A GLOBAL PROFILE OF YEAST GENES INVOLVED IN *AGROBACTERIUM*-YEAST GENE TRANSFER

TU HAITAO

(B. Agri.)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOLOGICAL SCIENCES

NATIONAL UNIVERSITY OF SINGAPORE

2010

ACKNOWLEDEMENTS

First of all, I would like to express my deepest gratitude to my supervisor, Associate Professor Pan Shen Quan, not only for giving me the opportunity to conduct this promissing project but also for his practical and professional guidance and encouragement throughout my PhD candidature.

My sincere gratitude also goes to Prof. Wong Sek Man and A/P Yu Hao for their patient instructions and general support to my PhD research. I also extend my appreciation to Ms Tan Lu Wee and Ms Xu Songci for their unselfish help and support to my PhD studies. I aso want to thank Mr Yan Tie and Ms Tong Yan for their technical assistance in fluorecent and confocal microscopy.

I would also like to thank the following friends and laboratory members who have helped me in different ways: Chang Limei, Hou Qingming, Seng Eng Khuan, Guo Minliang, Li Xiaobo, Zhang Li, Tang Hock Chun, Alan John Lowton, Sun Deying, Qian Zhuolei, Sheng Donglai, Yu Hongbing, Li Mo, Niu Shengniao, Zhang Xin, Yang Qinghua, Nguyen Cong Huong, Haripriya Bathula, Chen Zikai, Wang Bingqing, Li Xiaoyang, Lim Zijie, Anthony Tran, etc.

Moreover, I must thank my parents, my sister and my girlfriend for their moral support and persistent encouragement in the hard years of my research and life.

Finally, I gratefully acknowledge the financial support provided by National University of Singapore.

TABLE OF CONTENTS

SUMMARY

Agrobacterium tumefaciens can transfer a piece of DNA (transferred DNA or T-DNA) into natural host plant cells as well as a wide range of laboratory hosts of eukaryotic cells, including *Saccharomyces cerevisiae*. The *Agrobacterium*-eukaryote gene transfer process involves a number of genes of both bacterial and eukaryotic origins. Many of the *A. tumefaciens* factors involved in the process have been well characterized. However, much remains unknown about the host genes and factors involved in T-DNA transfer.

In the current project, a novel system has been developed that can rapidly identify *S. cerevisiae* genes involved in the *Agrobacterium*-yeast gene transfer. Using this system, we screened the entire yeast knockout mutant library and identified 209 yeast genes that are important for the T-DNA transfer. Mutations at some of these genes reduced the transformation efficiencies dramatically, while mutations at other genes increased the efficiencies significantly. The studies revealed for the first time that several host processes such as TCA cycle and posttranslational modifications are involved in *Agrobacterium*-yeast gene transfer pathways. In particular, the glycosylation pathway components like the *OST3* gene and the components of oligosaccharyltransferase (OST) complex positively regulate the *Agrobacterium*-mediated transformation (AMT) process.

Ost3p is the gamma subunit of the oligosaccharyltransferase (OST) complex of the endoplasmic reticulum (ER) lumen, which catalyzes N-linked glycosylation of

nascent proteins. Human homolog of yeast *OST3*, *N33,* the candidate tumour suppressor, has been found to be involved in many important cellular biological processes and human disorders. Examinations of other components of the OST complex revealed that some other components also affect the AMT efficiency to some extent. This result revealed the involvement of the OST complex and probably the glycosylation process in the *Agrobacteirum*-eukaryote gene transfer process.

Further studies showed that Ost3p neither glycosylates VirD2 nor regulates the VirD2 nuclear targeting; however, Ost3p physically interactes with VirD2. These results indicate that the effect of Ost3p on AMT process is not directly through glycosylation of VirD2 or on the VirD2 nuclear targeting. Interestingly, OST3 was found to be important in the VirE2 import. In addition, two downstream glycoproteins were found to be absent in *ost3*Δ mutant under the *Agrobacterium*-yeast co-cultivation conditions. All these data imply that the effects of *OST3* on AMT process may be due to the effect of its downstream glycoproteins, which probable affect the VirE2 importing process.

The study provides the first evidence showing the *OST3* gene and the OST complex are important for *Agrobacterium*-yeast gene transfer process and may provide additional clues to cancer therapy. The global profile of the yeast genes involved in the AMT process facilitates a systemic approach to explore the molecular events involved in the process.

LIST OF MANUSCRIPTS

Tu H. and Pan S.Q. (2010) The yeast oligosaccharyltransferase complex regulates *Agrobacterium*-yeast T-DNA transfer. (Manuscript in preparation)

Tu H. and Pan S.Q. (2010) Yeast TCA cycle genes are important for *Agrobacterium*-yeast T-DNA transfer. (Manuscript in preparation)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	microgram(s)
µl	microliter(s)
µm	micrometre
A	adenosine
aa	amino acid(s)
Amp	ampicillin
AMT	*Agrobacterium*-mediated transformation
ARS	autonomous replication sequence
AS	acetosyringone
AYGT	*Agrobacterium*-yeast gene transfer
bp	base pair(s)
BSA	bovine serum albumin
C- terminal	carboxyl terminal
C	cytidine
Cb	carbenicillin
Cef	cefotaxime
CM	co-cultivation medium
ConA	lectin concanavalin A
DMSO	dimethylsulfoxide
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deooxyribonucleoside triphosphate
dsDNA	double-stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetet acetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
EX	Exocellular
FISH	fluorescent *in situ* hybridization
g	grams or gravitational force
G	guanosine
GFP	green fluorescence protein
GUS	β-D-glucuronidase
h	hour(s)
His	histidine
HRP	horseradish peroxidase
IM	inner membrane
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase(s) or 1000 bp
kDa	kilodalton(s)
Km	kanamycin
LB	Luria-Bertani medium or lift border

Leu	leucine
LiAc	lithium acetate
M	molar
MALDI	matrix-assisted laser desorption/ionization
MCS	multiple cloning site(s)
Met	methionine
mg	milligram(s)
µ	micro-
min	min(s)
ml	milliliter(s)
mM	millimole
Mpf	mating pair formation system
mw	molecular weight
N- terminal	amino terminal
N	aspiragine
n	nano-
NLS	nuclear localization signal
nm	manometer
nt	nucleotide(s)
OD	optical density
OM	outer membrane
ORF	open reading frame
OST	oligosaccharyltransferase
p	pico-
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl floride
PP	periplasm
R/r	resistant/resistance
RB	right border
RBS	ribosome-binding site(s)
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per min
sec	second(s)
S	sensitive/sensitivity
SAP	shrimp alkaline phosphatase
SD	standard deviation or synthetic dropout
SDS	sodium dodecyl sulfate
Spect	spectinomycin
ssDNA	single-stranded DNA
T	thymidine or threonine
T4SS	type IV secretion system
Tc	tetracycline
TCA cycle	tricarboxylic acid cycle
T-DNA	transferred DNA
	thiogalactoside
TOF	time of flight
Trp	trptophan
UV	ultraviolet
V	voltage
V/V	volume per volume
W/V	weight per volume

CHAPTER 1. LITERATURE REVIEW

The soil-borne *Agrobacterium tumefaciens* (*A. tumefaciens*) is a phytopathogen of many plant diseases, through transfering a piece of its DNA (Transferred DNA or T-DNA) into plant cells and causes neoplastic growths on host pants (crown gall or hairy root). Naturally, *A. tumefaciens* can only affect most of dicotyledonous plants and some of the monocotyledonous plants. Later on a lot efforts have been put on the pathogenicity research and host range of *A. tumefaciens* has been extended to other trans-kingdom species such as yeast (Bundock *et al.*, 1995), fungi (de Groot *et al.*, 1998), and mammalian cells (Kunik *et al.*, 2001).

After decades of intensive studies, the pathogenic factors and infection process have become relatively clear. Wounded plant cells release some signal chemicals, such as phenolics, sugars, and acetosyringone, which can be sensed by the *A. tumefaciens* membrane-spanning VirA/VirG two-component system (Loake *et al.*, 1988; Bourret *et al.*, 1991; Nixon *et al.*, 1986). The activation of the VirA/VirG two-component system triggers the expression of a series of virulence (*vir*) genes, which are located on the tumor inducing (Ti) plasmid, and thus the production of virulence proteins. One fragment of the transferred DNA (T-DNA) of the Ti-plasmid is processed by endonuclease VirD2 with the help of VirD1 (Albright *et al.*, 1987). Then the single stranded linear T-DNA and the VirD2 form a T-complex, which is transferred to host cells through a type IV secretary channel.

As compared to the relatively clear pathogenic factors, only few host factors involved in the T-DNA transfer process were reported. The main reason for the relatively lagging research on the host factors may be the lack of good host models and screening methods. Since the successful development of *A. tumefaciens*-mediated

transformation of *Saccharomyces cerevisiae* (*S. cerevisiae*) system by Bundock *et al.* in 1995, it has become an ideal model for studying host factors because yeast is the simplest eukaryote that can be readily manipulated and has a relatively small genome size. Moreover, the genome data and many gene libraries are available, which can facilitate rapid gene identification and characterization.

Although *Agrobacterium*-yeast gene transfer technology has been studied for about 15 years, understanding of basic general gene transfer process and the *Agrobacteium* gene transfer system are still very important. This chapter gives an overview of the general biological features of *A. tumefaciens* and *S. cerevisiae*, the basic *A. tumefaciens*-mediated transformation (AMT) process of *S. cereciasie,* and the current studies of host factors involved in the AMT process.

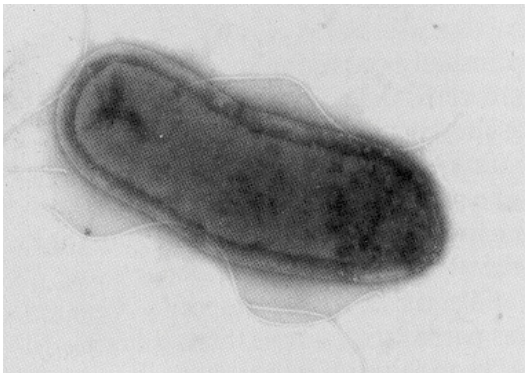
1.1. Introduction of *Agrobacterium tumefaciens*

1.1.1. Taxonomy of *Agrobacterium*

The genus *Agrobacterium* belongs to Bacteria kingdom, proteobacteria phylum, alpha proteobacteria class, rhizobiales order, and rhizobiaceae family. According to its plasmid-borne pathogenicity and host range (Kerr *et al.*, 1978), the genus *Agrobacterium* has been divided into several species: *A. radiobacter* is avirulent (Conn, 1942); *A. tumefaciens* causes crown gall disease (van Larebeke *et al.*, 1975; Watson *et al.*, 1975); *A. rhizogenes* causes hairy root disease (Willmitzer *et al.*, 1980; Tepfer, 1984); *A. rubi* causes cane gall disease (Starr and Weiss, 1943); and *A. vitis*, causes galls on grape and some other plant species (Otten *et al.*, 1984). However, sometimes this classification is not very accurate because it only based on the disease symptomology and host range. Firstly, curing the Ti plasmid in *A. tumefaciens* strain, such as pTiC58 in *A. tumefaciens* C58, can change the strain to nonpathogenic.

Secondly, simply introducing the Ti plasmid from *A. tumefaciens* to *A. rhizogenes* can alter the disease symptom on plants, such as from a crown gall formation to a hairy root formation disease (Costantino *et al.*, 1980). Thus, the term "species" becomes meaningless here. Therefore, another genus *Agrobacterium* classification system, the "biovar" system, was created by Keane *et al.* based on growth and metabolic characteristics (Keane *et al.*, 1970). According to this system, most *A. tumefaciens* and *A. rubi* belong to biovar I, *A. rhizogenes* stains belong to biovar II, and *A. vitis* stains belong to biovar III. The detailed classification is listed in Table 1.1. In addition to these two systems, another taxonomic classification system for *Agrobacterium* has been proposed by Young and *et al.* (Young *et al.*, 2001). More importantly, the completion of the whole genome sequencing of *A. tumefaciens* C58 may provide molecular basis and a better starting point for reclassification of the *Agrobacterium* strains in the future (Goodner *et al.*, 1999; Goodner *et al.*, 2001).

1.1.2. Biological features of *A. tumefaciens*



A. tumefaciens was first discovered and identified as the plant crown-gall disease pathogen by Smith and Townsend in 1907 (Smith and Townsend, 1907). They had described the biological characters of *A. tumefaciens* in details. *A. tumefaciens* is gram negative, non-gas-forming, and aerobic soil-borne bacterium without forming endospores. *A. tumefaciens* is rod shaped with rounded ends, usually about 0.6-1 μm in diameter and 1.5-3.0 μm in length and may exists singly or in pairs. It is also a motile organism with 1 to 6 peritrichous flagella (Smith and Townsend, 1907). In carbohydrate-containing culture, *A. tumefaciens* cells produce large amounts

of extracellular polysaccharides, giving colonies a voluminous, slimy appearance. On agar plate, colonies are usually round, smooth, and semitransparent. After several days growth on rich medium, colonies become viscous and milky. *A. tumefaicens* can grow at a wide temperature range from 5 ^{o}C to 46 ^{o}C and the optimal temperature for growth is 30 ^{o}C (Bell, 1990). Under optimal conditions, *A. tumefaicens* cells divide every 40 min.

A. tumefaciens is the causal pathogen of crown-gall disease in over 140 species of dicotyledonous plants in more than 60 different plant families. *A. tumefaciens* may live saprophytically in soil for up to two years. When a nearby host plant is wounded, *Agrobacterium* chemotactically moves into the wound site. The bacterium then inserts a piece of its T-DNA located on its Ti plasmid into the host cells' chromosomes (Schell and van Montagu, 1977), causing overproduction of cytokinins and auxins which are plant growth regulators, and opines which serve as nutrients for the *Agrobacterium* (Agrios, 1988). This resulting tissue continues to grow and a tumor is formed on the root or stem of the plant. Agrobacteria occupy the intercellular spaces of the gall. The tumor is not protected by an epidermis thus susceptible to secondary invaders. Degradation of the tumor by secondary invaders may release *A. tumefaciens* cells back into the soil.

Table 1.1. Taxonomy of *Agrobacterium.*

(Adapt from Young, 2008)

Species names based on natural classification			Species names based on pathogenicity	
After Young et al. (2001)	After Holmes and Roberts (1981); Bradbury (1986); Holmes (1988); Moore et al. (2001)	After Keane et al. (1970); Kerr and Panagopoulos (1977); Panagopoulos et al. (1978)	After Allen and Holding (1974); Skerman et al. (1980)	After Kersters and De Ley (1984)
R. larrymoorei[a]	*A. larrymoorei*[b]	NR[c]	NR	NR
R. radiobacter (Ti)	*A. tumefaciens*[d] (tumorigenic)	*A. radiobacter* biovar *tumefaciens* (biotype 1)	*A. tumefaciens*	*A. tumefaciens* (biovar 1)
R. radiobacter (Ti)	*A. tumefaciens*[d] (tumorigenic)	*A. radiobacter* biovar *tumefaciens* (biotype 1)	*A. tumefaciens*	*A. tumefaciens* (biovar 1)
R. radiobacter (Ri)	*A. tumefaciens*[d] (rhizogenic)	*A.* radiobacter biovar *rhizogenes* (biotype 1)	*A. rhizogenes*	*A. rhizogenes* (biovar 1)
R. radiobacter	*A. tumefaciens* (non-pathogenic)	*A. radiobacter* biovar *radiobacter* (biotype 1)	*A. radiobacter*	*A. radiobacter* (biovar 1)
R. rhizogenes (Ti)	*A. rhizogenes* (tumorigenic)	*A. radiobacter* biovar *tumefaciens* (biotype 2)	*A. tumefaciens*	*A. tumefaciens* (biovar 2)
R. rhizogenes (Ri)	*A. rhizogenes* (rhizogenic)	*A. radiobacter* biovar *rhizogenes* (biotype 2)	*A. rhizogenes*	*A. rhizogenes* (biovar 2)
R. rhizogenes	*A. rhizogenes* (non-pathogenic)	*A. radiobacter* biovar *radiobacter* (biotype 2)	*A. radiobacter*	*A. radiobacter* (biovar 2)
R. rubi[b]	*A. rubi*[b]	*A. radiobacter* biovar *tumefaciens* (biotype 2)	*A. rubi*	*A. rubi*
R. vitis (Ti)	*A. vitis* (tumorigenic)[e]	*A. radiobacter* biovar *tumefaciens* (biotype 3)	NR	*A. tumefaciens* (biovar 3)
R. vitis	*A. vitis* (non-pathogenic)	NR	NR	NR

[a] Oncogene designations are indicated when necessary for clarity; [b] Only tumorigenic (Ti) capability has been reported for this species; [c] NR = not recorded; [d] The correct name for this species is *A. radiobacter* (see Young et al., 2006; Sawada et al., 1993); [e] Moore et al. (2001)

1.1.3. *Agrobacterium* genome

From late 1990s, large portions of the *Agrobacterium* genome, including the entire Ti plasmid, had been sequenced by many research groups (Barker *et al.*, 1983; Slightom *et al.*, 1986; Thompson *et al.*, 1988; Rogowsky *et al.*, 1990; Suzuki *et al.*, 2000; see review on Gelvin, 2009). Finally in 2001, two research groups, Goodner *et al.* and Wood *et al.*, have completed the whole nucleotide sequence of the nopaline-type strain *A. tumefaciens* C58 indepently (Goodner *et al.*, 2001; Wood *et al.*, 2001). It was found that the C58 genome has a total of 5.67 Mb (Hamilton and Fall, 1971), including 4 replicons (Allardet-Servent *et al.*, 1993): a circular chromosome, a liner chromosome, a pAtC58 plasmid (also called the "cryptic plasmid"), and a pTiC58 plasmid (Goodner *et al.*, 2001; Wood *et al.*, 2001).

Around 5500 genes, some of which are RNA genes, were found to be encoded by *A. tumefaciens* C58. Most of the genes essential for its survival are located on the circular chromosome. During evolution process some essential genes have migrated to the linear chromosome. The ends of the linear chromosomes are protected by a telomere that forms a covalently closed hairpin (Goodner *et al.*, 2001). Most of the genes important for its pathogenesis as well as the T-DNA itself are located on the Ti plasmid. It is believed that some genes essentail for opine catabolism, the energy source of *A. tumefaciens*, are located on pAtC58 (Vaudequin-Dransart *et al.*, 1998), which is important for its pathogenesis and survival on host plant. The complete genome composition is listed in Table 1.2. The whole-genome sequence of *A. tumefaciens* C58 provides a basis for more extensive analyses of this important phytopathogen. Sequence analysis revealed that C58 has 308 pathways, 1199 enzymatic reactions, 17 transporters, 54 protein complexes, 3735 transcription units, and 57 tRNAs (Goodner *et al.*, 2001; Wood *et al.*, 2001).

In addition to the biovar I *A. tumefaciens* C58 strain, DNA sequence analysis of other *Agrobacterium* species has been conducted, such as *A. radiobacter* biovar II strain K84 and the *A. vitis* biovar II strain S4 (Slater *et al.*, 2009). Comparison of the genome sequences of several agrobacterial species has opened the door for the study of the evolutionary process of these organisms and provided information on systems involved in pathogenesis, symbiosis, and biological control (Gelvin, 2009). One important finding is that chromosomes may evolve from plasmids in many of these bacteria (Setubal *et al.*, 2009). The genome sequences of more *Agrobacterium* species will continue to increase, leading to substantial insights into the evolutionary history and function of plant-associated microbes (Setubal *et al.*, 2009).

Table 1.2. *A. tumefaciens* C58 genome composition

Replicon	Total Genes	Protein Genes	RNA Genes	Size (bp)
Circular chromosome	2,833	2,785	48	2,841,490
Linear chromosome	1,895	1,876	19	2,075,560
pAtC58	543	543	0	542,780
pTiC58	198	198	0	214,234
Total	5,469	5,402	67	5,674,064

(Adapt from http://www.*Agrobacterium*.org/, by Romero P. and Karp P.D.)

1.1.4. A brief history of research on *A. tumefaciens*

In 1853, Fabre and Dunal wrote the first report discribing the crown gall disease (Fabre and Dunal, 1853). In the late 19th and early 20th century, crown gall disease was a serious problem in horticultural industry in both America and Europe (Galloway, 1902). Later it was found that this disease greatly reduced the production of many horticultural crops (Kennedy, 1980), including cheery (Lopatin, 1939), apple (Ricker *et al.*, 1959), and grape (Schroth *et al.*, 1988). Although *Agrobacterium vitis* was identified as the causal agent of crown gall in grape in 1897 (Cavara 1897), the causal agent for crown gall disease of other plant species was still unknow. Smith and Townsend suspected bacteria as the causal agent for the crown gall disease; however, their speculation could not be proved due to experimental design and limited understanding of bacteria as plant pathogen (Smith and Townsend, 1911). At that time no one knew the exact causal agent of the crown gall disease. In 1907, after conducting a series of critical investigation, Smith and Townsend finally confirmed the bacterial origin of crown gall disease (Smith and Townsend, 1907). They named the bacterium as *Bacterium tumefaciens* and later renamed it to *Agrobacterium tumefaciens (A. tumefaciens).* Smith's work built solid foundation for later *Agrobacterium*-related studies. Thereafter, the intense interests and studies of *A. tumefaciens* began.

In 1940s and 1950s, White and Braun made several milestone discoveries concerning the *Agrobacterium*-mediated transformation of plants cells. They demonstrated that only transient exposure to *A. tumefaciens* could cause persist of tumor inducing phonotype even in the absence of the causing agent *A. tumefaciens* and thereafter proposed an *Agrobacterium*-derived tumor-inducing principle (TIP) (Braun, 1947; Braun and Mandle, 1948). Later opines were identified exclusively in

tumor tissue after affected by *A. tumefaciens* (Lioret, 1956). However, the TIP was still elusive until molecular techniques provided the first clue that crown gall tumors contained DNA from *A. tumefaciens* origin (Schilperoort *et al.*, 1967). In 1974, a large "tumor-inducing" (Ti) plasmid was indentified to be essential for *A. tumefaciens* virulence and the TIP is probably a component of the Ti plasmid (Zaenen *et al.*, 1974; van Larebeke *et al.*, 1974). Southern hybridization revealed that a DNA fragment on Ti plasmid is present in the genome of crown gall tumors and the genes required for tumor formation and opine production are encoded by Ti plasmid, which led to the discovery of T-DNA (transferred DNA), a Ti plasmid fragment tranferred from *A. tumefaciens* to plant cells (Chilton *et al.*, 1977; Chilton *et al.*, 1978; Depicker *et al.*, 1978). In 1980, opine concept was proposed by Guyon *et al.* that the synthesis of opines by transformed plant cells creates an ecological niche and exclusive carbon and nitrogen source for the infecting *Agrobacterium* (Guyon *et al.*, 1980).

In the following years, *A. tumefaicens*-mediated transformation had been extensively studied. In 1983, Zambryski *et al.* constructed a versitile Ti plamid vector, which allows the introduction of any DNA of interest into plant cells, thus created first transgenic plant using *A. tumefaciens* as a vector (Zambryski *et al.*, 1983). The healthy transgenic plants are capable of transmit the disarmed T-DNA including the foreign genes to their progeny. Later, effective selective techniques had been invented for accurate selection of transformed plant cells (Bevan, 1984). For example non-plant antibiotic-resistance genes like bacterial kanamycin-resistance gene could be driven by a plant-active promotor to function efficienctly in plant cells. The success of using *A. tumefaciens* as a gene vector to create transgenic plants and efficient selection methods had made the *A. tumefaciens* as a powerful tool for creating transgenic plants and thus a promising candidate for crop improvement. In the 1990's,

maize, a monocot and previously thought to be recalcitrant to *A. tumefaciens* infection, was successfully transformed by *A. tumefaciens* (Chilton, 1993).

From then on, many agronomically and horticulturally important plant species were transgenically improved by *A. tumefaciens*, and the list of plant species that can be transformed by *A. tumefaciens* grows daily (for review see Gelvin, 2003). At present, many economically important crops, such as corn, soybeans, cotton, canola, potatoes, and tomatoes, were improved by *A. tumefaicens*-mediated transformation and their transgenic varieties are growing worldwide (Valentine, 2003). Some of the important crops, such as maize (Ishida *et al.*, 1996), rice (Chan *et al.*, 1992; Chan *et al.*, 1993; Hiei *et al.*, 1994; Dong *et al.*, 1996; Rashid *et al.*, 1996; Toki, 1997), barley (Tingay *et al.*, 1997), and wheat (Cheng *et al.*, 1997), were transgenically modified to be antibiotic or herbicide resistance. In the meanwhile, besides plants cells, the species susceptible to *A. tumefaciens*-mediated transformation has been extended to other species, ranging from other bacteria, virtually all classes of plants(Lacroix *et al.* 2006), yeast (Bundock *et al.*, 1995; Piers *et al.*, 1996), fungi (de Groot *et al.*, 1998), and even mammalian cells (Kunik *et al.*, 2001).

Back to 1980's, while the host species suspectible to *A. tumefaciens*-mediated transformation grows daily, the studies on molecular basis inside the *A. tumefaciens*-plant transformation had also been explored exclusively. In 1984, sequence analysis revealed that two T-DNA gene products that mediate oveproduction of auxin and cytokinin are important for plant tumorigenesis (Klee *et al.*, 1984; Lichtenstein *et al.*, 1984). In 1986, Stachel and Nester (Stachel and Nester, 1986), and other researchers identified the VirA/VirG two component regulatory system which controls virulence (*vir*) gene expression and also discovered a single stranded DNA intermediate (T-strand) during the transformation process (Stachel *et al.*, 1985, Stachel *et al.*, 1986,

Stachel and Zambryski, 1986; Stachel *et al.*., 1987). Those landmark works set the basis for work on the virulence region for the next two decades. Due to intensive work by a lot of researchers, many plant genes involved in *A. tumefaciens* transformation have been identified and their roles on the gene transfer process have gradually been elucidated (Usami *et al.*, 1987; Sahi *et al.*, 1990; Zupan, *et al.*, 2000; Gelvin, 2000; Tzfira and Citovsky, 2002). Finally, the whole genome sequencing of the nopaline-type strain *A. tumefaciens* C58 opened the door for more extensive analyses of this biological and agricultural important phytopathogen (Goodner *et al.*, 2001; Wood *et al.*, 2001). At the same time, another species, *Saccharomyces cerevisiae*, has been successfully transformed by *A. tumefaciens* and used a modern model for studying the host factors involved in the T-DNA transfer process inside host cells for its fast growth rate, ease of manipulation, and variety of commercially available strain libraries (Bundock *et al.*, 1995; Piers *et al.*, 1996; Roberts *et al.*, 2003).

In addition to the application as a gene transfer vector to create transgenic plants, *A. tumefaciens* is also a wonderful model system to study other biological processes, including detection of host signaling chemicals (Winans, 1992), intercellular transfer of macromolecules (Christie, 2001), importing of nucleoprotein into plant nuclear (Ziemienowicz *et al.*, 2001), and interbacterial chemical signaling via autoinducer-type quorun sensing (Newton and Fray, 2004).

1.2. *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is the only known species, which is capable of trans-kingdom gene transfer. With the advance of understanding and genetic modification, *A. tumefaciens* has become a powerful gene-delivery vector to transfer genetic materials to other kingdoms of species, including its natural host-plant cells, as well as other organisms such as yeast (Bundock *et al.*, 1995; Piers *et al.*, 1996),

Fungi (de Groot *et al.*, 1998), and mammalian cells (Kunik *et al.*, 2001). The following sections briefly review the *Agrobacterium*-mediated transformation of other eukaryotic organisms.

1.2.1. *Agrobacterium*-mediated transformation of plant

Since it has been identified as the pathogen agent of plant crown gall disease at 1907 (Smith *et al.*, 1907), *A. tumefaciens*-plant T-DNA transfer process and molecular basis of the T-DNA transfer have been intensively studied. As the natural plant pathogen, *A. tumefaciens* was found to infect many perennial horticultural crops and significantly reduced their crop yield, including cheery (Lopatin, 1939), apple (Ricker *et al.*, 1959), and grape (Schroth *et al.*, 1988). Although the natural hosts for *A. tumefaciens* are dicots, it was later found that some of monocots were able to be transformed by *A. tumefaciens*. In 1993, maize, a monocot plant species, which was thought to recalcitrant to *A. tumefaciens*-mediated transformation, was successfully transformed by *A. tumefaciens* (Chilton, 1993). Nowadays, the list of plant species that can be genetically transformed by *A. tumefaciens* grows daily (Gelvin, 2003). Naturally, its host range covers various species of plant kingdom and more that 600 types of plants (Gelvin, 2003), including 56% of the gymnosperms and 58% of angiosperms including 8% of monocotyledons.

Other than the host range, extensive studies have been focused on mechanism and molecular basis inside the transformation process. Pioneer studies have revealed that the crown gall pathogenisis is initially caused by a tumor-inducing (Ti) plasmid (van Larebeke *et al.*, 1974; Zaenen *et al.*, 1974). Later studies futher confirmed that the bacterial encoding genes for tumor formation were located within the Ti plasmid, which was referred as the transferred DNA (T-DNA) (Chilton *et al.*, 1977; Chilton *et*

al., 1978; Depicker *et al.*, 1978). The whole T-DNA transfer process was later explored out after extensive research. Firstly, wounded plant cells release some signal chemicals, such as phenolics, sugars, and acetosyringone, which can be sensed by the nearby *A. tumefaciens'* membrane-spanning VirA/VirG two-component system (Loake *et al.*, 1988; Bourret *et al.*, 1991; Nixon *et al.*, 1986). The activation of the VirA/VirG two-component system triggers the expression of a series of virulence (*vir*) genes, which are located on the Ti plasmid, and thus the production of virulence proteins. One fragment of the T-DNA of the Ti-plasmid is processed by endonuclease VirD2 with the help of VirD1 (Albright *et al.*, 1987). Then the single stranded linear T-DNA and the VirD2 form a T-complex, which is transferred to host cells through a type IV secretary channel. After successful transferring into plant cells, T-DNA encodes enzymes for synthesis of plant hormones such as auxin and cytokinin. Accumulation of plant hormones causes uncontrolled cell proliferation, thus forms tumors at the wound sites on the stems, crowns and roots. More details about the transformation process will be discussed in the later section (Section 1.3).

In 1980s, it was demonstrated that the only sign for T-DNA is the left border and right border, thus wild-type T-DNA coding region can be replaced by any DNA sequence without any effect on its gene transfer ability. Therefore, understanding of the molecular basis of T-DNA production and the transfer process leads to the establishment of *A. tumefaciens*-mediated transformation as an important transgenic biotechnique. In 1983 first transgenic plant was created by *A. tumefaciens*-mediated transformation (Zambryski *et al.*, 1983). It was proved that the integration and expression of foreign T-DNA in plant cells did not interfere with the normal plant cell growth and could transmit the disarmed T-DNA, including the foreign genes, to their

progeny (Zambryski *et al.*, 1983). Since then, *A. tumefaciens* had become a modern gene delivery vector to create transgenic organisms.

To date, *A. tumefaciens*-mediated transgenic technique has been widely used in agriculture and horticulture. Many economical important crops were modified by *Agrobacterium*-mediated transgenic technology, such as tobacco (Lamppa *et al.*, 1985), rapeseed (Charest *et al.*, 1988), potato (Stiekema *et al.*, 1988), maize (Chilton, 1993), rice (Hiei *et al.*, 1994), soybean (Chee and Slightom, 1995), pea (Schroeder *et al.*, 1995), wheat (Cheng *et al.*, 1997), and barley (Tingay *et al.*, 1997).

1.2.2. *Agrobacterium*-mediated transformation of yeast

After decades of intensive studies, the bacterial pathogenic factors and infection process have become relatively clear. Almost all of virulence (*vir*) genes and some of chromosome genens are important for whole pathogenic process, such as host signal recognition, *vir* gene induction, T-DNA process, type IV secretion channel (T4SS) formation, and T-DNA delivery. Meanwhile, it is believed that besides bacterial factors, some host factors may also play roles in the T-complex transfer and integration process inside host cells. However, host factors involved in the *Agrobacterium*-mediated T-DNA transfer process remains unclear. One major problem is the slow growth rate, long life cyle, limited library source, and the difficulties in manipulation of the plant system. Hence plant mutants are not so easily to be tested out for their impacts on the *A. tumefaciens*-mediated transformation.

In 1995, Bundock *et al.* reported that the budding yeast *Saccharomyces cerevisiae,* belonging to another kingdom other than plant, can be transformed by *A. tumefaciens* under laboratory conditions (Bundock *et al.*, 1995). The successful T-DNA transfer from *A. tumefaciens* to *S. cerevisiae* provides an important eukaryotic

model for host factor studies involved in the *A. tumefaciens*-yeast T-DNA transfer process because of the advantages plant system cannot provide. *S. cerevisiae* has relative rapid growth rate replicating every 90 min under optimal conditions, which significiantly reduces the waiting period as compared to plant system. *S. cerevisiae* is easy to handle, very similar to bacterial manipulation. In addition, *S. cerevisiae* has a relatively small genome (about 5.7 Mb and about 5,800 genes) and whole genomic sequence of *S. cerevisiae* is available online. Moreover, different kinds of libraries are commercially available, such as gene deletion library, GFP fusion library, TAP-tagged gene library, and etc. All of those features assure *S. cerevisiae* as an ideal model organism to study host factors involved in the *A. tumefaciens*-yeast T-DNA transfer process.

Similar to *A. tumefaciens*-plant T-DNA transfer, requirements for T-DNA transfer to yeast cells relies on the sufficient *vir* gene induction, T-DNA borders, *vir* genes, and T-DNA processing process, which suggests that *A. tumefaciens*-yeast transformation process shares common T-DNA transfer mechanism with *A. tumefaciens*-plant transformation process. As indicated in their studies, plant wound signal, the phenolic *vir* gene inducer acetosyringone (AS) is absolutely required for AMT of yeast process (Bundock *et al.*, 1995; Piers *et al.*, 1996). Although plasmid transfer from *A. tumefaciens* to yeast cells can also be achieved by conjugative transfer which is regulated by yeast *tra* system (Sawasaki *et al.*, 1996), border sequences and T-DNA processing process is required for the AMT process (Piers *et al.*, 1996). In the plasmid trasmission, even the whole Ti plasmid is not required, so clearly the two kinds of gene transfer process share different mechanisms. In addition, similar to plant gene trasfer, *vir* mutant strains, such as *virD1/virD2, virA, virG, virE2, and some of the virB* mutants showed attenuated or no T-DNA transfer ability

to yeast (Bundock *et al.*, 1995; Piers *et al.*, 1996), which also indicates the T-DNA process and the T4SS are indispensable for AMT to yeast. However, chromosome virulence genes, such as *chvA, chvB*, and *exoC,* important for *A. tumefaciens*-plant cell attachment and gene transfer in the *A. tumefaciens*-plant gene tranfer are not important in the *A. tumefaciens*-yeast gene transfer system (Piers *et al.*, 1996).

