (Figure 3.6A). To stabilize GFP11 in yeast cell, the GFP11 coding sequence was cloned into another yeast expression vector pYES2 to make a DsRed-GFP11 fusion protein (pYES2-DsRed-GFP11); and expression of this fusion protein was under the control of yeast *GAL1* promoter to test the sensitivity of this Split-GFP system in yeast cells (Figure 3.6A). After induction with galactose in culturing medium, the DsRed-GFP11 would spontaneously bind to GFP1-10 and restore the green fluorescence in yeast cells (Figure 3.6B).

Yeast strain BY4741 were transformed with both pACT2A-GFP1-10 and pYES2-DsRed-GFP11 following standard lithium acetate transformation protocol as described in Chapter 2; SD Ura^- Leu^- agar plate was used to select the positive transformants. Single colony of the positive transformants was inoculated into liquid SD Ura^- Leu^- medium. After overnight culturing at 30 °C, cells were harvested and washed twice with 0.9% NaCl solution followed by sub-culturing into SD Gal/Raf Ura^- Leu^- medium. Cells were then harvested at different time point for fluorescence reporter detection under a confocol microscope.

The DsRed signals could be detected as early as 2 hours post galactose induction together with green fluorescence (Figure 3.7A), indicating that the Split-GFP system is a fast detection tool suitable for live yeast cells.

As a control, no green fluorescence could be detected in yeast BY4741 cells expressing only GFP1-10 (BY4741 with pACT2A-GFP1-10) (Figure 3.7B) or DsRed-GFP11 (BY4741 with pYES2-DsRed-GFP11) (Figure 3.7C).

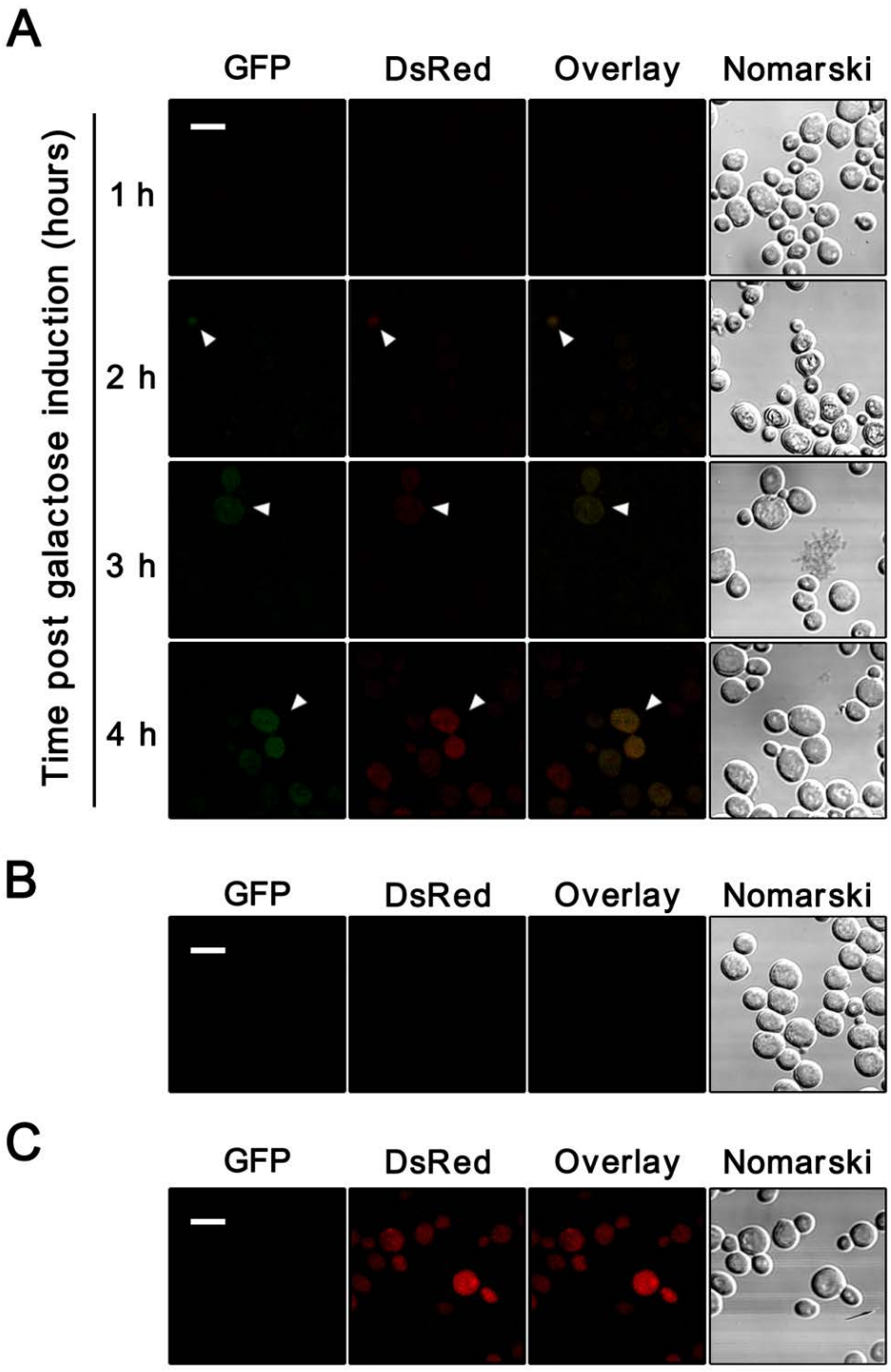


Figure 3.7. Development of Split-GFP system in yeast cells.

Time course studies revealed the instant binding of Split-GFP components in yeast cells (A). No green fluorescence was detected in yeast cells either without GFP1-10 (B) or GFP11 (C). Pictures were taken using a confocol microscope. Scale bars represent 5 μm.

3.4. Localization of *Agrobacterium* VirE2 protein in yeast cells

3.4.1. General strategy of *Agrobacterium* VirE2 protein labeling

The small GFP11 fragment containing only 16 amino acid residuals was used to label VirE2, minimizing the perturbation introduced by protein tagging.

To search for an appropriate position for VirE2 tagging, the GFP11 was fused to VirE2 at both N-terminus and C-terminus. Besides, a permissive internal site of VirE2 from *A. tumefaciens* A348 (Thr39) has been shown to be able to tolerate small peptide insertion (Zhou *et al.* 1999), the homologous site in *A. tumefaciens* EHA105 (Thr55) has also been tested in this study (Figure 3.8).

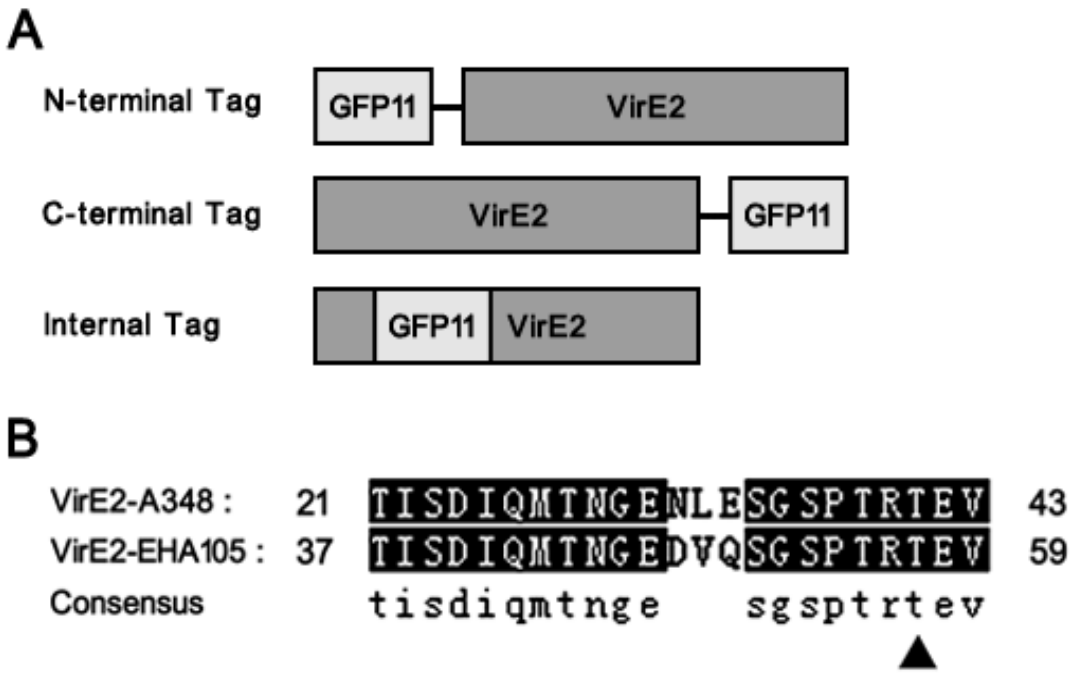


Figure 3.8. Schematic diagram of *Agrobacterium* VirE2 labeling strategy.
(A). GFP11 was used to tag VirE2 at three different sites, including N-terminus, C-terminus and a permissive site. (B). Sequencing comparison of VirE2 between EHA105 (upper row) and A348 (lower row) around the permissive site (arrowed) region.

3.4.2. Labeling of *Agrobacterium* VirE2 protein with GFP11

As described above, to find out an optical tagging position, three different types of tagged VirE2 were generated, including N-terminal tagged, C-terminal tagged and Internal tagged respectively.

To create an N-terminal tagged VirE2, the GFP11 coding sequence together with a linker sequence (GAT GGA GGG TCT GGT GGC GGA TCA ACA AGT) were inserted at downstream of VirE2 start codon. The resulting fusion sequence was then inserted into the yeast expression vector pHT105 to make pHT105-GFP11-VirE2 (Figure 3.9A).

A C-terminal tagged VirE2 was generated similarly. GFP11 coding sequence together with linker sequence (GAT GGA GGG TCT GGT GGC GGA TCA ACA AGT) were inserted at upstream of VirE2 stop codon. The resulting fusion sequence was subsequently inserted into pHT105 to generate pHT105-VirE2-GFP11 (Figure 3.9A).

The internal tagged VirE2 was generated using standard overlapping PCR method (Figure 3.10). The GFP11 coding sequence was synthesized onto the primer set b + c and inserted at the permissive site of EHA105 *virE2*. And the resulting sequence was used to generate pHT105-VirE2::GFP11 (Figure 3.9A).

To make a full-length GFP labeling control, the GFP coding sequence was inserted at the N-terminus of VirE2 coding sequence with a linker (GGT GGG GGA GGC TCT GGA GGG GGT GGA TCT GGT GGA GGT GGG TCA). The fusion sequence was then inserted into pHT105 to produce pHT105-GFP-VirE2 (Figure 3.9B).

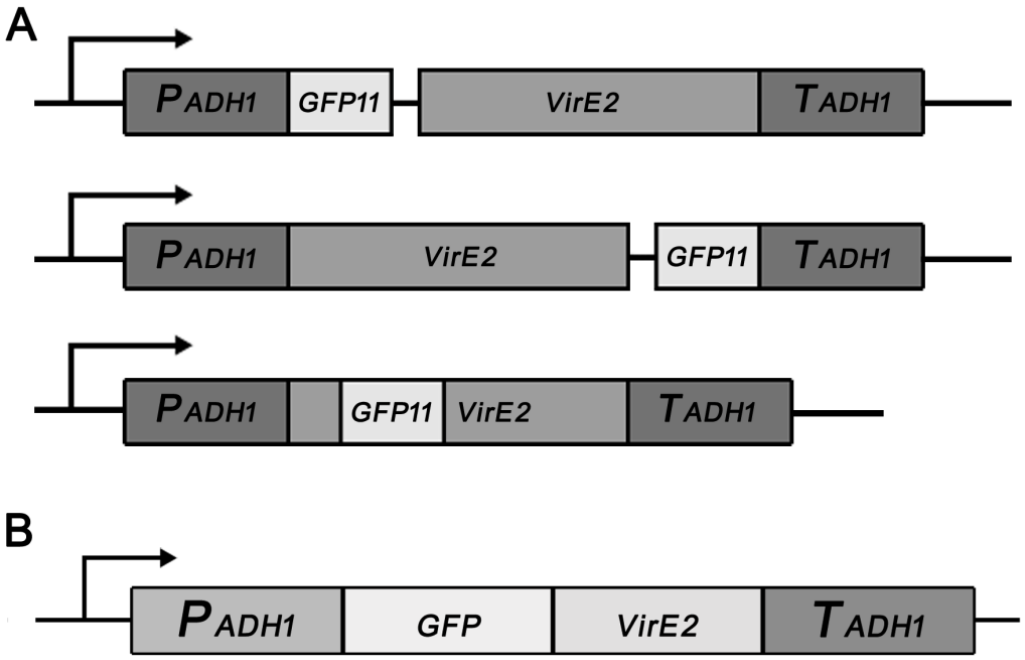


Figure 3.9. Schematic diagram of transgenic expression of VirE2 in yeast.

(A). GFP11 was used to tag VirE2 at N-terminus, C-terminus or a permissive site. The resulting sequences were inserted into yeast expression vector pHT105 thus the expression was under the control of yeast *ADH1* promoter. (B). The full-length GFP labeled VirE2 was expressed in pHT105 as a control.

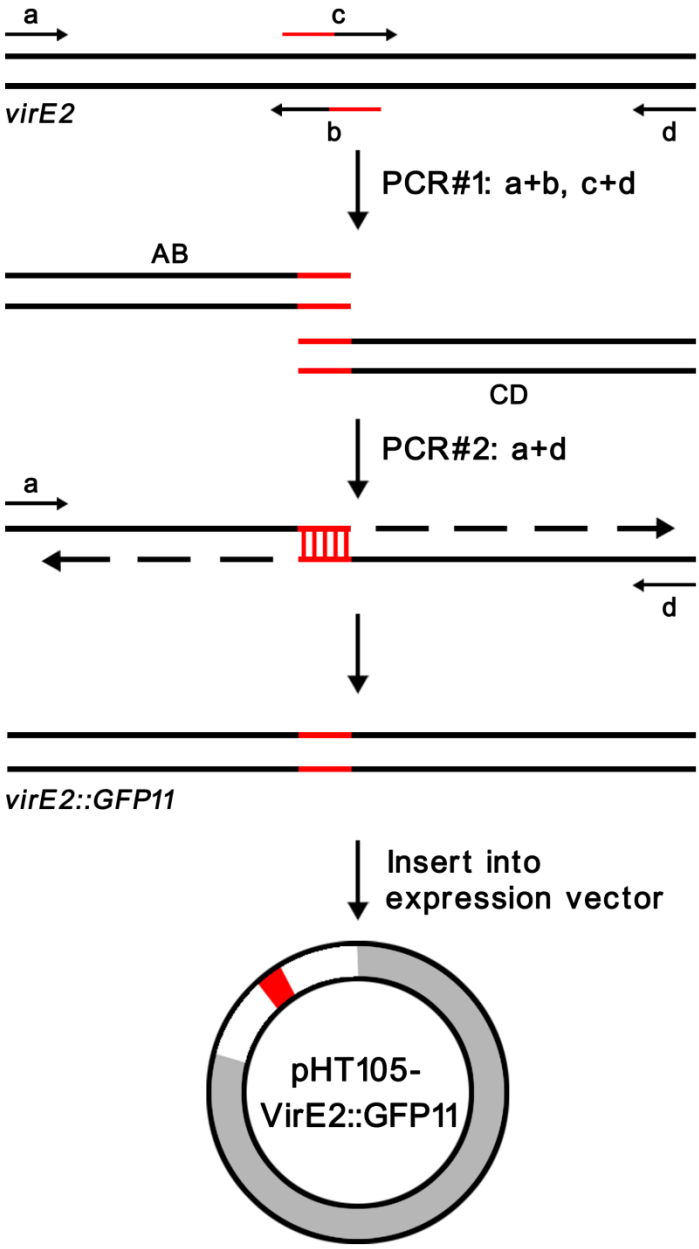


Figure 3.10. Schematic diagram of internal labeling of VirE2.

GFP11 coding sequence was synthesized onto the primer set b and c. In the first round of PCR, primer set a + b and c + d was used to amplify the upstream and down sequence of the permissive site respectively. In the second round of PCR, primer set a + d was used to amplify the *virE2::GFP11* and the resulting fusion sequence was inserted onto yeast expression vector pHT105.

3.4.3. Localization of *Agrobacterium* VirE2 protein in yeast cells

To localize VirE2 protein inside yeast cells, both GFP11 tagged VirE2 and GFP1-10 were expressed together in the same cell. A 1.2 kb *HIS3* cassette was inserted into pACT2A-GFP1-10 to generate pQH04-GFP1-10, thus the *HIS3* could be used as the selection marker.

Yeast BY4741 strain was transformed with pQH04-GFP1-10 together with different labeled VirE2 expression vector (pHT105-GFP11-VirE2, pHT105-VirE2-GFP11, or pHT105-VirE2::GFP11) respectively using lithium acetate transformation. The positive transformants were selected using SD Ura^- His^- agar plate. Single colonies of positive transformants were inoculated into corresponding liquid medium; cells were harvested after overnight culture and stained with DAPI at 28 °C for 10 minutes prior to observation under a fluorescence microscope.

As shown in figure 3.11, three different types of labeled VirE2 were localized exclusively inside yeast cytoplasm using Split-GFP system, which is similar to the behavior of full-length GFP tagged VirE2. Interestingly, in yeast cells, expressing of GFP11-VirE2, VirE2::GFP11 or GFP-VirE2 resulted in “long chain” structures with green fluorescence. This indicates that VirE2 molecules could self-aggregate when over expressed, which is consistent with previous *in vitro* studies showing self-aggregation of VirE2 in a “head to tail” manner (Frenkiel-Krispin et al. 2007; Dym et al. 2008). However, the self-aggregation of C-terminal tagged VirE2 seems to be impaired by the protein tag, though still could aggregated together in a different way to form small dots, indicating that the intact C-terminus is crucial for interactions between VirE2 molecules. As a control, no GFP fluorescence could be detected in yeast cells expressing untagged VirE2 and GFP1-10 (BY4741 containing pHT105-VirE2 and pQH04-GFP1-10).

Interestingly, although containing two putative nuclear localization signals, all of these four tagged VirE2 proteins were exclusively localized in the cytoplasm and those aggregated proteins presented a randomly dispersed manner inside host cells.

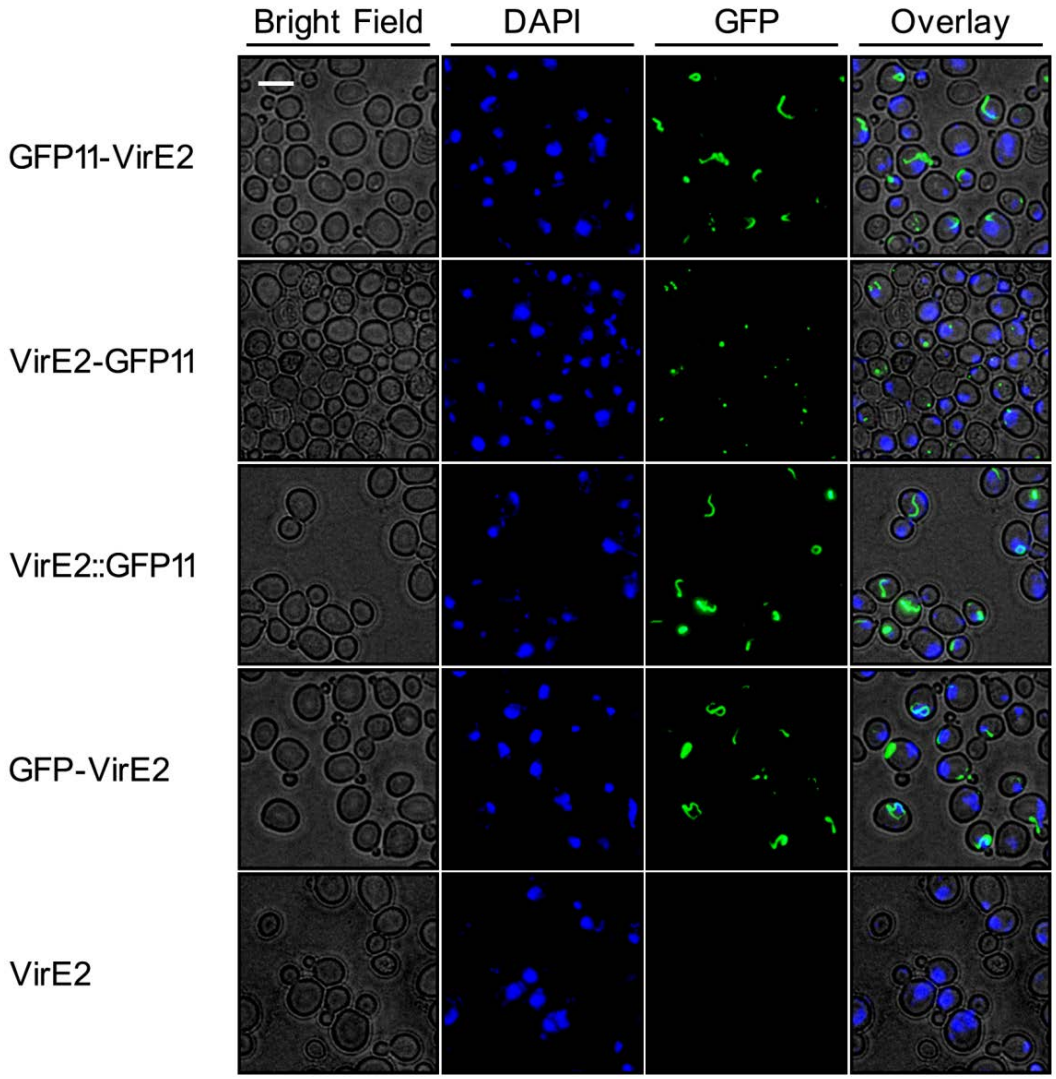


Figure 3.11. Localization of GFP11 labeled VirE2 in yeast cells.