Although *A. tumefaciens*-yeast gene transfer shares very similar meachanism with *A. tumefaciens*-plant gene transfer, their T-DNA integration processes are different. In plant species, T-DNA integration occurs predominantly via non-homologous recombination (illegitimate) (Offringa *et al.*, 1990), however, in yeast system, introduced T-DNA predominantly integrates into genome via homologous recombination (Bundock *et al.*, 1995; Bundock and Hooykaas, 1996). If T-DNA contains certain homologous sequence to yeast genome, it will replace the homologous region in yeast genome. If no homologous region exists between yeast and T-DNA, T-DNA will insert into yeast genome randomly (van Attikum *et al.*, 2001). The non-homologous recombination of T-DNA implies the involvement of the host factors in the T-DNA integration process (Bundock *et al.*, 1995). Indeed, it was later found that two yeast proteins, Rad52 and Yku70 are the key regulators in both of the homologous recombination and non-homologous recombination T-DNA integration process (van Attikum and Hooykaas, 2003).

Until now, T-DNA transfer passway inside host cells and host factors involved in the T-DNA transfer process remain unclear. Yeast provides as a good eukaryotic organism to study the transport, targeting, and integration of the T-DNA in the host cells.

1.2.3. *Agrobacterium*-mediated transformation of fungi

Similar to yeast cells, some filamentous fungal species were also successfully transformed by *A. tumefaciens* (de Groot *et al.*, 1998). It was demonstrated that *A. tumefaciens*-mediated transformation of fungal cells shares almost the same genetic requirements with *Agrobacterium*-yeast T-DNA transfer. Furthermore, T-DNA integration process in fungal cells is also regulated by homologous or non-homologous recombinations (de Groot *et al.*, 1998; Gouka *et al.*, 1999). Because of the simplicity, high transformation efficiency, stable transformants, and randomly T-DNA insertion, *Agrobacterium*-mediated transformation was used as a tool to perform large scale mutagenesis and study functional genomics in fungi (Michielse *et al.*, 2005).

1.2.4. *Agrobacterium*-mediated transformation of mammalian cells

With the daily expansion of *Agrobacterium* host range, in the year 2001, the species susceptible to *Agrobacterium*-mediated transformation was finally extended to another kingdom-mammalian cells (Kunik *et al.*, 2001). It was shown that *A. tumefaciens* was able to deliver and further integrated T-DNA into the HeLa cell genome. Further studies proved that T-DNA tumorgenesis important genes such as *virA, virB, virG, virD, virE, chvA,* and *chvB* were crucial for *A. tumefaciens*-Hela cell gene transfer, which indicates that *A. tumefaciens*-mediated HeLa cell transformation follows a similar mechanism to *A. tumefaciens*-mediated trasnformation of plant cells (Kunik *et al.*, 2001).

Although *Agrobacterium*-HeLa cell transformation process shares many common features with *Agrobacterium*-plant cell gene transfer process, they do have several different aspects. Firstly, *A. tumefaciens*-plant T-DNA transfers at room temperature, normally below 28 ^{o}C. In addition, the *vir* genes cannot be induced at a temperature higher that 32 ^{o}C because such as high temperature may change the

confirmation of VirA and thus inactivates its regulating properties (Jin *et al.*, 1993). As contrast, it was found that *A. tumefaciens* was still able to transform HeLa cells at 37^{o}C. Such a high temperature may also destabilize the VirB pilli and disable the transforming capability of *A. tumefaciens* for plant cells. Furthermore, it was demonstrated that *vir* genes induction was not required for *Agrobacterium*-HeLa cels gene transfer. In plant system, without sufficient *vir* gene induction, the T-DNA transfer machinery cannot be established and thus *Agrobacterium* cannot transfer DNA into plant cells. Therefore, the machinery inside *Agrobacterium*-HeLa cell T-DNA transfer process is still a mystery and requires further studies.

1.3. General process of *A. tumefaciens*-mediated T-DNA transfer

Understanding *A. tumefaciens*-mediated T-DNA transfer process is very crucial in studying host factors involved, T-DNA transport pathway inside host cells, and T-DNA integration mechanism. The general process of *A. tumefaciens*-mediated T-DNA transfer is comprised of several critial steps: (1) Sensing of plant signals. Plant chemical signals are captured by the agrobacteial VirA/VirG two-component system, which regulats the transcription of *vir* genes. (2) *vir* gene induction. *Vir* genes are transcripted upon the activation of VirA/VirG two-component system. (3) Attachment of *Agrobacterium* to host cells. *Agrobacteria* move toward and attach to host cells, which facilitate the T-DNA transfer process. (4) T-DNA processing and T-complex formation. T-DNA is nicked by VirD1/VirD2 from Ti plasmid and forms a single-strand (ss) linear T-strand, which later forms a T-complex with VirD2 and VirE2. (5) T-DNA transfer through type IV secretion system. *A. tumefaciens* cells attach to host cells then inject the T-complex to host cells through a VirD4/VirB type IV secretion system (T4SS). (6) Nuclear targeting and T-DNA integration. After transfering into host cells, T-DNA targets to host cell nuclear and integrates into host genome. This

section will discuss the general T-DNA transfer process in detail. The critical infection steps are summarized in Fig 1.1.

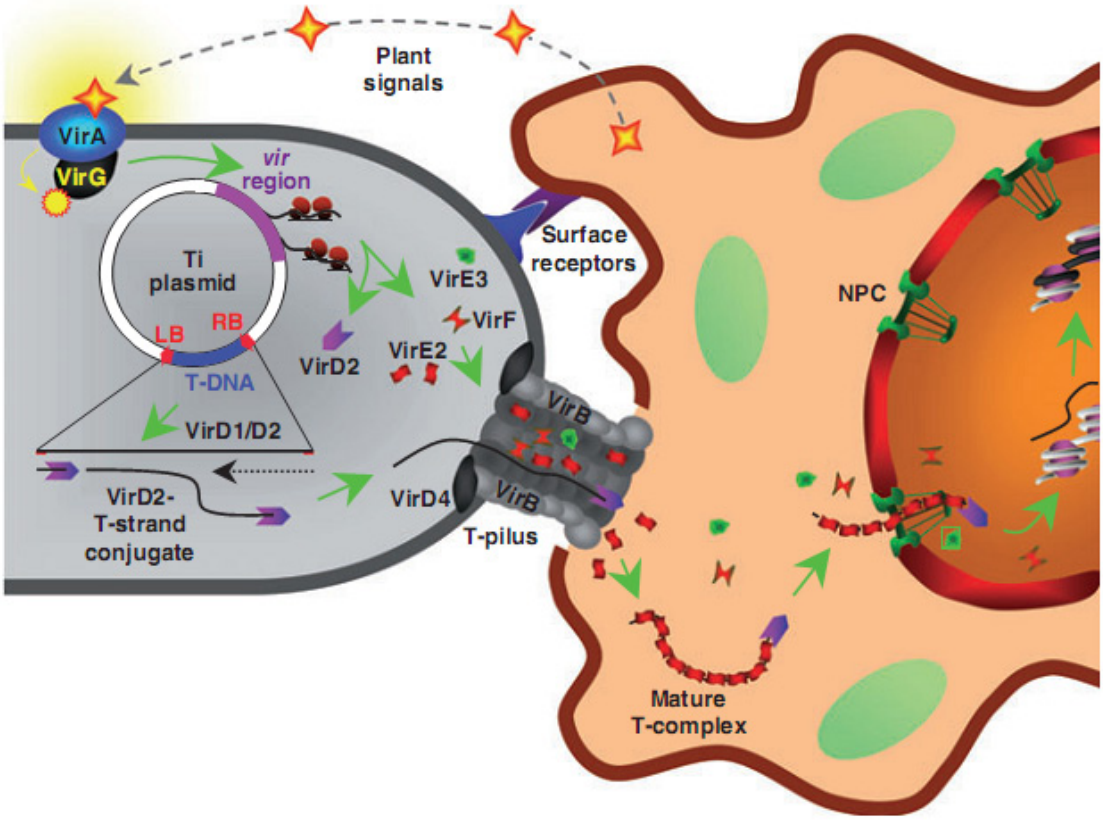


Figure 1.1. Summary of critical steps of the *Agrobacterium* infection process. The infection process starts from signals recognition from plant cells by the *Agrobacterial* VirA/VirG two-component system, which leads to the activation of the *vir* genes on the Ti plasmid and the attachment of *Agrobacteria* to plant cells. The T-strand is then nicked by virD2/VirD1 from T-DNA region and exported to plant cells via a VirB/D4 secretion channel together with VirD2, VirE2, VirE3, and VirF. The T-complex is then directed to host nuclear and integrates into host genome with the help of some host proteins. (Cited from Citovsky *et al.*, 2007)

1.3.1. Sensing of plant signals

A. tumefaciens is a motile organism and has peritrichous flagellae. It possesses a sensitive chemotaxis system, which responded to some sugars and amino acids and was attracted to these signals (Loake *et al.*, 1988). The motility and chemotaxis is critical to *A. tumefaciens* pathogenesis under natrual conditions (Hawes and Smith, 1989). It was found that genes responsible for most of the chemotactic responses in *A.*

tumefaciens seemed to be encoded on chromosome (Parke *et al.*, 1987; Loake *et al.*, 1988). Chemicals extracted from plant root cultures are strong attractants for *Agrobacterium* and some compounds possess *vir*-inducing activity. By screening some plant cell metabolites, researchers found that some plant phenolic compounds, such as acetosyringone (AS) and hydroxy-acetosyringone, are strong *vir* gene inducer (Bolton *et al.*, 1986; Stachel *et al.*, 1985). Most of the *vir*-inducing phenolic compounds are needed to make a plant cell wall polymer, the lignin. On the other hand, when plant phenolic compounds are absent or present in very low concentration, some monosaccharides, such as glucose, arabinose, galactose, xylose and fucose, may play crucial role in *vir* gene induction. Most of the *vir* gene induction sugars are monomers of plant cell wall polysaccharides. Later, it was proved that the regulation pathway for *vir* gene induction by phenolics is different from the pathway induced by sugars, which is mediated by a chromosomal gene encoded protein ChvE (Cangelosi *et al.*, 1990).

1.3.2. *vir* gene induction

Many genes are involved in the *A. tumefaciens* infection process, however, most of the genes required for T-DNA transfer are located on the *vir* region of Ti plasmid. The *vir* region consists of about 25 genes, including at least 6 essential operons (*virA, virB, virC, virD, virE,* and *virG*) and 2 non-essential operons (*virF and virH*). Each *vir* operon encodes different number of proteins. *virA, virF,* and *virG* encode one protein each; *virC, virE,* and *virH* encode 2 proteins each; *virD* encodes 4 proteins and *virB* encodes 11 proteins. Only *virA* and *virG* are constitutively expressed. The expression of all other *vir* operons is induced by plant phenolic compounds and some monosaccharides at acid environment and the *vir* gene expression is tightly regulated by the VirA/VirG two-component system. In vegetative growth *Agrobacterium*, only

virA and *virG* are highly expressed. However, in the presence of induction condition, the expressions of other *vir* genes are significantly induced to high levels (Engstrom *et al.*, 1987).

Besides plant signals, the inducers, infection environmental factors are also important, such as pH and temperature. The *vir* gene induction occurs at low pH, ranging from pH5.0 to 5.8, and does not happen at neutra pH. Athough it is hypothesized that the low pH may increase the membrane permeability, the mechanism of *vir* gene regulation by low pH remains unknow. ChvG, a chromosomally encoded protein, was identified as a global pH sensor which is important for the expression of *virB* and *virE* genes (Li *et al.*, 2002). Temperature also affects *vir* gene induction. The *vir* genes could not be induced at temperature higher than 32 ^{o}C because it may cause the conformational change of VirA and inactives its regulation function (Jin *et al.*, 1993). Moreover, high temperature may also destabilize the VirB pilli and thus affect the stability of T4SS.

The induction of *vir* genes by phenolic compounds and sugars is tightly regulated by membrane-spanning VirA/VirG two-component system (Bourret *et al.*, 1991). The presence of plant signals and acidic environment may induce autophosphorylation of VirA, a transmembrane receptor kinase. VirA activates the cytoplasmic VirG by transfering its phosphate to VirG. The activated VirG binds to *vir* box enhancer elements present in the promoters of the *virA, virB, virC, virD, virE*, and *virG* operon, and upregulates the transcription of these operons (Winans, 1992).

1.3.3. Attachment of *Agrobacterium* to host cells

To accomplish the T-DNA transfer to host cells, *A. tumefaciens* must contact and attach to host cells first. The attachment of *A. tumefaciens* to host cells occurs

prior to or concurrently with the *vir* gene induction process. The binding of *Agrobacterium* cells to host plant cells may require specific receptors that may exist on both bacterial and host cells (Lippincott and Lippincott, 1969). Many bacterial genes were found to be important in the attachment of *Agrobacterium* to plant cells (Reuhs *et al.*, 1997; Matthysse *et al.*, 2000). Suprisingly, all the genes reported to be involved in the attachment step are chromosomal genes.

Genes in two chromosomal regions of *A. tumefaciens*, which regulate a two-step attachment process (Matthysse and McMahan, 1988), are involved in the attachment of *Agrobacterium* to plant cells. The first binding step is loose and reversible because the bound bacteriual cells could be easily washed off from the binding sites by shear forces, such as washing or vortexing of co-cultivation cells. Genes involved in this step were identified to locate on the *att* gene region (more than 20 kb in size) of the bacterial chromosome. Gene mutations in this region cause avirulence of *Agrobacterium*.

Two different kinds of mutants may occur in the *att* gene region. Mutations in the first group occur in the genes homologous to ABC transporters and transcriptional regulater as well as some closely related downstream genes. Signal exchange between bacteria and host cells may be changed in this group of mutants. The attachment and virulence caused by this group can be easily restored by the supplementing of conditioned medium (Reuhs *et al.*, 1997; Matthysse and McMahan, 1998; Matthysse *et al.*, 2000). On the contrary, some mutants occurred in the *att* region cannot be reversed by the addition of conditioned medium. This group of genes is homologous to transcriptional regulator, ATPase, and biosynthetic genes, which may affect the synthesis of surface molecules important in the attachment of bacterial to host cells. (Reuhs *et al.*, 1997; Matthysse and McMahan, 1998; Matthysse *et al.*, 2000).

In contrast, the binding in the second step is tight because the bound bacteria cells cannot simply be removed from plant cell surface by shear forces. Cellulose fibrils genes (*cel* genes) were identified to be responsible for this binding step, which requires the synthesis of cellulose fibrils by bacteria and recruits larger numbers of bacteria to the wound sites. The *cel* genes are located on the bacterial chromosomal region, near but not contiguous with the *att* gene region (Robertson *et al.*, 1988). Bacterial mutants deficient in synthesis of cellulose showed attenuate virulence (Minnemeyer *et al.*, 1991).

In addition to *att* genes and *cel* genes, some othe chromosomal virulence genes, suchas *chvA, chvB,* and *pscA (exoC)* were suggested to indirectly involve in the bacterial attachment to host cells (Cangelosi *et al.*, 1987; Douglas *et al.*, 1982; O'Connell and Handelsman, 1989). These genes are important in the synthesis, processing, and export of a cyclic β-1, 2-glucan, which was supposed to involve in the bacterial attachment to plant cells. *chvA, chvB,* and *pscA (exoC)* mutants caused 10-fold decreased attachment betwwen *Agrobacterium* and mesophyll cells and significantly reduced virulence (Douglas *et al.*, 1985; Kamoun *et al.*, 1989; Thomashow *et al.*, 1987). ChvB is involved in the synthesis of the cyclic β-1, 2-glucan (Zorreguieta and Ugalde, 1986) and the virulence of chvB is temperature sensitive (Banta *et al.*, 1998). ChvA is involved in the export of the cyclic β-1, 2-glucan from cytoplasm to the periplasm and extracellular fluid (Cangelosi *et al.*, 1989; de Iannino and Ugalde, 1989).

1.3.4. T-DNA processing and T-complex formation

Following the *vir* gene induction, a series of downstream events occur. A linear single-stranded (ss) DNA fragment, the T-DNA or T-strand is produced by VirD2/VirD1. T-DNA is flanked by 2 highly homologous 25-bp direct repeat

sequences, the left border (LB) and the right border (RB). Because the T-DNA borders are the only elements required for T-DNA, thus any DNA fragment between LB and RB can be transferred into host cells. The RB is required for efficient tumorgenesis and acts in a polar fashion, directing the transfer of sequences to its left (Shaw *et al.*, 1984; Wang *et al.*, 1984). However, the LB is not necessary for tumor formation (Joos *et al.*, 1983). An enhancer (or overdrive) sequence, which enhances the T-DNA transfer efficiency, was found to be present near many T-DNA RB but not LB (Peralta and Ream, 1985; Peralta *et al.*, 1986).

T-strand is produced by the cleavage of VirD2/VirD1 on the bottom strand (the coding strand) of T-region at same positions between position 3 and 4 from the left end of each border (Albright *et al.*, 1987; Jayaswal *et al.*, 1987; Wang *et al.*, 1987). The T-strand production initiates at the T-DNA RB and elongates from 5' to 3' (Sheng and Citovsky, 1996). VirD2 may have nickase activity because purified VirD2 alone can cleave a single-stranded T-DNA border sequence *in vitro* (Pansegrau *et al.*, 1993; Jasper *et al.*, 1994). For cleavage of double-stranded (ds) border sequences, both VirD1 and VirD2 are required (Filichkin and Gelvin 1993; Yanofsky *et al.*, 1986; Scheiffele *et al.*, 1995). After cleavage of T-DNA border sequences, the ss gap of the T-region is repaired by a newly synthesized DNA strand. Meanwhile, VirD2 still attaches to the 5'-end of the ss T-Strand covalently. The association of VirD2 with the 5'-end of the ss T-strand is important for preventing the exonucleolytic attack to the 5'-end of the T-strand and labels the 5'-end as the leading end during the entire transfer process (Durrenberger *et al.*, 1989). Site-direct mutations study suggested that cyrosine 29 of VirD2 is required for the association of VirD2 with the 5'-end of T-strand (Vogel and Das, 1992). Meanwhile, Mg^{2+} is required for the VirD2 cleavage activity of border sequence.

The nopaline type VirD2 has 447 amino acids (aa) with a molecular weight (mw) of 49.7 kDa. The N-terminus has 90% homology from different *Agrobacterium* species, while only 26% homology was found in the C-terminus (Wang *et al.*, 1990). About 50% of VirD2 C-terminal sequence is dispensible for its endonuclease activity. However, there are two short nuclear localization sequences (NLS) present in the C-terminal region, which are thought to guide the T-complex to the plant nucleus. Sequence and functional domain comparison revealed that a conserved 14-residue motif, which is believed to coordinate the essential cofactor Mg^{2+} and is crucial for the endonuclease activity of VirD2 (Ilyina and Koonin, 1992).

VirD1 may assist the endonuclease activity of VirD2 through its interaction with the RB. Mutagenesis study of VirD1 indicated that aa 45~60 is important for VirD1 activity and this region is a potential DNA-binding domain (Vogel and Das, 1994). The interaction between VirD1 and RB could induce local double helix DNA destabilization and provided a single-strand loop substrate for VirD2 (Ghai and Das, 1989). Although VirD2 alone is enough for ss T-DNA borders, VirD1 is supposed to be important for the cleavage of T-borders on Ti plamids or supercoiled DNA substrates by VirD2.

In addition to VirD2/VirD1, two other Vir proteins, VirC1 and VirC2 were suggested to be involved in the T-DNA processing. VirC1 and VirC2 bind to the overdrive site of octopine-type Ti plasmid and enhance T-DNA border cleavage by the VirD2/VirD1 endonuclease (Toro *et al.*, 1989). Mutagenesis studies suggested that VirC proteins were important for *Agrobacterium* virulence (Stachel and Nester, 1986). Moreover, some studies suggested that VirC proteins may also play a role after T-DNA processing, possibly in T-DNA export (Zhu *et al.*, 2000b).

After processing and assiciation with VirD2, the T-strand is naked thus susceptible to nuclease degradation. To prevent nucleolytic degradation, the ss T-strand is coated by VirE2 forming the T-complex, composed of the T-strand DNA containing the 5'-associated VirD2 and coated VirE2 along its length (Gietl *et al.*, 1987; Sen *et al.*, 1989; Howard and Citovsky, 1990; Zupan and Zambryski, 1995). VirE2 is a single-stranded DNA-binding protein that can non-sequence-specifically and strongly bind single-stranded DNA, which suggests that VirE2 coats the T-strand along its length (Christie *et al.*, 1988; Citovsky *et al.*, 1988; Citovsky *et al.*, 1989). Plant cells expressing VirE2 could successfully transform *virE2* deficient *A. tumefaciens*, suggesting that VirE2 protein might function primarily in plant cells but not in *A. tumefaciens* cells (Citovsky *et al.*, 1992). In addition, *virE2* mutants could transfer T-DNA into plant cells, indicating that VirE2 is not essential for the export of T-DNA (Yusibov *et al.*, 1994). These evidences suggest that the coating of T-DNA by VirE2, which is not necessary for the tumorgenesis, may not occur inside the bacterial cells. VirE2 also has nuclear localization sequence, thus another possible function of VirE2 is to guide the nuclear import of T-DNA inside host cells (Ziemienowicz *et al.*, 1999; Ziemienowicz *et al.*, 2001).

Another VirE protein, VirE1, is essential for the export of VirE2 to plant cells and the export of VirE1 is independent (Binns *et al.*, 1995; Sundberg *et al.*, 1996). A VirE1-deficient mutant having normal amounts of VirE2 and T-strand is not infectious, however, the VirE1-deficient mutant can be complemented by the coinfection with a strain producing both VirE1 and VirE2 but lacking T-DNA. *In vitro*, VirE1 can strongly bind VirE2 and prevent VirE2 from self-aggregation. In addition, VirE1 may prevent VirE2 from binding to T-strand because VirE1 shares the same binding domain of VirE2 with single-stranded T-strand (Sundberg and

Ream, 1999). VirE1 decreases *virE2* translation without affecting its transcription, suggesting *virE1* regulates efficient translation of *virE2* (Zhao *et al.*, 2001). The fact that VirE1 is not required for the recognition of translocation signal of VirE2 by the type IV secretion system and translocation of VirE2 into plant cells indicates that VirE1 affects the export of VirE2 via stabilizing VirE2 by preventing it from premature bindings in the bacterial cell before translocation into plant cells (Vergunst *et al.*, 2003).

1.3.5. T-DNA transfer to host cells via a T4SS

After proper processing and packaging, the T-complex is transferred to host cells via a type IV secretion system (T4SS) together with some effectors, such as VirE2, VirF, and VirE3 (Cascales and Christie, 2003; 2004). Agrobacterial T4SS is highly homologous to the conjugal transfer (tra) system of IncN plasmid pKM101 (Burns, 2003; Christie and Vogel, 2000). T4SS is also known as the mating pair formation (Mpf) apparatus. The T4SS is normally composed of 11-13 core proteins, forming a pore or channel, through which DNA and proteins are delivered from the donor cell to recipient cell. T4SS is a big family with increasing members, among which the *A. tumefaciens* VirB/VirD4 T4SS is the best characterized system (Cascales and Christie, 2003; Christie and Cascales, 2005).

T-DNA is transferred as an ssT-strand-protein complex. As stated in the previous section, the VirD2 associates to the 5'-end of the ssT-strand and thus forms the VirD2-ssT-strand complex in *A. tumefaciens* cell right after the T-strand processing. On the other hand, VirD2-ssT-strand-VirE2 is important for T-complex tranfer in host cell but the coating of T-DNA by VirE2 in bacterial cell is not required for tumor formation. Thus, the question is whether T-DNA is transferred from *A. tumefaicens* to host cell in the form of VirD2-ssT-strand complex or in the form of

VirD2-ssT-strand-VirE2 complex (Zupan and Zambryski, 1997). Three models were proposed to depict the T-DNA transfer from *Agrobacterium* to plant cell (please read Zupan and Zambryski, 1997 for review).

The first model, the most accepted model, is that VirE2 binds to the ssT-strand inside *A. tumefaciens* cell and T-DNA is transferred in the form of VirD2-ssT-strand-VirE2 complex (Christie *et al.*, 1988; Citovsky *et al.*, 1989; Zambryski, 1992; Citovsky *et al.*, 1997). However, most of the recent data appear to support the second model, in which VirE2 could be exported independtly and then coats the naked VirD2-ssT-strand complex inside plant cell (Simone *et al.*, 2001; Schrammeijer *et al.*, 2003; Atmakuri *et al.*, 2003; Cascales and Christie, 2004). Nevertheless, same questions still need to be addressed in the second model, such as how does the export apparatus distinguish VirE2 and the naked VirD2-ssT-strand to regulated their independent export if VirE2 uses the same VirB/D4 export channel, and how VirE2 is maintained at high concentration in order to favor the efficient coating of VirE2 to ssT-strand. In order to incorporate the features of the first two models, an intermediate model for the T-DNA transfer from *Agrobacterium* to host cell was proposed. In this model, both VirE2 and naked ssT-strand are possibly localized near the export apparatus. The ssT-strand might be exported in partially to completely VirE2-coated state. In the meanwhile, free VirE2 could also be exported via the same export apparatus and would bind the ssT-strand during the transfer and entry into the plant cell so that the ssT-strand could be completely coated by VirE2 before the ssT-strand reaches plant cell cytoplasm. In addition, free VirE2 molecules might also enter the plant cell cytoplasm. To better understand the T-DNA transfer model, more studies are needed to support the existing models or new models are proposed.

Agrobacterial T4SS consists of 11 VirB proteins (VirB1 to VirB11) and VirD4. In the pass decade, extensive work has been done to identify components of T4SS, the interactions between components, and steps in the transporter assembly pathway. The proposed functions of the VirB/D4 proteins are summarized in Table 1.3. The model of *Agrobacterial* T4SS is proposed in Fig 1.2 by Chen *et al.* (Chen *et al.*, 2005).

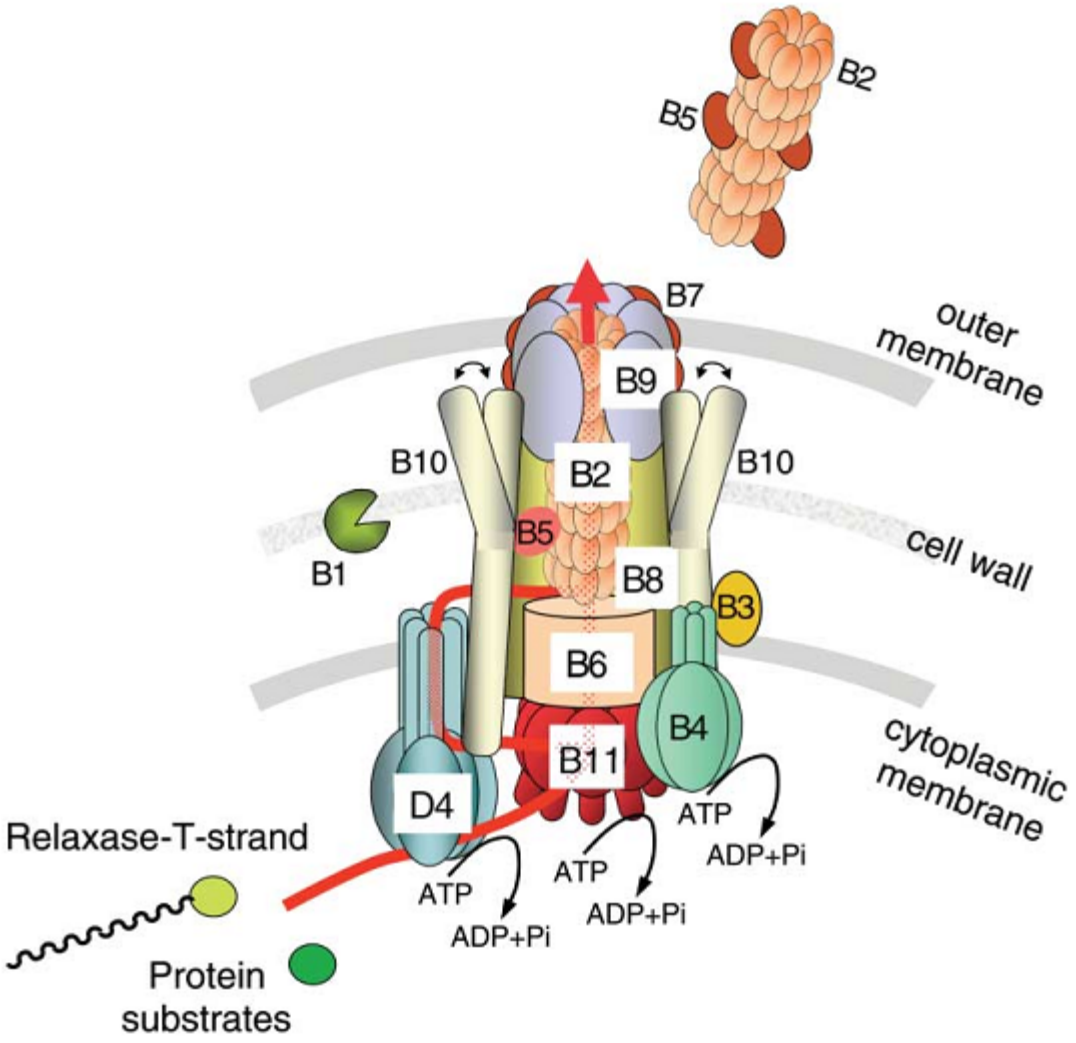


Figure 1.2. The model of *Agrobacterium* T4SS. Adapt from Chen *et al.*, 2005.

In this channel, VirB1 has glycosidase activity, which resides in its N-terminal lytic transglycosylase motif (Mushegian *et al.*, 1996). This lysozyme-like activity is

important for the hydrolysis of peptidoglycan layer of bacterial membrane and the formation of a channel in the bacterial membrane so that other VirB proteins can penetrate the peptidoglycan layer to assemble a multiprotein apparatus large enough to transfer nucleoprotein complexes. VirB2 is a pro-pillin with 121 aa, which is quickly processed to be a T-pilin with 74 aa, which forms a cyclized T-pillin subunit between its N-terminus Gln 48 and C-terminal Gly 121 (Eisenbrandt *et al.*, 1999; Jones *et al.*, 1996). VirB3 and VirB4 show highly homologous to the IncF protein TraC and TraL, which are essential for pilus assembly but not pilus structure components (Jones *et al.*, 1994). Moreover, VirB3 specific localization mediated by VirB4 may be important in the mobilization of the cyclic T-pillin subunit VirB2 to the cell exterior (Zupan *et al.*, 2000). VirB5 is a minor component of T-pilus, functioning as auxiliary structural proteins in pili T4SS (Schmidt-Eisenlohr *et al.*, 1999). VirB5 cellular level is affected by other Vir proteins, which prevent it from cellular degradation by protein-protein interactions (Schmidt-Eisenlohr *et al.*, 1999).

VirB6~VirB10, together with three ATPases VirB4, VirB11, and VirD4, are the main channel components (Christie, 1997; Christie and Vogel, 2000). Oligomerization of VirB6, which spans the inner membrane several times through its multiple membrane-spanning domains, is essential for the formation of functional pore (Christie, 1997; Das, 1998). VirB6, together with VirB8 and VirB10, forms the inner membrane sub-complex. VirB7, localized in the outer membrane, covalently attaches to a lipid moiety and interacts with VirB9 via disulfide bond (Fernandez *et al.*, 1996a). VirB7 and VirB9 can form homodimers and thus form a heterotetramer, which is essential for the stability of other VirB proteins during T4SS formation (Anderson *et al.*, 1996; Fernandez *et al.*, 1996b; Christie and Vogel, 2000). VirB8,

Table 1.3. Overview of *Agrobacterium* Vir proteins

Vir proteins	Length (aa)	Proposed function
VirA	829	Phenolic sensor of the VirA/VirG two-component regulatory system
VirB1	245	Peptidoglycan hydrolase; channel assembly
VirB2	121	Cyclized pilin subunit
VirB3	108	Requires VirB4 for stability
VirB4	789	ATPase; Homomultimer; Energy for substrate export & pilus biogenesis
ViB5	220	Pilus subunit; chaperone (?)
VirB6	295	Assembly factor; Channel subunit
VirB7	55	Stabilized VirB9 by disulphide crosslink; VirB7-VirB9 dimer stabilizes other proteins
VirB8	237	Assembly facotr; Bridge between subcomplex; IM & OM VirB channel subunit
VirB9	293	OM pore (?);VirB7-VirB9 dimer stabilizes other proteins
VirB10	377	Bridge between IM & OM subcomplexes; channel subunit
VirB11	344	ATPase; homomultimer; energy for substrate export & pilus biogenesis
VirC1	231	Putative “overdrive” binding protein; enhancement of T-DNA transfer (?).
VirC2	202	Putative “overdrive” binding protein; enhancement of T-DNA transfer (?).
VirD1	147	Required for T-DNA processing *in vivo* and dsT-DNA border nicking *in vitro*.
VirD2	447	T-DNA border-specific endonuclease; Putative “pilot protein” that leads the T-strand through the T4SS and into host cell; Nuclear targeting of the T-strand; Protection of the T-strand from 5’ exonucleolytic degration; T-strand integration into the plant genome.
VirD3	673	unknown

VirD4	668	ATPase, coupler of DNA processing and transport systems
VirE1	63	Required for VirE2 export; Chaperone for VirE2
VirE2	556	Formation of the T-complex; Protectionof the T-strand from nucleolytic degradation; nuclear targeting of the T-strand; Passage of the T-strand through the nuclear pore complex
VirE3	485	Mimics plant VIP1, acting as an adapter between VirE2 and karyopherin α and bring VirE2 into the host cell nucleus
VirF	312	Host range factor; possible interction with Skp1 proteins to regulate plant cell division cycle
VirG	241	Phenolic response regulator of the VirA/VirG two-component regulatory system
VirH1	419	Putative cytochrome P450 enzyme
VirH2	391	Putative cytochrome P450 enzyme
VirJ & AcvB	456	Putative T-strand binding protein; T-strand export from *Agrobacterium* (?); possible periplasmic chaperones

Modified from Cascales and Christie, 2003; Lai and Kado, 2000; Gelvin, 2000.

* Amino acids in *A. tumefaciens* C58 strain. Abbreviations: IM, inner membrane; OM, outer membrane; PP, periplasm; EX, Exocellular.

VirB9, and VirB10 interact with each other. VirB10 may link inner membrane and outer membrane VirB subcomplexes (Beaupre *et al.*, 1997).

VirB11 were thought to mediate T4SS apparatus assembly or function through dynamic ATP-dependent conformational changes through its ATPase activity, thus function as a gating protein at the inner membrane (Christie, 1997; Savvides *et al.*, 2003).

VirD4, as an inner membrane protein with potential DNA binding ability and ATPase activity, is a coupling protein which links the T-complex and T4SS transporter. VirD4 contains an N-terminal-proximal region and a C-terminal cytoplasmic domain, both of which are required for the polar localization of VirD4

and essential for T-DNA transfer (Das and Xie, 1998; Cascales and Christie, 2003). VirD4 forms a large oligomeric complex (Kumar and Das, 2002). VirD4 can recruit VirE2 to the cell polers and has weak interactions with VirD2-T-strand complex (Cascales and Christie, 2004). It was proposed that VirD4 might recruit T-complex to the T4SS through interactions with the T-complex protein and coordinate the passage of T-complex via T4SS channel through the interaction with VirB10 (Cascales and Christie, 2003; Llosa *et al.*, 2003).

VirE2 may also play a role in T-DNA transfer. It might assemble as a receptor or transport channel at the plant plasma membrane to dock the T-DNA transfer system and translocate the incoming VirD2-T-strand complex, VirE2 and VirF substrates (Dumas *et al.*, 2001). More interestingly, low level of VirE2, VirD2, and VirF could be exported to the extracullular milieu by *A. tumefaciens* via a virB-independent pathway (Chen *et al.*, 2000). This pathway might be used to assemble the VirE2 receptor or transport channel at the plant plasma membrane. This model may be account for the broad host range of *A. tumefaciens* because *A. tumefaciens* can deliver its own receptor to target cells (Christie, 2001; Dumas *et al.*, 2001).

1.3.6. Nuclear targeting and the T-DNA integration

Although when and where VirE2 coats the ssT-strand remains unclear, it is clear that the T-DNA exists in the form of VirD2-ssT-strand-VirE2 complex inside host cells (Citovsky *et al.*, 1992). In order to deliver the T-DNA to target cell, after successful transfer into the host cells, the T-complex must cross the host cytoplasm, target to the nucleus, penetrate the nuclear membrane, and finally reach the nucleolus. Since the VirD2-ssT-strand-VirE2 complex is a huge protein complex, which is about 50,000 kDa, much bigger then the 60 kDa size limit of the nuclear pore. Thus, the T-complex nuclear transport must be an active nuclear import process, which requires a

specific nuclear localization signal (NLS). NLS-binding proteins can recognize NLSs and guide the NLS-containing protein or protein complex to nuclear pores where active nuclear import occurs (Silver, 1991).

Sequence analysis revealed that VirD2 has two NLSs. The first VirD2 NLS, which is located in residues 396~413, plays a role but is not essential in T-DNA transfer. Because although this NLS could guide VirD2 to plant nuclei (Howard et al, 1992; Tinland *et al.*, 1992), mutation of this NLS attenuated but did not abolish tumorigenesis (Shurvinton *et al.*, 1992; Rossi *et al.*, 1993). Another NLS in residues 32~35 is not required for T-DNA nuclear localization (Shurvinton *et al.*, 1992). However, interestingly, the C-terminal ω domain, resided in residues 419~423, is important for tumorigenesis but does not have a nuclear localization activity.

Besides VirD2, another T-complex component VirE2 also has two NLS, both of which can direct the nuclear localization of fusion protein. However, genetic evidence is not availabe due to the overlapping between the two NLSs with the DNA binding domains. When VirE2 binds to ssT-strand, the NLSs of VirE2 may be inactive. Thus, it was proposed that VirE2 may function not in the nuclear localizaion, but in mediating the passage of T-strand through the nuclear pores (Ziemienowicz *et al.*, 2001).

In addition to VirD2 and VirE2, some host proteins are believed to be involved in the T-complex nuclear localization inside host cells, which will be discussed in the later sections. After translocated into host nuclelus, the ssT-strand of the T-complex will integrate into plant genome, which is the final step of the T-DNA transfer. In plant system, DNA sequence analysis of the T-DNA integration junction sites revealed that the T-DNA integration sites were more variable than the juctions created by transposon insertions, retroviruses, and retrotransposons (Gheysen *et al.*, 1987).

This random integration indicates that the T-DNA integration occurs via illegitimate recombination (or non-homologous recombination) (Ziemienowicz, 2001). To date, the random T-DNA integration into plant genome has been used as a mutagen to create genome-wide insertion mutation libraries of plant system (Valentine, 2003).

Before integration, the ssT-strand of the T-complex may need to convert to double-stranded DNA (dsDNA) and the coating VirE2 may need to be displaced at the same time. It was supported by the evidence that transient expression of T-DNA is much higher than the expression of stably integrated T-DNA and the conversion of the ssT-strand to a transcription form requires the conversion from single-stranded form to a double-stranded form (Narasimhuhu, *et al.*, 1996).

Some mobile DNA elements such as transposons and retroviruses encode enzymes for the integration themselves; however, agrobacterial T-DNA does not have such kind of enzymes. Therefore, the T-DNA integration process must be mediated by imported agrobacterial proteins or by host factors. Agrobacteial T-DNA integrating into plant genome is an illegitimate recombination process, which incorporates filler DNA into the repair sites. Although the molecular mechanism of the T-DNA integration process is largely unknown, some clues suggest that VirD2 is very important for the T-DNA integration process. A VirD2 point mutation at residue 129 (change from Arg to Gly) affected the fidelity of the 5'-end T-DNA integration (the right junction of the T-DNA insertion (Tinland *et al.*, 1995). In addition, analysis of the T-DNA integration junctions indicated that VirE2 is important for integration fidelity at the 3'-end but not the 5'-end of the T-DNA (Rossi *et al.*, 1996).

Although the mechanism of T-DNA integration process is still not clear, two general T-DNA integration models have been proposed. In the first model, the double-stand break repair and integration model, the ssT-stand is firstly replicated to a

double-strand form in the plant nuclear then integrates into double-strand breaks in the host genome. This model requires a non-homologous end-joining (NHEJ) process. In the second model, the strand-invasion model, T-strands find and invade microhomologous region between T-DNA and plant genome sequence. VirD2, attached to the 5'-end of the T-strand, generates a nick in one plant DNA strand. Then T-strand ligates into the nicked plant DNA strand. The complementary strand of the T-strand is synthesized during replication, resulting in insertion of a double-stand T-DNA into plant genome (Tinland and Hohn, 1995; Tzfira *et al.*, 2004). The first model better explains the observations that inverted repeat of T-DNA copies are frequently found in transgenic plants and that different T-DNAs introduced from different *Agrobacterium* strains into the same plant frequently link together after integration (de Buck *et al.*, 1999; de Neve *et al.*, 1997; Krizkova and Hrouda, 1998).

In contrast to the plant T-DNA integration via illegitimate recombination, in the *A. tumefaciens*-mediated *S. cerevisiae* T-DNA transfer system, the T-DNA integration occurs via homologous recommbination (Bundock *et al.*, 1995). Both of the two kinds of T-DNA integration modes require the involvement of host factors. Extensive studies have been focused on screening of the host factors involved in the T-DNA transfer as well as the integration process. Although some host factors involved in the T-DNA integration, which will be discussed later, have been identified, the integration mechanism remains unclear. More host factors needs to be identified to dissect the T-DNA transfer process as well as the T-DNA integration mechanism.

1.4. Host factors involved in *Agrobacterium*-mediated gene transfer

Besides plant species, *A. tumefaciens* can infect a broad range of host species, including yeast, fungal, and mammalian cells. It is also the first known species which can infect inter-kingdom species. The host range determination or recognition is a

complex process. Until to date, the molecular basis for the host range of *A. tumefaciens* is not clear. However, some *vir* loci, such as *virC, virF*, and *virH* were found to determine the host range of some plant species (Yanofsky and Nester, 1986; Regensburg-Tuink and Hooykaas, 1993; Jarchow *et al.*, 1991). However, it is believed that many factors including bacterial factors and host factors are involved in the *A. tumefaciens* host range.

In addition, after the T-complex is delivered into host cell, it needs to target to the nuclear, penetrate the nuclear pore, and finally integrate into plant nucleolus before successful T-DNA located gene expression, which is a highly complex process. During this process, the T-complex also needs to escape from the DNase and proteinase hydrolytic degradation. In addition to the effect of some *vir* proteins, host factors also play important roles in the entire T-DNA transfer process, particularly inside host cell. Although not as clear as the molecular basis and functions of *vir* proteins on the T-DNA transfer process, some host factors have been identified to be involed in this process. This section briefly reviews the known host factors involved in the T-DNA transfer process. For more detailed reviews of host factors involved in the entire T-DNA transfer process, please read Citovsky *et al.*, 2007 and Gelvin, 2000 and 2010.

1.4.1. Host factors involved in the *Agrobacterium*-host cell attachment

In addition to bacterial proteins, some host factors are crucial for the *Agrobacterial*-plant cell attachment. Previous work have found some plant factors important for bacterial attachment and *Agrobacterium*-mediated transformation, including a vitronectin-like protein (Wagner and Matthysse, 1992), a plant surface proteinaceous substance (Neff and Binns, 1985; Neff *et al.*, 1987), and a rhicadhesin-binding protein (Swart *et al.*, 1994). Vitronectin are specific mammalian receptors

used by different pathogenic bacteria (Burridge *et al.*, 1988). It was shown that human vitronectin and antivitronectin antibodies could inhibit *Agrobacteria*-plant tissue attachment and *Agrobacterium* mutants, which cannot attach to plant cell, reduced binding to vitronectin (Wagner and Matthysse, 1992). Therefore, plant vitronectin-like proteins were suggested to be involved in *Agrobacterium* to host cells attachment (Wagner and Matthysse, 1992). However, this assumption was argued by a paper regarding the role of the vitronectin-like protein in the attachment step and the entire *Agrobacterium*-mediated transformation process (Clauce-Coupel *et al.*, 2008).

In addition, several *Arabidopsis* ecotypes, such as B1-1 and Petergof, which are recalcitrant to *Agrobacterium*-mediated transformation, showed weak binding *Agrobacterium* cells, which is the early stage of the binding (Nam *et al.*, 1997). Screening of *Arabidopsis* T-DNA insertion mutants of the ecotype Ws revealed that some mutants, such as *rat1* and *rat3,* which are resistant to *Agrobacterium* transformation (named *rat* mutants), could not bind *A. tumefaciens* on the cut root surface (Nam *et al.*, 1999). *Arabidopsis arabinogalactan* protein, AtAGP17, which is deficient in *rat*1 and localized to plant cell walls or secreted into the apoplast, was confirmed to be important for transformation (Gaspar *et al.*, 2004). In addition, *rat*4 mutant encoding the cellulose synthase-like protein CsA-09 and *rat10* mutant encoding a plant cell wall β-expansin were reported to have reduced *Agrobacterium*-mediated transformation efficiency (Zhu *et al.*, 2003b; Ziemienowicz *et al.*, 2000).

1.4.2. Plant cell surface receptors

After the successful attachment to host cells, the *Agrobacteria* T4SS connects *Agrobacterium* cell to host cells and the T4SS itself serves as a transport channel to facilitate the T-DNA and relevant Vir protein transport. The T-pilus may bring the bacterial and host membranes together for T-DNA and Vir protein transport or may

be a conduit for transport (Backert *et al.*, 2008). VirB2, a processed cyclic protein is a major pilin protein (Eisenbrandt *et al.*, 1999; Lai and Kado, 1998). The VirB5 is a minor component located on the pilus tips (Aly and Baron, 2007). As the T-pilus components, VirB2 and VirB5 are potential targets for host surface receptors.

Using a processed VirB2 and an *Arabidopsis* cDNA library, a yeast two-hybrid screen assay was conducted to identify potential plant surface receptors (Hwang and Gelvin, 2004). Three reticulon domain-like (RTNL) proteins VirB2-interacting proteins BTI1, BTI2, and BTI3 (also called AtRTNLB1, AtRTNLB2, and AtRTNLB4) as well as a Rab8 GTPase, specifically interacted with VirB2 and with each other but not with other Vir proteins (Hwang and Gelvin, 2004). BT1 is a plasma membrane protein, whereas BTI2 and BTI3 are endoplasmic reticulum (ER) proteins (Marmagne *et al.* 2004; Nziengui *et al.*, 2007). Antisense and RNA interferenced BTI1 and AtRAB8 transgenic plants showed reduced *A. tumefaciens*-mediated transformation, which implies the involvement of the BTI1 and AtRAB8 in the transformation process. Interestingly, the expression of BTI1 is significantly increased after *Agrobacterium* infection (Hwang and Gelvin, 2004), indicating a possible positive feedback communication between *Agrobacterium* and plant cells. However, no experiment data show binding between the BTI proteins to the T-pili. The role of the T-pilus in the transformation process and host surface receptors remains unclear, thus further studies are required to resolve this question (Kado, 2000).

1.4.3. Host factors involved in nuclear targeting

After the VirD2-ssT-strand and the Vir effector proteins enter plant cytoplasm, The T-complex must transfer through the cytoplasm and target to the nucleus. Both VirD2 and VirE2 proteins contain nuclear localization signal (NLS) sequences, which are critical for T-complex nuclear targeting. Deletion of the VirD2 C-terminal

bipartite NLS could not completely block the transformation process (Shurvinton *et al.*, 1992). This may be complemented by the VirE2, which coats the entire ssT-strand. The two VirE2 bipartite NLS sequences probably help the nuclear targeting of the T-complex even in the absence of VirD2 NLS (Gelvin, 1998). In addition, many host proteins may interact with the T-complex to form super-T-complexes, which facilitates the T-complex nuclear targeting.

The importin α protein Impa-1 (also called AtKAPα) is the first identified plant protein to be involved in the T-complex nuclear targeting in a yeast-two-hybrid screening of *Arabidopsis* proteins that interact with the VirD2 NLS domain (Ballas and Citovsky, 1997). Impa-1 and all other tested importin α family members were reported to interact both with VirD2 and VirE2 (Bakó *et al.*, 2003; Bhattacharjee *et al.*, 2008; Lee *et al.*, 2008). In addition, VirE3, which is also transported into host cells, interacts with yeast Impa-1 and Impa-4 *in vitro* (Garcia-Rodriguez *et al.*, 2006). Importin α proteins are encoded by a multigene family, the *IMPA* family. RNAi data showed that *IMPA1*, *IMPA2*, or *IMPA3* did not affect *Agrobacterium* infection on *Arabidopsis*; however, disruption of the expression of *IMPA4* significantly reduced the infection efficiency (Bhattacharjee *et al.*, 2008). In addition to importin α protein family, importin β-like transportins may also be involved in the T-DNA transfer process (Zhu *et al.*, 2003a).

In addition to importin protein families, VIP1 and VIP2, two VirE2 interacting proteins, were identified in a yeast two hybrid screen for VirE2 binding host proteins (Tzfira *et al.*, 2001). VIP1 specifically binds to VirE2 but not to VirD2. VirE2 could localize to mammalian nuclus with the coexpression of VIP1 (Guralnick *et al.*, 1996). These results show that VIP1 can help VirE2 nuclear import in host cells.

Interestingly, *Agrobacterium* VirE3 can mimic VIP1 function in facilitating VirE2 nuclear targeting when VIP1 level is low in plant cells (Lacroix *et al.*, 2005).

VirD2 is a phosphoprotein and the phosphoralation of the T-complexes may play a role in the nuclear targeting. It was shown that VirD2 binds to and is phosphorylated by a cyclin-dependent kinase-activating kinase, CAK2Ms (Bakó *et al.*, 2003). In addition, VirD2 also binds to a protein phosphatase 2C (PP2C), which catalyzes dephosphorylation of VirD2 (Tao *et al.*, 2004). It was observed that a PP2C deficient *Arabidopsis* mutant is susceptible to *Agrobacterium*-mediated transformation (Tao *et al.*, 2004).

1.4.4. Host factors involved in T-DNA integration

The T-DNA integration into plant genome is through non-homologous end-joining (NHEJ) process even in the presence of extensive homology with the plant genome (Offringa *et al.*, 1990). Mutagenesis analysis revealed that in yeast model the NHEJ effector proteins Yku70, Rad50, Mre11, Xrs2, Lig4, and Sir4 are crucial for the integration of the T-DNA into yeast genome via a non-homologous (illegitimate) recombination (NHR) pathway (van Attikum and Hooykaas, 2003). However, Rad 51 and Rad52, but not those NHNJ proteins (Rad50, Mre11, Xrs2, Lig4 or Yku70) are essential for T-DNA integration by homologous recombination (HR) (van Attikum *et al.*, 2001). Yku70 and Rad 52 are the kernel effectors for T-DNA integration by HR or NHR respectively. In addition, another DNA repair effector, Rad54, enhanced gene targeting frequency in transgenic plants (Shaked *et al.*, 2005).

However, the role of another key participant of NHEJ, KU80, in T-DNA integration process is controversial. One piece of evidence showed that T-DNA could not integrate into genome of *Arabidopsis KU80* insertional mutants in somatic cells

and KU80 could bind dsT-DNA in *Agrobacterium*-infected plants (Li *et al.*, 2005b), which suggests that KU80 may play an important role in the T-DNA integration process. However, on the other hand, KU80 was not so crucial or indispensable for T-DNA integration during the transformation of germ-line cells (Friesner and Britt, 2003; Gallego *et al.*, 2003). Similarly, the role of another NHEJ protein, the *Arabidopsis* LIG4, in the T-DNA integration process is also controversial. Data showed that LIG4 was not required for T-DNA integration in somatic *Arabidopsis* cells (van Attikum *et al.*, 2003); however, it was important for T-DNA integration in germ-line cell (Friesner and Britt, 2003). These arguments may be due to the fundamemtal differences between host factors requirement in T-DNA integration in somatic cells and in germ-line cells (Zhu *et al.*, 2003a; Ye *et al.*, 1999).

In addition to those NHEJ proteins, DNA-packaging proteins may also be involved in the T-DNA integration process. Histone proteins play a major role in T-DNA integration process. It was found that *Arabidopsis* histone H2A is essential for T-DNA integration in somatic cells and H2A expression level is higher in *Agrobacterium*-infection susceptible tissues (Mysore *et al.*, 2000; Yi *et al.*, 2002). H2A may function in directing the T-complex to the integration sites (Li *et al.*, 2005a; Loyter *et al.*, 2005) and may also be involved in relaxing the host DNA structure (Mysore *et al.*, 2000). The *Arabidopsis rat5* mutant, containing an insertion in the C-terminal untranslated region of the histone *HTA1*, is susceptible to transient *A. tumefaciens*-mediated transformation but resistant to stable transformation (Mysore *et al.*, 2000; Zhu *et al.*, 2003a). *HTA1* is one of the histone H2A gene family containing 13 members.The fact that *rat5* mutant is resistant to tranformation indicates that there is no functional redundancy among the *HTA* genes. On the contrary, histone proteins showed functional redundancy in *Agrobacterium*-mediated transformation (Yi *et al.*,

2006). The observation that only *HTA1* gene could respond to *Agrobacterium*-mediated infection suggests the special properties of *HTA1* in *Agrobacterium*-mediated transformation process.

In addition to their role in T-DNA nuclear targeting, VIP1 and VIP2 proteins are also important for T-DNA integration. A VIP1 C-terminal truncation mutant could still interact with VirE2 and direct T-DNA to plant nuclei; however, it was defective in multimerization and interacting with histones, thus decreased T-DNA integration into plant genome (Li *et al.*, 2005a). Molecular and genetic data suggested that T-DNA integration process was blocked in *vip2* mutants (Anand *et al.*, 2007). VIP2 functions like a transcriptional regulator. Microarray analysis indicated that low levels of histone transcription were found in *vip2* mutants (Anand *et al.*, 2007). Because of the importance of histone proteins in T-DNA integration process, the alteration of histone transcription profile indicates that VIP2 is crucial for regulating the expression of genes important in *Agrobacterium* T-DNA integration.

1.5. Purposes and significance of this study

Agrobacterium-yeast gene transfer is an ideal model to study the gene transfer and protein trafficking mechanism during the T-DNA transfer into the yeast nucleus. However, host factors involved in the T-DNA transfer process is still unclear, which is due to lack of an effective and efficient transformation protocol and a high throughput screening system. Although previous studies (Robberts *et al.*, 2003; Zhu *et al.*, 2003a; Crane and Gelvin, 2007; Kawai *et al.*, 2004) have developed several screening systems for host factors, their methods are either limited in scope or not efficient enough. In addition, although their studies identified some host factors, more unknown host genes involved in *Agrobacterium*-yeast gene transfer need to be found.

In order to understand the T-DNA transfer process inside yeast cells, this project aims to achieve the following goals:

(1) to develop an effective *Agrobacterium*-mediated transformation of yeast protocol. The new protocol would be different from the one described by Bundock (1995) and should be able to handle dozens or even hundreds of samples each time, which would be especially suitable for large scale screening.

(2) to develop a holistic host gene screening system, which may be able to cover most of the yeast genes involved in the *Agrobacterium*-yeast gene transfer. The new screening system should be able to detect mutants that enhance transformation efficiencies as well as the mutants that reduce transformation efficiencies so that we may know the transformation efficiency of almost every yeast gene.

(3) to dissect T-DNA trafficking pathways inside eukaryotic cells and generate a global profile of yeast genes involved in *Agrobacterium*-yeast gene transfer. If the new screening system is successfully developed, we may be able to identify hopefully most yeast factors, which may play roles in the *Agrobacterium*-yeast gene transfer. After grouping those genes according their functions or gene processes, some of the genes may be closely related, which implies the involvement of the specific function or gene process in the gene transfer process. Therefore, a T-DNA transfer pathway or pathways could be found according to the closely related host factors.

(4) to develop *Agrobacterium*-yeast gene transfer as a model for studying nucleoprotein trafficking processes. Because some of the *Agrobacterium*

proteins, such as the VirD2 and VirE2 of the T-complex, are also transported into yeast cells and finally into the nucleus, the *Agrobacterium*-yeast T-complex transfer system may be used as a model to study the nucleoprotein trafficking inside the yeast cells.

This thesis focuses on the screening of host factors involved in the *Agrobacterium*-yeast gene transfer process. Other systems such as *Agrobacterium*-plant gene transfer processes are beyond the scope of this study. *Agrobacterium tumefaciens* strains and *Saccharomyces cerevisiae* strains were used in this study, other species of *Agrobacterium* and yeast are not considered in this study. In addition, yeast gene knockout libraries and yeast essential gene knockdown libraries, which cover about 85% of yeast genome, were used in this study. Some inviable mutants were not tested in this study.

CHAPTER 2. MATERIAL AND METHODS

2.1. Strains, plasmids, antibiotics, primers and culture media

A. tumefaciens stains, *E. coli strains*, and *S. cerevisiae* strains used in this study are listed in Table 2.1. Plasmids used in this study are listed in Table 2.2. Primers used in this sduty are listed in Table 2.3. *Agrobacteria*, *E. coli*, and yeast media used in this study were prepared as described in Table 2.4. *E. coli* stains were grown at 37 ^{o}C in Luria-Bertani (LB) broth or on agar medium (Sambrook and Russell, 2001). *A. tumefaciens* strains were grown at 28 ^{o}C in Mannitol Glutamate Luria (MG/L) or in Induction Broth (IB or IBPO4) (Caneglosi *et al.*, 1991; Piers *et al.*, 1995). *S. cerevisiae* strains were grown in Yeast extract Peptone Dextrose (YPD) or SD drop-out complement medium at 30 ^{o}C (Piers *et al.*, 1995). Bacterial and yeast cultures were supplemented with proper antibiotics to maintain plasmids when necessary. The preparation and concentration of antibiotics and other solutions used in this study are listed in Table 2.5.

Bacterial and yeast stains were kept at 4 ^{o}C refrigerator for short term (within 2 weeks) storage and in relevent medium containing 15% steriled glycerol at -80 ^{o}C freezer for long term storage (over than 5 years) (Sambrook and Russell, 2001).

Table 2.1. Bacterial and yeast stains used in this study

Stains	Relevant characteristic (s)	Source or reference
E. coli		
DH5α	EndA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 (argF-lacZYA) U169 ϕ80dlacZ ΔM15	Bethesda Research Laboratory
A. tumefaciens		
EHA105	Wild type, nopaline strain containing pTiBo542 harbouring a T-DNA deletion	Hood *et al.*, 1993; Chen et al., 1991
MX243	Octopine-type *virB* mutant strain	Stachel *et al.*, 1986
WR1715	Harbors a Ti plasmid with 70% of $virD_2$ deleted (aa 94-388).	Shurvinton *et al.*, 1992
EHA105 VirE2::S11	16 aa GFP small fragment S11 was inserted in to the position between 54 aa and 55 aa of VirE2	Lab collection
S. cerevisiae		
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystem
R1158	*MATa his3-1 leu2-0 met15-0 URA3*:: CMV-tTA	Open Biosystem
ost2	*pOST2*:: kanR-tet07-TATA *URA3*:: CMV-tTA *MATa his3-1 leu2-0 met15-0*	Open Biosystem
*ost3*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 OST3*:: *kanMX4*	Open Biosystem
*ost4*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 OST4::kanMX4*	Open Biosystem
*ost5*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 OST5::kanMX4*	Open Biosystem
*ost6*Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 OST6::kanMX4*	Open Biosystem
swp1	*pSWP1::kanR-tet07-TATA URA3::CMV-tTA MATa his3-1 leu2-0 met15-0*	Open Biosystem

wbp1	*pWBP1::kanR-tet07-TATA URA3::CMV-tTA MATa his3-1 leu2-0 met15-0*	Open Biosystem
SRP1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal SRP1 gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
OST1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *OST1* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
OST3-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal OST 3 gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
OST4-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *OST4* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
OST5-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *OST5* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
OST6-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *OST6* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
STT3-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *STT3* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
SWP1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *SWP1* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem
WBP1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, C-terminal *WBP1* gene is fused onto TAP-*HIS3MX6* tag	Open Biosystem

2.2. DNA manipulations

2.2.1. Plasmid DNA preparation from *E. coli*

E. coli DH5α strain was routinely used as the host strain for cloning experiments. Minipreparation of *E. coli* DH5α plasmid DNA was performed using HiYield Plasmid Mini Kit (Real Genomics). The protocol was described as follows according to the manufacturer's manual. Briefly, 2 to 5 ml of DH5α culture was grown over night at 37 ^{o}C incubator. Collect 1.5 ml of the overnight culture into 1.5 ml eppendorf tube by microcentrifugation at maxium speed (eppendor 5417C; 14,000 rpm) for 1 min. Repeat the harvest step up to 10 ml of overnight culture and double the usage of PD1, PD2, and PD3 if the plasmid is low copy number. Add 200 µl of PD1 Buffer (RNase A added) to the eppendorf tube and resuspend the vortexing or pipetting. Add 200 µl of PD2 Buffer and mix gently by inverting the tube about 10 times. Incubate for 2 min at room temperature until lysate clears. Add 300 of PD3 Buffer and mix immediatedly by inverting the eppendorf tube 10 times. Centrifuge for 3 min at full speed. Place a PD colume in a collection tube. Apply up to 800 µl of the supernatant to the PD column. Centrifuge at 6,000 g for 30 sec. Discard the flow-through and place the PD colume back in the collection tube. Repeat the previous 3 steps if the supernatant if more that 800 µl. Add 400 µl of W1 buffer in the PD column. Centrifuge at 6,000 g for 30 sec. Discard the flow-through and place the PD column back. Add 600 µl of wash buffer (ethanol added) and centrifuge at 6,000 g for 30 sec. Centrifuge again for 2 min at full speed. Transfer the PD column onto a new eppendorf tube. Add 50 µl of Elution Buffer or water in the center of the column matrix. Stand for 2 min at room temperature and centrifuge for 2 min at full speed to elute purified DNA. Concentration of plasmid DNA was determined by using NANODROP 2000 Spectrophotometer (Thermo Scientific).

Table 2.2. Plasmids used in this study

Plasmids	Revant characteristic(s)[a]	Source or reference
pYES2-OST3	pYES2 carrying a full length *OST3* gene flanked by *Kpn*I sites	This study
pAVD2	pACT2 carrying a full length VirD2 gene at *EcoR*I site	Lab collection
pCB301	Binary vector; Km^R	Xiang *et al.*, 1999
pCB301-5GFP	pCB301 carrying 5 copies of N-termial EGFP fragment; EGFP is not expressed.	This study
pDONR223-TUSC3	pDONR223 carrying a full length human *TUSC3* cDNA sequence; $Spect^R$	Open Biosystems
pEGFP209	2μ replicon; *LEU2*; *EGFP* under P_{ADH1} contro; Amp^R	Lab Collection
pEGFP209-EB*	Derivative of pEGFP209; carrying 3 copies of EGFP fragment; EGFP is not expressed.	This study
pHT101	Derivative of the binary vector pCB301, ligated at *Sal*I site with pACT2, in which the *GAL4AD* gene is replaced by EGFP reporter gene at *Hind*III site; Km^R, Amp^R, 2μ origin, *LEU2*.	This study
pHT105	Yeast expression vector; 2 μ replicon; *ADH1* promoter, *ADH1* terminator; *URA3*, Amp^R	This study
pHT105-GFP	pHT105 carrying an *EGFP* reporter gene flanked by *Bam*HI sites	This study
pHT105-OST3	pHT105 carrying a full length *OST3* gene flanked by *Kpn*I sites	This study
pHT105-OST3pro	pHT105 carrying a full length *OST3* gene with its natural promoter and terminator flanked by *Bam*HI sites	This study
pHT105-TUSC3	pHT105 carrying a full length human *TUSC3* cDNA sequence flanked by *Bam*HI sites	This study
pHT106	Yeast expression vector; 2 μ replicon; *EGFP* selectiom marker; *URA3*, Amp^R	This study

pHT110	Ligation of pCB301-5GFP and pEGFP209-EB* at SalI sites; carrying 8 copies of N-terminal GFP fragment; EGFP is not expressed.	This study
pQH04-S1-10	Yeast expression vector pQH04 carrying S1-10 split-GFP big fragment at ADH1 promoter. 2 μ replicon; *HIS3*, Amp^R.	Lab collection
pRS426	YE-type (episomal) shuttle vector; T3, T7 promoters; 2 μ replicon; *lacZ*, *URA3*, Amp^R	Christianson TW *et al.*, 1992
pRS426-OST3pro	pRS426 carrying a full length *OST3* gene with its own promoter and terminator flanked by *Bam*HI sites	This study
pYES2	Yeast expression vector; 2 μ replicon; *GAL1* promoter, *CYC1* terminator; *URA3*, Amp^R	Invitrogen
pYES2-GFP	pYES2 carrying a *GFP* reporter gene	Lab Collection
pYES2-GFPVirD2	pYES2 carrying a GFP-N terminal VirD2 fusion sequence	Lab Collection
pYES2-TUSC3	pYES2 carrying a full length human *TUSC3* cDNA sequence flanked by *Bam*HI sites	This study

[a]: Amp, ampicillin; Km, kanamycin; Spect, spectinomycin

Table 2.3. Primers used in this study

Primers	**Sequences**	**Origin**
HT101-4	5’ CTCGACCAAT TCCGATCATA 3’	pHT101
HT101-5	5’ CTTACAGACA AGCTGTGACC 3’	pHT101
OST3R-*BamH*I	5’CGGGATCCTTCGGTGGACTAGGCAG CCTCACA 3’	*S. cerevisiae* Chromosome XV: 483269
OST3F-*BamH*I	5’CGGGATCCGAGGAAGCGGACTATCC ACATTGT 3’	*S. cerevisiae* Chromosome XV: 481437
OST3UP	5’CACTGAACCACACGCGTCCGCATCA AACTCTTCCTCCCAAACAGATTGTACT GAGAGTGCAC 3’	*S. cerevisiae* Chromosome XV: 481993

OST3DN	5’TTCCATCTCTTTGTTATGGTGAAAAC AAAAAAAAAAACTGACCTGTGCGGTA TTTCACACCG 3’	*S. cerevisiae* Chromosome XV: 483129
OST3F	5’ GAGGAAGCGGACTATCCACATTGT3’	*S. cerevisiae* Chromosome XV: 481437
OST3R	5’ TTCGGTGGACTAGGCAGCCTCACA 3’	*S. cerevisiae* Chromosome XV: 483292
pRS426F	5’ GATGTGCTGCAAGGCGATTA 3’	pRS426: 1894
pRS426R	5’ GCTTCCGGCTCCTATGTTGT 3’	pRS426: 2229
VirD2F	5’ CCGGAATTCTGGATGCCCG 3’	*A.tumefaciens* linear chromosome: 167280
VirD2R	5’ CCGGAATTCGGTCCTTCCT 3’	*A.tumefaciens* linear chromosome: 169995
L10	5’ TGTTGTCTCACCATATCCGCA 3’	pTH74: 286
pRS426-MCS	5’ CCAAGCTTTGCAAAGGTACCTCGA GATATCGAATTCCTGCAGCC 3’	pRS426: 2046
GFP-*BamH*I-F	5’CGCGGATCCATGTCTAAAGGTGAAG AATT 3’	pHT101: 8319
GFP-*BamH*I-R	5’CGCGGATCCTTATTTGTACAATTCAT CA 3’	pHT101: 9050
OST3-*Kpn*I-F	5’CGGGGTACCATGAATTGGCTGTTTTT GGT 3’	*S. cerevisiae* Chromosome XV: 482035
OST3-*Kpn*I-R	5’CGGGGTACCACTTATTTGAATGGTGC CGATA 3’	*S. cerevisiae* Chromosome XV: 483087
OST3INF1	5’ CGTCCAATTCCGATACATCT 3’	pHT105-OST3: 959
OST3INR	5’AGTCTGTTGCTGGACATTGCCA 3’	pHT105-OST3: 1357
pDONR223F	5’ GTTTTCCCAGTCACGACGTT 3’	pDONR223: 517
pDONR223R	5’ AATACGACTCACTATAGGGG 3’	pDONR223: 3018