Agrobacterium VirE2 from EHA105 was labeled at N-terminus, C-terminus or permissive site using GFP11 and expressed inside yeast cells. The N-terminal labeled VirE2 using full-length GFP was used as the control of traditional labeling. Yeast cells expression VirE2 alone was used as the negative control. The yeast nucleus was indicated by DAPI staining. Pictures were taken using a fluorescence microscope. Scale bar represents 5 μm.

3.5. Study of *Agrobacterium*-delivered VirE2 in yeast cells

As mentioned above, traditional labeling methods using full length fluorescent proteins have various limitations thus seems not suitable to study VirE2 during natural transformation process.

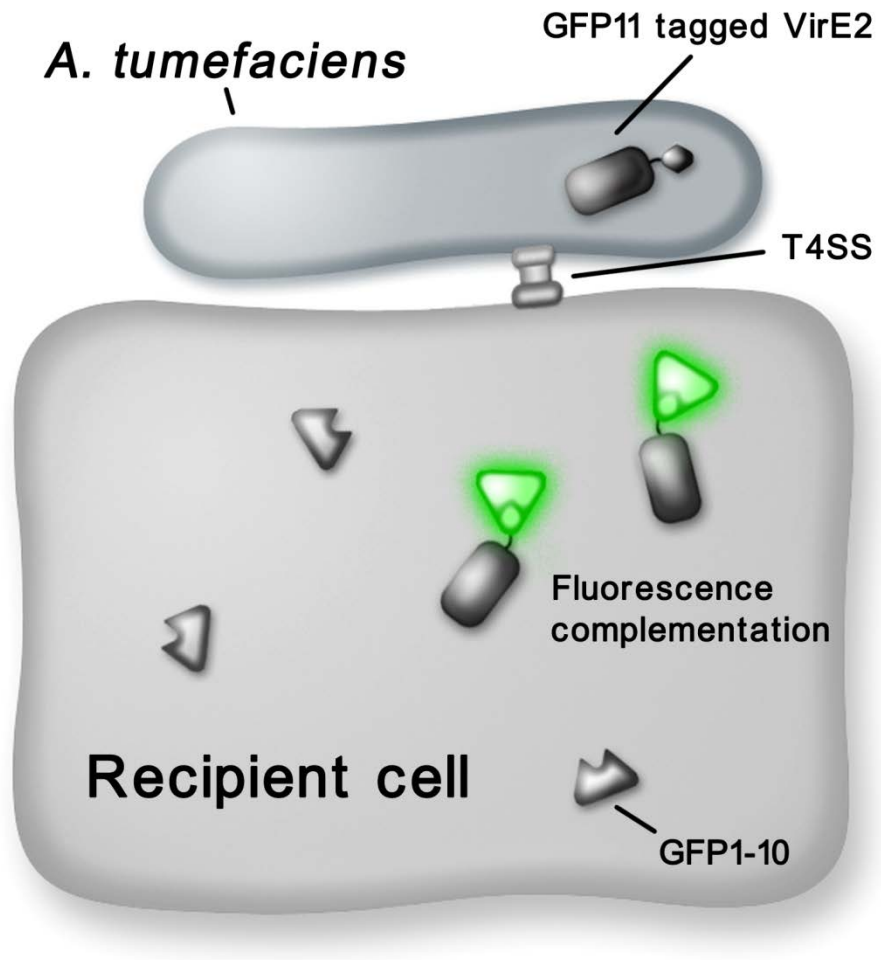


Figure 3.12. Schematic diagram of *Agrobacterium*-delivered VirE2 detection.

GFP11 labeled VirE2 is expressed inside *Agrobacterium* cells while the GFP1-10 is expressed in recipient cells. The two parts can bind to each other upon translocation of VirE2 into recipient cells and the restored GFP_{comp} fluorescence could be observed.

The Split-GFP system adopted in this study labeled the VirE2 with a small peptide GFP11 and thus introduced less perturbation in VirE2 function as well as the delivery process. The big fragment GFP1-10 will be constantly expressed inside host cells; it binds to the GFP11 labeled VirE2 once translocated into host cell and restores the fluorescence again; thus the signals of VirE2-GFP_{comp} could be detected (Figure 3.12).

3.5.1. Construction of *Agrobacterium* VirE2 labeling mutants

To study the bacteria-delivered VirE2 during natural transformation, the virulence protein delivery process should not be disturbed; thus VirE2 was labeled with the small GFP11 tag and expressed inside bacterial cells.

In order to maintain a more natural condition, the tagged VirE2 coding sequence was inserted back onto Ti plasmid to replace the original *virE2*, ensuring that it is controlled by the original expression cassette.

A *sacB*-based gene replacement strategy as described above was adopted to generate GFP11 tagged *virE2* strain in EHA105. Firstly, the *GFP11-virE2* coding sequence together with a 202 bp upstream sequence was cloned into pEX18TcKm to generate pEX18TcKm-GFP11-VirE2. The *virE2-GFP11* coding sequence together with a 1025 bp downstream sequence was inserted into pEX18TcKm to produce the pEX18TcKm-VirE2-GFP11. Similarly, pEX18TcKm-VirE2::GFP11 was obtained by cloning the *virE2::GFP11* into pEX18TcKm. These three plasmids were then used to generate *Agrobacterium* mutants EHA105*GFP11-virE2*, EHA105*virE2-GFP11* and EHA105*virE2::GFP11* respectively.

3.5.2. Virulence assay of *Agrobacterium* VirE2 labeling mutants

To confirm that the GFP11 tag does not affect the function of VirE2, the virulence of VirE2 tagged strains were firstly tested.

AMT of yeast was performed as described in Chapter 2. Briefly, *A. tumefaciens*

strains containing pHT101-2A were co-cultivated with yeast BY4741 for 24 h at 20 °C. Transformants were subsequently selected using SD Leu$^-$ agar plate. Transformation efficiency was then determined as the ratio of transformants number to the recipients number.

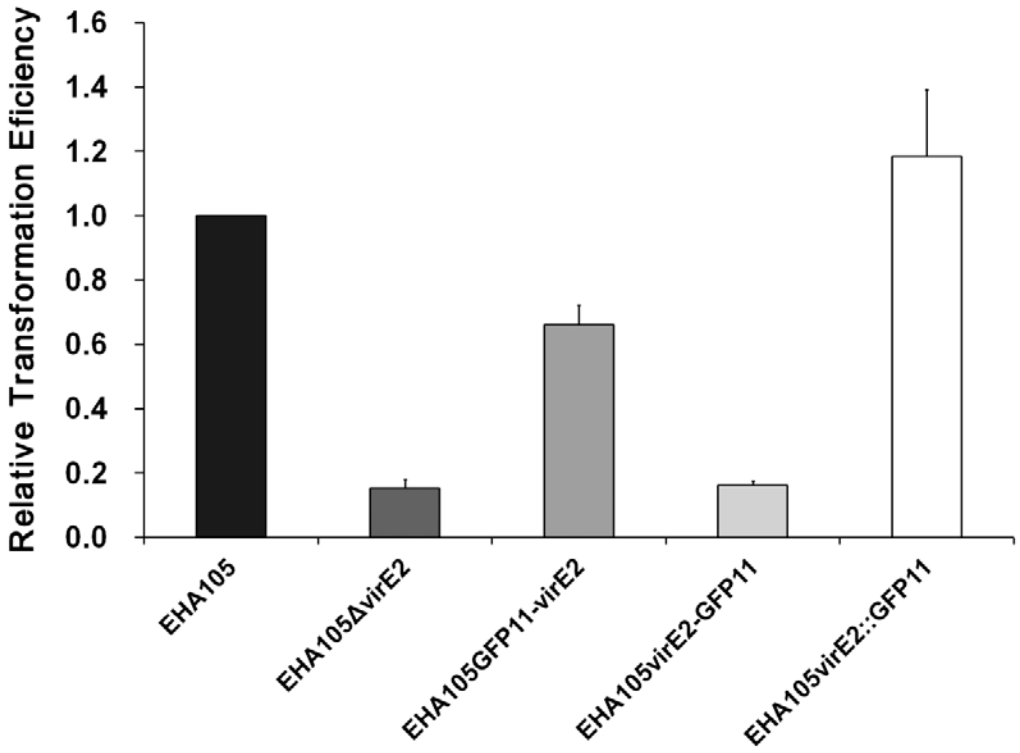


Figure 3.13. Virulence assay of GFP11 labeled *Agrobacterium* VirE2 mutants in yeast.

Virulence of three GFP11 tagged *Agrobacterium* strains (EHA105*GFP11-virE2*, EHA105*virE2-GFP11* and EHA105*virE2::GFP11*) were tested in AMT of yeast. The wild type EHA105 strain and *virE2* deletion mutant were used as positive and negative control respectively.

As shown in Figure 3.13, EHA105*virE2::GFP11* shows fully restored virulence compared with the *virE2* deletion mutants, indicating that GFP11 tagging at the permissive site does not affect the function of VirE2 during transformation process.

On the other hand, the C-terminal tagged VirE2 failed to restore its function in AMT, which might resulted from the previous observation that the C-terminal labeled VirE2 performed not well in self aggregation (Figure 3. 11).

Though still functional, EHA105*GFP11-virE2* only partially restored its virulence in the AMT assay, probably because the N-terminal labeling could affect the function of VirE2. A similar result has also been observed that the N-terminal labeling of VirE2 with full length GFP resulted in non-functional VirE2 when transgenicly expressed in plant cells (Bhattacharjee *et al.* 2008).

3.5.3. Detection of *Agrobacterium* VirE2 during natural AMT process

As the internal labeled VirE2 restored its original function in virulence assay, the mutant EHA105*virE2::GFP11* was chosen for further studies.

To detect the *Agrobacterium* delivered VirE2 in yeast cells, the GFP1-10 was constantly expressed inside yeast cells under the control of yeast *ADH1* promoter. Yeast strain BY4741 containing pQH04-GFP1-10 was co-cultivated with EHA105*virE2::GFP11* at 20 °C. Cells were harvested after 24 hours and observed using a fluorescence microscope.

As shown in figure 3.14, green fluorescent signals were able to be detected inside yeast cells after co-cultivation with EHA105*virE2::GFP11*.

As the control, no GFP fluorescence could be detected when using either unlabeled *Agrobacterium* strain EHA105 or yeast strain without GFP1-10 (Figure 3.15A-B).

To confirm the detected signals come from the translocated VirE2 proteins, an *Agrobacterium virD4* deletion mutant EHA105*virE2::GFP11ΔvirD4* were also used as the control. As VirD4 interacts with VirE2 inside *Agrobacterium* and is responsible

for VirE2 recognition prior to delivery into host cells, deletion of *virD4* gene should block the VirE2 translocation (Atmakuri *et al.* 2003). To generate the *virD4* deletion mutants, a 679 bp upstream sequence and a 649 downstream sequence were amplified and separately cloned into pEX18TcKm to generate pEX18TcKm-EHA105VD4KO, the resulting plasmid was then used to produce EHA105*virE2::GFP11ΔvirD4* similarly as described above.

Deletion of the *virD4* from *Agrobacterium* resulted in the abolishment of GFP fluorescence inside yeast cells, thus confirming that the detected signals came from the translocated VirE2 proteins (Figure 3.15C). And this is the first time that bacteria delivered VirE2 protein was visualized inside live host cells.

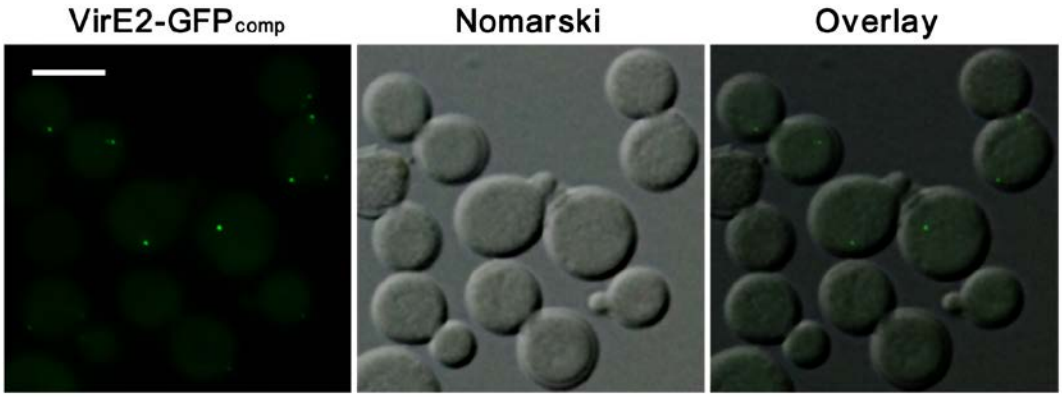


Figure 3.14. Detection of *Agrobacterium* delivered VirE2 in yeast cells.

GFP11 tagged VirE2 was expressed inside bacterial cells while GFP1-10 was expressed in yeast cells. GFP fluorescence was successfully detected upon binding of GFP1-10 with translocated VirE2. Pictures were taken under a fluorescence microscope. Scale bar represents 5 μm.

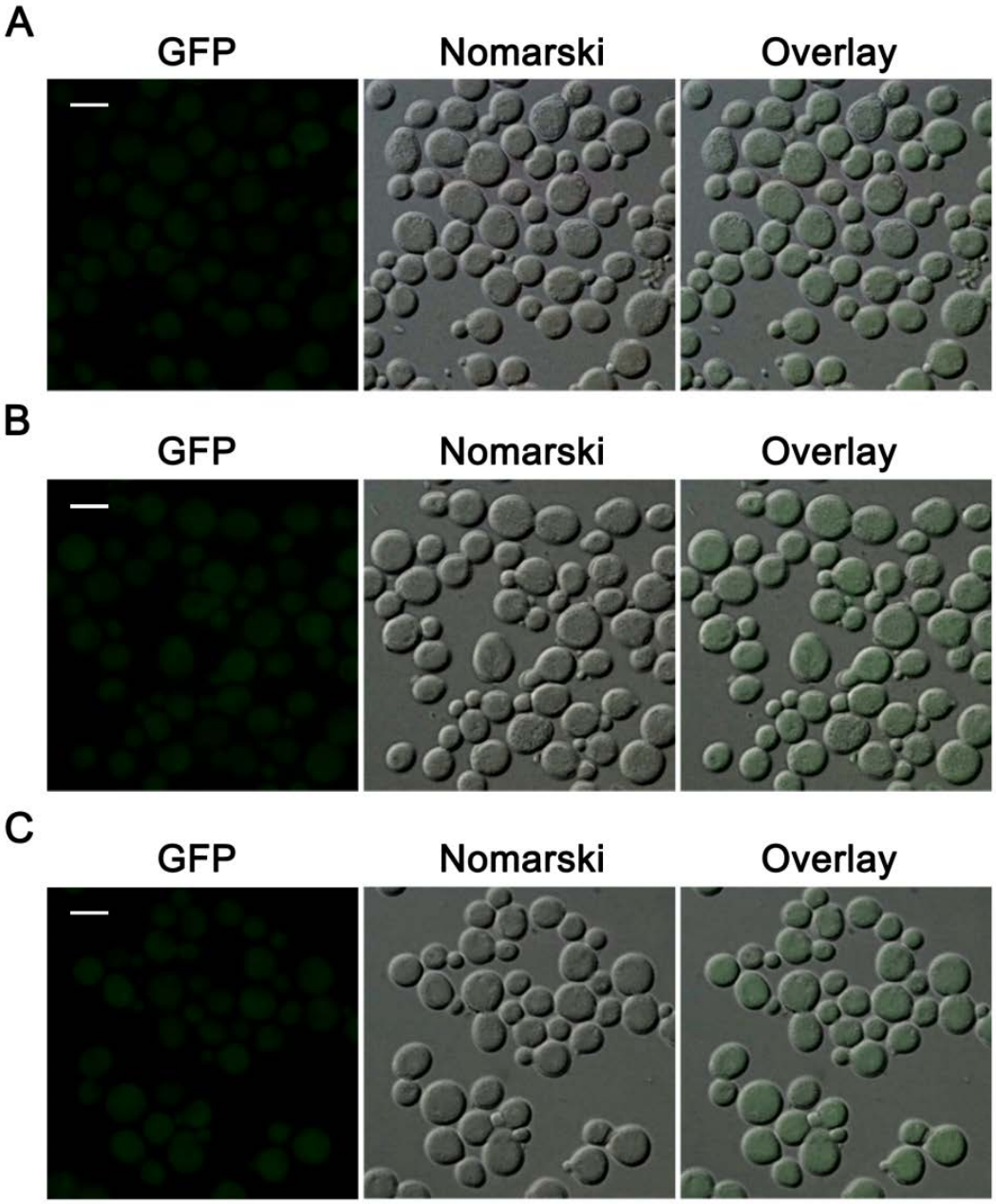


Figure 3.15. GFP fluorescence is not detected in yeast when omiting any Split-GFP component or deletion of *virD4*.

No GFP fluorescence could be detected when using unlabeled EHA105 (A) or yeast strain without GFP1-10 (B). Deletion of *virD4* in EHA105*virE2::GFP11* also abolished the GFP signals in yeast cells. Pictures were taken using a fluorescence microscope. Scale bars represent 5 μm.

As a control, a full length GFP labeled *Agrobacterium* mutant EHA105*GFP-virE2* was also used in VirE2 translocation assay. This *virE2* mutant EHA105*GFP-virE2* was constructed similarly as described. In brief, an N-terminal labeled GFP-VirE2 coding sequence together with a 202 bp upstream sequence was inserted into pEX18TcKm to generate pEX18TcKm-GFP-VirE2; this plasmid was subsequently used to generate the EHA105*GFP-virE2* mutant using similar strategy as for the other *Agrobacterium* mutants.

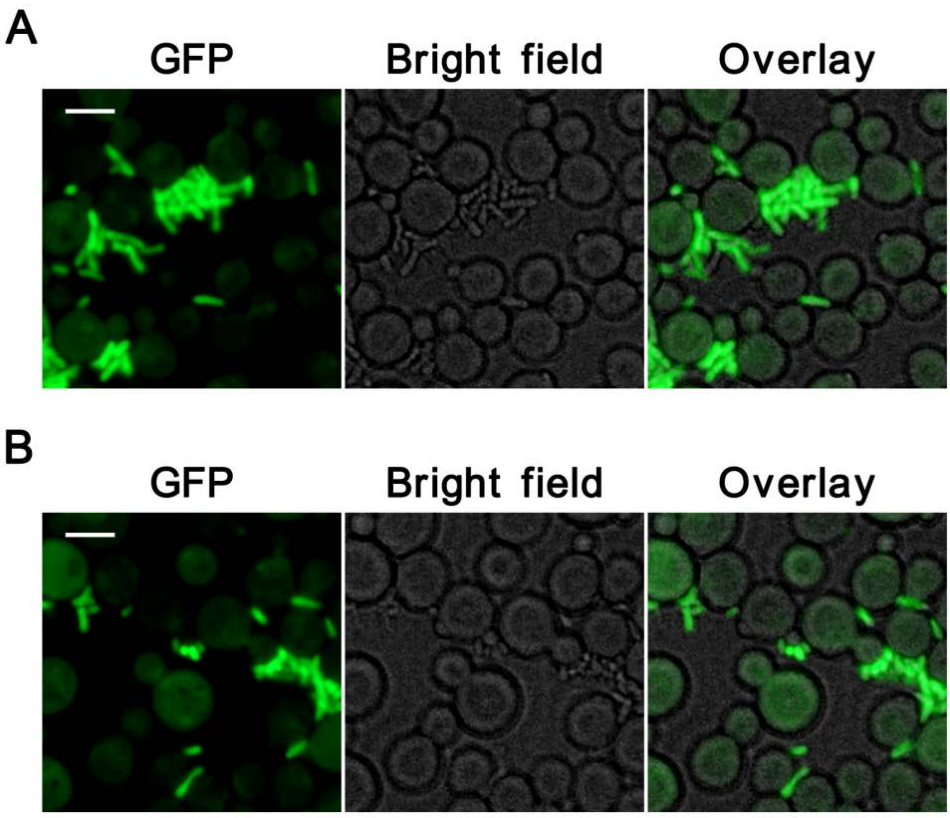


Figure 3.16. Full length GFP labeled VirE2 failed to be delivered by *Agrobacterium*.