TUSC3-*BamH*I-F	5’CGGGATCCATGGGGGCCCGGGGCGCTCCTTCACGCCGTAGGCAAGCGG 3’	pDONR223: 655
TUSC3-*BamH*I-R	5’CGGGATCCTTGGCTTCATTTAATTAAAAAGCTATAAGGATAGCCGTGG 3’	pDONR223: 1736
TUSC3-INF1	5’ GTATGGTGGACTATGATGAGGG 3’	pTH105-TUSC3: 1083
TUSC3-INF2	5’ GTCTTTGCTATGACTTCTGGC 3’	pTH105-TUSC3: 1432
pHT105-F1	5’ TTCCTCGTCATTGTTCTCGT 3’	pHT105: 560
pHT105-R1	5’ GCACAGATGCGTAAGGAGAAAA 3’	pHT105: 1096
AopB-F	5’GAAGAATTCGAACTTGACGCCGATACC 3’	*A.tumefaciens* circular chromosome: 1120592
AopB-R	5’AGGCTGCAGACATGCGTATTTTCG 3’	*A.tumefaciens* circular chromosome: 1121265
TRP1-F	5’ TCACAGGTAGTTCTGGTCC 3’	*S. cerevisiae* Chromosome IV: 461856
TRP1-R	5’ TTCTTAGCATTTTTGACGA 3’	*S. cerevisiae* Chromosome IV: 462510
GFP-F	5’ GATAAGGCAGATTGAGTGGA 3’	pHT101: 9011
GFP-R	5’ TTATTCACTGGTGTTGTCCC 3’	pHT101: 8426

Table 2.4. Media used in this study

Media	Preparation (1 L)	Reference
For *E. coli*[a,c]		
LB (Luria broth)	Tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; pH 7.5	Sambrook *et al.*, 1989
LB rich medium	10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose.	New England Biolabs
SOC	Tryptone, 20 g; yeast extract, 5 g; NaCl, 0.5 g; 10 ml of 250 mM KCl; pH 7.0,sterilize by autoclaving and add 5ml of filter-sterilized 2 M $MgCl_2$; 20mM glucose	Sambrook *et al.*, 1989
SOB	Tryptone, 20 g; yeast extract, 5 g; NaCl, 0.5 g; 10 ml of 250 mM KCl; pH 7.0,sterilize by autoclaving and add 5ml of filter-sterilized 2 M $MgCl_2$.	Sambrook *et al.*, 1989
TB	10 mM PIPS, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250mM KCl;	Sambrook *et al.*, 1989
For *A. tumefaciens*[a,c]		
MG/L	LB, 500 ml; mannitol, 10 g; g sodium glutamate, 2.32; KH_2PO_4, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; biotin, 2 μg; pH 7.0.	Cangelosi *et al.*, 1991
AB[b] (Minimal medium)	20 × AB salts, 50 ml; 20 × AB buffer, 50 ml; 0.5% glucose 900 ml (autoclaved separately before mix together).	Cangelosi *et al.*, 1991
IB[b] (Induction Medium)	20 × AB salts, 50 ml; 20 × AB buffer, 1 ml; 0.5 M MES (pH 5.5), 4 ml; glucose, 18 g in 941 ml RO H_2O (autoclaved separately before mix together).	Cangelosi *et al.*, 1991
$IBPO_4$[b] (Induction Medium)	20 × AB salts, 50 ml; 20 × AB buffer, 1 ml; 1 M KH_2PO_4 (pH 5.0), 8 ml; glucose, 18 g in 941 ml RO H_2O (autoclaved separately before mix together).	Piers *et al.*, 1996
20 × AB salts	NH_4Cl, 20 g; $MgSO_4 \cdot 7H_2O$, 6 g; KCl, 3 g; $CaCl_2$, 0.2 g; Fe $SO_4 \cdot 7H_2O$, 50 mg.	Cangelosi *et al.*, 1991
20 × AB buffer	K_2HPO_4, 60 g; NaH_2PO_4, 23 g; pH7.0.	Cangelosi *et al.*, 1991

0.5 M MES	MES, 97.6 g; pH5.5.	Cangelosi *et al.*, 1991
1M KH_2PO_4	KH_2PO_4, 136.08g; pH5.0	Piers *et al.*, 1996
For *S. cerevisiae*[d]		
YPD	Difco peptone, 20 g; yeast extract, 10 g; glucose, 20 g	Clontech user manual
SD (broth)	Minimal SD base, 26.7 g; appropriate drop-out supplement	Clontech user manual
SD (agar)	Minimal SD base, 46.7 g; appropriate drop-out supplement	Clontech user manual
CM[b] (co-cultivation medium)	IBPO4; histidine,20 mg; leucine 60 mg; methionine 20 mg; uracil 20 mg.	Piers *et al.*, 1996

[a]: For solid media, 15 g agar was added

[b]: Autoclaved separately before mix together

[c]: Sterilized by autoclaving at 121 ^{o}C for 20 min

[d]: Sterilized by autoclaving at 121 ^{o}C for 15 min

Table 2.5. Antibiotics and other stock solutions used in this study

(Sambrook *et al.*, 2001; Cangelosi *et al.*, 1991)

Antibiotics or solutions	Preparations	Stock Con. (mg/ml)	Working Con. in bacterial (μg/ml)	Working Con. in yeast (μg/ml)
Ampicillin (Amp)	Dissolved in dH_2O, filter sterilized	100	100	--
Kanamycin (Km)	Same as above	100	50	--
Carbenicillin (Cb)	Same as above	100	100	--
Spectinomycin	Same as above	50	50	--
G418	Same as above	100	5	100
Cefotaxime (Cef)	Same as above	100	100	--
IPTG	Same as above	24	24	--
Proteinase K	Same as above	20	50	50
Acetosyringone (As)	Dissolved in dimethyl sulfoxide.	100 mM	--	--
X-Gal	Dissolved in dimethyl sulfoxide	20	20	--
RNaseA	Dissolved in 10 mM Tris-HCl (pH7.5) and 15 mM NaCl	10	20	100

2.2.2. Genomic DNA preparation from *A. tumefaciens*

A. tumefaciencs genomic DNA was prepared as described by Charles and Nester (1993). 50 ml of overnight *A. tumefaciens* culture was harvested by centrifugation at 5000 rpm for 10 min and the supernatant was discarded. Washed the cells once with 4 ml of TES (10 mM Tris-HCl, 25 mM EDTA, 150 mM NaCl, pH 8.0), and resuspended in 4 ml of TE buffer (10 mM Tris-HCl, 25 mM EDTA, pH 8.0). Cells were lysed by the addition of 500 µl of 5 M NaCl, 500 µl of proteinase K (5 mg/ml), and 500 µl of 10% SDS and incubated at 68 ^{o}C for 30 min. The lysate was extracted once with 1:1 phenol-chloroform and then once with chloroform alone by centrifugation at 12,000 rpm for 15 min. Genomic DNA was precipitated by adding 7.5 M ammonium acetate to the final concentration of 2 M and then 2 volumes of cold absolute ethanol. The DNA pellet was washed twice with 70% ethanol and vacuum dried. Genomic DNA was dissolved in 1 ml of distilled water and stored at 4 ^{o}C.

2.2.3. Minipreparation of *S. cerevisiae* DNA

Yeast total DNA extraction was prepared according to David and Deniel (2005) with some modification. 5 ml of yeast overnight culture was collected in a clinical cenrifuge at 6,000 rpm for 5 min. Discard the supernatant. Resuspend the pellets in 0.5 ml of Lyticase buffer (1 M sorbitol, 0.1 M Na_2EDTA, pH7.5) and transfer to a 1.5 ml eppendorf tube. Add 20 µl of 5 U/µl of Lyticase in to the suspension and incubate for 1 h at 37 ^{o}C waterbath. Centrifuge at 6,000 rpm for 5 min. Discard the supernatant. Resuspend the cells in 500 µl of 50 mM Tris-Cl pH7.4, 20 mM Na_2EDTA. Add 50 µl of 10% SDS, mix well and incubate for 30 min at 65^{o}C. Add 200 µl of 5 M potassium acetate and place the microfuge tube in ice for 1 h. Add 640 µl of 100% chloroform to the mixture. Mix well and centrifuge at full speed for 5 min to separate the solution. Transfer the supernatant to a new eppendorf tube and add same volume of 100%

isopropanol. Mix well and precipitate at room temperature for 5 mimutes. Centrifuge at full speed for 1 min. Discard the supernatant and vacuum dry the pellet for 10 min. Resuspend the pellet in 300 µl of TE (pH 7.4).

2.2.4. Preparation of *E. coli* DH5α competent cells

High efficient competent cells were prepared as described previously (Inoue *et al.*, 1990). *E. coli* DH5α cells were inoculated from -80 ^{o}C stock and grown overnight on LB plates at 37 ^{o}C. Single colony was inoculated into 5 ml of SOB broth medium for 6-8 h at 37 °C with shaking. Then the 1 ml culture was subcultured in 250 ml of fresh SOB medium in a 1-liter conical flask. The cells were cultured at room temperature (about 20°C) with shaking to an OD_{600} of 0.5-0.7. Chill the culture on ice for 10 min then centrifugation at 4,000 rpm for 5 min at 4 °C. Resuspend the cells in 30 ml of ice-cold TB buffer (10 mM PIPES, pH 6.7; 55 mM $MnCl_2$; 15 mM $CaCl_2$; 250mM KCl; all components except $MnCl_2$ were dissolved and autoclaved; 1M $MnCl_2$ solution was filter-sterilized and added to make TB buffer; store at 4 °C) and then incubated on ice for 10 min. Cells were collected by centrifugation at 4,000 rpm for 5 min at 4 °C and resuspended in 5 ml of ice-cold TB buffer. Glycerol was added to the final concentration of 15% and the cells were aliquoted to pre-chilled sterile eppendorf tubes at 50 µl each. Then immediately fast freezed the competent cells by immersing the tightly closed tubes in the liquid nitrogen. The competent cells were kept at -80 °C freezer until needed.

2.2.5. Amplification of DNA by Polymerase Chain Reaction (PCR)

DNA fragments were amplified by polymerase chain reaction which was carried out using a thermal cycler (GeneAmp® PCR system 9700, Applied Biosystems) in a 200 µl PCR tube. The PCR mixture (50 µl) is normally composed of:

10 × PCR buffer (without Mg^{2+})	5 μl
25 mM Mg^{2+}	3 μl
Primer 1 (10 uM)	2 μl
Primer 2 (10 uM)	2 μl
dNTPs (10 mM each)	1 μl
Template DNA	20-100 ng
Taq DNA polymerase	1 unit

Add distilled water to a final volume of 50 μl

The PCR were run following the program below:

1 cycle	95 °C for 2 min
28-35 cycles	95 °C for 30 sec
	Annealing at (Tm-5) °C for 30 sec
	Extension at 72 °C for 1 min per kb
1 cycle	72 °C for 5 min
1 cycle	Hold at 16 ^{o}C until ready for collection

2.2.6. DNA digestion and ligation

The restriction enzymes were purchased from Promega and Fermentas. DNA digestion and ligation were conducted following the instructions of the manufacturers. Digestion reaction system includes digestion buffer, restriction enzyme(s), DNA and water. The digestion mixture was normally incubated at 37 °C (except for some

special enzymes) for 3 h or so. Usually, around 1 µg of plasmid DNA was used for digestion. For vectors digested with a single enzyme, dephosphorylation was carried out to prevent further self-ligation by addition of 1 µl of shrimp alkaline phosphatase (SAP) in the digestion mixture. Digested vectors and fragments used for ligation were extracted by running through an 1% agrose electrophoresis and purified using HiYield Gel/PCR DNA Fragments Extraction Kit (Yeastern Biotech, Cat. No.: YDF 10), (see section 2.2.7).

Ligation was carried out by incubating the mixture of 1 unit of T4 DNA ligase (Fermentas/Yeastern Biotech), vector DNA, insertion DNA, ligase buffer and water at 16 ^{o}C for 2-4 h or overnight. 10 µl system was used for ligation. Proper ratio of vector and insert DNA was considered for best ligation efficiency. 3:1-5:1 of vector and insert DNA was normally used. After ligation, ligase was inactivated by incubating the mixture at 65 ^{o}C for 10 min. Then 5 µl was added for the further transformation process (see section 2.2.8).

2.2.7. Agarose gel electrophoresis and DNA purification

PCR products and digested DNA fragments for ligation and transformation were usually purified by agrose gel electrophoresis. Usually, 0.8-2 % TAE (1% TAE: 0.04M Tris-acetate, 0.001 M EDTA, pH 8.0) agrose gel along with a standard DNA marker (Fermentas #SM0331) were used for electrophoresis. After electrophoresis, the gel slice containing the desired DNA bands were excised and extracted by HiYield Gel/PCR DNA Fragments Extraction Kit (Yeastern Biotech, Cat. No.: YDF 10) following the instructions provided by the manufacturer. Briefly, agrose gel slice (up to 300 mg) containing desired DNA fragments were cut and transferred to an eppendorf tube. 500 µl of DF buffer were added and the tube was incubated in a 55

°C waterbath for 15 min to dissolve the gel completely. Invert the tubes every 3 min. 800 μl of the mixture was transferred to a DF column in a Collection tube. Centrifuge at and discard the flow-through. The column was then washed once with 500 μl of Wash Buffer and centrifuged at 6,000x g for 30 sec. Centrifuge again at full speed for 2 min to dry the colume matrix. The column was placed into a clean 1.5-ml tube. To elute DNA, 15 μl of Elution Buffer or wather was applied to the center of the column matrix and stand for 2 min to dissolve the DNA. Finally, the column was centrifuged at full speed for 2 min.

If pure DNA fragments are not required after PCR reaction, the PCR products can also be cleaned up by the same HiYield Gel/PCR DNA Fragments Extraction Kit following manufacturers' protocol. Briefly, 5 volume of DF Buffer was added into 1 volume of PCR product after PCR reaction. The following centrifugation, washing, and DNA elution steps are the same as the HiYield Gel DNA Fragments Extraction Kit.

2.2.8. Transformation of *E. coli* DH5α cells

Plasmids or ligation products were introduced into *E. coli* DH5α cells by transformation for plasmid amplification or selection (Sambrook *et al.*, 1989). Frozen competent cells (50 μl) were thawed on ice for 5 min after taking out from -80 ^{o}C stocks. Plasmid (50-100 ng) or ligation product (5 μl) was added and gently swirled the mixture. The tube was then incubated on ice for 30 min. The mixture of cells and DNA were heat-shocked at 42 °C in a waterbath for 90 sec. Put the tube on ice immediately for 2 min. 900 μl of fresh SOC medium were added to the mixture. The culture was then transferred to a 14 ml cell culture tube and incubated at 37 °C for 1 h with shaking. The cells were collected and spread onto LB agar plates containing

appropriate antibiotic(s) or substrate(s). Colonies usually appear after 12-16 h incubation at 37 °C.

2.2.9. Transformation of *A. tumefaciens* cells by electroporation

Plasmids were introduced into *A. tumefaciens* by electroporation according to Cangelosi *et al.* (1991). *A. tumefaciens* was inoculated onto MG/L agar plates and grew overnight at 28 ^{o}C. About 10^{8} agrobacterial cells were scraped from the agar plates with a sterile wooden stick (Fisher Scientific) and transferred to an eppendorf tube. The cells then were washed twice with cold water and once with cold 10% glycerol. The centrifugation was done at 10,000 rpm for 1 mimutes and supernatant was removed. The cell pellet was resuspended in 50 µl of cold glycerol. About 0.1-1 µg plasmid DNA was added to the agrobacterial suspension and was gently mixed. The mixture was incubated on ice for 2 min and then transferred to a pre-chilled 0.2 cm BioRad electroporation cuvette. The moisture on the outside of the cuvette was wiped away with a tissue paper and the competent agrobacterial cells were electroporated using the Gene Pulser II Electroporation System (BioRad). The system was set at 25 µF capacitance, 2.5 KV voltages, and 400Ω for the pulse controller. The electroporation duration for *Agrobacterium* is ususally about 8-9 s. 1 ml of MG/L broth was added to the electroporation cuvette immediately then was transferred to a 14 ml cell culture tube. The electroporated cells were recoverred at 28 ^{o}C incubater with shaking for 1 h. Then the cells were harvested by centrifugation and spread onto MG/L agar plates with proper selectable antibiotics. Colonies usually appeared at the third day.

2.2.10. DNA sequencing

Plamids DNA for sequencing were prepared by using the HiYield Plasmid Mini Kit (Real Genomics) (See section 2.2.1) and PCR DNA fragments used for sequencing were purified by the HiYield Gel/PCR DNA Fragments Extraction Kit (Yeastern Biotech). The DNA sequencing reaction mixture includes the following components:

5 × Sequencing Buffer	2 µl
BigDye^{TM} Ready Mix (V 3.0)	2 µl
Primer (10 uM)	0.5 µl
Template DNA (100-250 ng)	2 µl
Deionized H_2O	3.5 µl
Total Volume	10 µl

The DNA sequencing was usually performed using the program as follows:

96 ^{o}C for 1 min	1 cycle
96 ^{o}C for 10 sec	25 cycle
50 ^{o}C for 5 sec	
60 ^{o}C for 1 min	
60 ^{o}C for 5 min	1 cycle
4 ^{o}C	Hold

After the sequencing PCR, the sequencing mixture was transferred into a clean 1.5 eppendorf tube. 1.5 µl of 3 M sodium acetate (pH 4.6), 31.25 µl of non-denatured 95% ethanol, and 7.25 µl of deionized H_2O were added to the mixture and votexed briefly. The tube was left at room temperature for 15 min to precipitate the product.

After precipitation, the pellet was collected by centrifugation at full speed for 20 min and removed the supernatant. The pellet was washed twice by 500 µl of 70 % ethanol and centrifuged at full speed for 10 min. After the supernatant were carefully removed from the tube, the pellet was dried in a vacuum concentrator for 10 – 15 min and stored at – 20 ^{o}C if necessary. The pellet was ready to be submitted for running electrophoresis.

2.3. General protein techniques

2.3.1. Buffers for protein manipulations

Buffers used in protein manipulations are listed in Table 2.6 according to Sambrook and Russel (2001).

2.3.2. SDS-PAGE gel electrophoresis

Protein profiles were analyzed using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970) according to their molecular weights. The preparation of polyacrylamide gels and the separation of protein were following the instructions of Molecular Cloning of Sambrook *et al.* (1989). The electrophoresis apparatus used in this study was the Mini-Protean III Electrophoresis Cell (Bio-Rad). The apparatus was assembled according to the manufacturer's instructions. The monomer stock solution of 30% Acrylamide/Bis Solution 29:1 was purchaed from Bio-Rad and stored in the dark at 4 ^{o}C. APS (10%) solution was freshly prepared before each use. Separating gel buffer (1.5 M Tris-HCl, pH 8.8) and stacking gel buffer (0.5 M Tris-HCl, pH 6.8) were prepared and stored at room temperature. Tank buffer was prepared as a 10 × stock solution (0.25 M Tris-HCl, 1.92 M glycine, 1% SDS, pH8.3) and stored at room temperature. 1 × Tank buffer was diluted from 10 × stock solution before use. The protein loading samples were

Table 2.6. Buffers used in protein manipulations

Name	Components (for 1 L)	pH adjustment
10 × Tris-buffered saline (10 × TBS)	0.2 M Tris base 1.5 M Sodium chloride	Adjust pH to 7.6
1 × TBST	0.1% Tween-20 (v/v) in 1 × TBS	N.A.
10 × Tank buffer	0.25 M Tris 1.92 M glycine 0.1% SDS	N.A.
10 × Transfer buffer	72.636 g Tris 35.658 g Glycine 4.625 g SDS	Adjust pH to 8.3
1 × Transfer buffer	80 ml 10 × Transfer buffer 200 ml Methanol 720ml H_2O	N.A.
Separating gel buffer	1.5 M Tris-HCl	Adjust pH to 8.8
Stacking gel buffer	0.5 M Tris-HCl	Adjust pH to 6.8
2 × SDS gel-loading buffer	100 mM Tris-HCl (pH 6.8) 2% SDS 0.1% Bromophenol blue 20% Glycerol	N.A.
1 × SDS gel-loading buffer	2 × SDS gel-loading buffer : 1 M Dithiothreitol : Protein sample = 5: 1: 4	N.A.
Staining solution	0.25 g Coomassie Brilliant blue R 400 ml methanol 70 ml acetic acid	N.A.
Destaining solution I	400 ml methanol 70 ml acetic acid	N.A.
Destaining solution II	70 ml Acetic acid 50 ml Methanol	N.A.

prepared by mixing 5 volume of 2 × Sample loading buffer (100 mM Tris-HCl, pH6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol), 1 volume of 1 M DTT and 4 volume of protein sample. The sample mixture was then boiled for 10 min before loading. 10% SDS-PAGE gel was usually used in this study for the analysis of proteins unless otherwise specified. A maximum of 10 µl of protein sample was loaded per lane to the 15-well protein gel and 15 µl of protein sample to the 10-well protein gel. An unstained or pre-stained protein standand was loaded to each SDS-PAGE gel. Usually the SDS-PAGE gel was run at a constant voltage of 50 V until the all protein sample ran into the stacking gel and then a constant voltage of 100 - 120 V was used until the bromophenol blue reached the bottom of the gel. It usually takes about 2-3 h. The completed SDS-PAGE protein gel could be visualized by Coomassie blue stain or Silver stain, or could be used for western blot analysis.

2.3.3. Coomassie blue staining of SDS-PAGE protein gel

After running SDS-PAGE, protein profiles can be displayed by staining by Coomassie brilliant blue R-250 as reviewed by Sasse and Gallagher (2004) and Steinberg (2009). The Coomassie blue protein staining followed the protocol described by Sambrook and Russel (2001). The solutions required for Coomassie blue staining was prepared as described in Table 2.6. Briefly, after running the SDS-PAGE electrophoresis, the protein gel was immersed in at least 5 volumes of staining solution and left for staining on a slowly rotating flat rotator for 4 h or overnight. The staining solution was then discarded and replaced with destaining solution I. After the gel had been destained for 2 – 4 h, destaining solution I was removed and replaced with destaining solution II. The destaining solution II was changed if necessary until the gel background was clear.

2.3.4. Silver staining of SDS-PAGE protein gel

In addition to Coomassie Blue R staining, silver staining can also be used to detect proteins separated by SDS-PAGE. Silver stain is a more sensitive than Coomassie Blue R stain, with detection capacity down to 0.3 – 10 ng level (Switzer *at al.*, 1979). In this study, silver staining was performed following the protocol described by Celis *et al.* (2006). Buffers used in silver staining were listed in Table 2.7. After electrophoresis, SDS-PAGE gel was soaked into the fixing buffers and shaked at 60 rpm for 2 h or overnight. After removing the fixing buffer, the gel was washed 3 times with washing buffer (35% ethanol) for 20 min each at 60 rpm. The gel was then sensititized with sensitizing buffer for 2 min with shaking. Wash the gel 3 times with H_2O for 5 min each. After discarding H_2O, the gel was stained by silver nitriate staining buffer for 20 min with shaking. Wash the gel twice with Milli-Q water for 1 min each after removing the staining buffer. The gel was then developed with developing buffer. The developing was stopped by stopping solution until the clear protein bands were shown. The developing duration varies from several min to several h, which depends on the protein concentration. After scanning the protein gel image, the gel can be stored at 4 ^{o}C in water or 1% acetic acid for future analysis or protein identification.

Table 2.7. Buffers used in silver staining

Buffers	Components	1 L
Fixing Buffer	50% Methanol 12% Acetic acid 0.05% Formalin	500 ml Methanol 120 ml Acetic acid 500 µl Formaldehyde (35%) H_2O (Milli-Q) top-up to 1 L
Washing Buffer	35% Ethanol	175 ml Ethanol 825 ml H_2O
Sensitizing Buffer	0.02% $Na_2S_2O_3$	0.315 g $Na_2S_2O_3 \cdot 5H_2O$ H_2O top-up to 1 L
Staining Buffer	0.2% $AgNO_3$ 0.076% Formalin	2 g $AgNO_3$ 760 µl Formaldehyde (35%) H_2O top-up to 1 L
Developing Buffer	6% Na2CO3 0.05% Formalin 0.0004% $Na_2S_2O_3$	60 g Na2CO3 500 µl Formaldehyde (35%) 20 ml Sensitizing Buffer H_2O top-up to 1 L
Stop Solution	50% Methanol 12% Acetic acid	500 ml Methanol 120 ml Acetic acid 379.5 ml H_2O

2.3.5. Western blot analysis

After separated by SDS-PAGE gel electrophoresis, proteins were transferred to an Immun-Blot™ PVDF membrane (Bio-Rad) by electrophoresis using an Electro-Blot Unit (Scie-Plas, EB10). The PVDF membrane was soaked in methanol for 1 min before covering onto the protein gel and bubbles between PVDF membrane and protein gel were carefully removed. The transfer buffer used in this study was 1× Transfer Buffer (48 mM Tris, 38 mM Glycine, 0.37 (W/V) SDS, pH8.3, 20% (V/V) Methanol). Transfer was performed at 200 mA constant current for 2-3 h or at 60 mA constrant current for overnight at 4 ^{o}C. After the transfer was completed, the PVDF membrane was incubated with 10% non-fat milk in TBST buffer (20 mM Tris, 150 mM NaCl, pH7.5, and 0.1% (V/V) Tween-20) at room temperature for 1 h on a orbital shaker to block the remaining non-specifi protein-binding sites on the PVDF membrane. After blocking, the PVDF membrane was washed 3 times with TBST buffer for 10 min each on an orbital shaker. Then the PVDF membrane was incubated with primary antibody (1: 5,000 diluted in TBST buffer) for 1 h at roome temperature with shaking. After binding with the primary antibody, the PVDF membrane was washed 3 times with TBST buffer for 10 min with agitating to remove unbound primary antibody. The PVDF membrane was then incubated with second antibody (normally the HRP IgG secondary antibody in this study unless otherwise specified, 1: 10,000 diluted with TBST buffer) for 1 h at room temperature with shaking. After washing 3 times (the same as above), the fluorescent signals produced by the HRP-conjugated secondary antibody were detected by Supersignal® West Pico Chemiluminescent Substrate (Pierce) and developed on a BioMax XAR Film (Kodak) using an X-ray developer.

2.4. *A. tumefaciens*-mediated transformation (AMT) of *S. cerevisiae*

A. tumefaciens-mediated transformation (AMT) of *S. cerevisiae* protocol used in this study was followed by Bundock (1995) and Piers (1996) with modifications. Media, chemicals, and solutions used in the AMT process were listed in Table 2.4 and Table 2.5. The detailed procedure of AMT was discribed in detail as follows. At day 1 evening, a fresh single colony of *Agrobacterium* stain, previously inoculated from -80 ^{o}C glycerol stock and grown at 28 ^{o}C, was inucolated into 1 ml of MG/L liquid with 100 μg/ml of Km from a MG/L Km100 agar plate. Then the *Agrobacterium* cells were grown at 28 ^{o}C with shaking for overnight.

At day-2 morning, the saturated Agrobacterial culture were subcultured into fresh MG/L broth Km100 with 100 times dilution and put back into the incubator until OD_{600} equals to 1.0. The cells were collected into a 2 ml eppendorf tube by centrifugation at 10,000 rpm for 1 min. After discarding the supernatant, the pellet was washed once with sterile water and twice with $IBPO_4$. The cells were then resuspended with 1 ml of $IBPO_4$ and the cell concentration was measured using the spectrophotometer. After calculation, the *Agrobacterium* cell density was adjusted to OD_{600} equals to 0.3 in $IBPO_4$ liquid with 100 μg/ml of Km and 200 mM of As. The *Agrobacterium* culture was then induced at 28 ^{o}C incubation shaker for about 18 h for induction of *vir* genes. Meanwhile, at day-2 evening, fresh single colonies of yeast strains including wild type strain and mutant strains, previously inoculated from -80 ^{o}C glycerol stock and grown at 30 ^{o}C, were inculated to 1 ml of YPD medium from YPD agar plate. The yeast culture tubes were put at 30 ^{o}C incubator for overnight.

At day 3 morning, the overnight yeast cultures were sub-cultured by taking 5×10^{6} yeast cells into fresh 2 ml of YPD liquid and put back into 30 ^{o}C incubation shaker for continuous growing until OD_{600} around 0.35, which takes about 3-4 h. Then 2×10^{6} yeast cells were taken from the sub-culture into 1.5 eppendorf tubes. The

yeast cells were spun down by centrifugation at 10,000 rpm for 1 min. After removing the supernatant, 2×10^8 induced *Agrobacterium* cells were added into the tubes. The tubes were mixed by vortex and cells were collected by centrifugation for 1 min at 10,000 rpm. The pellet was washed once with $IBPO_4$ liquid and resuspended by 100 µl of $IBPO_4$. The resuspension was dropped onto CM Km100 As100 agar plate and left in biological safety cabinet for air-dry. It usually takes about 30 min to 1 h. The plates were then incubated at 20 ^oC for co-cultivation for 24 h.

At day 4, after 24 h co-cultivation, the cells were scrapped from CM Km100 As100 agar plate using autoclaved wooden sticks and resuspended in 1,010 µl of sterile 0.9% NaCl. After competed resuspending by vortexing, 10 µl of the mixture were taken out for 10^5 times dilution by 0.9% NaCl and 100 µl of the diluted mixture was plated on SD Leu- agar plate with 100 µg/ml of Cefotaxime using 5 mm glass beads (Fisher Scientific). The remaining 1 ml of suspension was spun down and plated onto SD or YPD agar plate with 100 µg/ml of Cefotaxime using glass beads after 900 µl of supernatant was removed. The plates were then incubated in 30 ^oC incubator. Single colonies usually appeared after 2 days on SD or YPD agar recovery plate and after 3 days on SD Leu- agar selection plate. The *Agrobacterium*-yeast T-DNA transfer efficiency was calculated by dividing number of transformants, which is the colonies numbers on SD Leu- agar plate, by total transforming yeast numbers, which is the colonies number on SD Leu- agar plate multiples the dilution factor.

2.5. Lithium acetate-mediated transformation of *S. cerevisiae*

Plasmids and foreign linerized DNA fragments can be introduced into yeast cells by lithium acetate (LiAc)-mediated transformation protocol as discribed by Gietz and Schiestl (2007) with some modifications. A single colony of yeast strain was inoculated into liquid YPD or SD medium and grown overnight at 30 ^oC with shaking

at 200 rpm. Yeast cells were subcultured by 10-fold diltutions into fresh medium after they reach stationary phase (OD_{600}>3 in YPD or OD_{600}>1 in SD). The yeast culture was put back into 30 ^{o}C and continued to grow for about 3-4 h until OD_{600} reaches about 1.0. Then 2 ml of the yeast culture was collected by centrifugation at 10,000 rpm for 1 min. The cell pellet was then washed twice with sterile water and once with 100 mM LiAc. The yeast cells were resuspended in the following transformation mixture:

50% PEG 3350 (W/V)	240 µl
1 M LiAc	36 µl
Single-stranded carrier DNA (10 mg/ml)	5 µl
Plamid or linerized DNA fragment (0.1 – 10 µg)	10 µl

The mixture was mixed well by votex at maximium speed for 1 min and incubated at 30 ^{o}C for 30 min. 29 µl of DMSO was added to the mixture before heat shocking yeast in 42 ^{o}C waterbath for 15 min. After heat shock, the yeast cells were collected by centrifugation at 10,000 rpm for 15 sec and resuspended in sterile water. According to the type of foreign DNA molecules, appropriate amount of transformed yeast cells were spread on proper selection agar plate and incubated at 30 ^{o}C. Single colonies will normally appear at the third day.

2.6. Electroporation of *S. cerevisiae*

Foreign DNA can be introduced into *S. cerevisiae* cells by electroporation. A single colony of yeast strain was inoculated into 2 ml YPD broth and grown overnight at 28 ^{o}C with shaking at 200 rpm. Cells were harvested in a 1.5 eppendorf tube by centrifuge spin at 3000 rpm at 4°C for 5 min and keep cells on ice throughout the procedure. Wash cells twice with 1 ml ice-cold sterile ddH_2O and pellet at 3000 rpm

at 4°C for 5 min. Wash once with 1 ml ice-cold 1M Sorbitol and pellet at 3000 rpm at 4°C for 5 min. Resuspend cells with 40 µl of 1M ice-cold Sorbitol. Add <5 µl desalted DNA (0.1 to 5 µg) in the resuspension and transfer into a prechilled Biorad electroporation cuvette (0.2 cm). Tap contents to the bottom, making sure that the sample is in contact with both sides of the aluminum cuvette. The moisture on the outside of the cuvette was wiped away with a tissue paper and the competent agrobacterial cells were electroporated using the Gene Pulser II Electroporation System (BioRad). The system was set at 25 µF capacitance, 1.5 KV voltages, and 200 Ω for the pulse controller. The electroporation duration for *S. cerevisiae* is ususally about 4-5 sec. Add 1 ml of 1M Sorbitol (ice-cold) immediately and transfer to a sterile Eppendorf tube with a sterile Pasteur pipette. Spread proper volume of the cell suspension onto selective plates and grow at 28 ^{o}C for 3 to 4 days until single colonies appear.