Full-length GFP labeled VirE2 at either N-terminus (A) or permissive site (B) failed to be delivered into yeast cells after co-cultivation. Pictures were taken using a fluorescence microscope. Scale bars represent 5 μm.

Consistent with previous studies, no GFP signals could be detected inside host cells after co-cultivation of yeast with EHA105*GFP-virE2*, while the signals could only be observed inside *Agrobacterium* (Figure 3.16A). The full length GFP labeled VirE2 failed to be delivered into host cells by bacteria, probably because the GFP blocks the translocation of VirE2 through T4SS.

To further investigate whether the permissive site of VirE2 could tolerate larger peptide insertion, the full length GFP coding sequence was inserted at the permissive site to generate EHA105*virE2::GFP*. Similar as the VirE2 N-terminal labeled mutant EHA105*GFP-virE2*, EHA105*virE2::GFP* failed to deliver the tagged VirE2 into yeast cell (Figure 3.16B). All these indicate that compared with the traditional labeling approaches, the Split-GFP system is a more suitable detection system for VirE2 study during natural transformation process (Li *et al.* 2014).

3.6. Study of *Agrobacterium* delivered VirE2 during AMT process

3.6.1. The growth of bacteria and VirE2 expression level is not significantly perturbed by the GFP11 tag

Before detailed study of *Agrobacterium* delivered VirE2 using the Split-GFP system, the growth of the bacteria as well as the expression level of VirE2 were tested first to ensure the GFP11 tagging did not affect the bacterial growth and the VirE2 expression.

Single colony of EHA105*virE2::GFP11* from MG/L agar plate was inoculated into MG/L broth for overnight culture, the cells were then harvested and sub-cultured into fresh MG/L broth; the final concentration of the bacteria was adjusted to 1×10^8 cells/ml.

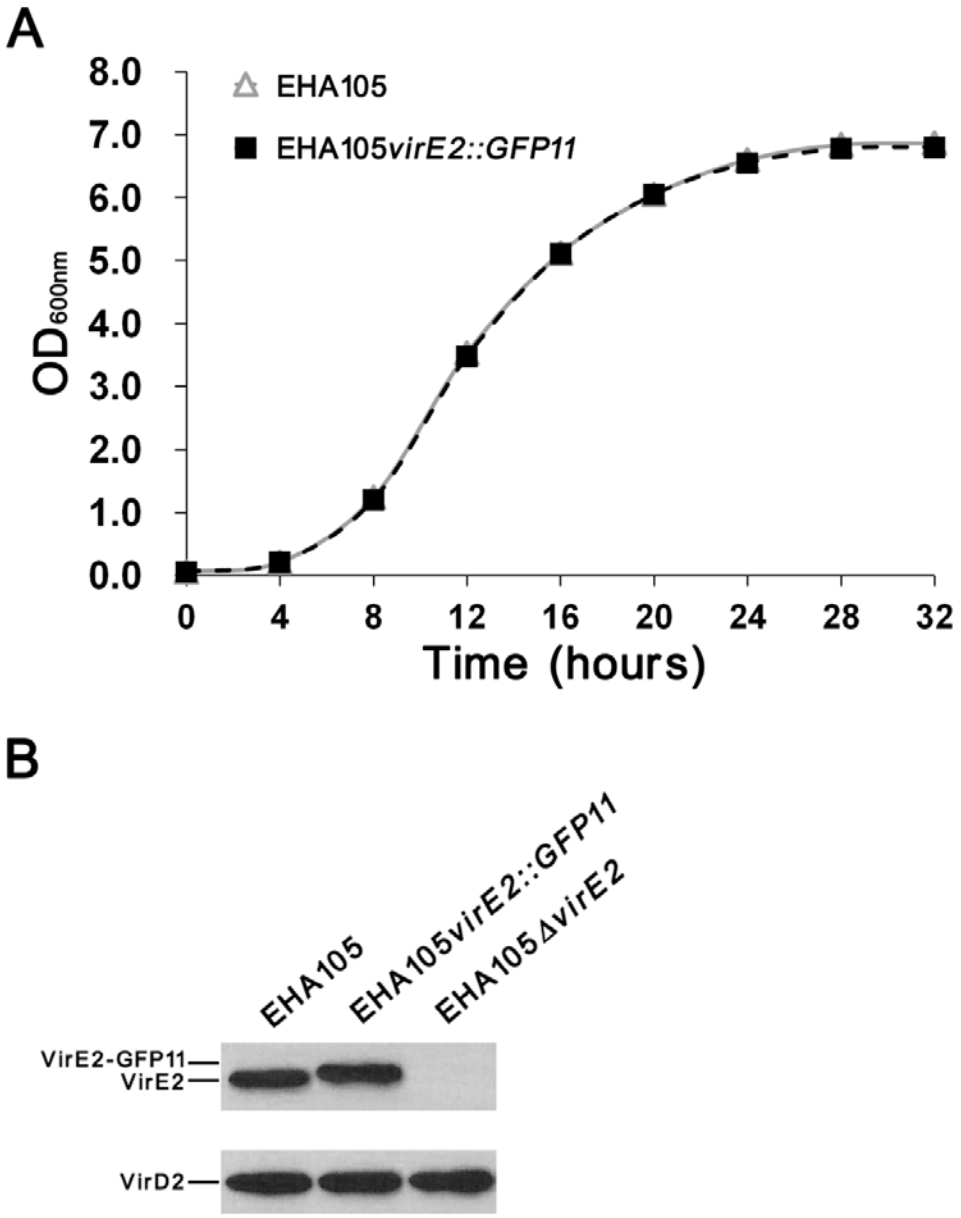


Figure 3.17. The Split-GFP system does not significantly affect bacterial growth and virulence protein expression.

(A). The growth curve of EHA105*virE2::GFP11* showed a similar pattern as the wild type EHA105. (B). The expression level of VirE2-GFP11 in EHA105*virE2::GFP11* is similar to that of VirE2 in EHA105 after AS induction. The VirD2 was used as a loading control.

OD_{600} of the cell culture was measured using a spectrophotometer at a 4 h time interval for 32 h. The wild type strain EHA105 was used as the control. The growth curve of GFP11 labeled mutant EHA105*virE2::GFP11* is similar compared with the wild type strain EHA105, which indicates that the small peptide labeling does not significantly affect the growth of the bacteria (Figure 3.17A).

As described above, to maintain a more natural situation, the tagged *virE2::GFP11* was used to replace the EHA105 *virE2* on the Ti plasmid. The expression level of both VirE2-GFP11 and intact VirE2 were measured in mutant and wild type strains to ensure that the expression level remains similar after tagging.

Both EHA105*virE2::GFP11* and EHA105 were inoculated into MG/L broth for overnight culture; the cells were harvested and then sub-cultured into fresh MG/L broth. Cells at early stage of log phase (OD_{600nm} = 1.0) were harvested and wash twice with $IBPO_4$. The cells were then sub-cultured into $IBPO_4$ with 200 μM AS at the final concentration of 3×10^8 cells/ml; induction of VirE2 expression was carried out by culturing the cells at 20 °C for another 20 hours. VirE2 expression level was analyzed by western blot using VirE2 antibody as described in Chapter 2. All the cells input were normalized based on OD_{600nm} of the bacteria culture. The VirD2 expression was also measured using western blot as the induction and loading control.

As shown in Figure 3.17B, the expression level of VirE2-GFP11 in EHA105*virE2::GFP11* is similar to that of VirE2 in EHA105, indicating that the expression level of VirE2 is not significantly perturbed by the GFP11 tag. As a negative control, no VirE2 expression could be detected in *virE2* deletion mutant.

Neither bacteria growth nor the VirE2 expression leveled is significantly changed in the GFP11 tagged mutant compared to the wild type strain EHA105; this suggests that the Split-GFP system is suitable for the study of VirE2 behavior in a relatively natural environment.

3.6.2. General study of *Agrobacterium* delivered VirE2 in yeast cells

Compared to the transgenic expression in yeast cells (Figure 3.11), VirE2 proteins were delivered into host cells in much smaller amount thus only spot-like signals could be detected inside yeast cells (Figure 3.14). And it tends to localized to the cell periphery region inside yeast (Figure 3.14, Figure 3.18A), indicating the VirE2 might has characteristics of membrane affinity.

To detect the early event of VirE2 translocation, a time course study was performed. Co-cultivation of EHA105*virE2::GFP11* with yeast BY4741 (pQH04-GFP1-10) was carried out as described above, yeast cells were harvested at different time post co-cultivation. Cells were washed twice with PBS and followed by staining with DAPI at 28 °C for 10 minutes. The cells were then observed under a fluorescence microscope for translocated VirE2 detection. As shown in Figure 3.18A, VirE2 could be detected inside yeast as early as two hours after co-cultivation, suggesting the protein delivery is a fast process in AMT.

To detect the possible movement of VirE2 inside host cells after delivery, yeast cells were washed off after 24 hours co-cultivation and washed twice with PBS prior to observation under a fluorescence microscope. However, no movement of VirE2 could be detected inside yeast cells (Figure 3.18B), probably because as a non-natural host, the yeast lacks the VirE2 interaction proteins such as VIP1 and VIP2, which also might be the reason that VirE2 failed to be localized inside yeast nucleus during AMT process.

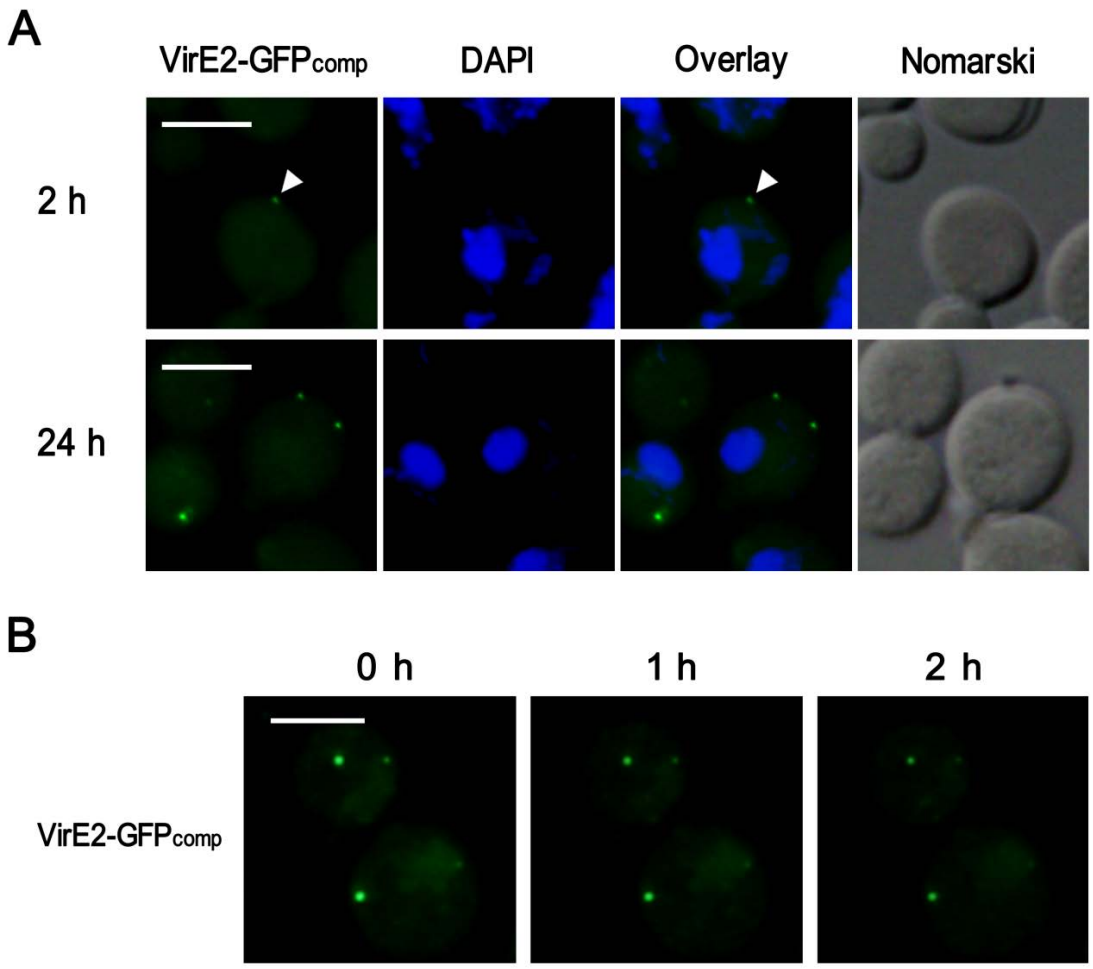


Figure 3.18. General study of *Agrobacterium* delivered VirE2 in yeast cells.

(A). *Agrobacterium* delivered VirE2 could be detected inside yeast cells as early as 2 hours post co-cultivation. The yeast nucleus was indicated by DAPI staining. (B). The *Agrobacterium* delivered VirE2 did not move inside yeast cytoplasm. 0 h represent 24 hours post co-cultivation. Pictures were taken using a fluorescence microscope. Scale bars represent 5 μm.

3.6.3. Study of VIP1 in yeast cells

As described above, no clear nucleus localization of VirE2 has been observed inside yeast cells either using transgenic expression (Figure 3.11) or upon bacteria delivery (Figure 3.18). Previous studies concerning *Agrobacterium*-mediated transformation of plants showed that the *A. thaliana* VIP1 was able to interact with VirE2 and is responsible to target it into plant nucleus (Tzfira *et al.* 2001). VIP1 functions as a transcriptional factor and is related in regulation of the pathogenesis-related gene PR1 expression; it was shown to display nuclear localization upon phosphorylation by the mitogen-activated protein kinase MPK3 (Djamei *et al.* 2007). Considering that the yeast cells do not encode any VIP1 homolog as that in plant, whether the cytoplasm localization in yeast results from lack of VIP1 homologs or not was examined in this study.

To test whether VIP1 could help VirE2 in nucleus targeting in yeast, the localization of VIP1 in yeast cells was studied first. VIP1 coding DNA sequence was amplified from *A. thaliana* cDNA and labeled with DsRed; the fusion gene was subsequently cloned into yeast expression vector pQH05 to generate pQH05-VIP1-DsRed. For VIP1-DsRed fusion gene expression, yeast cells containing pQH05-VIP1-DsRed were cultured in SD His^- liquid medium for overnight. Cells were then harvested and washed twice with PBS. DAPI staining was performed at 28 °C as described above prior to observation under a confocol microscope.

As shown in Figure 3.19A, *Arabidopsis* VIP1 could be localized inside yeast cell nucleus after transgenic expression, indicating that it could also be recognize by the yeast importin alpha homolog SRP1 followed by nucleus import (Tabb *et al.* 2000).

During plant transformation, *Agrobacterium* hijacks the VIP1 signaling pathway for VirE2 nucleus targeting (Bhattacharjee *et al.* 2008). To test whether VIP1 could mediate the VirE2 nucleus import in yeast cells, VIP1, GFP1-10 and VirE2-GFP11 were co-expressed together in the yeast cells. Yeast strain BY4741 was transformed with pQH05-VIP1, pACT2A-GFP1-10 and pHT105-VirE2::GFP11 together and the

positive transformants were selected with SD His$^-$ Leu$^-$ Ura$^-$ agar plate. Single colony was inoculated into SD His$^-$ Leu$^-$ Ura$^-$ liquid medium and cultured at 30 °C for overnight. Cells were then harvested and stained with DAPI at 28 °C for 10 minutes followed by observation under a confocol microscope.

Interestingly, though VIP1 could be localized inside yeast nucleus (Figure 3.19A), VirE2 stayed exclusively inside yeast cytoplasm even in the presence of VIP1 (Figure 3.19B). This indicates that other plant factors except VIP1 might also be needed for VirE2 nucleus uptake.

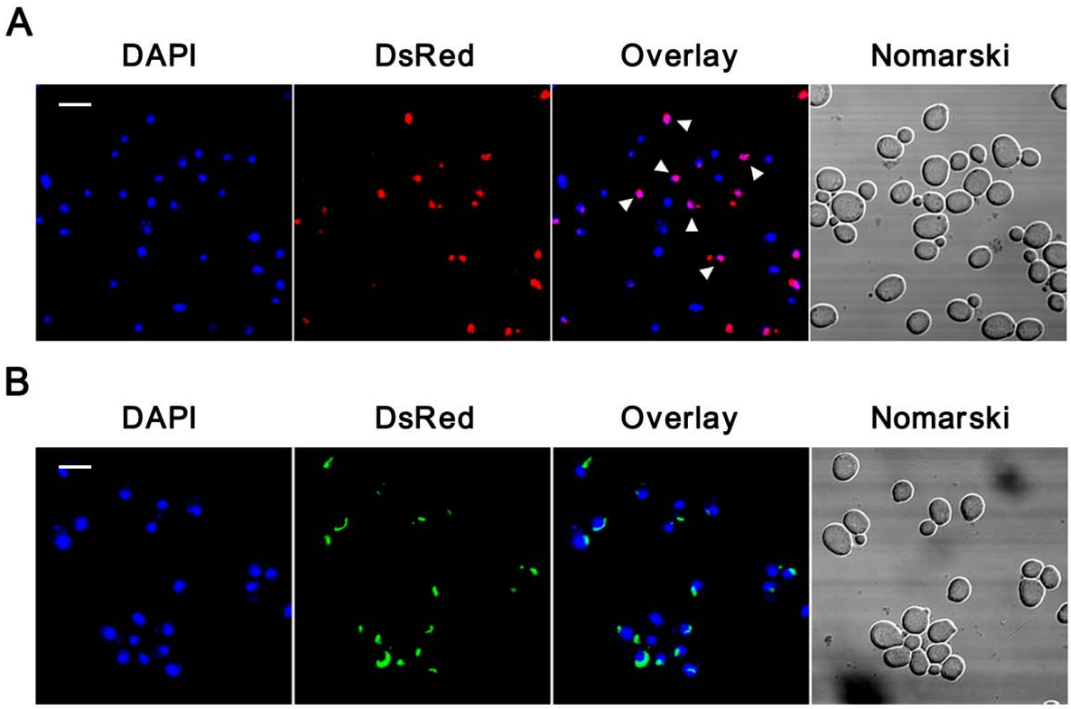


Figure 3.19. Study of VIP1 in VirE2 nucleus targeting process in yeast cells.

(A). *Arabidopsis* VIP1 was localized inside yeast nucleus (arrowed) upon expression. (B). Agrobacterium VirE2 stayed inside yeast cytoplasm in the presence of VIP1. The yeast nucleus was indicated by DAPI staining. Pictures were taken using a confocol microscope. Scale bars represent 5 μm.

3.6.4. Quantitative study of VirE2 delivery in AMT of yeast

Although the non-natural host yeast could be transformed by *Agrobacterium* under laboratory environment, it turns out to be less competent compared with natural host plant and the transformation efficiency remains low. However, a relatively high efficiency of VirE2 delivery was observed in this study. To better understand the AMT process, the efficiency of transient transformation, stable transformation and virulence protein delivery were compared.

The efficiency of transient transformation in yeast was measured using EGFP as an indicator. *Agrobacterium* strain EHA105 containing binary vector pHT101 was co-cultivated with yeast BY4741 at 20 °C; cells were harvested after 24-hour co-cultivation and subsequently analyzed by Becton-Dickinson (BD) Fluorescence Activated Cell Sorter (FACS) FACSAria II based on the intensity of EGFP transiently expressed in yeast cells (Figure 3.20A). The control experiment was performed similarly using *Agrobacterium* strain EHA105 containing binary vector pHT101-2A (without EGFP reporter) and yeast BY4741. To calculate the efficiency of stable transformation, partial of the cells were also plated onto SD Leu^- agar plate to select for stable transformants.

VirE2 delivery efficiency were calculated similarly after 24-hour co-cultivation using a fluorescence microscope, the efficiency was defined as the percentage of yeast cells with VirE2-GFP_{comp} signals.

The efficiency of transient transformation, stable transformation and virulent protein delivery of AMT were compared in Table 3.1. Although the efficiency of transient transformation as well as stable transformation of *Agrobacterium*-mediated transformation of yeast is relatively low, the VirE2 translocation efficiency reached a very high level, indicating that the *Agrobacterium* acts as an excellent protein transporter even in non-natural host.

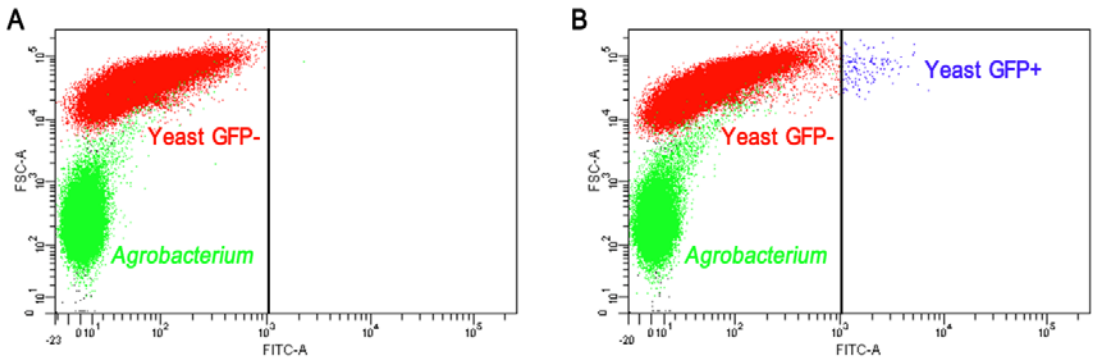


Figure 3.20. Transient transformation assay in yeast.

Yeast BY4741 was transformed with *Agrobacterium* EHA105 containing pHT101-2A (A) or pHT101 (B); the transient transformation efficiency was determined using a cell sorter.

Table 3.1. Comparison of transient transformation, stable transformation and VirE2 delivery in AMT of yeast.

Transient transformation efficiency (%)	Stable transformation efficiency (%)	VirE2 translocation efficiency (%)
0.2	0.4	50.9

3.6.5. Preliminary study of VirE2 degradation in yeast cells.

It has been shown that VirE2 undergoes VirF-mediated degradation in plant nucleus to facilitate integration of T-DNA into host genome(Tzfira *et al.* 2004).

A preliminary study of VirE2 degradation after delivered into yeast cells was carried out based on the percentage of VirE2 signals. Yeast BY4741 (pQH04-GFP1-10) cells were collected after co-cultivation with EHA105*virE2::GFP11* for 24 hours at 20 °C; the cells were then washed twice with PBS and re-suspended in PBS. Cells were kept at 20 °C and an aliquot of the cells was collected at an interval of 12 hours for totally 48 hours and observed under a fluorescence microscope for VirE2 signals counting.

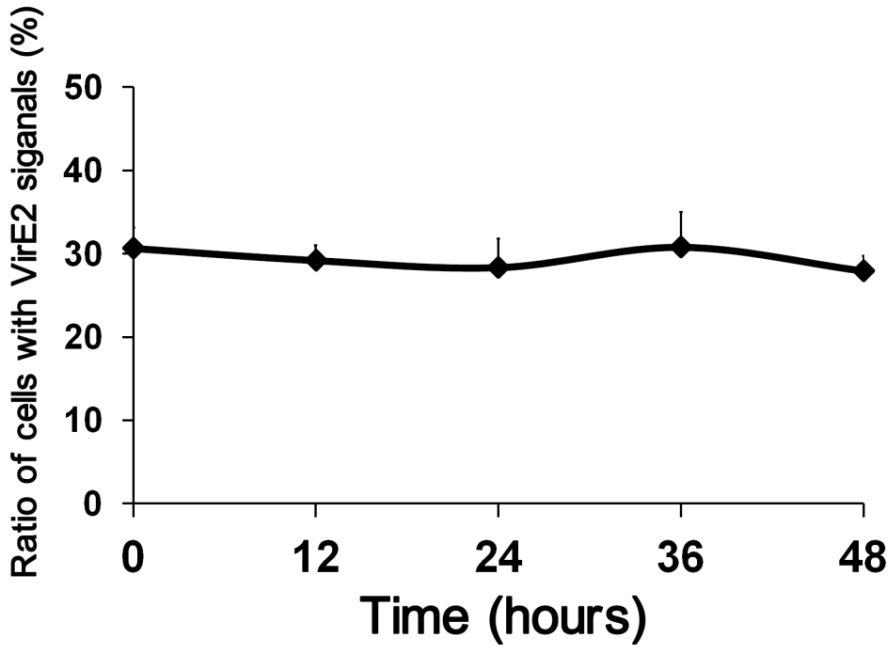


Figure 3.21. Degradation assay of VirE2 in yeast cells.

Degradation assay of VirE2 was carried out as described for 48 hours. Percentage of cells with VirE2 signals was shown with standard deviation.

As shown in Figure 3.21, VirE2 tends to be stable inside yeast cells after delivery within 48 hours. This probably results from the lack of VirE2 degradation related protein homologs in yeast cells.

3.7. VirE2 behavior study in plant cells

Compared with the non-natural host yeast, which could be transformed by *Agrobacterium* under laboratory condition, the natural host plants was also used for study of the VirE2 behavior considering VirE2 interaction partners such as VIP1 might be missing in the yeast cells.

3.7.1. Establishing Split-GFP system in plant cells

Function of VirE2-GFP11 from *Agrobacterium* strain EHA105 was tested in plant system using root transformation assay prior to VirE2 behavior study. As EHA105 is a disarmed strain thus does not induce grown gall in plants, the function of VirE2-GFP11 from EHA105 was then tested by using the tumor inducing strain A348.

To replace the VirE2 in A348 with VirE2-GFP from pTiBo542 background, the VirE2-GFP11 coding sequence from EHA105 was cloned into pEX18TcKm-A348VE2KO between the A348 *virE2* upstream and downstream sequences to make pEX18TcKm-105VirE2::GFP11, the plasmid was then used to generate the A348-*105virE2::GFP11* using similar replacement strategy as described. Similarly, the vector pEX18TcKm-105VirE2 was also made by cloning the VirE2 coding sequence from EHA105 into pEX18TcKm-A348VE2KO, which was then used to generate A348-*105virE2* as a control for root transformation assay.

Expression level of VirE2-GFP11 and VirE2 in A348-*105virE2::GFP11* and A348-*105virE2* were tested by western blot respectively (Figure 3.22D). The two *Agrobacterium* strains were cultured in MG/L medium for overnight. The cells were then subcultured into fresh MG/L medium till early log phase.

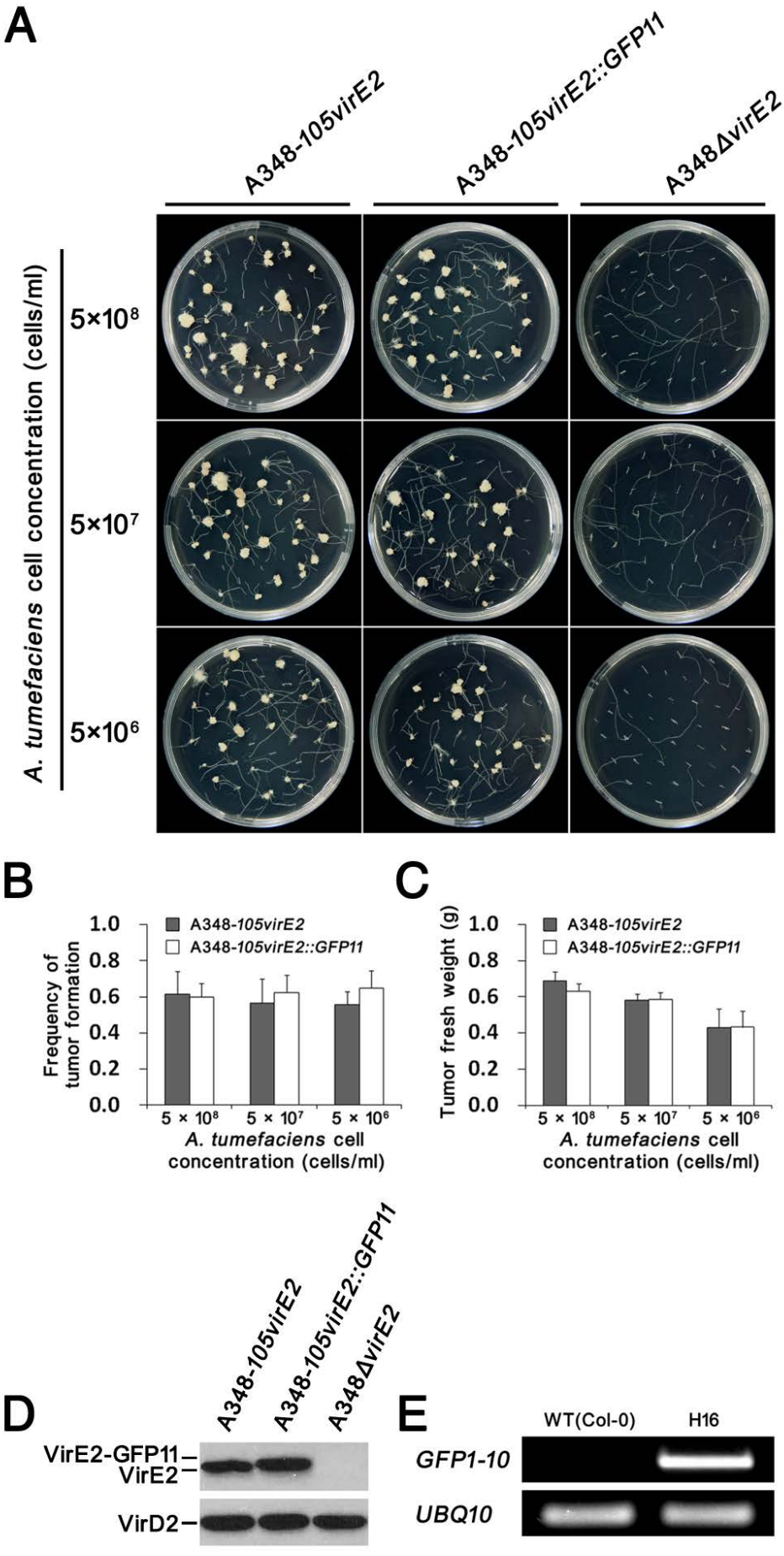

A
A348-105virE2
A348-105virE2::GFP11
A348ΔvirE2
A. tumefaciens cell concentration (cells/ml)
5×10⁸
5×10⁷
5×10⁶
B
Frequency of tumor formation
A348-105virE2
A348-105virE2::GFP11
1.0
0.8
0.6
0.4
0.2
0.0
5 × 10⁸
5 × 10⁷
5 × 10⁶
A. tumefaciens cell concentration (cells/ml)
C
Tumor fresh weight (g)
A348-105virE2
A348-105virE2::GFP11
1.0
0.8
0.6
0.4
0.2
0.0
5 × 10⁸
5 × 10⁷
5 × 10⁶
A. tumefaciens cell concentration (cells/ml)
D
A348-105virE2
A348-105virE2::GFP11
A348ΔvirE2
VirE2-GFP11
VirE2
VirD2
E
WT(Col-0)
H16
GFP1-10
UBQ10

Figure 3.22. GFP11 does not perturb the function of VirE2 in AMT of plants.

(A). The virulence of A348-*105virE2::GFP11* and A348-*105virE2* was compared using root transformation assay. A serial dilution of bacterial cells was used to test the function VirE2-GFP11 as indicated. The *virE2* deletion mutant was used as a negative control. Photographs were taken 4-5 weeks post transformation. (B). Quantification of the root transformation efficiency. Root transformation efficiency was defined as the ratio of the number of tumors to the number of the total root segments. (C). Quantification of the tumor fresh weight. Weights of the tumors from the same plate were measured after transformation. (D). Expression level of VirE2-GFP11 in A348-*105virE2::GFP11* was compared with that of VirE2 in A348-*105virE2* using western blot. The VirD2 was used as the control. (E). Confirmation of expression of GFP1-10 in the roots of *Arabidopsis* transgenic line H16. *GFP1-10* transcripts were detected using PCR based method. The *UBQ10* transcripts were used as the control.

Cells were collected and wash twice with $IBPO_4$ and re-suspended in $IBPO_4$ at the final concentration of 5×10^8 cells/ml. The induction was carried out at 20 °C for additional 20 hours. Cell input was adjusted by OD_{600} prior to western blot as described in Chapter 2.

As GFP1-10 binds to VirE2-GFP11 upon its translocation, the function of VirE2-GFP11 was tested in the presence of the big fragment GFP1-10. An *A. thaliana* transgenic line H16, which constantly expressing GFP1-10 under the control of CaMV 35S promoter, was used in the root transformation assay. The expression of GFP1-10 in the transgenic line was confirmed using PCR based method (Figure 3.22E). In brief, total RNA were extracted from the roots of 12-day old wild type *Arabidopsis* and transgenic line H16 using TRIzol(R)-based method as described in Chapter 2; the cDNA were then obtained using iScript cDNA SynthesisKit (Bio-Rad). Primers specific for *GFP1-10* transcript were used for PCR detection. The *UBQ10* transcript was detected as the control.

Arabidopsis root transformation was carried out as described in Chapter 2. To perform a more accurate virulence assay, a serial dilution of *Agrobacterium* as indicated was also included. Photographs were taken 4-5 weeks after transformation assay, the tumors from the same plate were also cut out and the fresh weights of the tumors were measured. No significant difference was observed between A348-*105virE2* and A348-*105virE2::GFP11* in the root transformation assay, indicating the GFP11 tagged VirE2 functioned similarly as VirE2 in the plant transformation process (Figure 3.22A-C).

All these indicate that the Split-GFP system is also functional in plants and is suitable to study the VirE2 behavior in plant system.

3.7.2. Study of nuclear localization signals in VirE2

To study the VirE2 behavior in AMT of plants, EHA105*virE2::GFP11* was used to transform transgenic *N. benthamiana* expressing GFP1-10 through agroinfiltration.

Agrobacterium cells were infiltrated to the underside of the *N. benthamiana* leaves as described in Chapter 2; and the leaf tissues were observed using a spinning disk microscope at 2 days post agroinfiltration. The transgenic *N. benthamiana* also expressed free DsRed to distinguish the cell borders and nucleus.

Different from that in yeast cells, *Agrobacterium*-delivered VirE2 localized in both cytoplasm and nucleus of the *N. benthamiana* epidermal cells (Figure 3.23B). As a control, no GFP signals could be detected in *N. benthamiana* epidermal cells either using unlabeled EHA105 or the *virD4* deletion mutant (Figure 3.24).

The nucleus localization of VirE2 in plant cells might results from the two putative nuclear localization signals (NLS) of VirE2 as well as the existence of interaction partner VIP1 homologs (Citovsky *et al.* 1992).

To study the potential role of the two putative nuclear localization signals in VirE2 nucleus targeting, relative VirE2 NLS mutants were constructed similarly as described for the other *Agrobacterium* mutants. In brief, NLS mutated VirE2-GFP11 coding sequences *virE2::GFP11nls1*, *virE2::GFP11nls2* or *virE2::GFP11nls1nls2* were generated with standard overlapping PCR and subsequently inserted into pEX10TcKm to produce pEX18TcKm-VirE2::GFP11NLS1M, pEX18TcKm-VirE2::GFP11NLS2M and pEX18TcKm-VirE2::GFP11NLSM respectively. These resulting plasmids were then used to generate the *Agobacterium virE2* mutants EHA105*virE2::GFP11nls1*, EHA105*virE2::GFP11nls2*, and EHA105*virE2::GFP11nls* using *SacB* based approach as described above. The eight conserved amino acid residuals of each NLS were replaced with alanines in the corresponding mutants as indicated (Figure 3.23A).

As shown in Figure 3.23C, VirE2-GFP_{comp} signals failed to be detected inside plant nucleus using VirE2 NLS1 mutated strain EHA105*virE2::GFP11nls1*, though the VirE2 still could localize to the periphery region of the nucleus (arrowed). This indicated that *Agrobacterium* delivered VirE2 might be targeted to the plant nucleus in a NLS dependent manner.

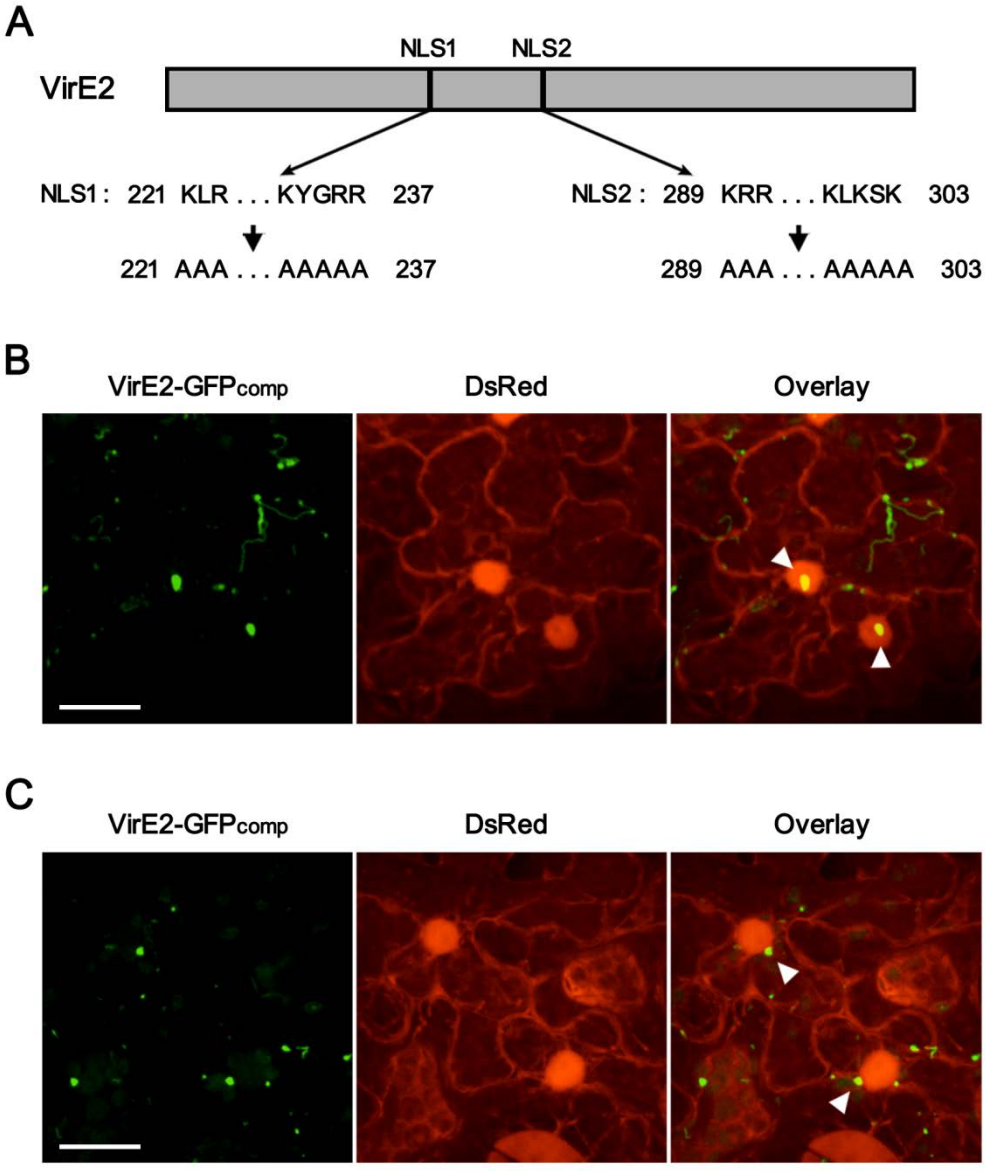


Figure 3.23. Study of putative nuclear localization signals of VirE2 in AMT of *N. benthamiana* epidermal cells.

(A). Schematic diagram of mutation of VirE2 nuclear localization signals. The conserved amino acid residues as indicated were replaced with alanines respectively. (B). *Agrobacterium* delivered VirE2 was localized in both cytoplasm and nucleus (arrowed) of *N. benthamiana* epidermal cells. (C). NLS1 mutated VirE2 delivered by *Agrobacterium* was localized exclusively inside the cytoplasm of *N. benthamiana* epidermal cells. Pictures were taken using a spinning disk confocol microscope. Scale bars represent 20 μm.