2.7. Cell imaging techniques

2.7.1. Cell fixation and DAPI staining

Cell culture was span down by centrifugation at 10,000 rpm for 1 min and washed twice with 1 ml of ddH_2O. After removing the supernatant, the cells were fixed by 500 µl of 4% paraformaldehyde at room temperature for 45 min. The cells were then washed twice with 1 ml of ddH_2O. The pellet was then treated with 500 µl of 70% ethanol for 5 min and washed twice with ddH_2O. The pellet cells were then resuspended in 10 µl ddH_2O. 5 µl of DAPI was added into the resuspension and incubated at room temperature for 15 min. The cell was then washed twice with 1 ml of ddH_2O. After resuspension in 20 µl ddH_2O, 3 µl of the cell resuspension was used for fluoresent or confocal microscopy.

2.7.2. Fluorescent microscopy

Olympus Fluoview FV1000 was used to detect image fluorescent signals of treated cells. The excitation light for DAPI signal was 405 nm and emission signal was detected using 475-525 nm bandpass filter. Signals for green fluorescent protein (GFP) were excited using 488 nm excitation light and emission light is detected using 515-560 nm bandpass filther. Cy3 signals were excited using 543 nm excitation light and emission light is detected using 565 nm longpass filter. The images for the green and red signals were superimposed in a computer by using Olympus Fluoview ver1.6b.

2.7.3. Confocal microscopy

Confocal microscopy was performed using a Carl Zeiss Laser Scanning Microscope (LSM) 510 Meta. The DAPI staining was excited using Diode laser (405 nm, 30.0 mW) and emission is detected by 475-525 nm bandpass filter. GFP Signals were excited using 488 nm excitation laser light and emission light is detected using 515-560 nm bandpass filther. Cy3 signal was excited using Helium Neon (HeNe) gas laser (543 nm, 1.2 mW) and emission is detected by 565 nm longpass filter. Co-localization image was captured via multi-track imaging.

CHAPTER 3. A GLOBAL PROFILE OF YEAST GENES INVOLVED IN *AGROBACTERIUM*-YEAST GENE TRANSFER

As reviewed in chapter 2, after decades of intensive research since the first report of gene transfer ability of *A. tumefaciens* in 1907 (Smith and Townsend, 1907), the *vir* gene functions, T-DNA processing process, and type IV secretion channel have been illuminated and become clear. Unfortunately, the T-DNA trafficking pathway and related host factors involved in the gene transfer process remains unknown. It is not clear how the T-DNA is transferred from outside of host cells to cytoplasm and finally to nucleus, how the T-DNA is protected inside host cells, and where the T-DNA is integrated into host genome. Although much effort has been endeavored on the searching of host genes involved in the T-DNA transfer, the number of identified host factors involved in the transformation process is still limited.

One possible reason for this dilemma is likely due to the low efficiency of existing host screening systems, or lack of an efficient screening system. Another reason is that plant, as the natural host for *A. tumefaciens*, is not an ideal model for host factor study because of its long life cycle and difficulties in mutagenesis study. This section firstly reviews existing screening systems for host factors involved in *A. tumefaciens*-mediated transformation and introduces the advantages of yeast in host factors study. In order to identify additional host factors important for AMT, a novel protocol for *A. tumefaciens*-mediated transformation of yeast was developed. Base on the new protocol, a new host factor screening system was developed and a large-scale screening of yeast genes involved in *Agrobacterium*-yeast gene transfer was explored.

3.1. Introduction

3.1.1. Screen systems to identify plant genes involved in AMT

As the natural hosts of *A. tumefaciens*, plants are the first choice to be used for host factor screening. During the past few years, different approches have been employed by scientists to identify plant proteins involved in the AMT process. Among hundreds of plant species, *Arabidopsis thaliana* is one of the most widely used species in plant gene screen for its availability of genome sequence and extensive genomic resources. In addition, transformation of many *Arabidopsis* ecotypes on either root or flower by *A. tumefaciens* is a common procedure in many applications (Clough and Bent, 1998; Valvekens *et al.*, 1988). Since root and the crown are the natural sites for *Agrobacterium*-mediated transformation, *Arabidopsis* root tissure is usually used for plant gene screen (Nam *et al.*, 1999).

The classic way to identify plant genes and proteins involved in the AMT process is to screen a plant gene mutant library and identify the mutant plants with altered phenotype after AMT. *Arabidopsis* mutants generated by T-DNA random insertion (Alonso *et al.*, 2003; Azpiroz-Leehan and Feldmann, 1997; Feldmann, 1991; Weigel *et al.*, 2000) and by RNA interference (RNAi) against specific target were used to identify *Arabidopsis* genes involved in AMT (Clauce-Coupel *et al.*, 2008). Over than 120 loci affecting transformation have been identified using this kind of approach, including the plants resistant to *Agrobacterium*-mediated transformation (*rat* mutants) (Nam *et al.*, 1999; Zhu *et al.*, 2003a) and the ones hypersusceptible to *Agrobacterium*-mediated transformation (Gelvin, 2010). In addition, a new strategy, virus-induced gene silencing (VIGS), has been used to identify proteins important for AMT both in *Nicotiana benthamiana* and in yeast (Anand *et al.*, 2007; Bundock *et al.*, 2002; Roberts *et al.*, 2003; van Attikum *et al.*, 2003; van Attikum and Hooykaas, 2003).

The second approach is to identify host factors having potential protein-protein interactions with *Agrobacterium* Vir proteins using yeast two-hybrid system. Some virulence effector proteins, including VirD2, VirE2, VirE3, and VirF, have been exported into host cells together with the ssT-strand. The VirD2-ssT-strand-VirE2 T-complex may interact with some host effector proteins to form supercomplexes during the T-complex transfer inside host cells. As components of the T-complex, VirD2 and VirE2 have been used as baits to fish out possible plant proteins, generated from plant cDNA libaries, in yeast cells (Bakó *et al.*, 2003; Deng *et al.*, 1998; Hwang and Gelvin, 2004; Tao *et al.*, 2004; Tzfira *et al.*, 2001). Recently, bimolecular fluorescence complementation (BiFC) assay was used to verify possible protein-protein interactions between *Agrobacterium* Vir proteins and plant proteins in plant cells (Citovsky *et al.*, 2006; Lee *et al.*, 2008).

In the third strategy, potential plant effector proteins were identified by comparing transcriptional profile between *Agrobacterium* infected plants and *Agrobacterium* non-infected plants (eg. Veena *et al.*, 2003; Deeken *et al.*, 2006; Lee *et al.*, 2009; Yi *et al.*, 2002; Yi *et al.*, 2006). Proteins either induced or repressed by *Agrobacterium*-mediated transformation and downstream proteins transcripted by previous identifed regulator important for AMT were identified.

Although the screening work is tedious and maybe not so efficient, some plant proteins important for AMT were identified and thus provided important clues for future studies. In addition, the fact that same genes and proteins were identified from different approaches further confirmed their values in the host factor studies on *A. tumefaciens*-mediated transformation.

3.1.2. Yeast as an ideal model for host factors study

Although many host factors important for AMT have been identified from plant, it is still far from enough for assembling the T-DNA transfer pathway(s) and understanding the molecular basis and biological processes involved in the T-DNA trafficking inside host cell. Thus, additional host factors need to be identified. However, some plant features may hinder efficient identification of host factors. For example, it ususally takes several months before we can get the seeds. In addition, it also takes several weeks for tumor formation after *Agrobacterium* infection. Thus, each batch of experiments takes very long time. Moreover, mutagenesis is another difficult task for plant and sometimes not possible.

In 1995, Bundock *et al.* successfully transferred T-DNA from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*, which became a good model for host factor study (Bundock *et al.*, 1995). Compared to plant system, yeast provides a lot of advantages for host factors indentification and related study. Yeast is the simplest eukaryote that can be readily manipulated, just similar to bacteria. Budding yeast *S. cerevisiae* replicates every 90 min at optimal conditions, which saves a lot of time. Moreover, Yeast has a relatively small genome size (only about 12 Mb and about 6, 000 genes) and yeast genome sequence is available since 1996. In addition, different kinds of gene libraries are available, which facilitate rapid gene identification and characterization, such as ORF deletion library (Giaever *et al.*, 2002), TAP-tagged library, and GFP-tagged library. Nowadays, yeast has been widely used as a model organism for *A. tumefaciens*-mediated transformation study, including host factors screening.

3.1.3. Host factors screening in yeast

Because of the importance of understanding the gene trafficking pathway and the host factors involved in the gene trafficking pathways, host facotors screen has become a hotspot research area in recent decade. However, most of the screen systems for host factors involved in *A. tumefaciens*-mediated T-DNA transfer were carried out in plant system. Since the developing of yeast as a good model for host factors study, several host factors screens have been explored.

In 2003, Robberts *et al.* tested 100,000 yeast transposon insertion mutants to search for yeast mutants supersensitive to *A. tumefaciens*-mediated transformation (Robbert *et al.*, 2003). To assist rapid identify supersensitive mutants, they adapted a replica-plating assay. Using this screen system, 24 supersensitive mutants, all of which were adenine deficient mutants, were identifed and a purine systhesis pathway, which positively regulates *A. tumefaciens*-mediated transformation process, were proposed. Although several supersensitive mutants were identified, the replica-plating method is still inefficient as compared to the total number of mutants they have used. In addition, this method is not holistic as it can only detect limited number of supersensitive mutants but not the transformation resistant mutants.

In 2004, another screening system was performed by Kawai *et al.* They used lithium acetate-mediated transformation of yeast to screen host factors related in the gene deliverey process. Although some gene candidates were identified in their assay, this kind of scenario cannot be applied to natural gene transfer systems and is totally different from *A. tumefaciens*-mediated T-DNA transfer system. In the *A. tumefaciens*-mediated T-DNA transfer system, the T-DNA is transferred naturally via *A. tumefaciens* type IV secretion channel; however, in the lithium acetate-mediated system, yeast cell wall is digested by chemicals and the DNA is forced into host cells

by heat shock. Furthermore, the chemical treatment may change some genetic property of the yeast. Thus, the genes found in the assay may share different gene transfer pathway from and thus could not be applied to *A. tumefaciens*-yeast gene transfer system.

In 2009, after or at the same time of the screen system was established in this study, a genome-wide host factor screen was conducted using yeast gene deletion library (Soltani, 2009). In this system, an *A. tumefaciens*-mediated T-DNA transformation efficiency profile of the yeast gene deletion library was obtained and compared with wild type strain, thus mutants with significantly alterred AMT efficiency was identified. This system was supposed to be able to identify both supersensitive mutants as well as resistant mutants, however, only 7 histone acetyltransferases and deacetylases related genes, which were previously reported to be involved in AMT process (Wu *et al.*, 2001; Mysore et al., 2000; Loyter *et al.*, 2005; Nagy and Tora, 2007), were identified.

Although previous studies identified some host factors and shed some light on the understanding the gene transfer process, their screening systems are either inefficient or not compatible with the *Agrobacterium* gene transfer system. Therefore, more additional host genes are still in urgent need to be identified. In order to achieve this goal, an efficient and holistic host factors screening system was developed in this study.

3.2. Construction of pHT101

To facilitate the high efficiency transformation of yeast, a binary vector, which can be replicated in *Agrobacterium* cell as well as in yeast cell, is needed. The super virulence *A. tumefaciens* strain EHA105, which contains a nopaline pTiBo542

harbouring a T-DNA deletion, was selected for the transformation assay. Thus, a T-DNA border sequences together with T-DNA should be included in the binary vector. Yeast replication origin sequence and yeast selction marker as well as yeast promoters are also needed for the new binary vector. To fullfil those requirements, a new *Agrobacterium*-yeast binary vector was designed.

pEGFP209 was first digested with *Sal*I and dephosphorylated with Shrimp Alkaline Phosphatase (SAP) then was inserted into *Sal*I digested pCB301 fragment. The orientation of the insertion was checked by digestion. The trans-orientation, as shown in Fig 3.1, was chosen to transform into the *A. tumefaciens* strain EHA105. The EHA105 harboring pHT101 plasmid was capable of transforming yeast strain *S. cerevisiae* (data not shown).

In this study, *S. cerevisiae* haploid strain BY4741 strain (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) was used as host strain for *A. tumefaciens*-mediated transformation and for host factors screening study. Leucine auxotrophic minimum medium was used for selection of *A. tumefaciens*-mediated yeast transformants. The transformants can also be visualized under UV light.

3.3. Development of a novel liquid transformation protocol

3.3.1. Introduction

There are two *Agrobacterium tumefaciens*-mediated transformation of *Saccharomyces cerevisiae* protocols, which were developed by Bundock *et al.* (1995) and by Piers *et al.* (1996) respectively. Both of the protocols need to co-cultivate the *A. tumefaciens* and *S. cerevisiae* cells on co-cultivation (CM) agar plates. After co-cultivation, the cells were collected by scraping off or washing off from the CM agar plate, which is tedious, time-consuming, and inefficient. More importantly,

considerable amount of cells is lost during the collecting process, which arbitrarily increases the variation of transformation efficiencies. Thus, the protocols are not suitable for large-scale screening. In order to explore an efficient large-scale screen, we developed a simple and efficient way to do the co-cultivation.

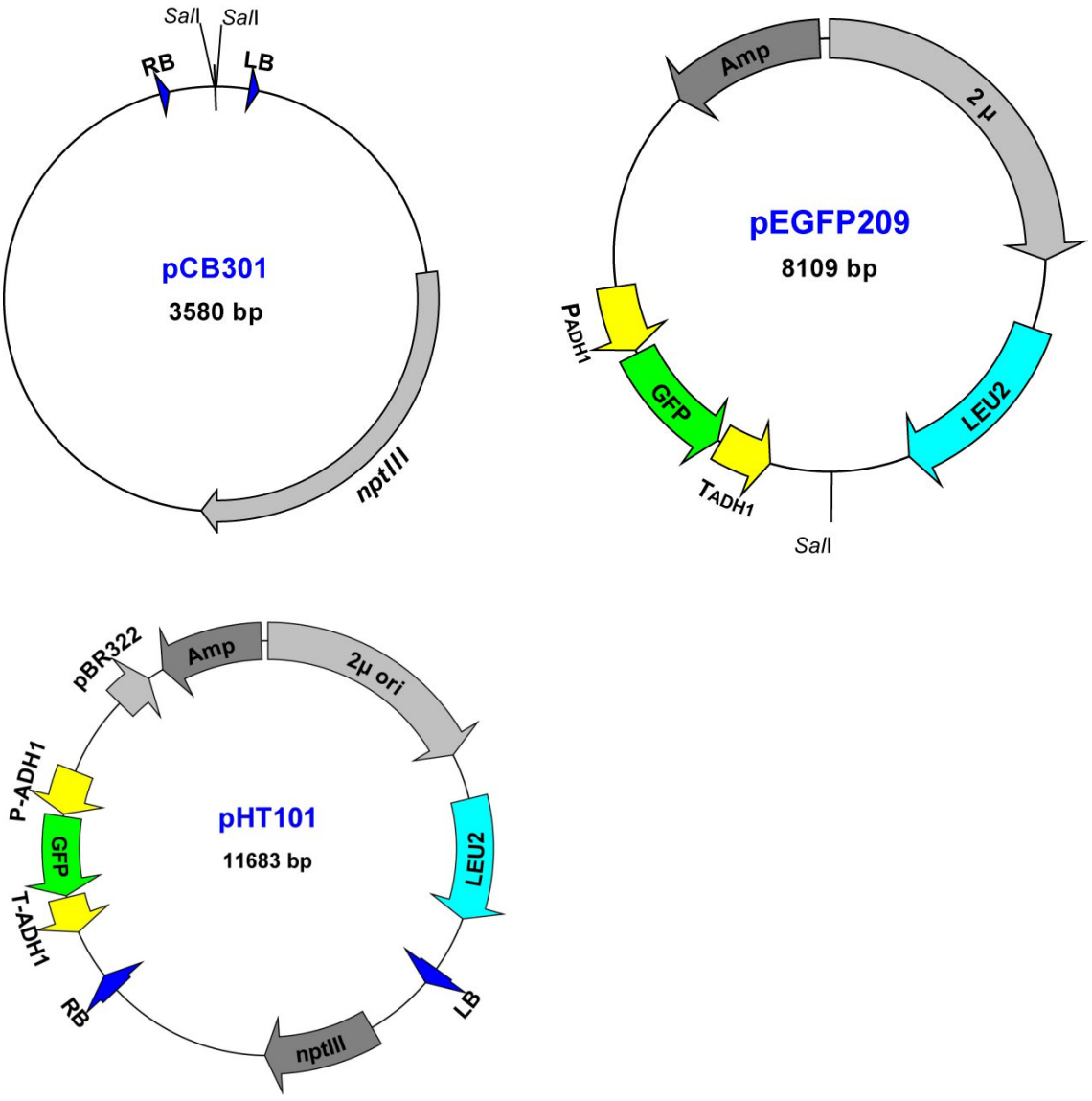


Figure 3.1. Plasmid maps for pHT101 construction. pCB301 and pEGFP209 were used to construct binary vector pHT101 by ligation at the *Sal*I site.

3.3.2. Methods

At day-1 evening, *A. tumefaciens* was inoculated from fresh MG/L plate to 1 ml MG/L broth with proper antibiotics and grown at 28 ^{o}C for over night. At day 2 morning, saturated *A. tumefaciens* culture were subcultured 100 times into fresh MG/L broth with antibiotics. At day-2 evening, the *Agrobacterium* culture was collected into 2 ml eppendorf tube by centrifugation at 10,000 rpm for 1 min when the culture reaches OD_{600} to 1.0. The cell pellet was washed 2 times with 1 ml of IBPO4. The cells were then diluted with IBPO4 with 100 μg/ml Km and 200 uM AS at a final concentration of OD_{600} equals to 0.3. The culture was induced at 28 ^{o}C incubator with shaking for 16-18 h. At the same time, *S. cerevisiae* was inoculated from fresh YPD agar plate to 1 ml of YPD broth and grown at 28 ^{o}C for over night. At day-3 morning, when yeast culture reaches OD_{600} 3.0, 25 μl of yeast culture (about 2.5E+06 cells) was mixed with 500 μl (about 2.5E+08 cells) of induced *Agrobacterium* culture in a 14 ml cell culture tube. The co-cultuvation mixture was incubated in 20 ^{o}C for 24 h. It should be noticed that it is critical not to shake the co-cultivation tube during the co-cultivation. At day-4, 100 μl of cocultivation mixture was dropped onto SD Leu- agar plate with 100 μg/ml Cef. At the same time, 100 μl of proper diluted mixture was dropped onto YPD agar plate with 100 μg/ml Cef. Colonies appeares after 3-day's incubation at 28 ^{o}C incubator.

Instead of co-cultivating the cells on the CM agar plates, this protocol co-tultivates *Agrobacterium* and yeast cells into a small volume of liquid co-cultivation medium at the same ratio as the agar plate co-cultivation. The co-cultivation was conducted without stirring or shaking of the *Agrobacteria* and yeast cells, which allows the attachment of the *A. tumefaciens* cells onto the *S. cerevisiae* cells. After co-

cultivation, the cells suspensions were dropped onto the selection and recovery agar plates instead of spreading the cells onto the plates.

Using this liquid co-cultivation protocol, *Agrobacterium* strain EHA105 carrying pHT101 plasmid can normally achieve 5.0E-04 transformation efficiency of yeast strain BY4741. As shown in Fig 3.2, this new protocol is able to detect high efficiency mutants (Fig 3.2 B red circle) as well as low efficiency mutants (Fig 3.2 B green circle) as compared to wild type strain.

3.3.3. Results

It is interesting to notice that dropping but not plating the co-cultivation mixture onto selection plate is critical for the successful transformation. When spreading the co-cultivation mixture from the same tube onto selection plate, no transformants appeared after 3~4 days growth (data not show). It is possible that as compared to separating cells on selection mediate in the plating method, a large amount of *Agrobacteria* and yeast cells in the relatively small droplet are still attached to each other thus facilitating T-DNA transfer from *Agrobacterium* cell to yeast cell. In addition, the selection medium also has minimal nutritions and weak acid pH, which are required for *vir* genes induction and T-DNA transfer (Engstrom *et al.*, 1987). Therefore, it is likely that the T-DNA transfer process continues on the selection medium in this liquid co-cultivation assay. In addition, because there was no transformant after co-cultivation mixture was spreaded onto selection plate in the liquid co-cultivation assay and transformants started to appear even at 0 h, it is possible that after liquid co-cultivation, the T-DNA transfer from *Agrobacterium* to yeast cell is not completed and may continues on selection plate.

Actually, this novel transformation procedure is the first liquid based co-

cultivation protocol in the world. Compared to the Bundock's or Piers' protocols, this new co-cultivation protocol is simpler and more convenient. More importantly, this protocol minimized the cell loss, which is inevitable in the previous protocols. In addition, this liquid co-cultivated transformation protocol generates about ten-fold higher transformation efficiencies, as compared to the previous methods. Thus, this protocol is suitable for large-scale screenings.

3.4. Development of a new high-throughput screening system

Because the liquid co-cultivation is very easy to handle and more importantly suitable for large-scale screening, we adapt this novel liquid co-cultivation based *A. tumefaciens*-mediated transformation of yeast protocol to a new genome wide screening system for host factors invovled in *Agrobacterium*-yeast T-DNA transfer process.

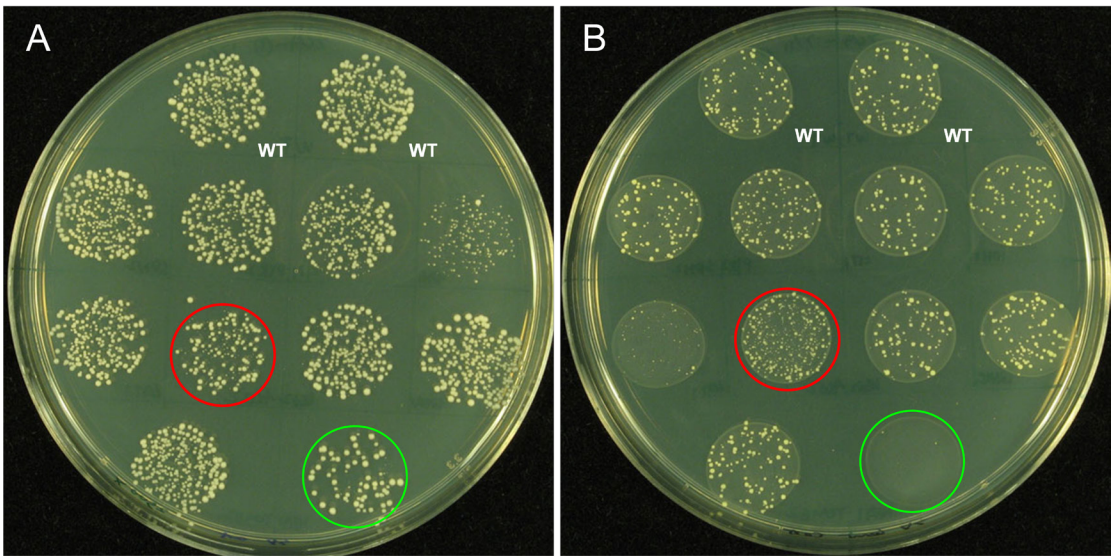


Figure 3.2. A liquid *A. tumefaciens*-mediated transformation of *S. cerevisiae*. Red circles represent high efficiency mutants. Green circles represent low efficiency plate. **A**: SD recovery plate. **B**: SD Leu- plate.

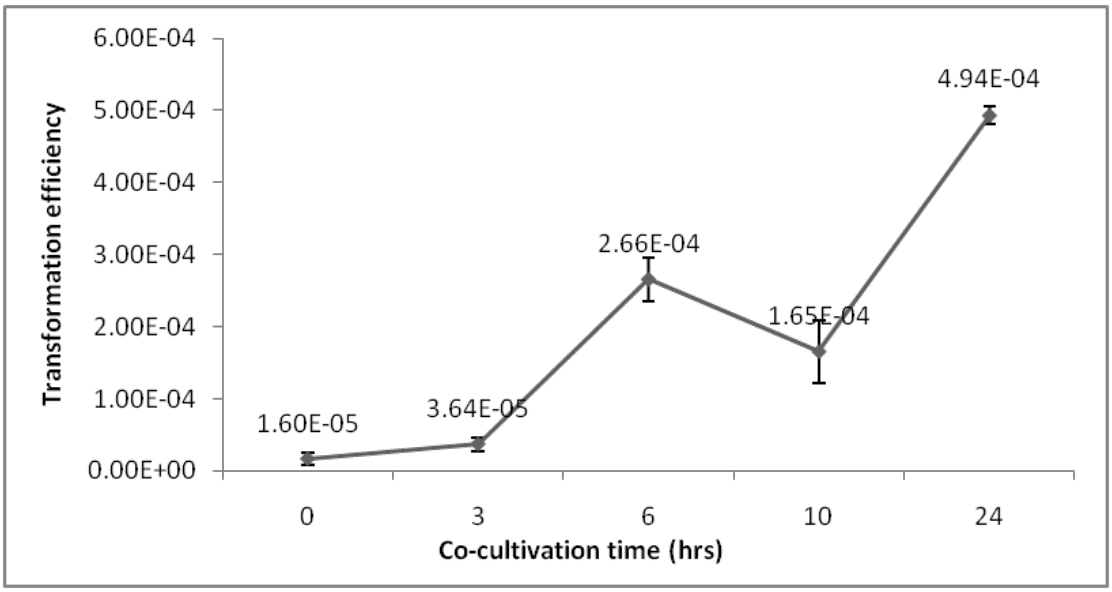


Figure 3.3. Time course study of liquid co-cultivation. Transformation efficiencies were calculated based on 0, 3, 6, 10, or 24 h co-cultivation of Agrobacteria and yeast cells.

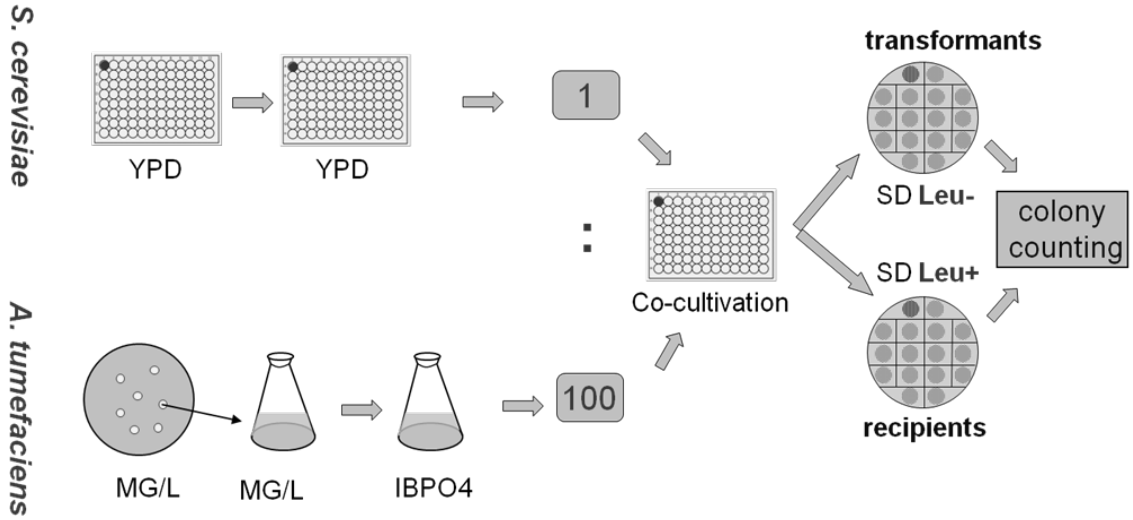


Figure 3.4. A protocol for large-scale screening of YKO strains. Yeast cells were inoculated from thawed YKO collection stocks which were stocked in 96-well microplates. Yeast and *Agrobacterium* cells were also co-cultivated in 96-well microplates by mixing 5 µl of yeast culture and 120 µl of induced Agrobacterium culture. Selection and recovery (1000 times dilution) were conducted by dropping 24 h co-cultivated mixture onto selection and recovery medium.

3.4.1. The YKO library screening system

For the YKO library, the host factor screening was explored in 96-well cell culture plate (Iwaki). The protocol is illustrated in Fig 3.4. The YKO strains were kept in 96-well glycerol stock at -80 ^{o}C. Firstly, 2 µl of thawed YKO strain from -80 ^{o}C was inoculated into 100 µl of fresh YPD broth in steril 96-well cell culture plate. The culture was incubated at room temperature (about 22 ^{o}C) for about 30 h at a robital shaker at 225 rpm in dark. Wild type strain BY4741 was inoculated into the 2 empty wells in the 96-well plate to serve as the positive controls. After 30 h incubation, most YKO strains were satuated. At the same time, *A. tumefaciens* strain EHA105(pHT101) were inoculated into MG/L medium and incubated at 28 ^{o}C for over night. After 100 times dilution and subculture, the *Agrobacterium* cells were collected and washed 3 times with IBPO4. The cells were then induced in IBPO4 with Km and AS and at a final concentration of OD_{600} equals to 0.3 for 18 h. 120 µl of induced *Agrobacterium* cells (OD_{600} around 0.5 and total cell number is about 5.0E+07 cells) were aliquoted into a new 96-well plate. 5 µl of yeast culture (OD_{600} around 3 and total cell number around 5.0E+05) were directly added into the same 96-well plate containing 120 µl of induced *A. tumefaciens* cells. The co-cultivation 96-well plate was put at a 20 ^{o}C incubator without shaking in dark. After 24 h co-cultivation, 100 µl of the co-cultivation mixture were directly dropped onto SD Leu-Cef selection plate, while 2 µl of the co-cultivation mixture were diluted 1,000 times and dropped onto SD Cef plate. Colonies showed up after 3-4 days incubation at 28 ^{o}C incubator. After conoly counting, transformation efficiency was calculated for each mutant strain.

3.4.2. The yTHC library screening system

The protocol for yTHC library screening was modified from the protocol for YKO library screening. Yeast strains were inculated to 100 µl of YPD in 96-well plate and incubated at room temperature for about 30 h in dark. Yeast strain R1158 derived from BY4741 was used as wild type strain in the yTHC library screening. Doxycycline was added into each well to a final concentration of 10 µg/ml and induced for another 18 h. Co-cultivation was mixed as the same cell number with YKO library screening and dropped onto CM co-cultivation plate with Km 100 µg/ml As 100 uM. After 24 h co-cultivation, cells were scrapped off from CM plate and resuspended in 120 µl of 0.9% NaCl. 100 µl of cell resuspension were directly dropped onto SD Leu- Cef selection plate, while 2 µl of cell resuspension were diluted 2,500 times before dropped onto SD Cef recovery plate. Colonies showed up after 3-4 days incubation.

3.5. Large-scale screening for host factors involved in *Agrobacterium*-yeast gene transfer

In order to perform a genome wide host factors screening, we obtained two *S. cerevisiae* mutant libraries from Open Biosystems, the yeast knockout (YKO) collection developed by Winzeler *et al.* (1999) and the Tet-promoters Hughes collection (yTHC) developed by Hughes Laboratory (Mnaimneh *et al.*, 2004), which totally cover 96.7% of the entire yeast genome. For the YKO collection, each yeast ORF was replaced with a KanMX cassetteusing homologous recombination strategy, which created about 5,800 ORF knockout mutants (Wach *et al.*, 1994; Winzeler *et al.*, 1999; Giaever *et al.*, 2002). The yTHC collection contains about 800 essential yeast genes, for which expression is regulated by a chemical named doxycycline. The endogenous promoter of each essential gene has been replaced with a Tet-titratable promoter in the genome. Therefore, the expression of the gene can be switched off by

the addition of doxycycline to the yeast's growth medium (Hughes *et al.*, 2000; Mnaimneh *et al.*, 2004).

Using the newly developed host factor screening systems, we perform the genome-wide screen for host factors involved in *Agrobacterium*-yeast gene transfer. After the first round screening, we selected out the mutants that had 2 times higher or lower transformation efficiencies, as compared to the wild type. Subsequently, these mutants were tested again to identify the mutants that consistently had higher or lower transformation efficiencies. After 3 rounds narrow down screening, mutants consistantly giving higher or lower transformation efficiencies were identified.

3.6. A global profile of yeast genes involved in *Agrobacterium*-yeast gene transfer

After four rounds screening, we finally narrowed down the genes that affected *A. tumefaciens*-mediated transformation of *S. cerevisiae*. We identified 209 candidate mutants that have at least 3 times higher or lower transformation efficiencies. Based on the known or proposed functions or biological processes involved of the identified genes, we grouped these genes into different categories. Thus, a global profile of yeast genes involved in *Agrobacterium*-yeast gene transfer was obtained. Interestingly, we found that many genes belong to the same groups, such as DNA replication and DNA repair related genes, RNA processing related genes, TCA cycle related genes, mitochondrial related genes, protein trafficking related genes, and etc. The summary of the screening results are listed in Table 3.1.

As can be seen in Table 3.1, interestingly, most of the biological processes were involved in the T-DNA transfer process, even though they may play different roles in the process. T-DNA transfer is a very complex process, such as cytoplasm trafficking, nuclear targeting, and T-DNA integration. Therefore, it is likely that proteins related

to these processes may affect T-DNA transfer. In addition, many genes involved in endocytosis and vesicle trafficking process were involved in the T-DNA process. More interestingly, all of them are supersensitive to *Agrobacterium*-mediated transformation, implying that the T-DNA trafficking pathway may compete with endocytosis and vesicle trafficking pathway. If the endocytosis and vesicle trafficking pathway is blocked, T-DNA may be transferred to host nuclear more efficiently. Surprisingly, some biological processes not related to gene transfer were found to be important in the T-DNA transfer process, such as TCA cycle, protein modification, and glycosylation, which prove the complexity of the T-DNA process.

This study is the first to give a global profile of yeast gene involved in *Agrobacterium*-yeast gene transfer process. 96% of yeast genome was tested for the *Agrobacterium*-mediated T-DNA transfer efficiency. Gene candidates important for T-DNA transfer were identified and possible T-DNA trafficking pathway was proposed. Further detailed studies will be carried out to several candidate genes.