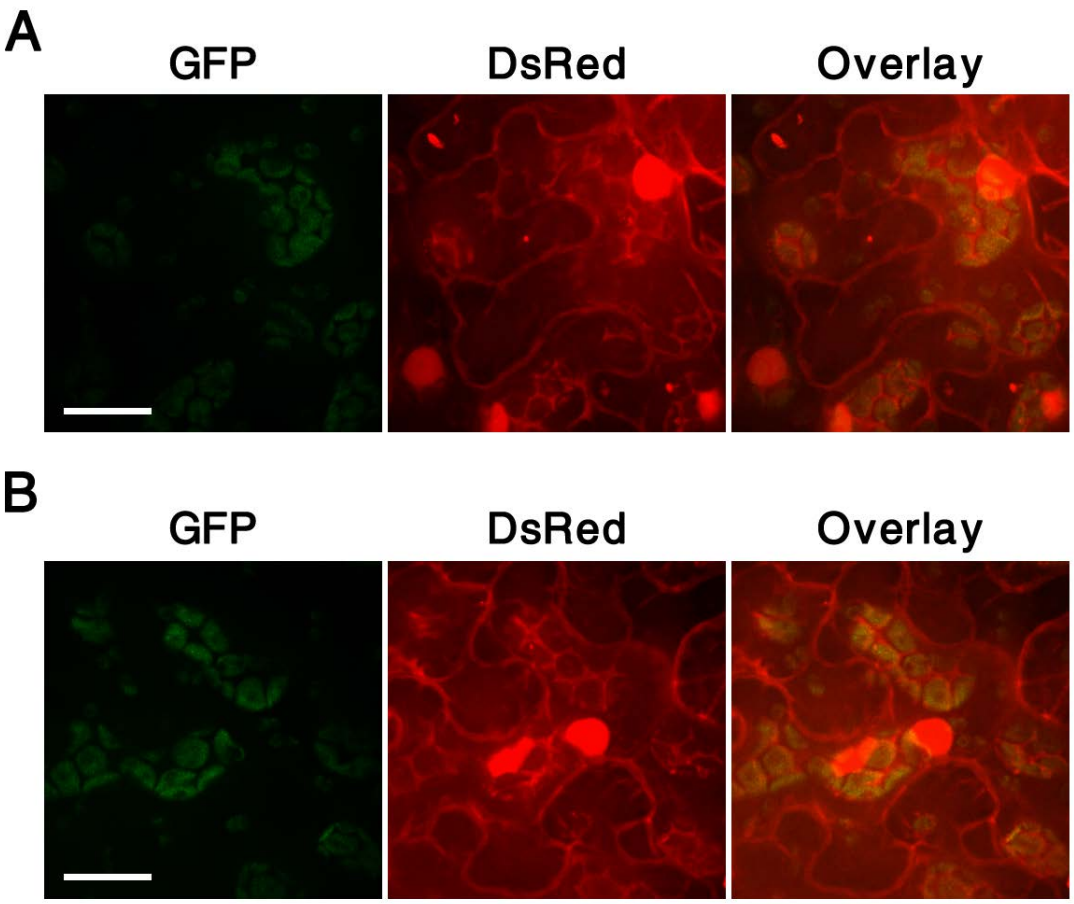


Figure 3.24. GFP fluorescence is not detected in *N. benthamiana* epidermal cells when omiting any split-GFP component or deletion of *virD4*.

No GFP fluorescence could be detected when using unlabeled EHA105 (A) or *virD4* deletion mutant of EHA105*virE2::GFP11* in *N. benthamiana* epidermal cells. Pictures were taken using a spinning disk confocol microscope. Scale bars represent 20 μm.

However, no VirE2-GFP_{comp} signal could be detected inside *N. benthamiana* leave cells using either EHA105*virE2::GFP11nls2* or EHA105*virE2::GFP11nls*, probably because the NLS2 region might also be important for VirE2-GFP11 secretion and/or its self-interaction.

3.8. Discussion

Agrobacterium tumefaciens has long been used as a genetic tool in biotechnology, its unique ability of efficient horizontal gene transfer makes it commonly used in genetic manipulation of natural host plants as well as other non-natural host organisms.

Except the transferred DNA fragment, this bacterium also delivers several virulence proteins into host cells, which facilitate the transformation process in a variety of respects including T-DNA uptake, cytoplasmic trafficking, nucleus targeting, T-DNA integration and expression. The effector VirE2 is the most abundant protein among these transferred virulence factors, which is a single stranded DNA binding protein and coats the T-DNA in host cytoplasm to protect it from degradation (Citovsky *et al.* 1988; Citovsky *et al.* 1992; Yusibov *et al.* 1994). Besides, the VirE2 might also help the uptake of T-strand into host cells and its nuclear targeting followed by genome integration (Duckely *et al.* 2005; Anand *et al.* 2007; Djamei *et al.* 2007). Thus VirE2 plays important roles through the whole transformation process and is indispensible for *Agrobacterium*-mediated transformation of plants. Moreover, deletion of VirE2 also resulted in dramatically attenuated virulence in transformation of yeast, which also implies the important role of VirE2 in *Agrobacterium*-mediated transformation of non-host organisms.

However, *in vivo* study of VirE2 remains difficult due to its vulnerability to protein tagging techniques. Secretion of VirE2 from *Agrobacterium* requires its C-terminal signal while its virulence function in host cells needs an intact N-terminus, making it hard to be studied *in vivo* using traditional labeling approaches

(Bhattacharjee *et al.* 2008). Previous research studied the VirE2 behavior by artificially introducing this protein into host cells either through direct uptake or transgenic expression, these introduced VirE2 might behaved differently from those natural transferred ones and these different approaches also resulted in conflicting observations (Gelvin 2010). Thus a milder labeling approach is needed for *in vivo* study of VirE2 behaviors.

This study has adopted a recently developed Split-GFP detection system. In this system, a modified GFP molecule is divided into two parts, the big fragment GFP1-10 (β strands 1-10) and the small fragment GFP11 (β strand 11); neither of these two fragments is fluorescent while spontaneously binding could happen when placed together and the GFP fluorescence will be restored. The GFP11 with 16 amino acid residuals was used for VirE2 tagging and an internal permissive site of VirE2, which has been shown to have small peptide insertion tolerance, turned out to be a suitable position for GFP11 labeling (Zhou *et al.* 1999). The VirE2-tagged *Agrobacterium* strain did not display significant perturbation in bacterial growth, protein expression and virulence, indicating this GFP11 tag did not affect the VirE2 function during transformation process thus is suitable for *in vivo* studies. The GFP11 tagged VirE2 was expressed inside the bacteria while the GFP1-10 was expressed in host cells, restored fluorescence could be detected upon VirE2 translocation. This Split-GFP system worked well in both natural host plants and non-natural host yeast; and this is the first time that bacteria delivered VirE2 were visualized inside live host cells. Taken together, this system is suitable for *in vivo* studies of VirE2 behavior during natural transformation process. Moreover, the VirE2 coats the T-DNA with 1 molecule to 19 bases to facilitate the T-DNA trafficking inside host cells (Citovsky *et al.* 1997); thus this system might also help to study the T-complex during AMT process as well.

Agrobacterium VirE2 protein contains self-interaction structures and could form “telephone cord’’ structure independent of single strand DNA *in vitro* (Frenkiel-Krispin *et al.* 2007; Dym *et al.* 2008; Bharat *et al.* 2013). Our observation

also confirmed such aggregated structure of VirE2 *in vivo*. The bacteria-delivered VirE2 in *N. benthamiana* epidermal cells formed filamentous structures in different lengths, indicating that the VirE2 could self-aggregate to form such structures inside host cytoplasm. On the other hand, only “dot-like” instead of filamentous structures could be observed when VirE2 was delivered into yeast cells. This might resulted from the limited cell size of yeast and relatively lower amount of translocated VirE2 considering that filamentous structures could also be observed when VirE2 was over-expressed inside yeast cells.

Agrobacterium-delivered VirE2 could be detected inside yeast cells as early as 2 hours post co-cultivation, suggesting that the virulence protein delivery is a very fast process. Moreover, the translocated VirE2 stably stayed inside yeast cells, indicating that the degradation of VirE2 might requires the involvement of specific plant factors.

Previous studies showed both cytoplasmic and nucleus localization of VirE2 using microinjection or transgenic expression (Gelvin 2010). In this study, the GFP11 labeled VirE2 localized to the nucleus of *N. benthamiana* epidermal cells; this also happened for the T-DNA deletion strain EHA105, suggesting that the VirE2 could be actively delivered into plant nucleus independent of T-strand. However, VirE2 delivered into yeast cells exclusively localized in the host cytoplasm, which might be due to the lack of VirE2 interaction partners in the non-natural host cells. It has been hypothesized that *Agrobacterium* VirE2 “hijacked” the MAPK3 regulated VIP1 signaling pathway for nucleus targeting in plant cells, thus the role of VIP1 was also tested in yeast cells. However, though the VIP1 could localized to the yeast nucleus, it failed to facilitate the VirE2 nucleus targeting in yeast cells, indicating other plant factors except VIP1 might also be involved in nucleus localization of VirE2. Although exclusively localized outside the nucleus, the bacteria-delivered VirE2 mainly stayed at the periphery regions of yeast cells. It has been shown that VirE2 has membrane affinity and could create channels in the artificial membranes (Dumas *et al.* 2001; Duckely *et al.* 2005); thus our observations also supported such membrane affinity *in*

vivo.

Using the Split-GFP system, a NLS dependent localization of VirE2 in plant nucleus was observed. The NLS represents a group of conserved signal sequences that could be recognized by importin α for nucleus transportation. Such observation differed from the hypothesis that VirE2 gets into plant nucleus in the help of host transcription factor VIP1 (Djamei *et al.* 2007). Some previous studies also showed that VirE2 directly interacted with several *Arabidopsis* importin α isoforms and the IMPa-4 is responsible for nucleus translocation of VirE2 (Bhattacharjee *et al.* 2008). These different observations suggest that VirE2 might abuse diverse host factors to achieve a higher efficiency of nucleus targeting to facilitate the transformation. The NLS dependent manner of VirE2 localization to plant nucleus supported the involvement of direction of VirE2 with plant importin α isoforms. However, the functional structure analysis of VirE2 has not been completed, and such observation might also due to an overlapping of NLS region with VIP1 binding domain of VirE2; thus further experiments are still needed to confirm this.

Using this Split-GFP system, the efficiency of VirE2 delivery by *Agrobacterium* was also calculated. Surprisingly, more than 50% of VirE2 translocation efficiency has been observed in the non-natural host yeast, though the transient and stable transformation efficiencies were relatively low. Some previous studies monitored virulence protein translocation from *Agrobacterium* to plant cells with the Cre Recombinase Assay for Translocation (CRAfT); by using this assay, up to 1% of yeast cells received Cre-VirE2 fusion protein during AMT (Vergunst *et al.* 2000; Schrammeijer *et al.* 2003). With this newly developed Split-GFP system, a much higher efficiency of VirE2 delivery has been observed; the Split-GFP assay introduced much smaller perturbation to the VirE2 molecule compared with the relatively big size of Cre (343 amino acid residuals) thus represented more natural and accurate results.

Agrobacterium has been used as a genetic manipulation tool for various species;

its host spans from fungi to plants and to mammalian cells and the host range is still expanding. However, although *Agrobacterium*-mediated transformation is a relatively conserved process, it happens in a much higher efficiency in natural host plants compared with the non-natural hosts. This difference probably results from the existence of host factors in plants from a long-term evolution process. Thus the previous studies that only focus on the DNA transfer by *Agrobacterium* might overlook its protein delivery ability. The VirE2 delivery assay in this study showed that for the non-natural host yeast, the virulence protein translocation still remained effective while the ultimate transformation turned out to be very low. In conclusion, not only the T-DNA transfer but also the protein delivery ability of *Agrobacterium* should be emphasized in the future especially for the non-natural hosts.

Chapter 4. Study of host Pmp3p in *Agrobacterium*-mediated transformation of yeast

4.1. Introduction

Previous studies of *Agrobacterium*-mediated transformation mainly focused on the bacteria part and the process inside bacteria has been well studied (Cascales *et al.* 2003); however, little is known about the host factors which are also believed to be essential in this process. Though several potential pathways as well as the host factors involved in AMT have been identified by previous work (Citovsky *et al.* 2007; Pitzschke *et al.* 2010), these connections still need to be further confirmed and the internal relationships are not very clear.

Using *Saccharomyces cerevisiae* as a model turns to be another new way to study the role of host proteins in AMT. As *Agrobacterium*-mediated transformation is a relatively conserved process among different host species, using yeast to study AMT is a convenient approach with the available manipulation approaches and its characteristic of easy for handling compared with plants.

4.2. A host Pmp3p affected *Agrobacterium*-mediated transformation in yeast

4.2.1. A yeast mutant *pmp3*Δ is more resistant to *Agrobacterium*-mediated transformation

Previous work in our lab has established a screening platform for identification of yeast mutants sensitive or resistant to *Agrobacterium*-mediated transformation. Based on the unpublished screening results, several yeast mutants were chosen from YKO deletion collection (Open Biosystems) for further confirmation. Among them, a yeast mutant *pmp3*Δ showed higher resistance to AMT compared with wild type strain

and consistently displayed decreased AMT efficiency (Figure 4.1A).

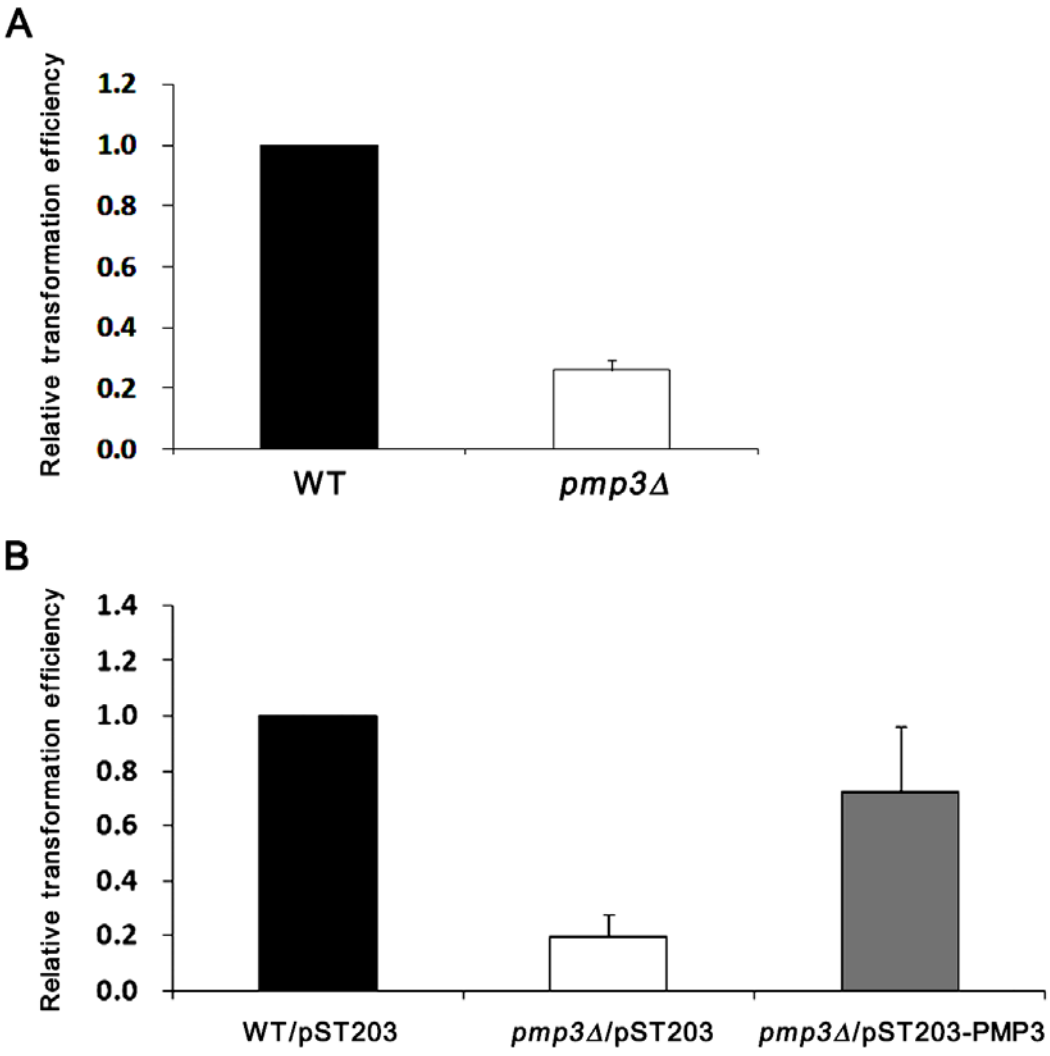


Figure 4.1. A yeast mutant *pmp3*Δ showed decreased transformation efficiency in AMT.

(A). Yeast mutant *pmp3*Δ is more resistant to AMT compared with wild type strain BY4741. (B). Complementation assay confirmed the important role of yeast Pmp3p in AMT process.

AMT of yeast was carried out as described in Chapter 2. In brief, *A. tumefaciens* EHA105 (pHT101-2A) was co-cultivated with either *pmp3*Δ or wild type yeast strain on co-cultivation agar medium ($IBPO_4$ agar medium supplemented with appropriate amino acids) at 20 °C for 24 hours. Positive transformants were subsequently selected using SD Leu^- agar plate. Transformation efficiency is defined as the ratio of the number of transformants to the number of recipients.

To confirm the role of host Pmp3p in AMT, complementation experiment was also carried out. The yeast *PMP3* was cloned into yeast expression vector pST203, together with its genomic upstream 691 bp (containing *PMP3* natural promoter) and downstream 86 bp (containing *PMP3* natural terminator) sequences, to generate pST203-PMP3. The vector pST203 uses *CEN6/ARSH4* as the replication origin, thus could maintain the copy number of the gene of interest similar to those of wild-type genes (1-2 copies per cell). The mutant *pmp3*Δ containing pST203 alone was used as a negative control. The decreased AMT efficiency observed for *pmp3*Δ could be partially rescued when harboring pST203-PMP3 (Figure 4.1B).

The above results confirmed that yeast Pmp3p might play an important role during AMT process.

4.2.2. Yeast Pmp3p is a membrane protein related to cellular ion homeostasis

Yeast *PMP3* encodes a small protein containing only 55 amino acids. Structural analysis with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) shows that Pmp3p is highly hydrophobic and bears two potential trans-membrane domains (Figure 4.2A).

To localize PMP3 protein inside yeast, an EGFP reporter was fused at C-terminus of Pmp3p and expressed from yeast *PMP3* genomic promoter and terminator (pST203-PMP3-GFP). As shown in Figure 4.2B, the Pmp3p displays membrane localization pattern inside both wild type strain and *PMP3* deletion mutant. The plasmid pST203-GFP expressing only GFP was used as a control.

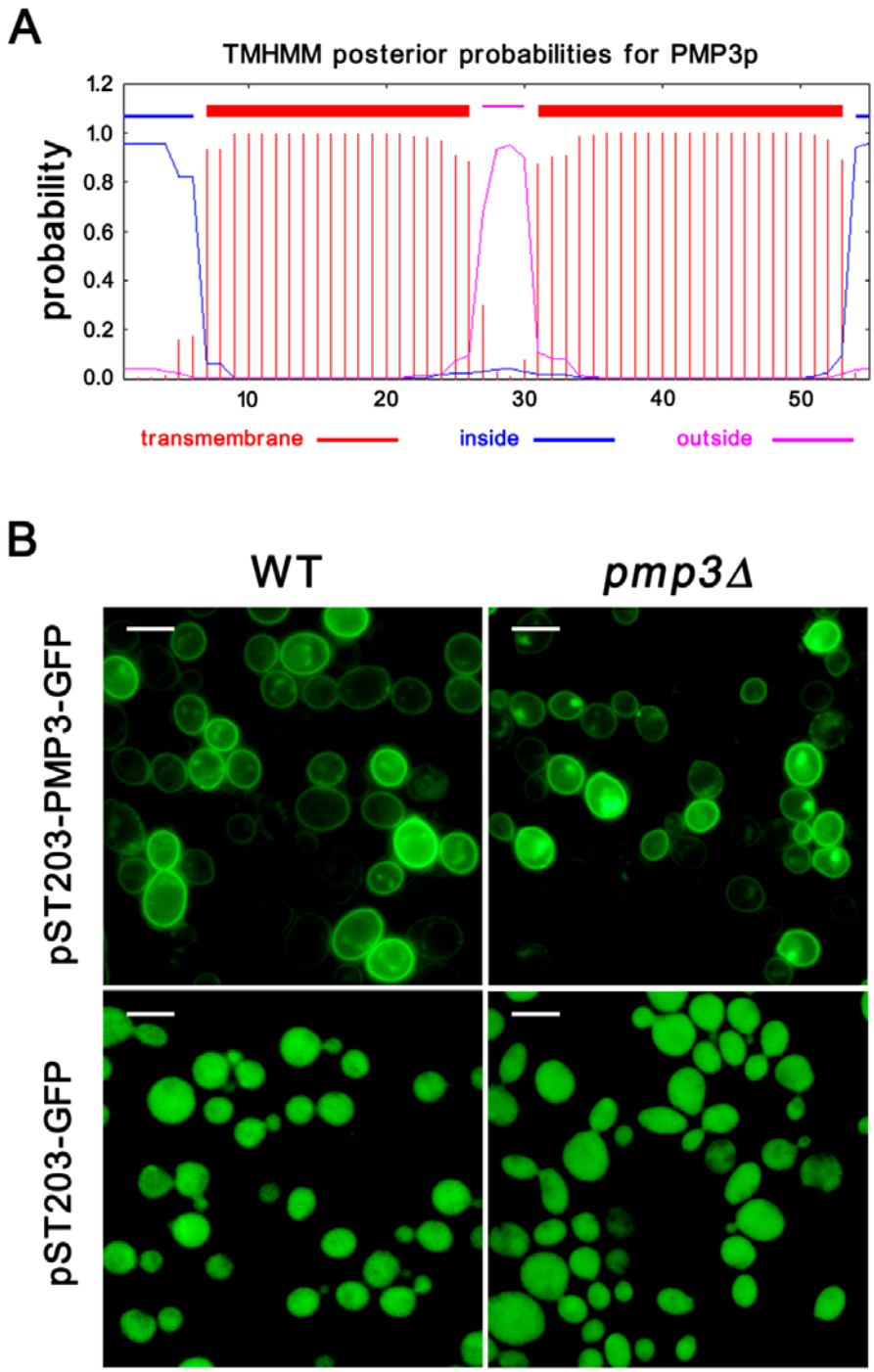


Figure 4.2. Plasma membrane localization of Pmp3p in yeast cells.