Table 3.1. A global profile of yeast genes involved in T-DNA transfer

Gene function	Higher efficiency mutants			Lower efficiency mutants			Total
	Non-essential	Essential	Sub-total	Non-essential	Essential	Sub-total	
Unknown function	18	1	19	14	0	14	33
Endocytosis and vesicle trafficking	17	6	23	0	0	0	23
Mitochondria	5	0	5	8	1	9	14
RNA processing	1	6	7	5	1	6	13
Biosysthesis	6	1	7	5	0	5	12
Transporters	8	0	8	2	0	2	10
Protein synthesis	5	0	5	4	1	5	10
Transcription	1	3	4	4	2	6	10
DNA modification and repair	5	0	5	4	1	5	10
TCA cycle and respiration	1	0	1	9	0	9	10
Cell Viability and stress resistance	8	0	8	1	0	1	9
Kinases and signal transduction	7	0	7	0	0	0	7
Cytoskeleton	5	0	5	0	0	0	5
Glycosylation	1	1	2	2	1	3	5
Protein modification	2	0	2	2	1	3	5
Ubiquitination and protein degradation	3	1	4	1	0	1	5
Nucleus	0	1	1	4	0	4	5
Transcription factors	3	0	3	2	0	2	5
Cell wall	2	0	2	2	0	2	4
Protein targeting	1	1	2	2	0	2	4
mRNA transport	1	1	2	2	0	2	4
Cytokinesis	2	0	2	0	0	0	2
Microtubule	0	0	0	1	0	1	1
Protein secretion	0	0	0	1	0	1	1
Post-transcriptional regulator	0	0	0	1	0	1	1
Mating response	1	0	1	0	0	0	1
Total	103	22	125	76	8	84	209

CHAPTER 4. INVOLVEMENT OF OST COMPLEX IN *AGROBACTERIUM*-YEAST GENE TRANSFER

In this study, we obtained a global profile of yeast genes involved in *Agrobacterium*-yeast gene transfer through a novel host factor screening system. As a result, we identified many candidate genes that may play important roles in the *Agrobacterium*-yeast T-DNA transfer process. Among those gene candidates, we found the *ost3Δ* mutant strain significantly decreased *A. tumefaciens*-mediated transformation efficiency. Ost3p is the gamma subunit of the oligosaccharyltransferase (OST or OT) complex of the endoplasmic reticulum (ER) lumen, which catalyzes asparagine-linked (N-linked) glycosylation of newly synthesized protein (Knauer and Lehle, 1999a). Ost3 protein is important for N-linked glycosylation of many proteins (Karaoglu *et al.*, 1995). Therefore, because of the involvement of *ost3*Δ in the *Agrobacterium*-yeast T-DNA transfer process, the OST complex and the glycosylation processes may be involved in the T-DNA transfer process. This chapter discusses the roles of the *OST3* gene, the OST complex, and the entire glycosylation processes in the *A. tumefaciens*-mediated transformation of *S. cerevisiae* process.

4.1. Introduction of the OST complex

4.1.1. N-linked Glycosylation

Eukaryotic cells are regulated by a series of cellular processes, including cell cycle control, energy production, protein processing and waste disposal. Compared with other cellular processes, glycosylation is probably the most complex and ubiquitous protein modification within the eukaryotic system (Baenziger, 1994). More than 70% of proteins processed by these processes are glycosylated (Gavel and von

Heijne, 1990; Imperiali and Hendrickson, 1995; Mononen and Karjalainen, 1999; Dempski and Imperiali, 2002). Glycosylation is a co-translation and post-translational modification process, linking saccharides to produce glycans that are attached to proteins, lipids, or other organic molecules. Glycoproteins have been found involving in many processes such as immune response (Opdenakker *et al.*, 1993), intracellular targeting (Gleeson *et al.*, 1994), signal recognition (Wagner *et al.*, 1994), and proper protein folding, stabilization, and solubility (Imperiali and Riekert, 1995; Riederer and Hinner, 1991; Alien *et al.*, 1995; Duranti *et al.*, 1995).

There are three kinds of protein carbohydrate modification: asparagine-linked (N-linked) glycosylation, O-linked glycosylation of serine or threonine, and the glycosyl-phosphatidyl inositol (GPI) derivatization of the C-terminus carboxyl group (Rudd and Dwek, 1991). N-linked glycosylation is catalyzed by a enzyme complex, the oligosaccharyltransferase (OST), and involves the en bloc transfer of the preassembled oligosaccharide $Glc_3Man_9GlcNAc_2$ from donor, dolichyl pyrophosphate, onto asparagine side chain within nascent polypeptide during translocation into the endoplasmic reticulum (ER) lumen (Knauer and Lehle, 1999a). In contrast, O-linked glycosylation involves direct post-translational transfer of single monosaccharides to threonine / serine residues in folded polypeptides. O-linked glycosylation involves several different enzymes; however, its peptides sequence secificities are not very clear (Wilson et al, 1991). Moreover, proteins can also be processed with a glycosyl phosphatidyl inositol (GPI) at the C-terminus. This modification anchors protein to membrane bilayers (Englund, 1993). GPI modification recognizes 6 C-terminal amino acids: Cys, Asp, Asn, Gly, Ala or Ser (Dwek, 1995).

4.1.2. The OST complex

Glycosylation process is catalyzed by a membrane associated multimeric enzyme, the oligosaccharyltransferase (OST, also called OT), localized in the ER lumen. OST recognizes a consensus sequence of Asn-X-Thr/Ser, where X can be any amino acid residue except for proline (Welply *et al.*, 1983; Silberstein and Gilmore, 1996; Knauer and Lehle, 1999a). It has been proven that in both yeast and mammalian that OST exists as a heteromeric, multisubunit complex in the ER membrane. In the past two decades, OST subunits in *Saccharomyces cerevisiae* have been identified and characterized (for review see Knauer and Lehle, 1999a; Dempski and Imperiali, 2002; Yan and Lennarz, 2002 and 2005a; Lennarz, 2007). *S. cerevisiae* OST contains 9 membrane-bounded subunits (Fig 4.1). Among them, five proteins (Ost1p, Ost2p, Stt3p, Wbp1p, and Swp1p) are essential for cell survival; Ost4p is essential for cell growth at 37 ^{o}C but not at 25 ^{o}C; Ost3p, Ost5p, and Ost6p are not essential for the viability of yeast cell but required for full enzyme activity. Ost6p is an isoform of Ost3p. Genetic, immunoprecipitation, and overexpression experiments show that the OST contains 3 subcomplexes: (Wbp1p, Swp1p, and Ost2p), (Ost1p and Ost5p), and (Stt3p, Ost4p, and Ost3p/Ost6p) (Karaoglu *et al.*, 1997; Spirig *et al.*, 1997; Silberstein *et al.*, 1995; Zufferey et al, 1995; te Hessen *et al.*, 1993; Yan and Lennarz, 1999). Proteins within each subcomplex also interact with each other (Park and Lennarz, 2000; Stagljar *et al.*, 1998).

In addition to yeast OST, many mammalian OST subunits have also been identified and characterized in recent years. Genome-wide searches showed that many of the mammalian OST subunits share high homologies with yeast OST subunits (Kelleher *et al.*, 2003), which is showed in Table 4.1 in details.

4.1.3. The properties of OST subunits

WBP1. Wbp1p was first identified as an essential ER type I glycoprotein that can bind wheat germ agglutinin (te Hessen *et al.*, 1991). Wbp1 protein has an N-terminal signal sequence, N-linked glycans, a transmembrane (TM) domain near its C-terminal, and a cytoplasmic tail containing a dilysine ER retention/retrieval motif (Silberstein *et al.*, 1992; Gaynor *et al.*, 1994; Fu and Kreibich, 2000). *WBP1* is important for OST activity both *in vitro* and *in vivo* (te Heesen *et al.*, 1992). Point mutations in *WBP1* lead to underglycosylation of yeast glycoproteins both *in vivo* and *in vitro* and destabilized the OST complex (te Heesen *et al.*, 1992; Karaoglu *et al.*, 1997). Yeast Wbp1p shares 25% identity and 50% similarity with the mammalian Ost48p (Table 4.1). It was proven that Wbp1p is the subunit that recognizes dolichol-pp-oligosaccharide (Pathak *et al.*, 1995; Bause *et al.*, 1997). In addition, the lumen part of the Wbp1 transmembrane domain is important for the Wbp1 function (Li *et al.*, 2003).

SWP1: Swp1p was first identified as an allele-specific suppressor of a Wbp1p mutation. Swp1p is 30 kDa in molecular weight and contains three transmembrane domains. Swp1p interacts with Wbp1p, Ss1p, and Stt3p (te Heesen et al, 1993; Chavan *et al.*, 2005; Dempski and Imperiali, 2004). In addition, because Swp1p lumenal domain was also found to interact with Ost1p, it may help Ost1p to form nascent polypeptide chains into the active site of the OST complex (Li *et al.*, 2003). Although exists as an essential gene product, the function of Swp1p is not well understood. Swp1p shares homology with mammalian ribophorin II (Knauer and Lehle, 1999a).

OST2: Ost2p, with a mass of 16 kDa, contains 2 or 3 transmembrane domains and a hydrophobic C-terminus (Silberstein *et al.*, 1995). Genomic disruption of yeast *OST2* gene is lethal. Conditional mutation of *ost2* strain leads to an

underglycosylation of soluble and membrane-bound glycoproteins because of its affects in OST activity (Knauer and Lehle, 1999a). Ost2p shares 45% identity with mammalian Dad1p, which is also essential in mammalian species. However, a temperature-sensitive mutant of Dad1 could not be complemented by yeast Ost2p (Makishima *et al.*, 1997). Disruption of DAD1 function affects the essential process of N-linked glycosylation, thus may indirectly lead to apoptosis (Nakashima *et al.*, 1993). It was shown that Ost2p strongly interacts with Wbp1p, therefore it may function in recognition of the Dol-PP-oligosaccharides together with Wbp1p (Dempski and Imperiali, 2004; Yan and Lennarz, 2005b).

OST1: Ost1p has a mass of 64 kDa, contains an N-terminal signal sequence, four potential N-X-T/S glycosylation sites, a single TM domain and a C-terminal cytosolic domain. Ost1p shares 28% identity and 58% similarity to mammalian ribohporin I. Ost1p can interact with all other OST subunits (Dempski and Imperiali, 2004). Ost1p is partially glycosylated in wild type cells therefore has 64/62/60 kDa glycoforms. Disruption of *OST1* gene caused underglycosylation of soluble and membrane-bound glycoproteins, and reduced OST activity *in vitro* (Silberstein, *et al.*, 1995). In addition, it was found that Ost1p was able to interact with glycosylatable photoreactive acceptor substrates, therefore it was hypothesized that Ost1p contains the peptide-bindng site of the OST complex (Yan *et al.*, 1999). However, it was disproved that the functional domain of Ost1p actually exists in its membrane-bound lumenal domain (Yan and Lennarz, 2002). All these data show that the lumenal domain of Ost1p functions in conveying nascent proteins into the active site on Stt3p for the N-linked glycosylation reaction.

OST5: Ost5p is a membrane protein with a mass of 9 kDa and 2 transmembrane domains. Although Ost5p is not essential for cell viability, it is required for maximal

activity of OST complex (Reiss *et al.*, 1997). Although putative Ost5p homologues have been found in metazoan organisms sequence database, biological evidence showing they are OST subunits is not available at present time (Kelleher *et al.*, 2003; Shibatani *et al.*, 2005). Until now, no function can be proposed for Ost5p in the OST reaction.

STT3: Stt3p was initially identified in a screen for yeast supersensitive to staurosporine, an inhibitor of protein kinase C (Yoshida *et al.*, 1992). Stt3p is the largest of the essential subunits in the OST complex, containing 12-13 TM domains and a C-terminal hydrophilic domain located in the ER lumen (Zufferey *et al.*, 1995). Stt3p is the most conserved protein across species, including *Entamoeba histolytica, Campylobacter jejuni, Drosophila melanogaster* and *Homo sapiens* (Gutiérrez *et al.*, 2000). In addition, Stt3p shares more than 50% identity to eukaryotic proteins from human, mouse, and *C. elegans*. Mutation in the *STT3* gene affects the substrate specificity of the OST complex both *in vivo* and *in vitro* (Zufferey *et al.*, 1995). In addition, depletion of Stt3p causes almost complete loss of OST activity and also affects the assembly of the OST complex (Zufferey *et al.*, 1995). During past 2 decades, many evidences have been proven the fact that Stt3p is the catalytic site of the OST complex. A Stt3p homologue in *C. jejuni,* PglB, is essential for glycosylation and it is the only OST subunit present in this bacterium (Wacker *et al.*, 2002). In the mammalian system, the Stt3p was found to be the only OST subunits that could interact with the glycosylation consensus sequence of newly synthesized peptides (Nilsson *et al.*, 2003). A recent paper showed that the parasitic protozoa *Leishmania major* homologues of yeast Stt3p was able to complement a deletion of the yeast *STT3* locus and restored the lethal phenotype of single and double deletions in essential

OST subunits (Nasab *et al.*, 2008). All of these data clearly proved Stt3p as the catalytic subunit of the OST complex and indispensable for OST activity.

OST4: *OST4* encodes a 36 amino acids protein, which is the smallest verified protein in yeast. *OST4* is not essential for yeast cell viability; however, *ost4Δ* mutants are temperature sensitive, grow poorly at 30 ^{o}C and are inviable at 37 ^{o}C (Chi *et al.*, 1996). In addition, *ost4*Δ mutants cause a severe underglycosylation of glycoproteins both *in vitro* and *in vivo* (Chi *et al.*, 1996). Analysis of the hydropathy profile of Ost4p revealed that Ost4p is wrapped in the hydrophobic core of the membrane. At present time, the specific function of Ost4p is not clear. Homologues of yeast Ost4p were found in fungi, vertebrate, nematode, insect, and plant (Kelleher and Gilmore, 2006). Human and mouse expressed sequence tags (EST) databases contain highly expressed mRNAs that are homologues to yeast Ost4p (Kelleher and Gilmore, 2006).

OST3*/*OST6: The Ost3 protein was first identified as a 34 kDa. The Ost6 protein, with a molecular weight of 32 kDa, is the isoform of Ost3p. Ost6 is the less abundant form of yeast OST complex lacking Ost3p and is believed to perform redundant function of the Ost3p (Spirig *et al.*, 2005; Chavan *et al.*, 2006). The *OST6* shares 21% weak sequence identity to *OST3*; however, they have a very similar topology particularly 4 C-terminal TM spans (Knauer and Lehle, 1999b). The Ost3 protein family has an N-terminal signal sequence, an N-terminal lumenal domain, and four predicted TM spans (Fetrow *et al.*, 2001; Karaoglu *et al.*, 1995). Disruption of either *OST3* or *OST6* only leads to a minor underglycosylation; however, an *ost3*Δ*ost6*Δ double mutant causes a severe underglycosylation of membrane-bound and soluble glycoproteins but does not have a temperature sensitive phenotype (Chavan *et al.*, 2006). In addition, overexpression of Ost6p can restore the underglycosylation in *ost3*Δ mutant, which suggests their similar roles of the two

proteins in the OST complex (Knauer and Lehle, 1999b). Interestingly, Ost3p and Ost6p have different interaction specific properties with Sbh1p (the β-subunit of the Sec61 translocon) and Sbh2p (the β-subunit of the Ssh1 translocon) (Yan and Lennarz, 2005b), which suggests that the 2 isoforms of the OST complex specifically interact with the 2 structurally similar translocon complexes. However, one piece of evidence showed that the *C. elegans OST3* gene (ZK686.3) is essential for *C. elegans* survival. Disruption of ZK686.3 by RNAi causes an embryonic lethal of *C. elegan* (Kamath *et al.*, 2003) due to a defect in cytokinesis (Gonczy *et al*., 2000), which implies other important roles of the *OST3*.

Sequence analysis revealed that Ost3 protein family are conserved among all fully sequenced vertebrate, nematode, fungi (except *E. cuniculi*), arthropod, and plant genomes (Kelleher and Gilmore, 2006). Interestingly, only the *Saccharomycetaceae* family of budding yeasts has the *OST3* isoform, the *OST6*. *OST3* and *OST6* share 20% sequenece identity to human *TUSC3* (also known as *N33*), which is a candidate tumour suppressor gene (MacGrogan *et al.*, 1996). All these evidence seems to imply that *OST3* plays important roles other than N-linked glycosylation.

Table 4.1. Comparison of yeast and human OST complexes

Yeast	**Protein size (kDa)**	***H. sapiens* subunit**	**Identity**	**Similarity**
Stt3p	82	Stt3	51%	65%
Wbp1p	45	Ost48	25%	50%
Swp1p	30	Ribophorin II	22%	49%
Ost1p	64/62	Ribophorin I	28%	58%
Ost2p	16	Dad1	45%	68%
Ost3p/Ost6p	34/32	N33	23%	41%
Ost4p	3.4	37 aa EST	36%	-
Ost5p	9	149 aa EST	38%	-

Knauer and Lehle, 1999a; Dempski and Imperiali, 2002; Kelleher and Gilmore, 2006

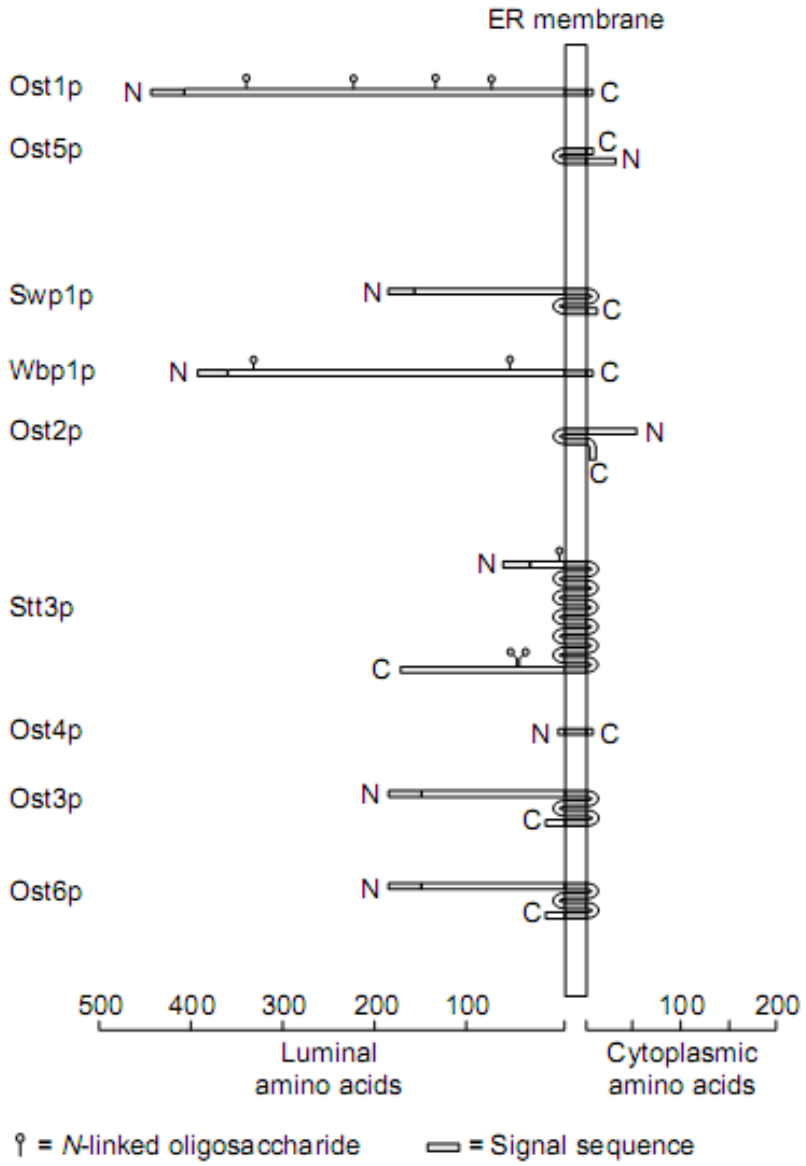


Figure 4.1. Predicted membrane orientations of yeast OST subunits. The topology is partially based on direct or indirect experimental evidence. The presentation is not to scale. The figure is adapted from Knauer and Lehle, 1999a.

4.1.4. OST related diseases

As stated previously, some OST subunits are essential for cell growth; thus, deletions of these essential components result in a lethal phenotype. Deletion of UDP-GlcNAc: dolichol phosphate N-acetyl-glucosamine-1-phosphate transferase, catalyzing the transfer of P-GlcNAc onto dolichol-P in embryonic cells, results in a lethal phenotype, which implies the importance of the glycosylation process in cell viability (Marek *et al.*, 1999). On the other hand, mutations of enzymes associated with the assembly or processing of N-linked sugar groups result in a broad range of disorders, including psychomotor retardation, nervous system deficienes, coagulation disorders and dysmorphic features (Matthijs and Jaeken, 2001). These diseases are called congenital disorders of glycosylation (CDGs). There are two types of CDGs. The type I CDGs (CDG1) are caused by mutations affecting the assembly of the tetradecasaccharide on the dolichol pyrophosphate donor. The type II CDGs (CDG2) are caused by mutations affecting N-linked processing proteins.

Several disorders of N-glycosylation haven been studied in the recent years. Defects of lipid-linked oligosaccharide (LLO) assembly and defects of N-glycan processing are resulted from abnormal transferrin glycosylation. Most genes on the N-glycosylation pathway encoding ER proteins have been found to be involved in glycosylation disorders; however, the OST complex seems different. Some recent data seem disagree with the assumption that defective of OST activity would cause abnormal transferrin glycosylation. Mutations in the *TUSC3/N33* gene, homology of the yeast *OST3*, did not affect the N-glycosylation of transferrin (Molinari *et al.*, 2008; Garshasbi *et al.*, 2008). The possible reason of the normal N-glycosylation of transferrin may be due to the partial rescue of *OST6*, which is homologue to *OST3* (Schwarz *et al.*, 2005; Knauer and Lehle, 1999b). However, even *OST6* and *OST3*

share similar funciton in OST activity, mutations in *TUSC3/N33* gene causes nonsyndromic mental retardation, which implies the unique function of *OST3* towards specific glycoproteins (Molinari *et al.*, 2008; Garshasbi *et al.*, 2008; Hennet, 2009). In addition, it also shows that *OST3* is important for proper glycosylation and functions of proteins in the central nervous system.

The *N33 (TUSC3)* gene is a tumor repressor candidate, which is located within a homozygously deleted region of a metastatic prostate cancer. *N33* is expressed in most nonlymphoid human tissues including prostate, lung, liver, and colon, as well as many epithelial tumor cell lines. Besides its role in metastatic prostate cancer, *N33* has been found to be involved in many important cellular biological processes and disorders, such as nonsyndromic autosomal recessive mental retardation (Molinari *et al.*, 2008; Garshasbi *et al.*, 2008), cellular magnesium uptake and vertebrate embryonic development (Zhou and Clapham, 2009), development of lymph node metastasis in larynx and pharynx carcinomas (Guervós *et al.*, 2007), and in ovarian carcinoma and survival (Pils *et al.*, 2005). Although its molecular basis and cellular mechanisim are not clear, the importance of *OST3* in cancers and disorders will prompt the search for specifically underglycosylated proteins and the better understanding of the mechanism involved in these biological processes.

4.2. Effects of *ost3*Δ on *Agrobacterium*-mediated transformation

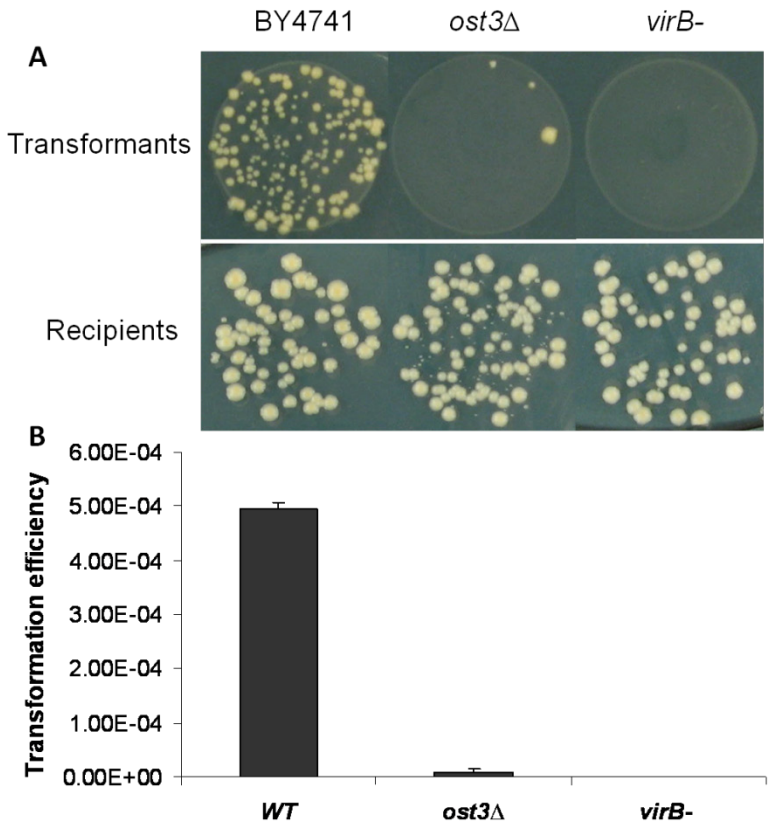
During the genome-wide screen of yeast factors involved in the *A. tumefaciens*-mediated tranformation of *S. cerevisiae*, we found that *ost3*Δ significantly decreased the AMT efficiency with about 50 folds reduced transformation efficiency (Fig 4.2 A and B). Since the screening is based on a liquid co-cultivation assay, we then further confirmed the effect of *OST3* on the AMT efficiency on co-cultivation (CM) plate. In addition, in the liquid co-cultivation assay, yeast cells were grown to stationary phase

(around 10^8 cells/ml) before co-cultivation with induced *Agrobacteria*; while in a standard CM plate-based co-cultivation assay, log phase yeast cells (around 10^7 cells/ml) were used. Thus, to compare the transformation efficiencies of both log phase and stationary yeast cells, we also tested the effect of *OST3* on the AMT efficiency at both log phase and stationary phase. As shown in Fig 4.2 C and D, compared with wild type yeast strain, both log phase and stationary phase *ost3Δ* significiantly reduced AMT efficiency and showed about 50 times reduced efficiency, which is consistant with the liquid-based co-cultivation result (Fig 4.2 A and B).

This result indicates that *OST3* plays an important role in *Agrobacterium*-yeast T-DNA transfer pathway. It also implies that Ost3 protein itself or its downstream proteins may help the T-DNA transfer process inside yeast cells. Since *OST3* is a subunit of oligosaccharyltransferase (OST) complex, which glycosylates nascient proteins on ER membrane, it is very likely that the OST complex and the glycosylation process play roles in the *Agrobacterium*-yeast gene tranfer process.

4.3. Complementation assay

In order to confirm that the effect of *ost3*Δ on AMT of yeast was indeed due to the deletion of the *OST3* gene, complementation assay was performed. In addition, as stated earlier, mammalian *TUSC3* shares high homologe to yeast *OST3*. *N33* gene also plays important roles in some human disorders including cancers. Hence, we also want to know whether human *TUSC3* can complement yeast *OST3* in *A. tumefaciens*-mediated transformation of yeast. *S. cerevisia* BY4741 genomic DNA was used as template to PCR the *OST3* ORF and *OST3* promoter and terminator regin. Human *N33* ORF clone was obtained from Open Biosystem (Cat. No.: OHS1770-9382574) in a pDONR223 vector. Several plasmids were constructed to perform the complementation assay (Fig 4.3).



C

Strain	Log phase-CM plate				Stationary phase-CM plate			
	Transformants	Recipients	Efficiency	Average	Transformants	Recipients	Efficiency	Average
BY4741	161	320000	5.03E-04	5.37E-04	143	320000	4.47E-04	5.83E-04
	195	440000	4.43E-04		128	240000	5.33E-04	
	385	580000	6.64E-04		308	400000	7.70E-04	
ost3Δ	5	1600000	3.13E-06	8.20E-06	7	920000	7.61E-06	1.05E-05
	8	1020000	7.84E-06		3	660000	4.55E-06	
	6	440000	1.36E-05		7	360000	1.94E-05	

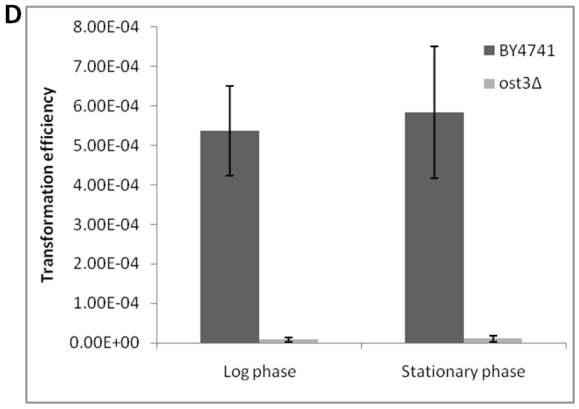


Figure 4.2. Effect of *OST3* on *Agrobacterium*-yeast T-DNA transfer. A and B: AMT was performed using a liquid-based co-cultivation assay. *virB*- mutant strain MX243 carrying a binary vector pHT101 was used a negative control. A, pictures were taken 4 days after dropping co-cultivated cells onto selection and recovery plates. B, the graphic was generated based on a triplicate assay. C and D: AMT was carried out base on a CM plate co-cultivation assay. Both log phase cells (around 10^7 cells/ml) and stationary cells (around 10^8 cells/ml) were used in the assay.

4.3.1. Plasmids construction

pHT105: In order to perform the complementation assay, a yeast expression vector containing yeast 2-micro origin, *URA3* selection marker, Ampicillin resistant gene, and *ADH1* promotor was constructed as follows. The general idea is to replace the Dsred gene in the pHT184 vector with a multiple coloning sits (MCS). The MCS region of PRS426 was synthesized into the primer pRS426-MCS. A 208 bp fragment was obtained by PCR reaction using primer pair pRS426-MCS and pRS426R. The PCR product was then digested with *Hind*III and *BamH*I and ligated to *Hind*III and *BamH*I digested bigger fragment of pTH184. The sequence of obtained vector, which was name pHT105, was confirmed by sequencing using primer L10.

pHT106: In order to whether the pHT105 can express yeast genes under the *ADH1* promotor, *EGFP* was introduced into the pHT105. The *EGFP* ORF was emplified by PCR using GFP-*BamH*I-F and GFP-*BamH*I-R from pHT101. The PCR product was digested with BamH*I* after gel extraction and inserted into *BamH*I linearized pHT105. The direction of the insertion of the resulting vector was confirmed by PCR using L10 and GFP-*BamH*I-R and also by analyzing the digestion pattern. In addition, the sequence of the pHT106 was then further confirmed by sequencing and no mutation was found. The vector pHT106 was then introduced into yeast BY4741 cell and GFP signal can be seen under fluorescent microscope when compared with BY4741 cells without pHT106 (data not shown). Hence, the expression of *EGFP* in pHT106 construction further confirmed the gene expression ability under the *ADH1* control of the pHT105.

pHT105-OST3: A 1073 bp fragment containing full length *OST3* ORF flanked by *Kpn*I digestion sites was emplified by PCR using OST3-*Kpn*I-F and OST3-*Kpn*I-R

from yeast genomic DNA. After purified by gel extraction, the PCR product was then digested by *Kpn*I and inserted into the *Kpn*I site of pHT105. After heat-shock of the resulting ligation product into DH5α strain, the direction of the vector was analyzed by PCR (L10 and OST3INR) and resctriction digestion patterns. The sequence of the plasmid pHT105-OST3 was further confirmed by sequencing using primer L10 and OST3INF1. The plasmid pHT105-OST3 was then transformed into *ost3*Δ mutant strain for complementation assay.

pHT105-OST3PRO-F: To better complement the *ost3*Δ mutant strain, *OST3* was introduced into pHT105 plasmids under its natural promoter and terminator. As the natural promoter and terminator are not clear, a 1873 fragment containing full length *OST3* ORF, 603 bp upstream of *OST3* ORF, and 207 bp downsteam of *OST3* ORF was obtained by PCR using OST3F-*BamH*I and OST3R-*BamH*I from yeast genomic DNA. After gel extraction, the PCR product was then digested with *BamH*I and inserted into the *BamH*I site of the pHT105. The directions of the resulting vectors were analyzed by PCR (L10 and OST3INR) and resctriction digestion patterns. However, the directions of all 4 resulting vectors containing the insertion are the forward directions with the ADH1 promotor and the vectos thus named pHT105-OST3PRO-F. The sequence of the plasmid pHT105-OST3 was further confirmed by sequencing using primer pHT105-F1, OST3-*Kpn*I-F, OST3INF1, and pHT105-R1. The plasmid pHT105-OST3 was then transformed into *ost3*Δ mutant strain for complementation assay.

pRS426-OST3PRO-R: A 1873 fragment containing full length OST3 ORF, promoter and terminator regions of *OST3* was obtained by PCR using OST3F-*BamH*I and OST3R-*BamH*I from yeast genomic DNA. After gel extraction, the PCR product was then digested with *BamH*I and inserted into the *BamH*I site of the pRS426. The

directions of the resulting vectors were analyzed by PCR (pRS426F, pRS426R, and OST3INR) and resctriction digestion patterns. A reverse plasmid was obtained and named as pRS426-OST3PRO-R. The sequence of the plasmid pRS426-OST3PRO-R was further confirmed by sequencing using primer pRS426F, OST3-*Kpn*I-F, OST3INF1, and pRS426R. The plasmid pRS426-OST3PRO-R was transformed into *ost3*Δ mutant strain for complementation assay.

pHT105-TUSC3: Human *TUSC3* ORF was emplified from Open Biosystem human *TUSC3* ORF clone vector pDONR233 using TUSC3-*BamH*I-F and TUSC3-*BamH*I-F. After gel extraction, the PCR product was digested with *BamH*I and inserted into *BamH*I linearized pHT105. The direction and sequence of the resulting pHT105-TUSC3 were confirmed by PCR and sequencing. The plasmid pHT105-TUSC3 was transformed into *ost3*Δ mutant strain for complementation assay.

pYES2-OST3: The full length *OST3* ORF was emplified by PCR using OST3-*Kpn*I-F and OST3-*Kpn*I-R from yeast genomic DNA. After purified by gel extraction, the PCR product was then digested by *Kpn*I and inserted into the *Kpn*I site of pYES2. The direction and sequence of the resulting pYES2-OST3 were confirmed by PCR and sequencing. The plasmid pYES2-OST3 was transformed into *ost3*Δ mutant strain for complementation assay.

pYES2-TUSC3: Human *TUSC3* ORF was emplified from Open Biosystem human *N33* ORF clone vector pDONR233 using TUSC3-*BamH*I-F and TUSC3-*BamH*I-F. After gel extraction, the PCR product was digested with *BamH*I and inserted into *BamH*I linearized pYES2. The direction and sequence of the resulting pYES2-TUSC3 were confirmed by PCR and sequencing. The plasmid pYES2-TUSC3 was transformed into *ost3*Δ mutant strain for complementation assay.

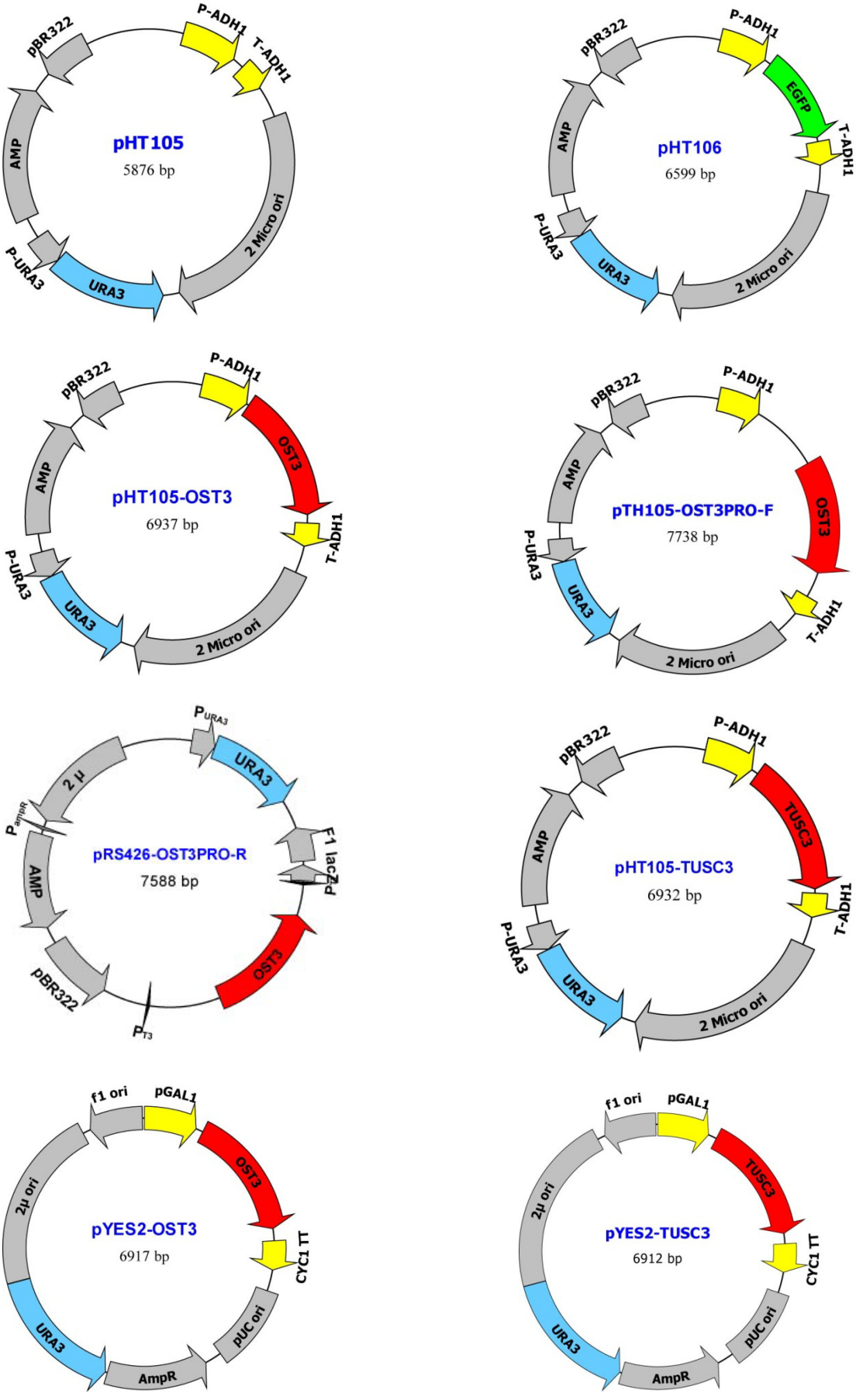


Figure 4.3. Plasmids for complementation assay.

4.3.2. Complementation assay

In order to test whether the induced AMT efficiency of *ost3Δ* can be restored by yeast *OST3* gene either under its natural promoter or strong promoters or by its human homology *N33* gene, complementation assays were conducted. Two sets of complementation assays were performed. The first sets of complementation assay used natural promoter of yeast *OST3* gene or yeast *ADH1* promoter and the assay was performed as follows.

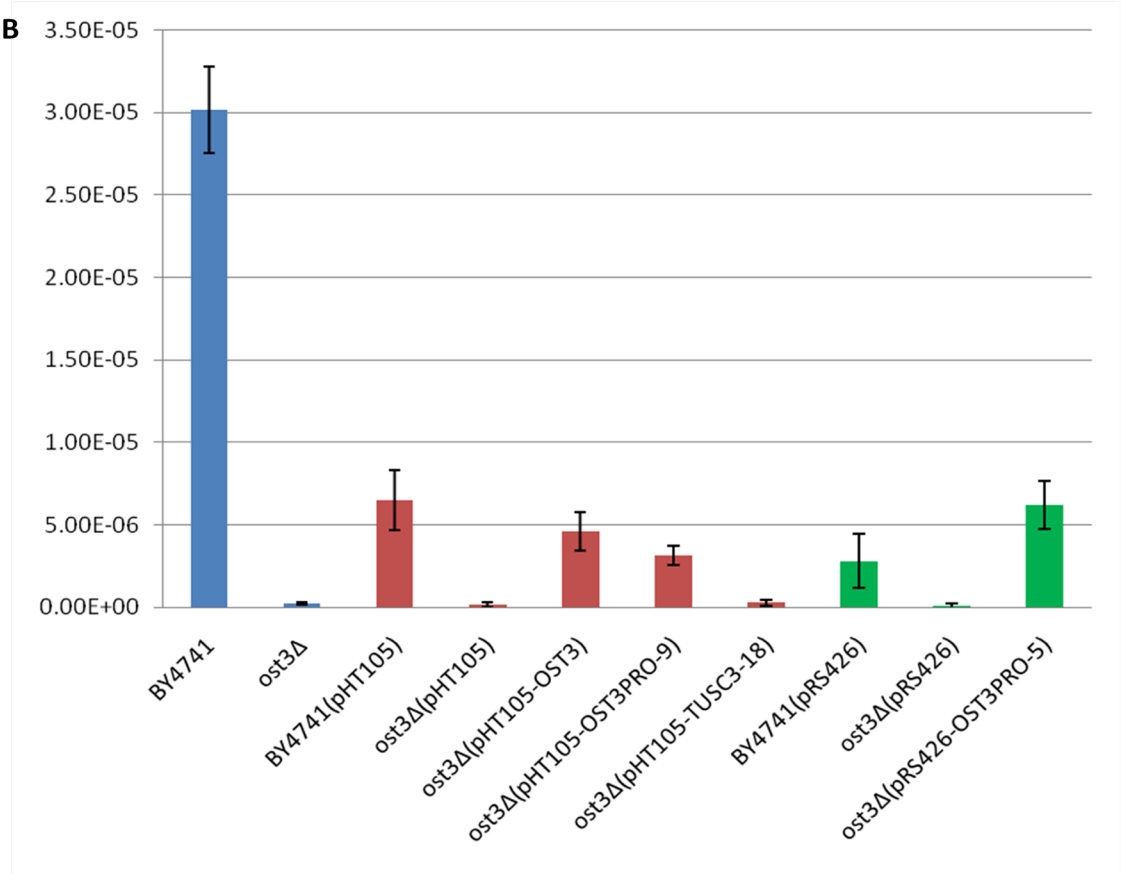
Wild type yeast strain BY4741 and *ost3*Δ were inoculated from newly grown YPD plates into 2 ml of SD broth in 14 ml culture tubes. The rest of yeast strains, including BY4741(pHT105), *ost3*Δ(pHT105), *ost3*Δ(pHT105-OST3), *ost3*Δ(pHT105-OST3PRO), *ost3*Δ(pHT105-TUSC3), BY4741(pRS426), *ost3*Δ(pRS426-OST3PRO), were inoculated from fresh SD Ura- plates into 2 ml of SD Ura- broth. After overnight growth and until cells were saturated, 2.0E+06 cells were collect by centrifugation. The pellets were washed with 1 ml KH_2PO_4 and mixed with 2.0E+06 induced *A. tumefaciens* cells. After centrifugation, the co-cultivation mixtures were resuspended with 100 µl of KH_2PO_4 and dropped onto CM plates with AS 100 µM and Km 100 µg/ml. After drying in biosafety cabinet (BSC) for about 30-60 min, the co-cultivation plates were incubated at 28 ^{o}C. After 24 h co-cultivation, cells were scrapped off from CM plates by steril wooden sticks. Yeast samples BY4741 and *ost3*Δ were plated onto SD Leu- Cef 100 µg/ml for selection and onto YPD Cef 100 µg/ml for recovery. All other yeast samples containing empty vectors and complementation vectors were plated onto SD Ura- Leu- Cef 100 µg/ml for selection and onto YPD Cef 100 µg/ml after proper dilution for recovery. Colonies appear after 3 days incubation at 28 ^{o}C. Transformation efficiency was calculated by dividing transformants number by total recipient number.

The second set of the complementation assay contains BY4741 stain, *ost3*Δ, and other vector-containing strains including BY4741(pYES2), *ost3*Δ(pYES2), *ost3*Δ(pYES2-OST3), and *ost3*Δ(pYES2-TUSC3). In this sets of vector-containing strains, the insertion *OST3* or *TUSC3* genes were driven by yeast *GAL1* promoter, which is induced in the presence of galactose and repressed by the addition of glucose in medium. For wild type strain BY4741 and *ost3*Δ, the complementation assay was done the same as the complementation assay set I. For the rest vector-containing strains, yeast cells were inoculated from fresh SD Ura- plates into 2 ml of SD Raf/Gal Ura- broth. The remanining assay was done similar to the complementation assay set I except that the co-cultivation was done on CM Gal As 100 μM and Km 100 μg/ml (glucose in CM was replaced by same amount of galactose), selection of transformants was done on SD Raf/Gal Ura- Leu- Cef 100 μg/ml, and recovery of recipients was done on SD Raf/Gal Leu- Cef 100 μg/ml.

A

Strains	Transformants	Recipients (5.0E+4 dilution)	Transformation efficiency	Average (1.0E-05)	SD (1.0E-05)	Folds
BY4741	313	195	3.21E-05			
	332	213	3.12E-05	**3.01**	0.26	**1.000**
	205	151	2.72E-05			
***ost3*Δ**	2	180	2.22E-07			
	1	176	1.14E-07	**0.02**	0.01	**0.007**
	2	125	3.20E-07			
BY4741(pHT105)	57	200	5.70E-06			
	59	226	5.22E-06	**0.65**	0.18	**1.000**
	74	172	8.60E-06			
***ost3*Δ(pHT105)**	2	158	2.53E-07			
	0	164	0.00E+00	**0.02**	0.01	**0.026**
	2	160	2.50E-07			
***ost3*Δ(pHT105-OST3)**	20	106	3.77E-06			
	15	74	4.05E-06	**0.46**	0.12	**0.704**
	24	81	5.93E-06			
***ost3*Δ(pHT105-OST3PRO-9)**	26	178	2.92E-06			
	17	90	3.78E-06	**0.31**	0.06	**0.481**
	17	126	2.70E-06			
***ost3*Δ(pHT105-TUSC3-18)**	3	120	5.00E-07			
	1	157	1.27E-07	**0.03**	0.02	**0.040**
	1	138	1.45E-07			
BY4741(pRS426)	35	150	4.67E-06			
	16	180	1.78E-06	**0.28**	0.16	**1.000**
	23	238	1.93E-06			
***ost3*Δ(pRS426)**	2	194	2.06E-07			
	0	237	0.00E+00	**0.01**	0.01	**0.025**
	0	150	0.00E+00			
***ost3*Δ(pRS426-OST3PRO-5)**	34	112	6.07E-06			
	40	168	4.76E-06	**0.62**	0.15	**2.212**
	45	117	7.69E-06			

B



C

Strains	Transformants	Recipients (5.0E+4 dilution)	Transformation efficiency	Average (1.0E-05)	SD (1.0E-05)	Folds
	984	123	1.60E-04			
BY4741	930	124	1.50E-04	**14.58**	1.68	**1.000**
	840	132	1.27E-04			
	34	153	4.44E-06			
***ost3*Δ**	48	163	5.89E-06	**0.51**	0.07	**0.035**
	35	142	4.93E-06			
	180	277	1.30E-05			
BY4741(pYES2)	239	202	2.37E-05	**3.47**	2.89	**1.000**
	560	166	6.75E-05			
	26	300	1.73E-06			
***ost3*Δ(pYES2)**	28	252	2.22E-06	**0.22**	0.04	**0.063**
	34	259	2.63E-06			
	568	263	4.32E-05			
***ost3*Δ(pYES2-OST3-10)**	233	347	1.34E-05	**2.17**	1.88	**0.626**
	131	308	8.51E-06			
	13	244	1.07E-06			
***ost3*Δ(pYES2-TUSC3-9)**	31	339	1.83E-06	**0.19**	0.09	**0.055**
	42	295	2.85E-06			

D

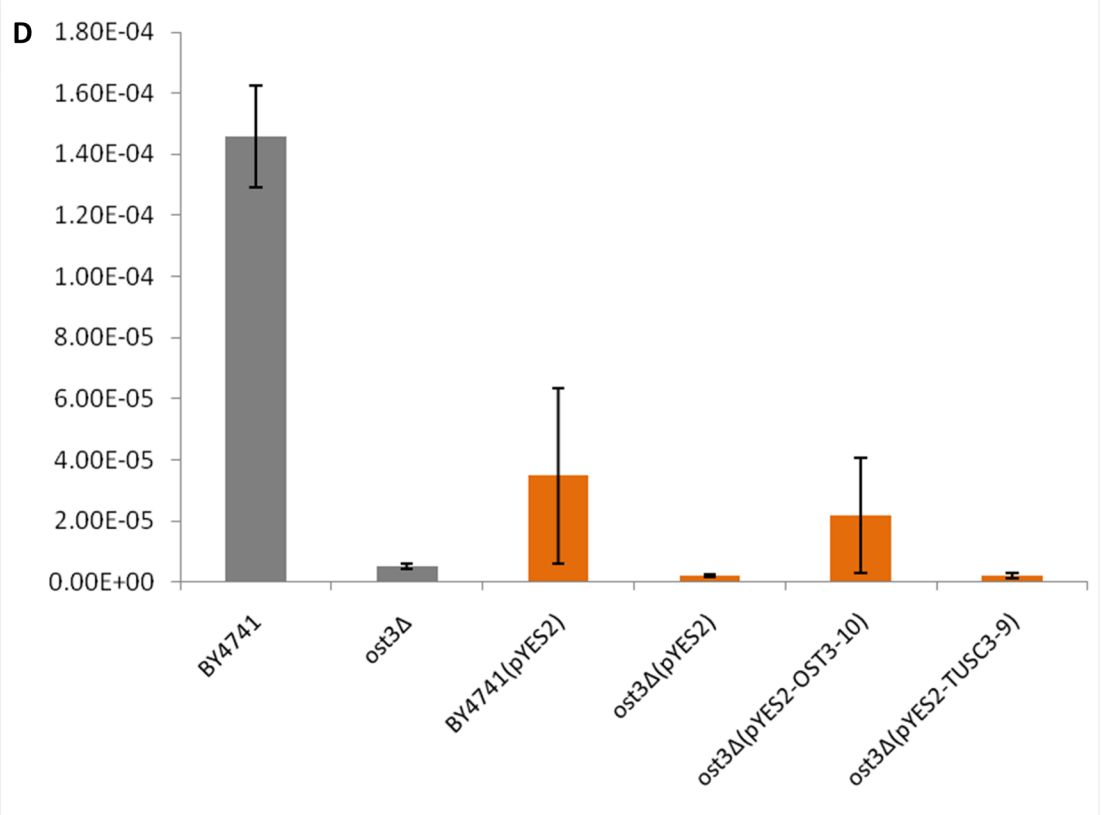


Figure 4.4. Complementation assay results. A and B: Complementation assay set I. C and D: Complementation assay set II.

4.3.3. Results

When compared with BY4741, the AMT efficiency of BY4741(pHT105), BY4741(pRS426), or BY4741(pYES2) was reduced from 5 to 10 times (Fig 4.4 A, B, C, and D). It is probably because of the effect of different media, including growing medium and induction medium, used for those strains. In both sets of complementation assays, SD broth was used for growing and normal CM plates for co-cultivation BY4741 strain, while for BY4741(pHT105) and BY4741(pRS426), SD Ura- was used for growing yeast cells; and for BY4741(pYES2), SD Raf/Gal Ura- was used for growing yeast cells and CM Gal was used for co-cultivation. Glucose and glactose belong to different metabolic pathways, which may affect the AMT efficiency. In different growth and co-cultivation medium, yeast cells may behave differently and at totally different stages, which may affect the *Agrobacterium*-mediated transformation process. On the other hand, those empty vectors may also influence the growth condition and stage of yeast cells; thus affect the AMT transfer process. All of those vectors contain the *URA3* selection marker, which produce uracil for yeast cells. Although the wild type BY4741 can get minimal uracil from medium, it is probably that the amount of the uracil supplemented in medium may be different the uracil synthesized by yeast cells. More importantly, the production of uracil will trigger the uracil synthesis pathway, which may change the growth condition and stage of yeast cells. As we know, the adenine synthesis pathway affects the AMT transfer process (Roberts, 2003). Although it is not proven, it is also possible that the uracil synthesis pathway may also be involved in the AMT process. Therefore, BY4741 carrying different empty vectors were included in the complementation assays for fair comparision.

From Fig 4.2, we can see *ost3*Δ carrying empty vectors had about 16 to 40 times reduced transformation efficiency compared with wild type BY4741 carrying empty vectors. However, when expressing yeast *OST3* gene under either native promoter or other promoters in different expression vectors, the AMT efficiencies of *ost3*Δ deletion mutant were restored from 50% to 200% (Fig 4.2). This result clearly showed that the decreased AMT efficiency of *ost3*Δ is indeed due to the deletion of *OST3* gene not other factors. Moreover, this low AMT efficiency can be complemented by introduction *OST3* gene into the mutant. Therefore, it supports the idea that *OST3* plays an important role in regulating the *A. tumefaciens*-mediated transformation process.

As shown in Fig 4.2, although human *TUSC3* shares high homology with yeast *OST3*, human *TUSC3* gene could not complement yeast *ost3*Δ in the AMT process. Although the high homology exists between yeast *OST3* and human *TUSC3*, their may play different roles in the glycosylation process or in the AMT process. Moreover, the AMT conditions for mammalian cells are very different from the conditions for plants and yeast cells, implying that the AMT process might be different in these systems. In addition, the role of *TUSC3* plays in the mammalian AMT process should be proved in the future.

4.4. OST complex is involved in the AMT process.

4.4.1. Materials and methods

Since *ost3*Δ significantly decreases AMT efficiency of yeast cells, we speculated other components of the OST complex and the OST complex itself or the glycosylation process may also be involved in the AMT process. Therefore, the AMT efficiencies of other components of the OST complex were examined. As reviewed in

the early section, in the OST complex, only the *OST3*, *OST4*, *OST5*, and *OST6* are not essetial for cell survival; while other components, including *STT3*, *SWP1*, *WBP1*, *OST1*, and *OST2*, are required for cell viability. We bought two sets of yeast gene collections, one is yeast gene knockout library (Openbiosystems) and the other is yeast essential gene knockdown library. In the two libraries, 7 of the OST components are present except for *stt3* and *ost1*. For the non-essential components of the OST complex, liquid co-cultivation based AMT and CM plated co-cultivation based AMT were performed. For those essential components, doxycycline induced AMT was performed.

4.4.2. Results

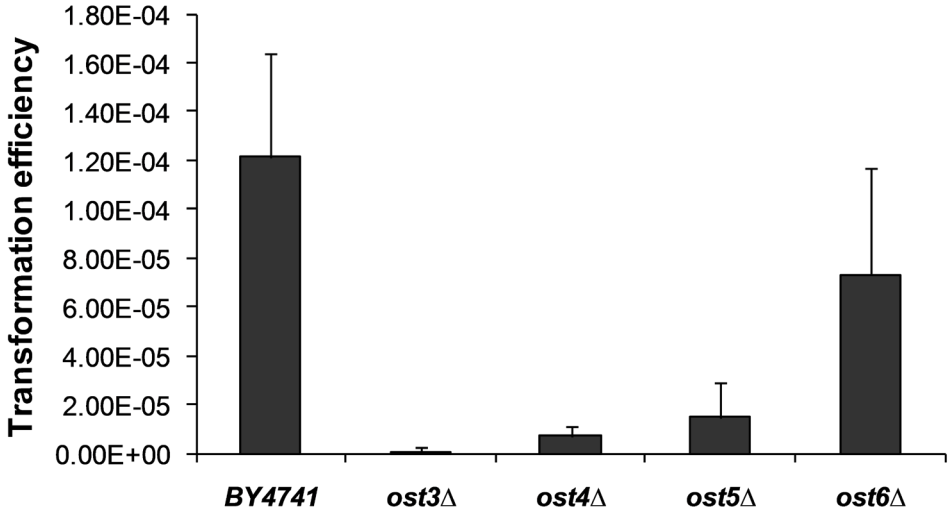
As shown in Fig 4.5, *ost3*Δ consistently showed 40 to 150 times reduced AMT efficiency at different assay conditions, which is consistant with previous screening and complementation results. Similar to *ost3*Δ, *ost4*Δ and *ost5*Δ also significantly reduced AMT efficiency; while *ost6*Δ and *ost2* reduced about 50% of AMT efficiency. However, other two essential components, *swp1* and *wbp1* showed similar transformation efficiency to the WT strains. Since other two essential components are crucial for cell survial and are not present in the two collect, we could not assess their roles on the AMT process. Nevertheless, these results clearly sugguest that the OST complex, particularly the *ost3*Δ is very important in the AMT process and may positively regulate the T-DNA tranfer process inside yeast cell. Interestingly, although *OST3* and *OST6* are isoforms and share very high homology, it seems they have different impacts on the AMT process. More importantly, *OST6* could not complement the AMT efficiency in the *ost3*Δ mutant strain, and vise versa. This result suggests the different function of *OST3* from *OST6*. Furtheremore, deletion of *ost3* only caused minor underglycosylation (Chavan *et al.*, 2006); however, deletion of

ost3 significantly reduce AMT efficiency (Fig 4.5), which suggests *OST3* plays an important role in the AMT process other than the glycosylation process.

A

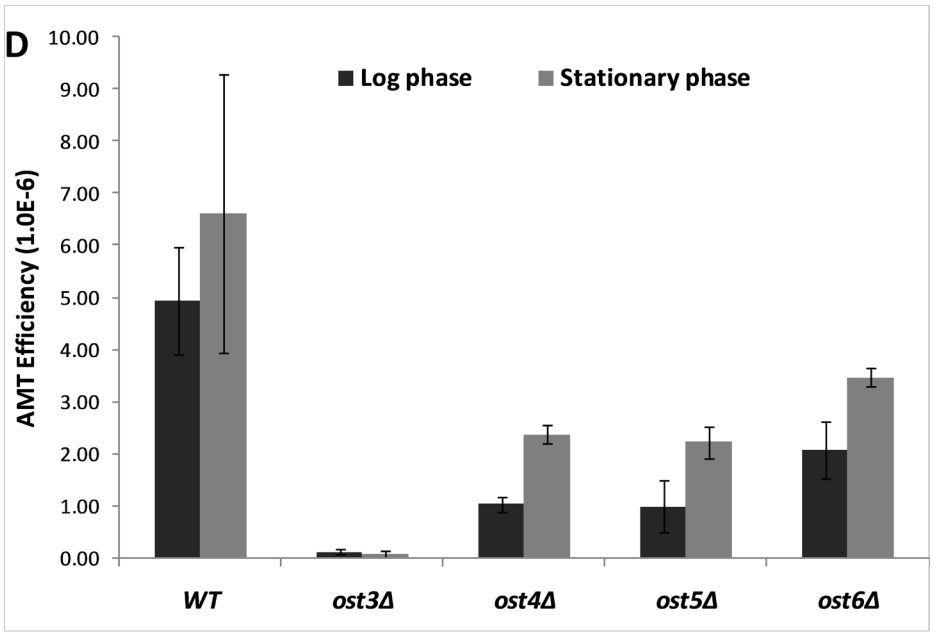
Strains	AMT efficiency
BY4741	1.21E-04 ± 4.27E-05
***ost3*Δ**	7.69E-07 ± 1.72E-06
***ost4*Δ**	7.08E-06 ± 4.30E-06
***ost5*Δ**	1.46E-05 ± 1.44E-05
***ost6*Δ**	7.30E-05 ± 4.37E-05

B



C

Strain	AMT efficiency (log phase)	AMT efficiency (Stationary phase)
WT	4.93E-06 ± 1.03E-06	6.60E-06 ± 2.67E-06
ost3Δ	1.19E-07 ± 4.69E-08	6.24E-08 ± 7.94E-08
ost4Δ	1.03E-06 ± 1.52E-07	2.37E-06 ± 1.72E-07
ost5Δ	9.86E-07 ± 4.92E-07	2.22E-06 ± 3.19E-07
ost6Δ	2.08E-06 ± 5.56E-07	3.47E-06 ± 1.77E-07



E

Strains	AMT efficiency
R1158	3.73E-04 ± 2.67E-05
swp1	3.72E-04 ± 3.48E-05
wbp1	3.12E-04 ± 3.49E-05
ost2	1.27E-04 ± 3.30E-05

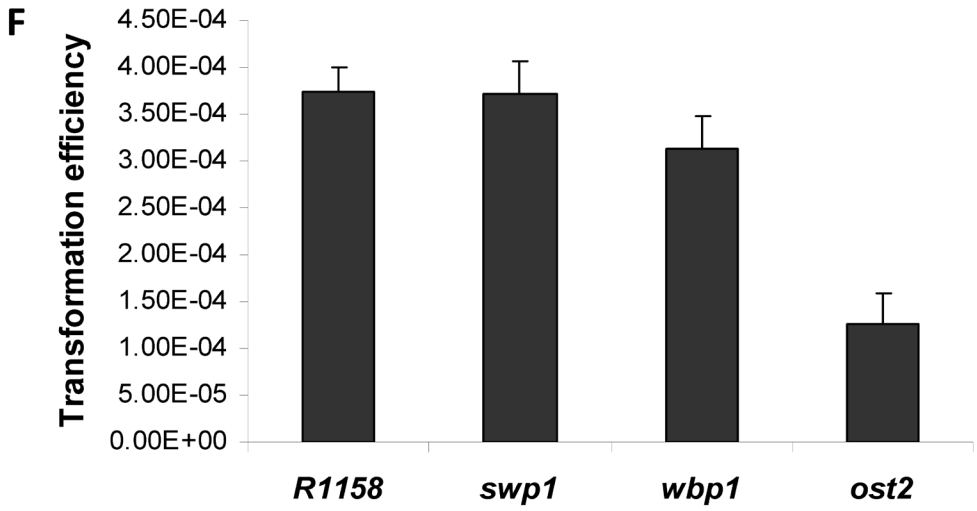


Figure 4.5. AMT efficiencies of the OST components. **A** and **B**: based on liquid co-cultivation AMT assay of non-essential OST components. **C** and **D**: based on CM plate co-cultivation AMT assay of non-essential OST components. Both log phase (1.0E+07 cells/ml) and stationary phase (1.0E+08 cells/ml) yeast cells were tested for AMT. **E** and **F**: based on CM plate co-cultivation AMT assay of essential OST components. Yeast cells were induced at 10 µg/ml Doxycycline in YPD for 18 h before co-cultivation with induced *A. tumefaciens* cells.

CHAPTER 5. THE ROLE OF *OST3* IN THE *AGROBACTERIUM*-YEAST GENE TRANSFER PROCESS

In the previous chapter, we have proved that *OST3* and the OST complex or glycosylation process are crucial for the *Agrobacterium*-yeast gene transfer. However, the molecular basis of the Ost3p and the glycosylation reaction in the T-DNA transfer process is not known. In this chapter, we tried to dissect the role of Ost3p in the T-DNA transfer process.

5.1. Lithium acetate-mediated transformation assay

5.1.1. Introduction

There are several ways to introduce foreign DNA into yeast cells, such as *Agrobacterium*-mediated transformation (AMT), lithium acetate (LiAc)-mediated transformation, and electroporation. In *Agrobacterium*-mediated transformation, T-DNA, the foreign DNA, is injected into yeast cell through *Agrobacterial* T4SS, which is a natural process. While for LiAc-mediated transformation, foreign DNA is delivered into chemical treated yeast by heat-shock, which is very different from the nature AMT process. Previously, we have shown that *ost3*Δ significantly decreased AMT efficiency. Currently, we wanted to know whether ***ost3***Δ affects the LiAc-mediated transformation or not and whether the *OST3* gene specifically regulates the AMT pathway not other DNA delivery pathways such as the LiAc-mediated pathway.

5.1.2. Materials and methods

Both circular plasmid and linear DNA were used for LiAc-mediated transformation of yeast. For circular plasmid LiAc-mediated transformation, 200 ng of pHT101 DNA, the same plasmid used for AMT experiment, was used to transform

5.0E+07 yeast cells. For linear DNA LiAc-mediated transformation, 130 ng of *Sal*I digested pGFP209, T-DNA fragment transferred to yeast cells in AMT experiment, was used to transform 8.0E+07 yeast cells. The detailed lithium acetate-mediated transformation protocol was described in previous section 2.5.

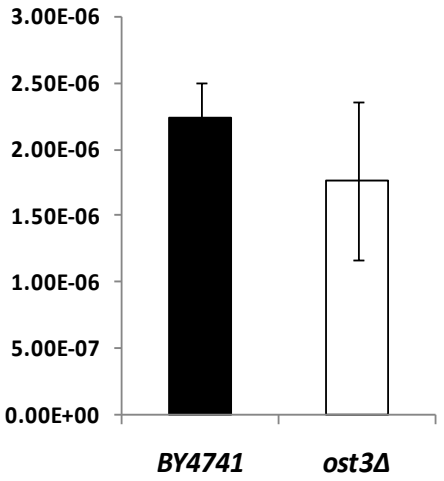
5.1.3. Results

As can be seen in Figure 5.1A and B, *ost3*Δ showed similar transformation efficiency in the LiAc-mediated transformation of circular plasmid DNA compared to WT strain. Moreover, for the linearized plasmid DNA, LiAc-mediated transformation efficiency of *ost3*Δ was not reduced but slightly increased when compared to WT strain. As a contrast, AMT efficency of *ost3*Δ was significantly reduced by at least 30 times. These results indicate that the effect of *OST3* on the AMT T-DNA transfer process is very different from the LiAc-mediated DNA transfer process. This difference is most likely due to the different mechanisms of the two kinds of transformation processes. For LiAc-mediated transformation, external DNA is forced into yeast cells, which have been treated by LiAc, by heat-shock. However, this physical and chemical mediated process is different from the natural *Agrobacterium*-yeast gene transfer process, in which the T-DNA is injected into yeast cells by *Agrobacterium* through a natural type IV secretion channel. Hence, it is reasonable that the two different processes display different results. Therefore, the effect of *ost3*Δ in the gene transfer process is specific for *Agrobacterium*-yeast T-DNA transfer.

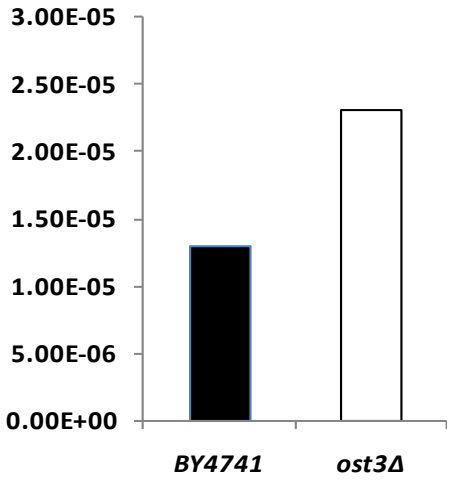
A

Strains	Transfor-mants	Recipients (1.0E+5 ×)	TE (1.0E-06)	TE Average (1.0E-06)	SD (1.0E-06)	Fold change
BY4741	57	225	2.53	2.23	0.26	1.00
	36	177	2.03			
	91	427	2.13			
ost3Δ	46	392	1.17	1.77	0.60	0.79
	101	427	2.37			
	73	415	1.76			

B



C



D

Strains	Transfor-mants	Recipients (1.0E+5 ×)	TE (1.0E-05)	TE Average (1.0E-05)	SD (1.0E-05)	Fold change
BY4741	302	299	1.01	1.29	0.40	1.00
	332	211	1.57			
ost3Δ	651	312	2.09	2.31	0.32	1.79
	620	244	2.54			

Figure 5.1. LiAc-mediated transformation. A and B: Circular plasmid pHT101 was used for LiAc-mediated transformation. **C and D:** *Sal*I digested pGFP209 fragment was used for LiAc-mediated transformation.

5.2. Glycosylation of VirD2 by OST complex

5.2.1. Introduction

VirD2 is one of the most important components in the whole *Agrobacterium*-mediated transformation process. As reviewed previously, after induction, VirD2/VirD1 cut the T-DNA from the lower strand of the T-plasmid. Then the VirD2 protein covalently attaches to the 5'-end of the ssT-DNA and leads the T-strand through the type IV secretion channel and to the nucleus of the host cell. Obviously, VirD2 plays key roles in the whole T-DNA process and transfer process. In this study, we found *OST3* gene, the OST complex, and probably the glycosylation process is important for T-DNA transfer inside yeast cells. Thus, it is possible that VirD2 may be glycosylated by *OST3* gene and the glycosylated form of VirD2 plays an important role for T-DNA trafficking. To verify this hypothesis, glycosylation of VirD2 was designed to compare the VirD2 protein profile in yeast cells.