(A). Structure analysis of yeast Pmp3p with TMHMM predicted two potential trans-membrane domains. (B). Localization of yeast Pmp3p with EGFP reporter. Pictures were taken using a fluorescence microscope. Scale bars represent 5 μm.

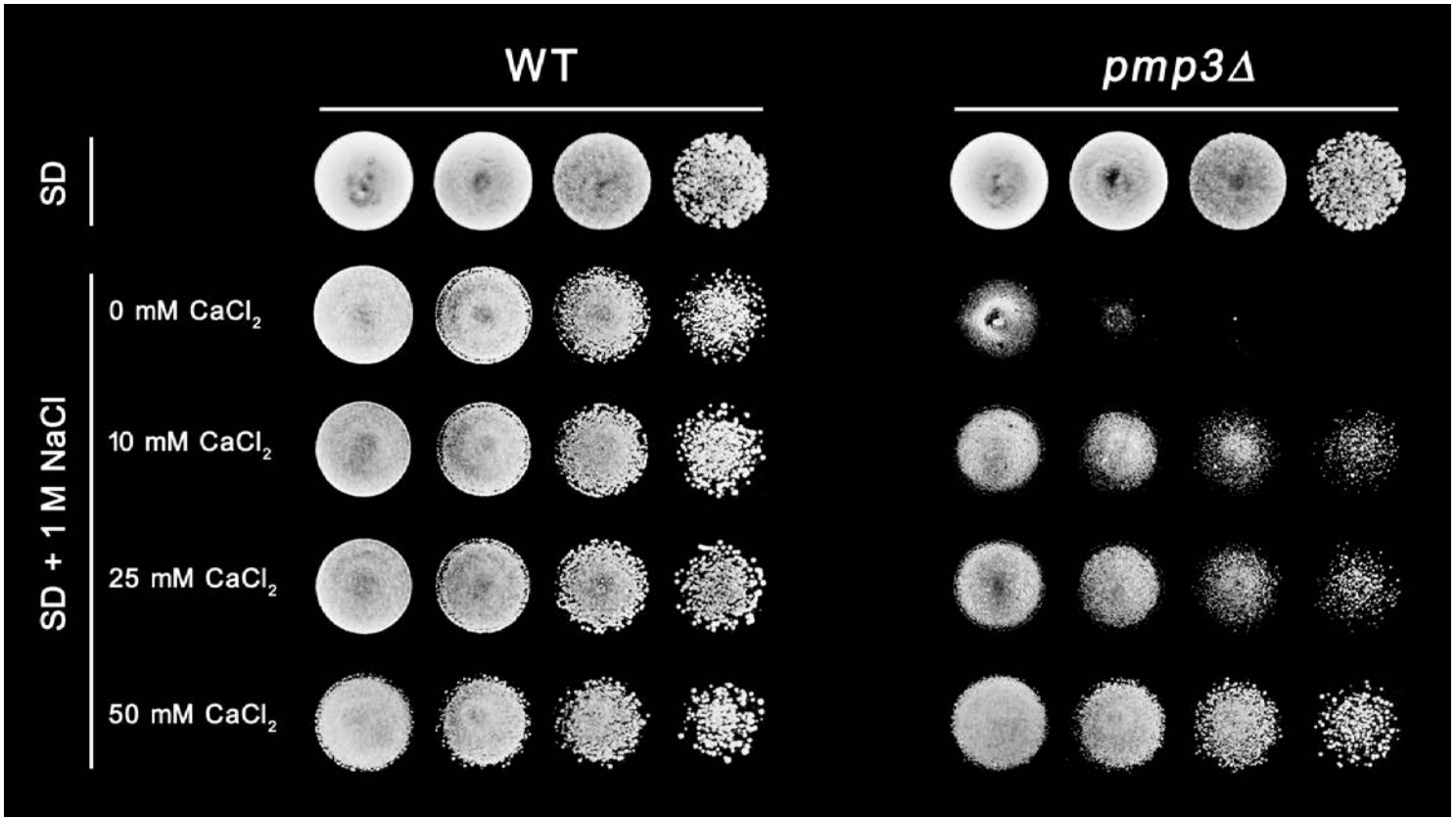


Figure 4.3. Pmp3p is required for cellular ion homeostasis.

Growth of serial 10-fold dilutions of overnight culture of wild type BY4741 and *pmp3*Δ on SD agar medium. Different concentration of NaCl and/or $CaCl_2$ were added as indicated.

Yeast mutant *pmp3*Δ showed a similar growth pattern compared with the wild type strain when growing on SD agar medium, indicating that the *PMP3* is not essential under normal growth condition (Figure 4.3). However, the deletion of *PMP3* confered sensitivity to environmental cation concentration as *pmp3*Δ grew less well in high salts condition compared to wild type strain (Figure 4.3). Interestingly, the sensitivity to high concentration of Na^+ could be reversed by divalent cations. As shown in Figure 4.3, the sensitivity of mutant *pmp3*Δ was attenuated by adding Ca^{2+} into the medium in a dose dependent pattern.

All these taken together indicated that the yeast protein Pmp3p might be related to membrane ion channels and play an important role in adjusting cellular ion homeostasis.

4.2.3. Resistance of *pmp3*Δ to *Agrobacterium*-mediated transformation displays a temperature dependent pattern

To find out whether the resistance of *pmp3*Δ to AMT is specific to *Agrobacterium* or not, the standard lithium-acetate (LiAc) based physical transformation method was used as a control. Lithium-acetate transformation of yeast was carried out as described in Chapter 2. The yeast cells were incubated with the same binary vector (pHT101-2A) used for the AMT experiment and treated with heat shock method; after which the cells were spread onto SD Leu^- agar medium or SD agar medium as used before for selection of transformants or recovery of recipients respectively. Transformation efficiency is determined as the ratio of the number of transformants to the number of recipients.

The result in Figure 4.4 shows no significant difference between the wild type and the mutant *pmp3*Δ, which suggests that the resistance-to-transformation phenotype of *pmp3*Δ is specific to AMT and is neither a consequence of the selection marker, nor a result of the features from the binary vector we use.

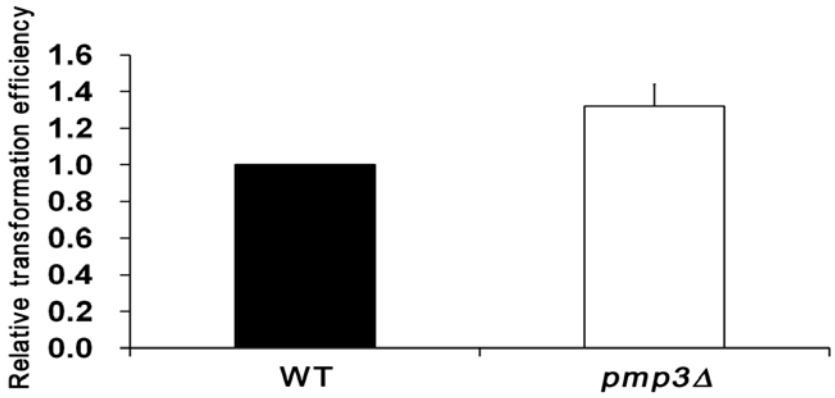


Figure 4.4. Comparison of lithium acetate transformation efficiency between *pmp3*Δ and wild type BY4741.

Yeast mutant *pmp3*Δ showed similar efficiency in lithium-acetate transformation compared to wild type strain.

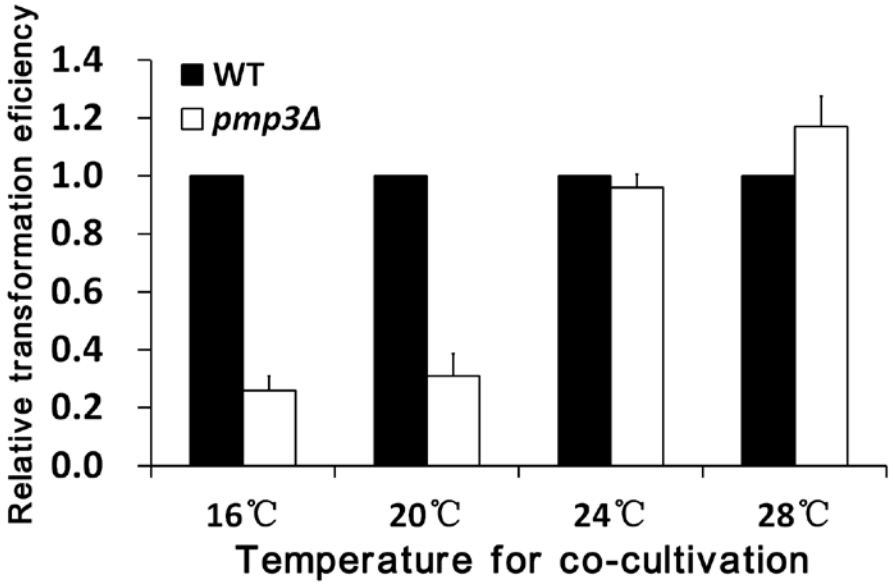


Figure 4.5. Yeast mutant *pmp3*Δ is resistant to AMT in a temperature dependent pattern.

Co-cultivation of yeast and *Agrobacterium* was performed at different temperature as indicated. *pmp3*Δ shows resistance to AMT when co-cultivated with *Agrobacterium* at lower temperature.

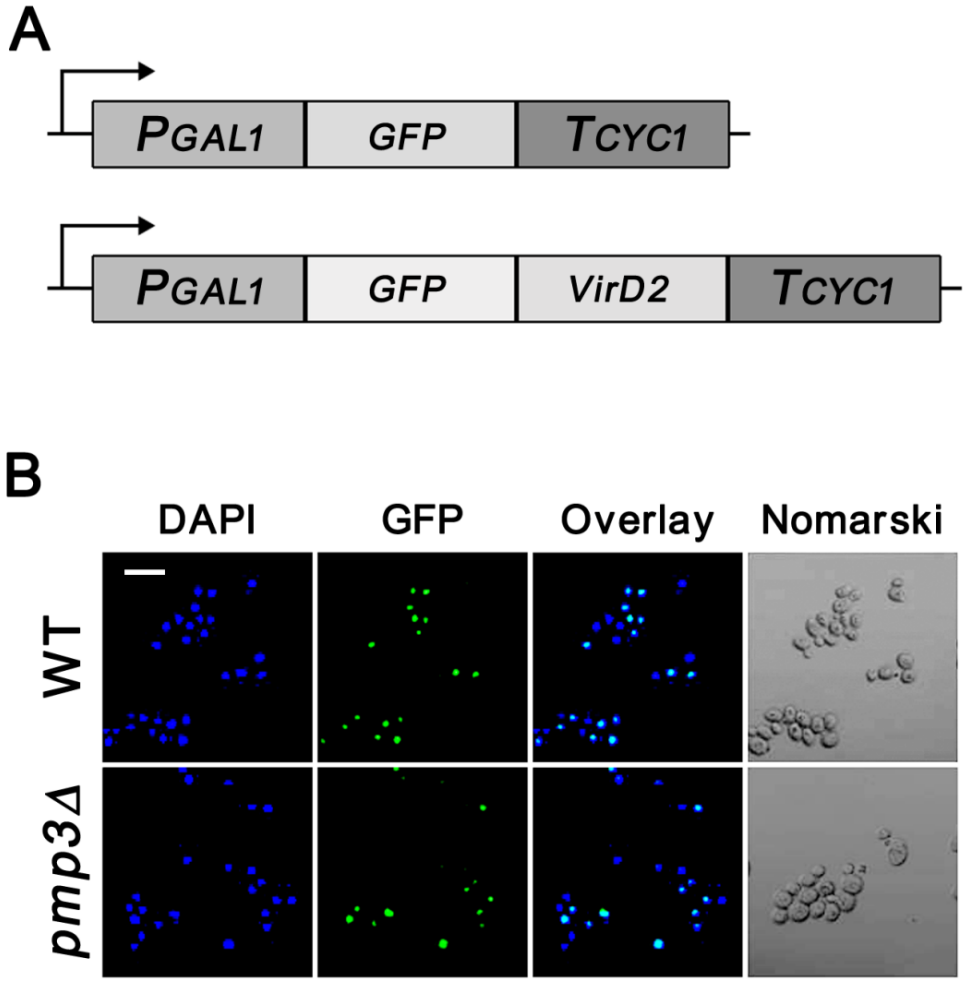
Besides, the resistance of *pmp3*Δ to *Agrobacterium*-mediated transformation also displayed a temperature dependent pattern (Figure 4.5). When co-cultivated with *Agrobacterium* at lower temperature (16 °C or 20 °C), the *pmp3*Δ was more resistant to AMT compared with wild type strain; however, the difference could be eliminated when the co-cultivation was carried out at relatively higher temperature (24 °C or 28 °C)

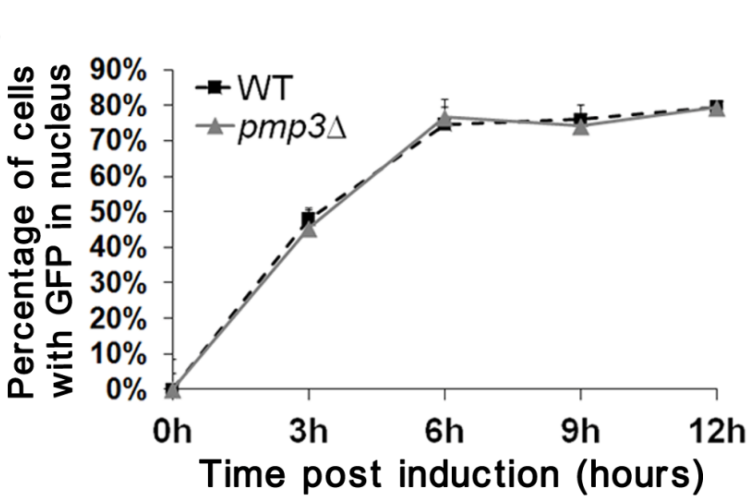
4.3. The VirD2 nucleus targeting process is not affected in yeast mutant *pmp3*Δ

Agrobacterium VirD2 belongs to the Vir protein family, which plays a crucial role in T-DNA formation as well as T-complex nucleus targeting in host cells (Tinland *et al.* 1992; Filichkin *et al.* 1993; Scheiffele *et al.* 1995). As nucleus targeting of T-complex happens inside host cytoplasm and is critical for successful transformation, whether the nucleus targeting of VirD2 is affected in *pmp3*Δ or not was tested.

Different from VirE2, relatively low copy number of VirD2 protein is transported into host cell which is difficult for detection. Thus an alternative way was used to study the cellular movement of VirD2 protein inside the host cell by transgenic expression.

The GFP-VirD2 fusion protein was directly expressed inside yeast cell using a yeast expression vector pYES2 (pYES2-GFP-VirD2). The expression is under the control of yeast *GAL1* promoter and could be induced by galactose. Yeast strain BY4741 and *pmp3*Δ harboring pYES2-GFP-VirD2 was grown in SD Ura^- liquid medium for overnight respectively; the cells were then harvested and washed twice with 0.9% NaCl solution followed by sub-culturing into SD Gal/Raf Ura^- liquid medium. Expression of the fusion protein was induced by galactose and yeast cells were collected at an interval of 3 hours for totally 12 hours after induction. Collected yeast cells were washed once with PBS, followed by fixing with 4% paraformaldehyde and incubating at 28 °C for 15 minutes.


A
PGAL1
GFP
TCYC1
PGAL1
GFP
VirD2
TCYC1
B
DAPI
GFP
Overlay
Nomarski
WT
pmp3Δ


C
Percentage of cells with GFP in nucleus
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
pmp3Δ
0h
3h
6h
9h
12h
Time post induction (hours)

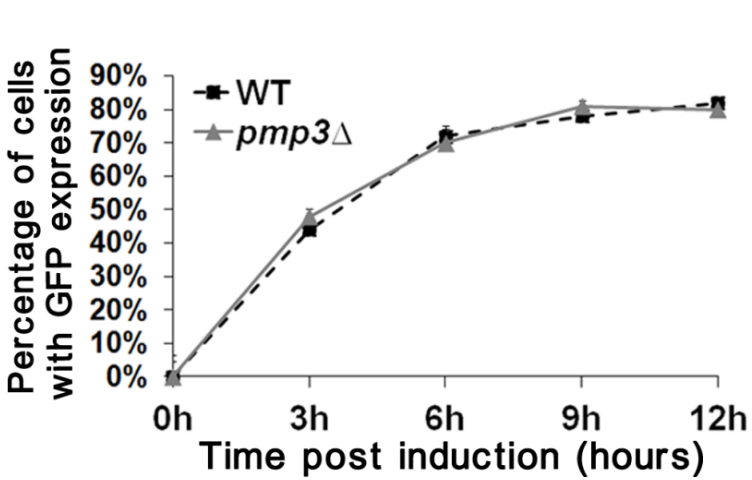

D
Percentage of cells with GFP expression
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WT
pmp3Δ
0h
3h
6h
9h
12h
Time post induction (hours)

Figure 4.6. VirD2 nucleus targeting process is not affected in yeast mutant *pmp3*Δ.

(A). Schematic diagram of plasmids used in VirD2 nucleus targeting study, the upper one represents pYES2-GFP and the lower one represents pYES2-GFP-VirD2. (B). Yeast cells were harvested and observed under a confocol microscope, the yeast nucleus was indicated by DAPI staining. Scale bar represents 10 μm. (C). Quantitative comparison of the ratio of cells with GFP signals inside nucleus between *pmp3*Δ and wild type strains. (D). Quantitative comparison of the ratio of cells with GFP expression between *pmp3*Δ and wild type strains.

The cells were then washed twice with PBS and stained with DAPI at 28 °C for 10 minutes. After washed twice with PBS, the cells were observed using a confocol microscope for GFP reporter detection. A yeast cell with GFP signals localized inside nucleus was counted as one with efficient VirD2 nucleus targeting. The ratios of cells with efficient VirD2 nucleus targeting were determined for both wild type strain and *pmp3*Δ.

No statistical difference was observed between the wild type strain and yeast mutant *pmp3*Δ (Figure 4.6B-C). To confirm that the transgenic expression level is not affected in *pmp3*Δ, the control experiment with the expression of GFP protein alone (pYES2-GFP) was also carried out simultaneously under the same condition as described above. No significant difference of the GFP protein expression was observed (Figure 4.6D). Combining with the result of VirD2-GFP nucleus localization, it suggests that the VirD2 localization activity was not affected in *pmp3*Δ.

4.4. Yeast mutant *pmp3*Δ showed an decreased competency to *Agrobacterium*-mediated delivery of VirE2

Agrobacterium translocates the DNA as well as protein substrates into host cells through the type Ⅳ secretion system, which is evolutionarily related to the bacteria conjugation process (Cascales *et al.* 2003). The proteins translocation represents an early event in the AMT process and is crucial for the transformation result. Thus whether the protein transfer process is affected in the yeast mutant *pmp3*Δ was tested.

As the *Agrobacterium*-delivered virulence proteins share a common VirB/VirD4 secretion channel to enter host cytoplasm, the VirE2 delivery was chosen as a representative for virulence protein translocation considering that it is the most abundant protein delivered by *Agrobacterium* during AMT (Engstrom *et al.* 1987). And the Split-GFP detection strategy developed above tends to be an efficient tool in quantitative study of VirE2 delivery.

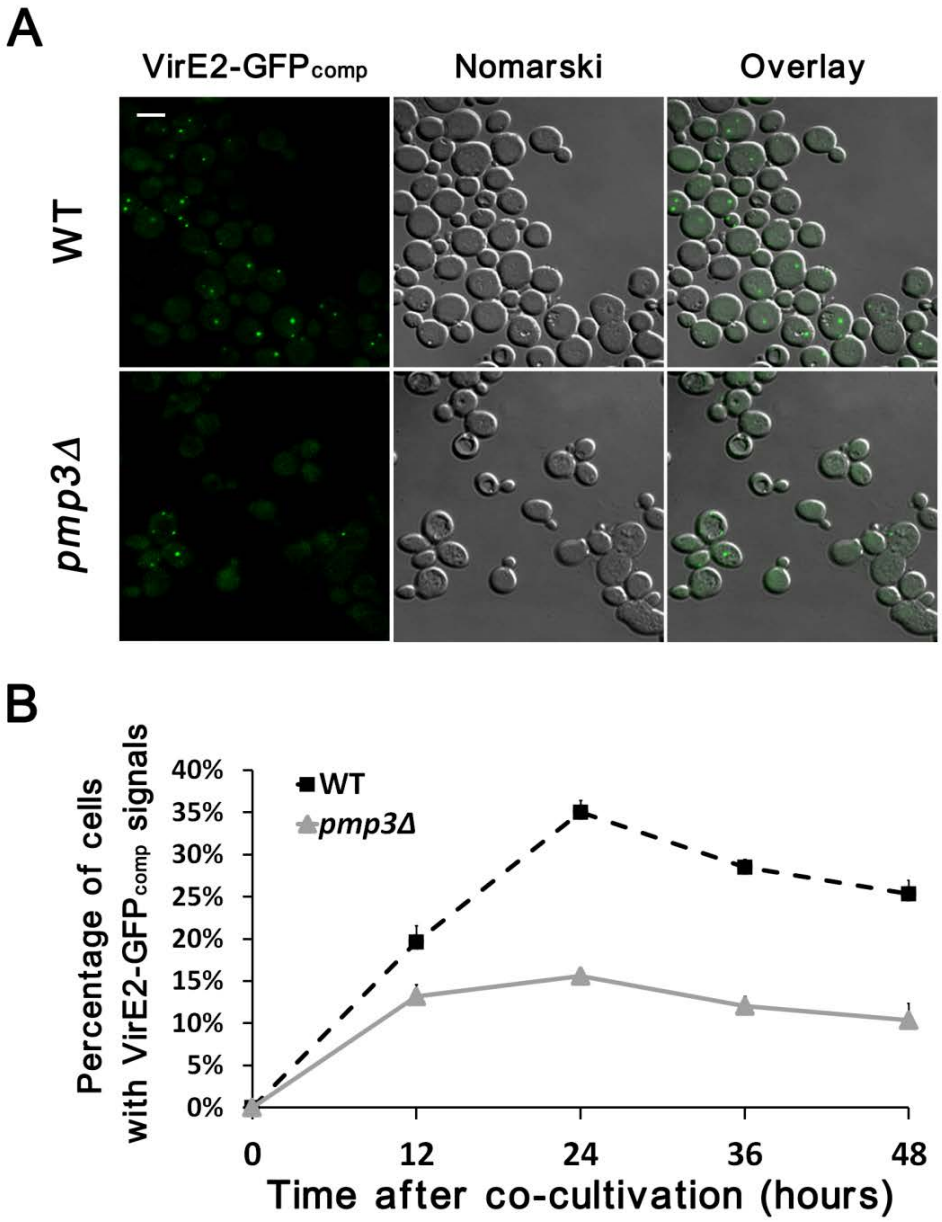


Figure 4.7. VirE2 translocation is affected in yeast mutant *pmp3*Δ during AMT process.

(A). Cells of *pmp3*Δ and wild type strains were harvested and observed under a fluorescence microscope. Scale bar represents 5 μm. (B). Quantitative comparison of the ratio of cells with VirE2-GFP$_{comp}$ signals between *pmp3*Δ and wild type strains in a time course study.

The AMT experiments were carried out as described above, *Agrobacterium* EHA105*virE2::GFP11* was co-cultivated with yeast *pmp3*Δ or wild type strain respectively. Co-cultivation spots on CM plates were wash off at an interval of 12 hours for totally 48 hours after co-cultivation; and the cells were observed under a fluorescence microscope. Percentages of yeast cells containing VirE2-GFP_{comp} signals were then compared between *pmp3*Δ and wild type.

As shown in Figure 4.7, ratio of cells containing VirE2-GFP_{comp} signals was calculated after recording using the fluorescence microscope; the ratio increased in both wild type strain and *pmp3*Δ with the elongation of co-cultivation time during the first 24 hours and reached a peak at 24 hours post co-cultivation. The slightly decreased efficiency after 24 hours might result from the increasing dividing speed of yeast cells. Interestingly, VirE2 protein delivered into the *pmp3*Δ showed a decreased pattern compared to the wild type strain at each time point.

This suggests that the VirE2 protein delivery process might be affected in the mutant *pmp3*Δ. Considering that both the T-DNA and other virulence proteins delivery share a conserved recognition and delivery mechanism to pass the host cell membrane, all these T4SS substrates delivery might also be affected and this probably could explain the decreased transformation efficiency displayed by *pmp3*Δ.

4.5. Discussion

The process of *Agrobacterium*-mediated transformation inside bacterial cell has been well studied (Cascales *et al.* 2003) while little is known about the host factors that are also believed to be essential in the transporting, nucleus targeting and T-DNA integration. Using *Saccharomyces cerevisiae* as a new model turns out to be another new way to study the role of host factors in AMT process (Bundock *et al.* 1995; Piers *et al.* 1996). The relatively short life cycle, easy manipulation, completely sequenced genome and available mutant library make the yeast a suitable model in AMT studies.

Plenty of useful studies have been carried out using *S. cerevisiae* to reveal the

role of host factors during AMT process, including purine synthesis related proteins (ADE1, ADE2, ADE4, ADE5,7, ADE6, and ADE8), non-homologous end-joining proteins (Yku70, Rad50, Mre11, Xrs2, Lig4 and Sir4), recombination/repair proteins (Rad51 and Rad52), histone acetyltransferases (GCN5, NGG1, YAF9 and EAF7) and histone deacetylases (HDA2, HDA3 and HST4) (Bundock *et al.* 1996; van Attikum *et al.* 2001; Roberts *et al.* 2003; Soltani *et al.* 2009).

In this study, a yeast membrane protein Pmp3p was shown to play an important role in *Agrobacterium*-mediated transformation of yeast; deletion of *PMP3* in yeast cells resulted in dramatically decreased transformation efficiency. In contrast, the mutant *pmp3*Δ displayed similar competence in LiAc based physical transformation, suggesting that the potential role of Pmp3p was specific for AMT.

Further experiments showed that nucleus targeting of VirD2, the pilot virulence protein which is mainly responsible for nucleus targeting of the T-complex, was not affected in the *pmp3*Δ mutant. However, VirE2 translocation assay showed that the *pmp3*Δ mutant was relatively less competent in receiving VirE2 during AMT process compared with the wild type strain BY4741. *Agrobacterium* delivers several virulence factors, including T-DNA and virulence proteins, into host cells during infection process; these virulence factors use the T4SS as a common secretion channel to get into host cytoplasm. Thus as the most abundant translocated virulence protein, the VirE2 could be used as an indicator to represent the T4SS secretion status. The decreased VirE2 translocation efficiency that has been observed in the *pmp3*Δ mutant might result from less efficient T4SS in AMT, which probably also lead to less acquisition of *pmp3*Δ in other virulence factors including the T-DNA. However, this still needs support from further experiments.

Yeast PMP3 protein is a small hydrophobic protein containing only 55 amino acids. It contains two potential trans-membrane domains in structure analysis, and this is further supported by the observation that the GFP tagged Pmp3p localized to the plasma membrane. Functional studies implied the potential role of Pmp3p in

regulating ion homeostasis of yeast especially in stressful growth conditions. The deletion of *PMP3* in yeast leaded to hypersensitivity to Na^+, which could be reversed by divalent cations in a dose dependent manner. Previous studies also hypothesized that the deletion of *PMP3* might lead to plasma membrane hyperpolarization and subsequent influx of monovalent cations (Navarre *et al.* 2000).

As a small membrane, the Pmp3p might not directly act as a receptor protein in either abiotic stresses perception or *Agrobacterium* attachment. However, this protein might affect the membrane status and plasma membrane potential directly or indirectly. Deletion of this protein in yeast might results in modification of plasma membrane compositions, which subsequently affect the initial attachment process of *Agrobacterium* thus affect the virulence factors translocation.

Chapter 5. Study of RCI2 family proteins in plant immunity responses

5.1. Introduction

Environmental stress tolerance of plants has long been an important research focus in agriculture studies, which mainly aims to develop optimal plant growth conditions as well as create stress-tolerant crops.

Plenty of useful results have been acquired from laboratory studies by imposing individual stress onto the tested plant; however, in the natural environment, the plants are usually exposed to a combination of different stress factors, including abiotic (cold, heat, salinity, drought, and nutrient stress) and biotic (viruses, bacteria, fungi, nematodes, and herbivorous insects) stresses. And plants have evolved to adapt to different combinations of various stresses by activating specific stress responses.

Interestingly, different responses of plants have been observed when encountering multiple stresses compared to each stress individually in both laboratory and field studies (Rizhsky *et al.* 2004; Mittler 2006). It has also been shown that the biotic and abiotic stresses could interact with each other both positively and negatively when simultaneously imposed onto the same plant (Atkinson *et al.* 2012). All of these imply the complexity of signaling pathways involved in plant stress responses.

Indeed, plant cells employ complex signaling networks in response to environmental stresses (Atkinson *et al.* 2012). Transcriptome analysis revealed that a variety of genes are regulated by both biotic and abiotic stresses, suggesting the existence of cross-talks between these signaling pathways (Kreps *et al.* 2002; Seki *et al.* 2002; De Vos *et al.* 2005; Swindell 2006; Kilian *et al.* 2007; Huang *et al.* 2008). The cross-talk and convergence of different signaling pathways of plant in response to various environment stresses might due to multiple roles of a variety of regulators

including transcription factors, MAPK cascades, heat shock factors, reactive oxygen species and small RNAs (Atkinson *et al.* 2012).

Plants adapt to diverse environmental conditions by activating both specific and general stress responses; and the reactions involved in biotic and abiotic stresses are interlinked within a broad network of signaling pathways. Study of controlling factors in stresses interactions could help to understand the underlying molecular basis as well as create super plants with broad-spectrum stress tolerance.

5.2. PMP3 protein family

5.2.1. PMP3 protein family in lower forms of eukaryotes and higher plants

The yeast PMP3 family represents a group of small molecular weight hydrophobic proteins that are highly conserved in lower forms of eukaryotes and higher plants (Figure 5.1).

These PMP3 family proteins has been shown to be related to stress tolerance, and the expression level of these genes in different plants could be transiently induced by various abiotic stresses such as low temperature, high salinity, dehydration, and exogenous abscisic acid (ABA); though it is relatively stable in yeast (Navarre *et al.* 2000; Medina *et al.* 2001; Inada *et al.* 2005; Medina *et al.* 2005; Morsy *et al.* 2005; Wang *et al.* 2006; Chang-Qing *et al.* 2008; Fu *et al.* 2012; Liu *et al.* 2012) (Figure 5.3B).

Figure 5.1. Sequence comparison of PMP3 family proteins.

PMP3 family protein sequences from different species including lower forms of eukaryotes and higher plants were compared using DNAMAN program. The accession numbers and sources are as follows: *PMP3* (*Saccharomyces cerevisiae*, X91499); *AcPMP3-1*, *AcPMP3-2* (*Aneurolepidium chinense*, AB161676, AB161677); *PutPMP3-1*, *PutPMP3-2*(*Puccinellia tenuiflora*, AB363567, AB363568); *OsLti6a, OsLti6b* (*Oryza sativa*, AY607689, AY607690); *BLT101*, *BLT101.2* (*Hordeum vulgare*, Z25537, AJ310995); *AtRCI2A, AtRCI2B* (*Arabidopsis thaliana*, AF122005, AF122006); *WPI6* (*Triticum aestivum*, AB030210). The numbers of residues are indicated to the right.

5.2.2. PMP3 family proteins in *Arabidopsis thaliana*

As one of the most commonly used model organisms for studying plant biology, *Arabidopsis thaliana* has a short life term and already has its entire genome sequenced. Moreover, changes in the plant could be easily observed, making it a very useful tool in studying plant stress tolerance and immunity.

Two tandemly organized homologous genes, *RCI2A* and *RCI2B*, of yeast *PMP3* were identified in *Arabidopsis* (Capel *et al.* 1997). Subsequent studies have identified another six new *Arabidopsis* genes (*RCI2C-H*) that showed high homology to *RCI2A* and *RCI2B* (Medina *et al.* 2007). These *PMP3* family proteins in *Arabidopsis* shared highly homologous regions (Figure 5.2) and their expression could be induced by various stress conditions including cold temperature, ABA, dehydration and high salinity (Medina *et al.* 2007) (Figure 5.3A).

Figure 5.2. Sequence comparison of *RCI2* family proteins in *A. thaliana*.

PMP3 family protein sequences in *Arabidopsis thaliana* were compared using DNAMAN program. The accession numbers and sources are as follows: *RCI2A* (AF122005); *RCI2B* (AF122006); *RCI2C* (AEE33434); *RCI2D* (AEC07522); *RCI2E* (AEE85790); *RCI2F* (AEE85792); *RCI2G* (AEE85439); *RCI2H* (AEE33434). The numbers of residues are indicated to the right.

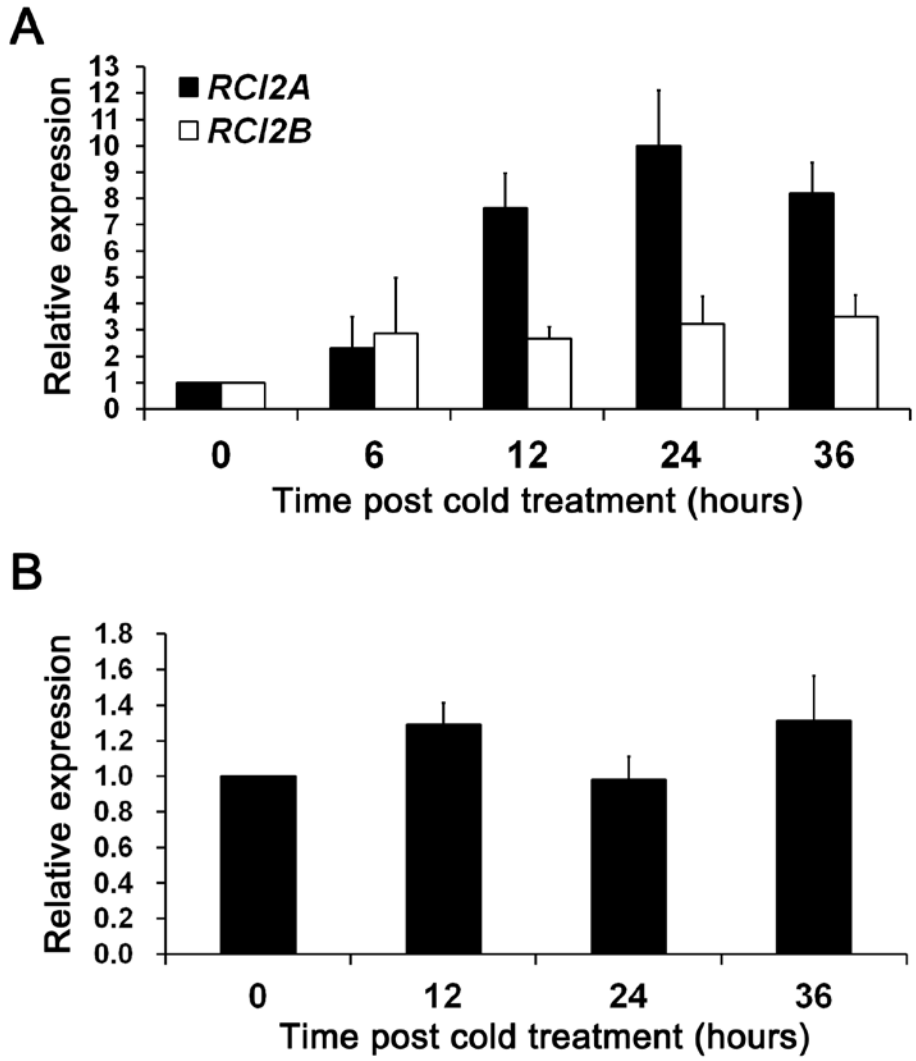


Figure 5.3. Expression patterns of *PMP3* family in response to cold treatment.

(A). Expressions of *RCI2A* and *RCI2B* in *Arabidopsis* leaves were induced by low temperature. The *Arabidopsis UBQ10* transcripts were used as the control. The primer sets UBQ10F/UBQ10R, RCI2AF/RCI2AR, RCI2BF/RCI2BR were used for *UBQ10*, *RCI2A* and *RCI2B* transcripts detection in qRT-PCR respectively. (B) Expression of *PMP3* in yeast is relative stable under cold treatment. The yeast *ACT1* transcripts were used as the control. The primer sets PMP3F/PMP3R and ACT1F/ACT1R were used for *PMP3* and *ACT1* transcripts detection in qRT-PCR respectively.

Besides, the *RCI2* family proteins could complement the yeast *PMP3* deletion mutant to a certain degree, indicating that the *PMP3* family proteins might shared conserved function in different species (Medina *et al.* 2007).

5.3. *Arabidopsis rci2a* mutant showed resistance to AMT

As shown in Chapter 4, yeast *pmp3*Δ mutant displayed decreased transformation efficiency in AMT assay. Thus as functional homologs, the possible roles of *Arabidopsis RCI2* family proteins in *Agrobacterium*-mediated transformation were also tested.

It has been shown previously that RCI2A is the most abundant expressed protein of the RCI2 family and it might play a major role in stress response in *Arabidopsis* (Mitsuya et al. 2005). Thus the role of RCI2A in AMT was tested in this study first.

The seeds of *Arabidopsis rci2a* mutant and wild type in Col-0 background were purchased from the *Arabidopsis* Information Resource (TAIR, http://arabidopsis.org). To confirm the elimination of *RCI2A* expression in the T-DNA insertional *rci2a* mutant, PCR based method was adopted. cDNA of *rci2A* mutant leaves was obtained as describd in Chapter 2; and specific primers were used to detect the *RCI2A* transcripts. As shown in Figure 5.4C, no *RCI2A* transcripts could be detected in homozygous *rci2a* mutant; the transcripts of *Arabidopsis UBQ10* were used as the loading control.

Arabidopsis root transformation assay was carried out as described in Chapter 2. Roots from 10-12-day-old *Arabidopsis* seedlings were cut into 3-5 mm fragments and co-cultivated with *Agrobacterium* strain A348 on 1/2 × MS agar plate. Tumor formation was carried out using the same plate with 100 μg mL^{-1} cefotaxime to kill the bacteria.

Interestingly, *rci2a* mutant displayed decreased transformation efficiency compared with wild type (Figure 5.4A-B), indicating that the RCI2A protein might be involved in *Arobacterium*-plant interaction.

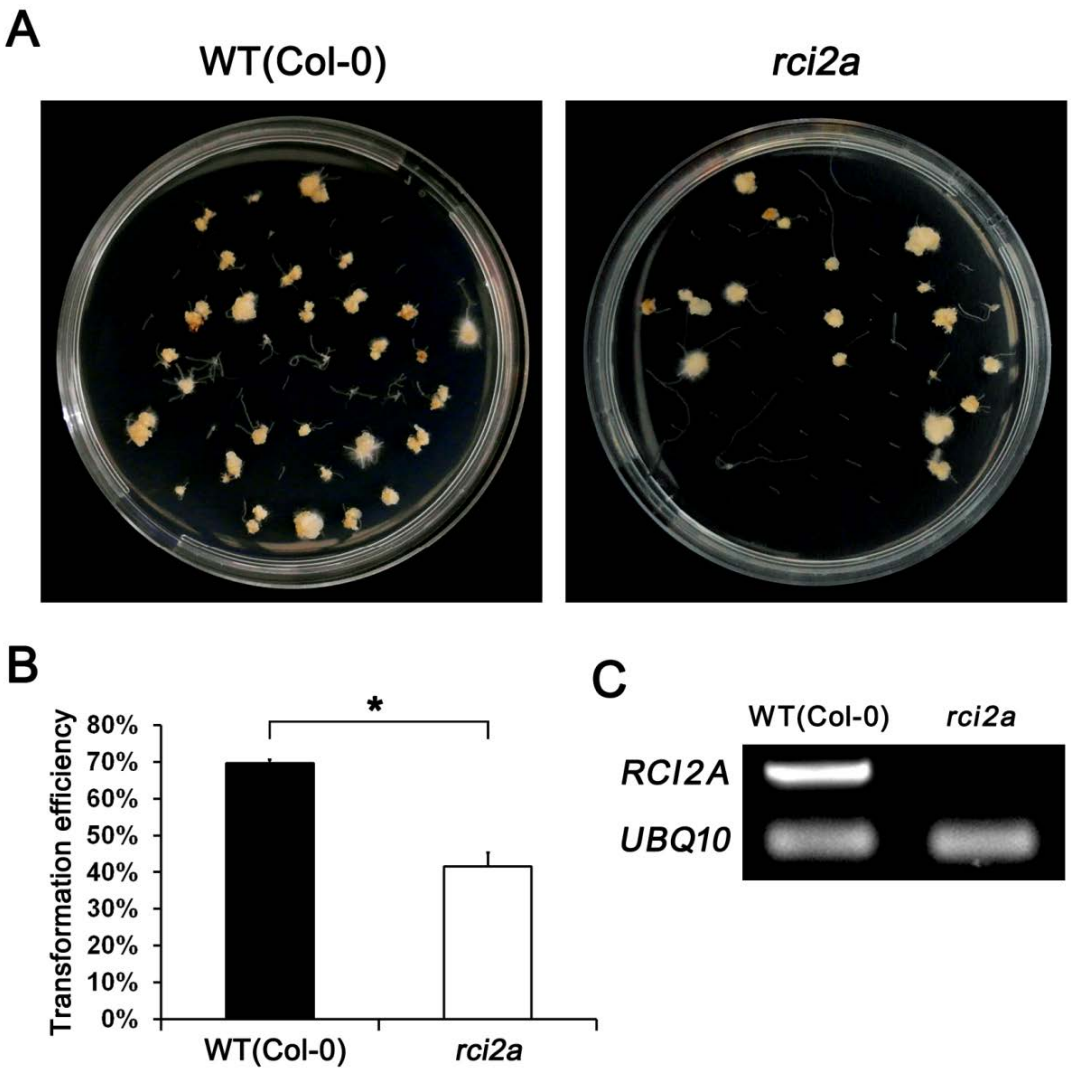


Figure 5.4. *Arabidopsis rci2a* mutant showed resistance to AMT.

(A). Root transformation assay of *Arabidopsis* rci2a mutant and wild type. 5×10^7 cells of *Agrobacterium* strain A348 were used in co-cultivation for each assay. Pictures were taken 4-5 weeks post co-cultivation. (B). Quantification of the root transformation efficiency. Root transformation efficiency was defined as the ratio of the number of root segments with tumors to the number of the total root segments. (C). Confirmation of the elimination of *RCI2A* expression in the T-DNA insertion *rci2a* mutant. cDNAs from both *rci2a* mutant and wild type were used for *RCI2A* transcripts detection. The *UBQ10* transcripts were used as loading control.

5.4. *Arabidopsis RCI2* family shows down regulated expression under biotic stress

As a group of genes concerning plant response to abiotic stress, it is interesting that the *RCI2* family is also related to *Agrobacterium*-mediated transformation. The possible response of *RCI2* family to biotic stress was also tested in this study.

5.4.1. *Arabidopsis RCI2* family showed down regulated expression pattern upon *Agrobacterium* infection

As a natural phytopathogen, *Agrobacterium* was used to impose biotic stress to the plants.

Agroinfiltration of *Arabidopsis* leaves was carried out as described in Chapter 2. In brief, *Agrobacterium* strain EHA105 was inoculated into MG/L medium for overnight culture. After sub-cultured into fresh MG/L to reach log phase (OD_{600} ~ 2.0), the cells were harvest and re-suspended in infiltration buffer at a final concentration of 5×10^8 cells/ml. The bacteria were infiltrated to the underside of 5-6-week-old *Arabidopsis* leaves (Col-0) using a syringe. The inoculated plants were then grown at 22 °C under a 16-hour photoperiod. Control experiments were carried out by infiltrating the infiltration buffer to a similar leaf from the same plant.

To detect the *RCI2* gene family transcription level, the leaves were cut out after 24 hours for total RNA extraction using TRIzol(R)-based method, qRT-PCR analysis was then carried out as described in Chapter 2. The transcripts of *Arabidopsis UBQ10* were used as the control.

Interesting, the expression of *RCI2* genes could also respond to biotic stress at transcription level. As shown in Figure 5.5A, the transcripts of *RCI2A*, *RCI2B*, *RCI2D*, *RCI2E* and *RCI2F* were down-regulated upon *Agrobacterium* infection. On the other hand, the *RCI2C*, *RCI2G* and *RCI2H* transcripts were found at very low levels in both *Arabidopsis* leaves and roots (data not shown), which was also in consistent with the previous observations (Medina *et al.* 2007).

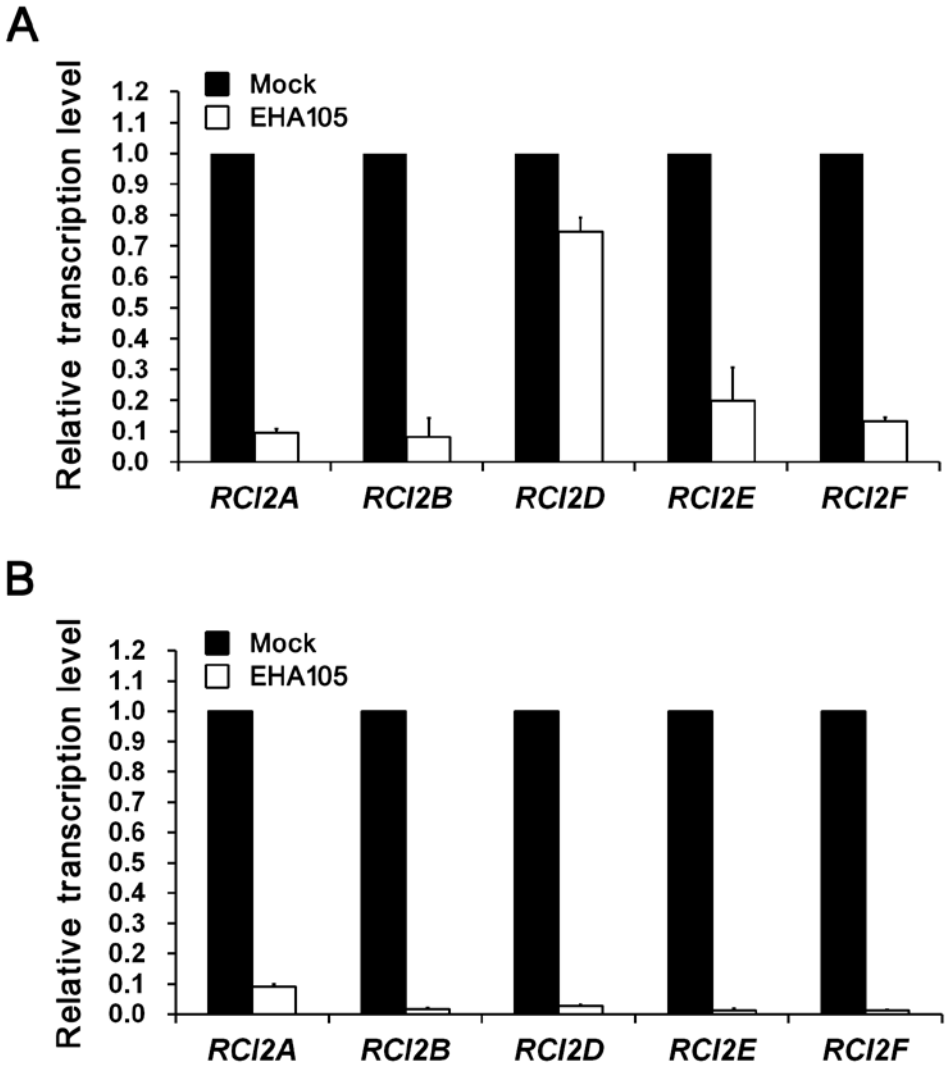


Figure 5.5. Down regulated expression pattern of *RCI2* family in *Arabidopsis* leaves and roots upon *Agrobacterium* infection.

(A). *RCI2* family displayed down regulated transcriptional level in *Arabidopsis* leaves 24 hours after *Agrobacterium* infection. Mock, infiltration buffer. (B). *RCI2* family displayed down regulated transcriptional level in *Arabidopsis* roots 24 hours after *Agrobacterium* infection. Mock, water. The *Arabidopsis UBQ10* was used as the control. The primer sets UBQ10F/UBQ10R, RCI2AF/RCI2AR, RCI2BF/RCI2BR, RCI2DF/RCI2DR, RCI2EF/RCI2ER and RCI2FF/RCI2FR were used for *UBQ10*, *RCI2A*, *RCI2B*, *RCI2D*, *RCI2E* and *RCI2F* transcripts detection in qRT-PCR respectively.

Besides, the expression of *RCI2* genes in *Arabidopsis* roots in response to *Agrobacterium* infection was also tested. *Arabidopsis* seeds (Col-0) were surface-sterilized with 15% bleach for 10-20 minutes and washed 4-5 times with H_2O. The seeds were then incubated at 4 °C for at least 2 days before germination on 1/2 × MS agar plates. The germination plates were then incubated at 22 °C under a 16-hour photoperiod for 10-12 days prior to *Agrobacterium* inoculation. *Agrobacterium* strain EHA105 was grown in MG/L to reach log phase (OD_{600} ~ 2.0), cells were then harvested washed twice with H_2O followed by re-suspension in 1 ml H_2O at the final concentration of 5×10^8 cells/ml. The bacteria suspension was then added onto the roots of the *Arabidopsis* seedlings for co-cultivation. The inoculated seedlings were then kept at 20 °C in dark. After 24-hour co-cultivation, the roots were cut out from the seedlings for RNA extraction. qRT-PCR analysis was then performed as described, the *Arabidopsis UBQ10* was used as a control.

Similar as the observation in leaves, expression of *RCI2* genes in *Arabidopsis* roots were also down-regulated upon *Agrobacterium* infection (Figure 5.5B).

5.4.2. *Arabidopsis RCI2* family showed down regulated expression pattern upon treatment with pathogen-associated molecular patterns

The above observations of the down regulated expression pattern of *RCI2* family upon *Agrobacterium* infection implied their roles in plant immunity responses. Interestingly, similar results have also been observed using an *Agrobacterium virD4* deletion mutant, which could not deliver either virulence protein or T-DNA into host cells. Agroinfiltration was carried out as described above using EHA105*ΔvirD4*, the down regulated expression pattern of *RCI2* family was also observed 24 hours after inoculation (Figure 5.6A).

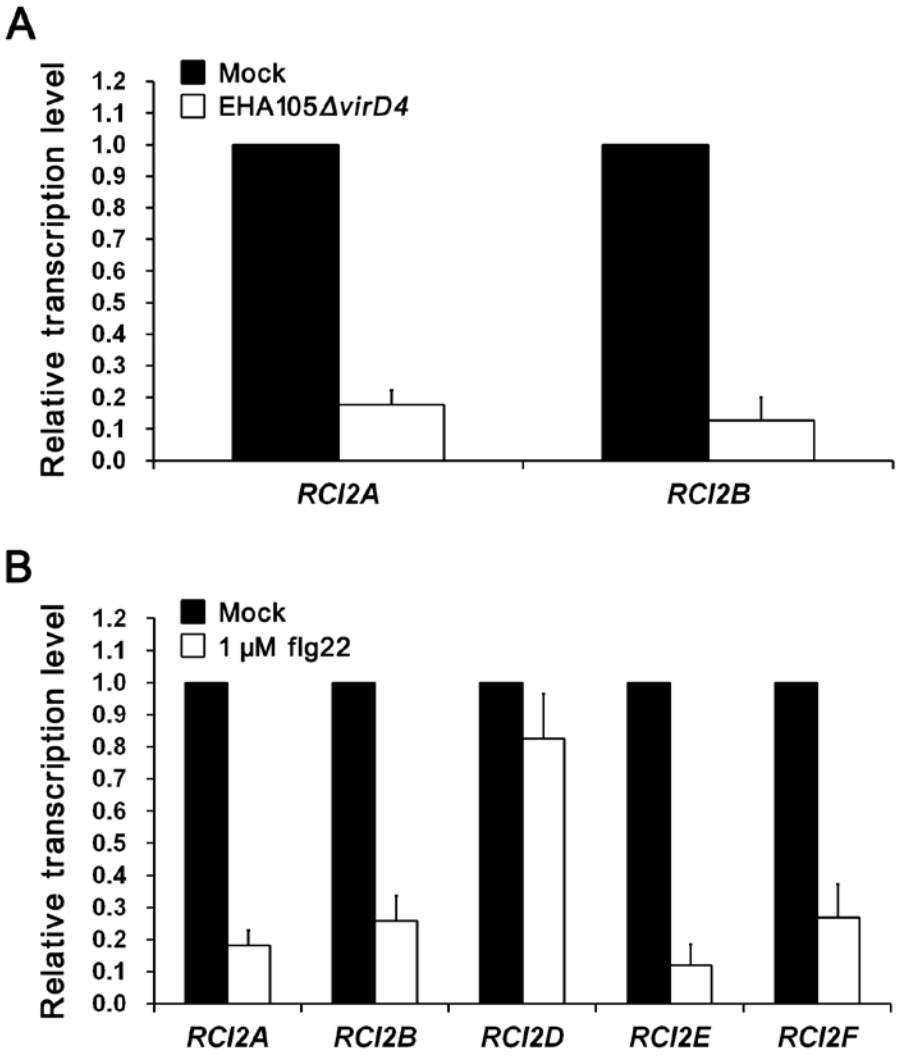


Figure 5.6. Down regulated expression pattern of *RCI2* family in *Arabidopsis* leaves could be induced by PAMPs.

(A). *RCI2A* and *RCI2B* displayed down regulated transcriptional level in *Arabidopsis* leaves 24 hours after agroinfection with EHA105*ΔvirD4*. Mock, infiltration buffer. (B). *RCI2* family displayed down regulated transcriptional level in *Arabidopsis* leaves 24 hours after infiltration with flg22. Mock, water. The *Arabidopsis UBQ10* was used as the control. The primer sets UBQ10F/UBQ10R, RCI2AF/RCI2AR, RCI2BF/RCI2BR, RCI2DF/RCI2DR, RCI2EF/RCI2ER and RCI2FF/RCI2FR were used for *UBQ10*, *RCI2A*, *RCI2B*, *RCI2D*, *RCI2E* and *RCI2F* transcripts detection in qRT-PCR respectively.

Plant immunity system could be activated through two separate ways, recognition of PAMPs by transmembrane surface receptors or recognition of pathogen effectors using NB-LRR proteins, and results in PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI) respectively. According to previous observations, the down regulation of *RCI2* genes expression might be part of the PTI responses.

The bacterial flagellin represents a typical elicitor of PTI and it triggered similar defense responses as PTI in various plants (Gomez-Gomez *et al.* 2002). To confirm that the regulation of *RCI2* genes results from PTI responses, a synthetic 22-amino-acid peptide (flg22) representing the conserved flagellin domain was adopted as the elicitor.

Synthetic flg22 peptide (AnaSpec) was dissolved in H_2O and infiltrated into 5-6-week-old *Arabidopsis* leaves at the final concentration of 1 μM. Total RNA was extracted 24 hours after infiltration as described above followed with qRT-PCR analysis.

As shown in Figure 5.6B, the *RCI2* genes displayed down regulated pattern after infiltration with flg22 similarly as agroinfiltration, confirming that such pattern might result from the PTI responses of plants.

5.5. Discussion

Yeast PMP3 family is a group of small hydrophobic proteins that are highly conserved in lower forms of eukaryotes and higher plants. Expression of these proteins could be transiently induced by various abiotic stresses including low temperature, high salinity, dehydration, and exogenous ABA; thus they are hypothesized to be required for abiotic stress tolerance of plants (Navarre *et al.* 2000; Medina *et al.* 2001; Inada *et al.* 2005; Medina *et al.* 2005; Morsy *et al.* 2005; Wang *et al.* 2006; Chang-Qing *et al.* 2008; Fu *et al.* 2012; Liu *et al.* 2012).

A. thaliana contains eight homologs of yeast PMP3 protein (RCI2A-H); and these proteins shared similar induced expression pattern upon abiotic stresses

treatment (Medina *et al.* 2007). Interestingly, results from this study indicated that this group of proteins might also be involved in plant responses to biotic stresses. In the root transformation assay, an *rci2a* insertional mutant of *A. thaliana* displayed less susceptibility to *Agrobacterium*-mediated transformation, implying its role in plant-*Agrobacterium* interaction.

Among these eight RCI2 family proteins, RCI2A, RCI2B, RCI2D, RCI2E and RCI2F showed detectable transcription level in both *Arabidopsis* leaves and roots; while the transcripts of *RCI2C*, *RCI2G* and *RCI2H* were at very low levels. Moreover, all the detectable transcripts of *RCI2* family (*RCI2A*, *RCI2B*, *RCI2D*, *RCI2E* and *RCI2F*) showed down regulated transcriptional pattern upon *Agrobacterium* infection, implying the active regulation of these genes upon biotic stresses. Interestingly, the regulation of this *RCI2* family by plant showed antagonistic pattern in response to either biotic or abiotic stresses, suggesting that the plants adapted to different stresses through the complex signaling network.

Although all the *RCI2* family genes were actively down regulated upon agroinfiltration, distinct patterns have also been observed among these individual genes and different plant tissues. In *Arabidopsis* leaves, the *RCI2D* transcripts were only slightly down regulated in the assay compared with the others while the difference was much smaller in the roots. These indicate that these *RCI2* family members might share both communal and distinct regulation mechanisms in different plant tissues. Moreover, the above regulation pattern could also be elicited by the flg22 peptide, suggesting the involvement of PAMP-triggered immunity responses in such regulations.

The PMP3 family is a group of conserved proteins that shared similar physiological functions in various species. Previous studies implied the role of these proteins in ion homeostasis thus they are probably related to the ion channels regulation on the plasma membrane. In plants, ion channels are a group of membrane proteins share various physiological functions, including ion homeostasis,

environmental stresses perception and signal transduction (Hedrich 2012). Various ion channels have been suggested to play important roles in signaling transduction and early responses in plant immunity system (Demidchik *et al.* 2007; Ma 2011; Moeder *et al.* 2011; Roelfsema *et al.* 2012). Active regulation of *RCI2* family in plants might be related to these membrane channels thus play a part in the immunity responses.

Chapter 6. Conclusions and future prospects

6.1. Conclusions

Agrobacterium tumefaciens has long been used as a genetic manipulation tool for plants and non-plant species because of its lateral-gene-transfer ability. Although the transformation process was well studied inside the bacteria, the part in the host cells remains unclear due to the complexity of the process which requires the participation of both bacterial and host factors. This study mainly focus on the transformation process inside recipient cells, including bacterial virulence factors trafficking as well as study of host proteins that is potentially involved in the AMT.

In this study, a novel VirE2 labeling approach involving a Split-GFP system was developed. This approach was shown to be suitable for study of VirE2 *in vivo*, thus turned out to be a new way to study this virulence factor during natural transformation process especially inside the recipient cells. By using this system, VirE2 behaviors in both natural host plant and non-natural host yeast have been successfully observed. *Agrobacterium*-delivered VirE2 localized in plant nucleus while it stayed cytoplasmic in yeast cells, indicating that specific host factors are necessary for nucleus targeting of VirE2 in recipient cells. Surprisingly, VirE2 delivery turned out to be a very efficient process in both natural host and non-natural host of *Agrobacterium*, though the transformation efficiencies differed considerably. The efficient VirE2 translocation by *Agrobacterium* suggests that this bacterium might be a much more efficient protein transporter rather than a genetic engineer concerning the non-natural hosts. Thus the protein delivery ability of *Agrobacterium* should be more emphasized in the future.

All these observations indicate that the newly developed Split-GFP system is suitable for VirE2 study inside host cells, which might also shed light on the study of other virulence factors.

On the other hand, this study also focused on the host factors involved in the AMT process. A yeast membrane protein Pmp3p was identified to play important role

during the transformation. This protein specifically affects the AMT results and subsequent experiments showed that the bacterial virulence protein delivery process was affected in the *PMP3* deletion mutant.

As a conserved membrane protein, the role of *PMP3* family in *Arabidopsis thaliana* was also examined in this study. Interesting, the *RCI2A* insertional mutant of *Arabidopsis* also displayed resistance to *Agrobacterium*-mediated transformation. Further experiments showed that the *RCI2* family members were actively adjusted upon *Agrobacterium* infection or flg22 treatment, implying their important role in plant immunity responses.

6.2. Future prospects

In this study, a novel *Agrobacterium* VirE2 labeling approach has been developed, which was proved to be suitable for VirE2 study *in vivo*. By using this Split-GFP tagging system, detailed VirE2 trafficking studies inside host cells are promising in the future, which may provide extremely useful knowledge for the mechanism of *Agrobacterium*-mediated transformation. Moreover, this Split-GFP system turns out to be a suitable approach for the study of translocated bacterial proteins, thus extension of this labeling approach to the other *Agrobacterium* virulence factors might also be possible.

On the other hand, preliminary studies of yeast PMP3 family proteins revealed their potential roles during AMT process. However, how these membrane proteins are involved in bacterial-host interaction still needs further investigations. Thus the mechanism concerning the role of these PMP3 family proteins need to be addressed in the future studies.

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