5.2.2. Materials and methods

A. tumefaciens VirD2 (pTiBo542 type) is driven under yeast ADH1 promoter in plasmid pAVD2. The pAVD2 was introduced into wild type yeast strain and OST component deletion strains. After expressing in yeast, yeast total protein was collected and prepared for western blot detection. *Agrobacterium* VirD2 antibody was used for detecting of native VirD2 protein as well as any glycosylated form of VirD2. Uninduced and induced *Agrobacterium* The detailed procedure for SDS-PAGE and western blot was stated in the previous section 2.3.

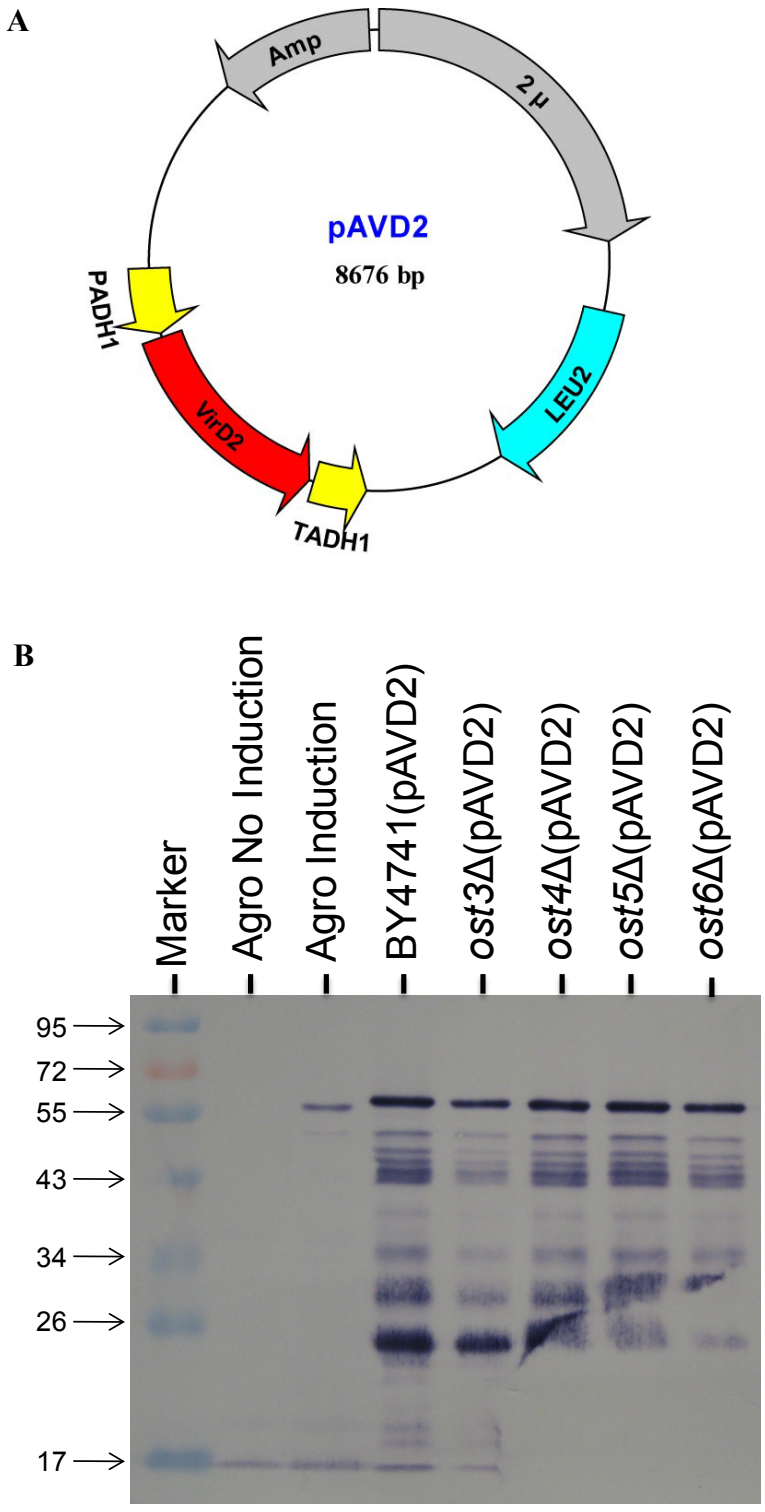


Figure 5.2. Glycosylation of VirD2 by OST complex. A: pAVD2 was used to express Agrobacterial VirD2 in yeast. **B**: Protein expression assay. Total cell protein extracts were used in this assay. 10% SDS-PAGE gel were used for electrophoresis. Rabbit VirD2 antibody were used for detecting VirD2 protein.

5.2.3. Results

As shown in Fig 5.1, the native agrobacterial VirD2 showed a 58 kD band, while VirD2 expressed in yeast showed a bit bigger band. In addition, there are VirD2 protein degrations, indicating that VirD2 undergoes many biological processes. However, when compare mutant strains carrying pAVD2 with wild type strains carrying pAVD2, we found that the VirD2 protein profiles are same, which means VirD2 did not undergo glycosylation process. Moreover, protein sequence analysis revealed that there is no N-X-S/T consensus sequencing of N-glycosylation in the pTiBo542 VirD2 plasmid, also indicating that VirD2 is not a glyprotein and should not be glycosylated by the OST complex. This result revealed that the effect of *OST3* gene on the T-DNA transfer process is not via the direct glycosylation of VirD2 protein.

5.3. The effect of *OST3* on VirD2 nuclear targeting

5.3.1. Introduction

Nuclear targeting is a critical step in the T-DNA transfer process. Sequence analysis revealed that VirD2 has two NLSs, which are important for T-DNA nuclear targeting (Howard et al, 1992; Tinland *et al.*, 1992). During the T-DNA trafficking inside yeast cell, VirD2 leads the T-complex from cytoplasm to nuclear. We then want to check whether *OST3* affect this T-DNA nuclear targeting process.

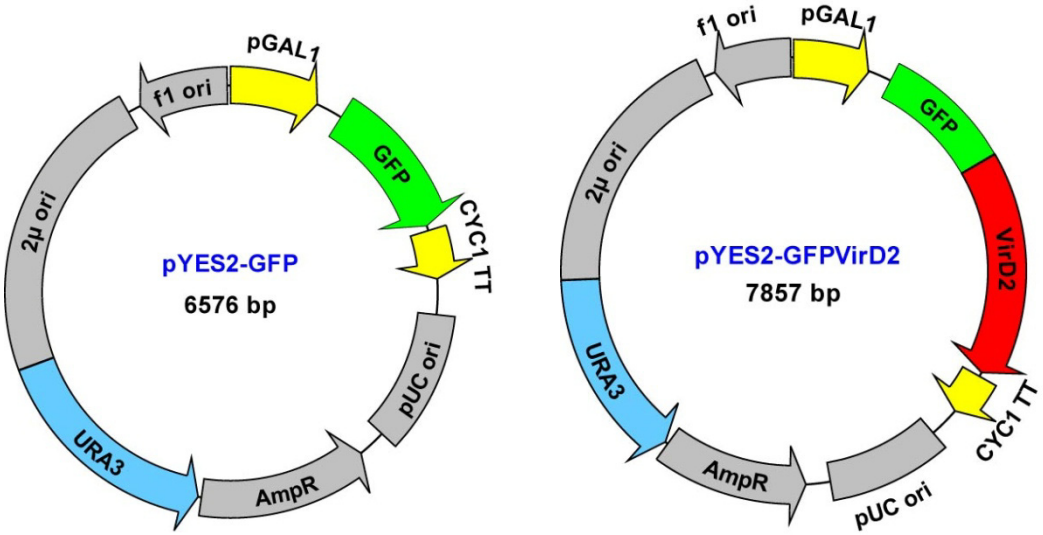
5.3.2. Materials and methods

In order to detect the effect of *OST3* gene on VirD2 nuclear targeting, GFPVirD2 fusion protein was used and expressed inside yeast cell driven by yeast inducible GAL1 promoter. The GAL1 promoter is repressed in the presence of gluocose, thus the GFP and GFPVirD2 gene cannot be expressed. In the presence of

galactose, the GAL1 promoter is turned on and its controlling gene GFP and GFPVirD2 will be expressed, thus green fluorescence can be visualized under fluorescent microscope.

Fresh yeast strains BY4741(pYES2-GFP), BY4741(pYES2-GFPVirD2), *ost3*Δ(pYES2-GFP), and *ost3*Δ(pYES2-GFPVirD2) were inoculated from SD Ura- plate into 2 ml of SD Ura- broth and incubated at 28 ^{o}C. Cells were collected into 1.5 eppendorf tube when OD_{600} reaches 1.0. Supernatant was discarded and the cells were washed twice with sterile ddH_2O and once with 1 ml of SD Raf/GAL Ura-. Cells were resuspended with 1 ml of SD Raf/GAL Ura- and transferred to 10 ml of SD Raf/GAL Ura- in a 50 ml flask. 1 ml of culture was taken out and used as 0 h time point sample. The rest culture was incubated at 28 ^{o}C with shaking. 1 ml of culture was taken out at 0 h, 3 h, 6 h, and 12 h. After fixation and DAPI staining (see Chapter 2.), GFP signals was detected and recorded using fluoresent microscopy.

A



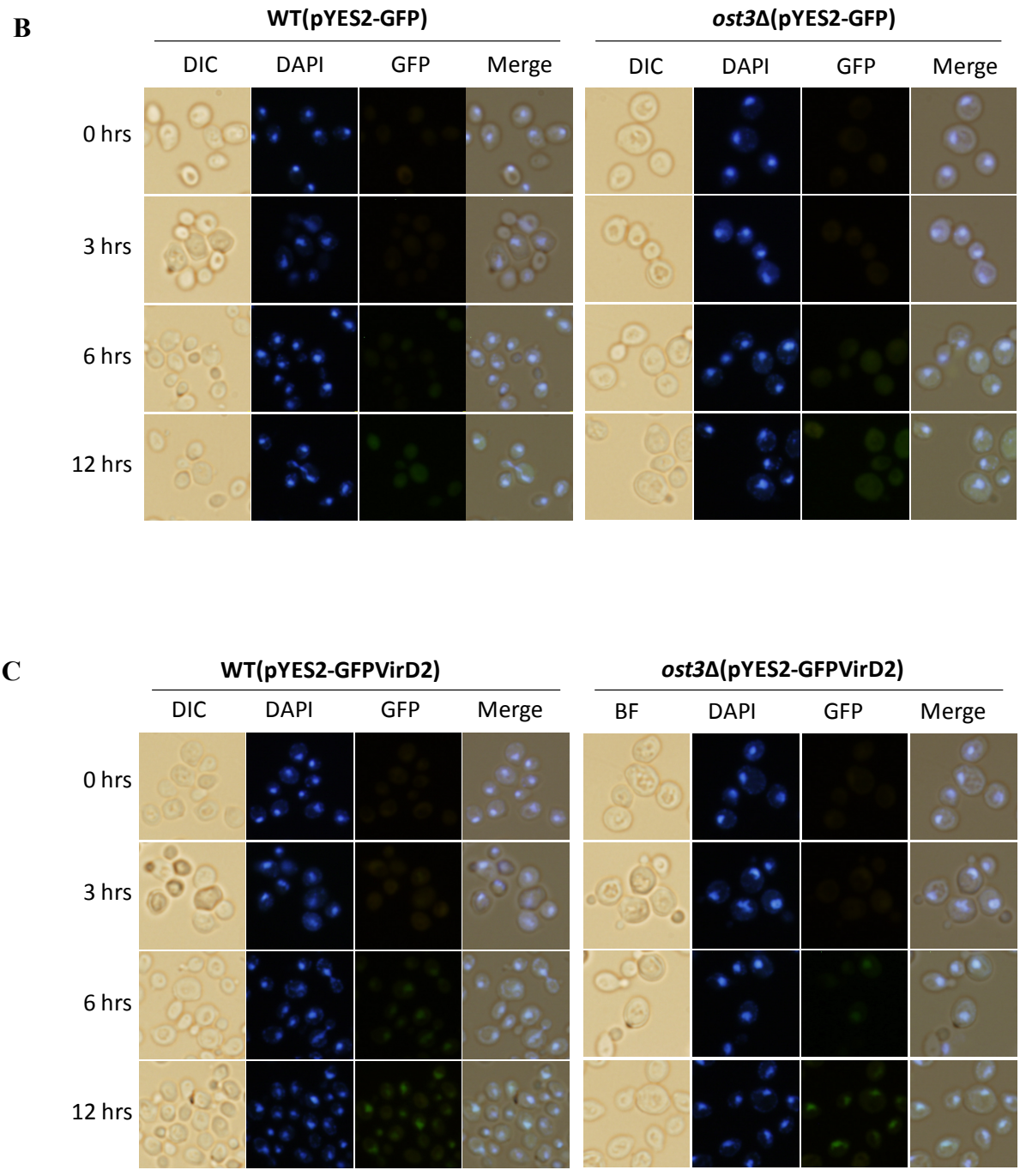


Figure 5.3. VirD2 nuclear targeting assay. A: plasids used to express GFP and GFPVirD2 proteins in yeast cell. **B** and **C**: time course study of WT and *ost*3Δ cells expressing GFP and GFPVirD2 respectively. Cells were collected 0, 3, 6, and 12 h after switching into SD Raf/GAL Ura- broth. Pictures were taken by fluoresent microscopy.

5.3.3. Results

From Fig 5.2B, we can see GFP started to express 6 h after induced by glactose and reached higher level expression at 12 h in both WT and *ost3*Δ cells. GFP was expressed all over the cells both in cytoplsm and nuclear. In addition, the GFP expression pattern and time points are similar between wild type and *ost3*Δ cells, indicating that the vector and *OST3* gene did not affect GFP expression. As shown in Fig 5.2 C, both wild type and *ost3*Δ cells started to express GFPVirD2 protein 6 h after induction and GFPVirD2 protein also started to localize in the nucleus at the same time. After 12 h inducing, all GFP signals colocalized with DIPI signals in the nucleus in both wild type and *ost3*Δ cells. This data prove that *OST3* does not affect VirD2 nuclear targeting process. Without Ost3p, VirD2 still can target and localize to yeast nucleus.

5.4. Co-immunoprecipitation of VirD2-binding OST components

5.4.1. Introduction

Since *OST3* does not affect VirD2 nuclear targeting, we further want to know whether *OST3* and the OST complex physically interact with VirD2 or not. Tandem affinity purification (TAP) is a good method to precipitation interaction protein thus we wanted to used the scTAP tagged yeast Ost3p and other OST components to precipitate VirD2 and test their potential interactions. The scTAP tagged yeast strains were brought from Openbiosystems to test whether they have interactions with VirD2 protein. The TAP method was described by Puig et al in 2001. The whole assay included severl parts: preparation of VirD2-expressing yeast cells, total yeast protein expression and quantification, co-immunoprecipitation of VirD2-binding scTAP tagged yeast proteins, and western blot analysis of the protein binding profile.

5.4.2. Preparation of VirD2-expressing yeast cells

Wild type yeast BY4741 strain and *OST3*-scTAP were inoculated into 1 ml of SD medium from fresh agar plates. VirD2-expression scTAP-tagged yeast strains and VirD2-expression BY4741 strain were inoculated into 1 ml of SD Ura- medium from fresh agar plates. Cells were allowed to grow at 30˚C overnight. Then the yeast cells were sub-cultured into 50 ml of same media in 250 ml sterile flasks and grew until satuated or OD_{600} reaches 1.0. Cells were harvested by centrifugation and washed twice with 20 ml ddH_2O. The pellets were carried with total protein extraction or kept at -80˚C for storage.

5.4.3. Total yeast protein expression and quantification

Total protein extraction was followed the protocol described by Rigaut et al (1999). After collecting VirD2-expressing yeast cells and other control samples, the pellets were re-suspended with 1.6 ml of ice cold cell freshly made lysis buffer A [10mM K-Hepes pH7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM Phenylmethylsulfonyl floride (PMSF), 0.5mM DTT, 1× EDTA free proteinase inhibitor cocktail (Sigma P8340)]. The cells were then passed through French Press three times at 1,000 PSI to break the cell wall and release the total proteins. The cells were collected using 2 ml eppendorf tube and put on ice. 400 µl of 1M KCl was added to reach final concentration of 0.2M. The protein extract was then centrifuged at 13,800 rpm for 1 h at 4˚C and the supernatant was harvested.

Protein concentration was quantified using Coomassie Plus Protein Assay (Pierce). A standard curve was prepared by a serial dilution of bovine serum albumin fraction V (Roche) according to manufacer's manual. The optical densities of protein

samples were measured using 595nm wavelength. Protein concentrations were calculated according to the equation generated from the protein standard curve.

5.4.4. Co-immunoprecipitation of VirD2-binding scTAP tagged yeast proteins

The TAP pull-down procedure used for co-immunoprecipitation of VirD2-binding scTAP tagged yeast proteins was modified from Rigaut *et al.* (1999) and Puig et al. (2001). 200 μL of Calmodulin Affinity Resin (Stratagene) was washed twice with 1 ml of IPP150 calmodulin binding buffer (10 mM β-mercaptolethanol, 10 mM Tris-Cl pH8.0, 150 mM NaCl, 1 mM Magnesium acetate, 1 mM Imidazole, 2 mM $CaCl_2$, 0.1% NP-40) at 1,000×g for 1 min at 4 ^{o}C and resuspended in 200 μl of IPP150 calmodulin binding buffer. Then mix 800μL of protein crude extract (adjusted to same amount of protein for each sample), 3 ml of IPP150 calmodulin binding buffer, and 1.5 μL of 2 M $CaCl_2$ in a 15 ml Falcon tube. Take out 1 ml of the mixture and save as the crude extract sample. 200 μl of washed calmodulin affinity beads was added into the mixture. The mixture was then incubated for 2 h at 4°C with gentle rotating to allow protein binding to calmodulin affinity beads.

The mixture was then spun at 1,000×g for 1 min at 4 ^{o}C and supernatant was removed. 1 ml of IPP150 calmodulin binding buffer was added to the mixture and put back onto the rotator and washed for 10 min. Repeat the washing for 3 more time to remove non-binding proteins. The washing buffer should be removed as clean as possible to remove non-binding crude extract proteins. After complete washing, 200 μl of IPP150 calmodulin elution buffer (10 mM β-mercaptolethanol, 10 mM Tris-Cl pH8.0, 150 mM NaCl, 1 mM Magnesium acetate, 1 mM Imidazole, 2 mM EGTA, 0.1% NP-40) was added to the beads and incubated on the rotator for 2 h at 4 ^{o}C to release the protein complex from beads. The elution was saved as the pull-down sample.

5.4.5. Western blot analysis of the protein binding profile

The crude extract samples and the pull-down samples were separated by 10% SDS-PAGE (see section 2.3.2 for detail). The protein profils were analyzed by western blot (see section 2.3.5 for detail) using Rabbit anti-$VirD_2$ antibody (from lab stock, by Guo *et al.*, 2007a and 2007b) as the primary antibody and anti-rabbit IgG HRP conjugate (Promega) as the secondary antibody. The western blotted protein profile image was developed on a BioMax XAR Film (Kodak).

5.4.6. Results

Agrobacterial VirD2 was expressed in yeast vector pAVD2 driving by yeast *ADH1* promoter. The VirD2 containing vector pAVD2 was introduced into yeast by lithium acetate-mediated transformation. Wild type yeast strain, wild type yeast strain expressing containing pAVD2, and OST3-scTAP tagged strain without pAVD2 were used as negative controls because only VirD2 interacting scTAP-tagged proteins could be detected. Meanwhile, GND1-scTAP containing pAVD2 and SRP1-scTAP containing pAVD2 were served as positive control. SRP1, the karyopherine alpha homolog, has been reported to mediate import of nuclear proteins and bind the nuclear localization signal of the substrate during import (Enenkel *et al.*, 1995; Tabb *et al.*, 2000). GND1, 6-phosphogluconate dehydrogenase, has been shown to be involved in the T-DNA transfer process and interact with VirD2 (Lin RE, unpublished data). VirD2 antibody was used to detect the presence of VirD2 after pull-down assay.

As shown in Fig 5.3, some of the OST components, including *OST1*, *OST3*, *OST5*, and *SWP1* could pull-down VirD2 after tandem affinity precipitation; while other components of OST complex, including *OST4*, *OST6*, *STT3*, and *WBP1* could not pull-down VirD2. This result indicates that some of the OST components may be

physically interacted with VirD2 during the T-DNA transfer process and further comfired their importance in the T-DNA transfer process. However, interestingly, not every OST component interacts with VirD2, implying their different roles in the T-DNA transfer process.

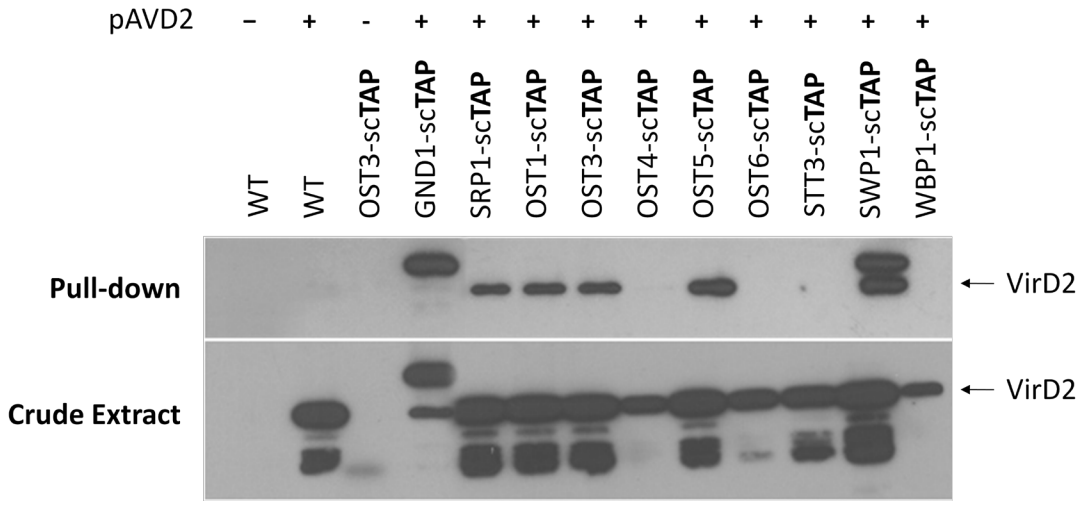


Figure 5.4. Pull-down of VirD2 by scTAP-tagged proteins. VirD2 antibody was used to detect the presence of VirD2 after TAP pull-down assay.

5.5. Fluorescent *in situ* hybridization of T-DNA in yeast.

5.5.1 Introduction

In order to dissect the role of *OST3* in the T-DNA transfer process in yeast, we wanted to know the spatial distribution of T-DNA inside yeast cells during the entire T-DNA transfer process. The spatial distribution of T-DNA in yeast during the transfer process was detected by fluoresccent *in situ* hybridization (FISH). The FISH protocol used in this study was developed and modified based on previous protocol developed for detection of T-DNA delivery in tobacco cells (Chang, unpublished

data) and from fluorescent *in situ* hybridization detection for mRNA, single RNA, and short probes in yeast or plants (Long *et al.*, 1995; Femino *et al.*, 1998; Levsky *et al.*, 2002; Guzzo *et al.*, 2000; Zenklusen *et al.*, 2008; Zenklusen and Singer, 2010).

5.5.2. Probe design

FISH probes are crucial for successful FISH experiments. To achieve single molecule sensitivity and allow the entry of FISH to yeast nucleus, 4 oligonucleotide probes each labeled with 4 fluorescent Cy3 dyes labeling the same region of the target T-DNA (Fig 5.4) and were synthesized for FISH (from IDT group). To ensure high specificity and strigent hybridization conditions, probes of around 50 nucleotides (nt) in length were designed. The total length of the probes region is about 250 bp. In order to hybridize T-DNA, the probes were designed to complement the T-DNA, which is the lower strand of the T-DNA on the binary vector pHT101. To prevent quenching of fluoresent signals, the Cy3 dyes in each probe were designed about 10 nt away from each other. Moreover, four internal "T"s (the bold "T"s in Fig 5.4B) in each probes were replace by Cy3 probes. Thus, each probe-containing region has 16 Cy3 labeling sites. These modified oligonucleotide probes were proved to specifically hybridize to T-DNA on the pHT101 by PCR (data not shown).

5.5.3 Construction of pHT110

Plasmid pGFP209-EB* and plasmid pCB301-5GFP were used to construct binary vector pHT110. Plasmid pGFP209-EB* contains a *BamH*I flanked 270 bp of N-terminal EGFP fragment, an *EcoR*I flanked 353 bp of N-terminal EGFP, and a frame shifted full length EGFP. Plasmid pCB301-5GFP contains 5 copies of EGFP fragments, including two copies of *BamH*I flanked 270 bp of N-terminal EGFP fragments, an *EcoR*I flanked 353 bp of N-terminal EGFP, an *EcoR*I flanked 270 bp of

N-terminal EGFP, and a frame shifted full length EGFP. Linearized pGFP209-EB* was ligated to pCB301-5GFP at *Sal*I site, forming plasmid pHT110. Binary vector pHT110 contains 8 copies of N-terminal EGFP fragments, thus can bind to 128 copies of Cy3-labeled probes. Plamids pHT110 should be able to increase the Cy3 signals strength, particularly for T-DNA detection by 8 times in FISH assay.

5.5.4 Sample preparation

AMT of yeast experiment was performed as described at section 2.4 from day 1 to day 3. The co-cultivated cells at different time point were washed off from co-cultivation plates using sterile water into a 1.5 ml eppendorf tube. Ten drops of cells were combined as one FISH sample. Cells were washed twice with 1 ml of PBS using a mini-centrifuge (Tomy PMC-060 Capsulefuge, generating 2,000 g) for 10 sec to remove *Agrobacterium* cells. The cell pellet was then fixed with 1 ml of 4% paraformaldehyde (for preparation, see Rodriguez-Lanetty *et al.*, 2004) for 45 min at room temperature. After fixing, yeast cells were washed twice with PBS and once with 1 ml of Buffer B (1.2 M sorbitol, 100 mM $KHPO_4$ pH7.5). The cells were then digested with Spheroplast Buffer (1.2 M sorbital, 100 mM $KHPO_4$ pH7.5, 20 mM β-mercaptoethanol, Lyticase 0.5 U/µl). 100 µg/ml RNase was added into the Spheroplast Buffer when doing the FISH of yeast DNA. The digestion was carried out at 30 ^{o}C for about 25 min. The progression of digestion was monitored by a phase contrast microscope (Zenklusen and Singer, 2010). After digestion, the spheroplast cells were collected by centrifugation for 3 min at 3500 rpm at 4 ^{o}C. The cells was washed once with 1 ml of cold Buffer B and resuspended with 500 µl of Buffer B. 100 µl of the resuspension was dropped onto a poly-L-lysine treated 18 mm coverslip face up into a 6-well tissue culture dish, one coverslip per well. After settling down for 1 h at 4 ^{o}C, 2 ml of Buffer B was slowly added into each well and removed using a

pipettor afterwards. A monolayer of immobilized cells will attach to the coverslip. 2 ml of 70% ethanol was slowly added into each well and the 6-well tissue culture dish was stored at least 3 h at -20 °C. Cells can be stored at -20 °C for at least 6 months.

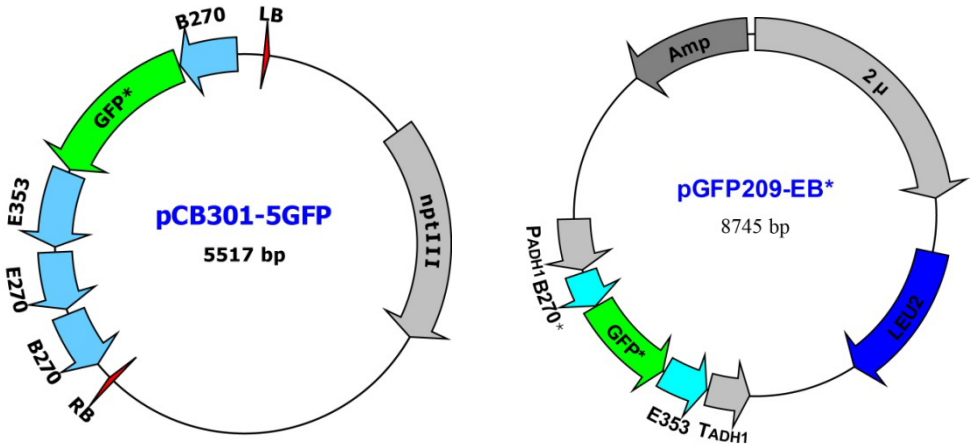
A

```
1    TTATTTGTAC AATTCATCCA TACCATGGGT AATACCAGCA GCAGTAACAA ATTCTAACAA
61   GACCATGTGG TCTCTCTTTT CGTTTGGATC TTTGGATAAG GCAGATTGAG TGGATAAGTA
121  ATGGTTGTCT GGTAACAAGA CTGGACCATC ACCAATTGGA GTATTTTGTT GATAATGGTC
181  AGCTAATTGA ACAGAACCAT CTTCAATGTT GTGTCTAATT TTGAAGTTAA CTTTGATACC
241  ATTCTTTTGT TTGTCAGCCA TGATGTAAAC ATTGTGAGAG TTATAGTTGT ATTCCAATTT
301  GTGACCTAAA ATGTTACCAT CTTCTTTAAA ATCAATACCT TTTAATTCGA TTCTATTAAC
361  TAAGGTATCA CCTTCAAACT TGACTTCAGC TCTGGTCTTG TAGTTACCGT CATCTTTGAA
421  AAAAATAGTT CTTTCTTGAA CATAACCTTC TGGCATGGCA GACTTGAAAA AGTCATGTTG  ← GFP Probe 2
481  TTTCATATGA TCTGGGTATC TAGAAAAACA TTGAACACCA TAAGTTAAAG TAGTGACTAA  ← GFP Probe 3
541  GGTTGGCCAT GGAACTGGCA ATTTACCAGT AGTACAAATA AATTTTAAGG TCAATTTACC  ← GFP Probe 4
601  GTAAGTAGCA TCACCTTCAC CTTCACCGGA GACAGAAAAT TTGTGACCAT TAACATCACC  ← GFP Probe 5
661  ATCTAATTCA ACCAAAATTG GGACAACACC AGTGAATAAT TCTTCACCTT TAGACAT
```

B

```
GFP Probe 2: 5' CTTCTGGCATGGCAGACTTGAAAAAGTCATGTTGTTTCATATGATCTGGG 3'
GFP Probe 3: 5' TTGAACACCATAAGTTAAAGTAGTGACTAAGGTTGGCCATGGAACTGGCA 3'
GFP Probe 4: 5' AAGGTCAATTTACCGTAAGTAGCATCACCTTCACCTTCACCGGAGACAGA 3'
GFP Probe 5: 5' GTGACCATTAACATCACCATCTAATTCAACCAAAATTGGGACAACACCAG 3'
```

C



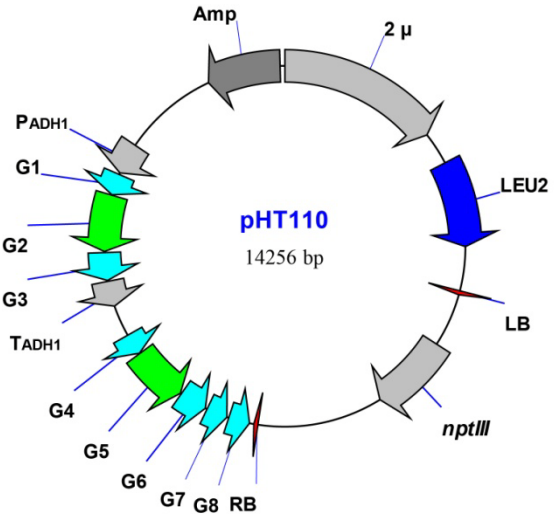


Figure 5.5. Probes design and plasmids construction for FISH. A: Distribution of GFP probes in the GFP gene region of pHT101. **B:** GFP probes for FISH. Bold "T"s are replaced by cyanine dye Cy3. **C:** Plasmid pHT110 was used as the bianary vector in the FISH assay. Plasmid pGFP209-EB* and plamids pCB301-5GFP were used for construction of pHT110.

5.5.5. Fluorescent *in situ* hybridization

The 6-well culture dish was taken out from -20 ^{o}C freezer. Ethanol was removed from the 6-well plate using a pipettor and samples were then rehydrated twice by adding 2 ml 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) at RT for 5 min. The samples were then washed once with 40% formamide, 2× SSC at RT for 5 min. Mix 20 ng of each probe with 20 µl of Hybridization Mixture (50% Formamide, 10% Dextran sulfate, 0.01% herring sperm DNA, 2× SSC) per sample and heat the mixture at 95 ^{o}C for 5 min then keep on ice immediately. In the meanwhile, denature the sample at 80 ^{o}C for 30 min and keep on ice immediately. Drop the Hybridization Mixture on the denatured sample and seal the sample in two incubation chambers (CoverWell^{TM}) to prevent evaporation and incubated for 24 h at 37˚C in a humid box. After 24 h hybridization, the slides were washed twice with 2 ml of 2 × SSC 0.1% Triton X-100 for 15 min at RT; washed once with 2 ml of 1 × SSC for 15 min at RT;

and washed once with 2 ml of PBS for 5 min at RT. After removing PBS, 100 µl of 1 × DAPI in PBS was applied at the centre of the coverslip at incubate at RT in dark for 10 min. Before mounting, dip coverslip in 100% ethanol and let them air dry for 10 min. Invert cells facing down onto 5 µl of Vetashield mounting medium (Vector) placed on a glass slide. The coverslips were then sealed with nail polish. The signal was detected using fluorescent or confocal microscope.

5.5.6. Results

There are many validated FISH protocol to hybridize mRNA in yeast cells, however, no procedure could be found to detect single copy of DNA in yeast cells. In order to monitor the spatial distribution of T-DNA in DNA in wild type yeast strain and *ost3*Δ strain, a novel sytem capable of detecting single copy of ssT-DNA in yeast cells. Zenklusen and Singer (2010) established a FISH method to detect single copy mRNA in yeast cell. Therefore, we wanted to adapt the FISH protocol for mRNA to detect single copy chromosomal DNA and cytoplasmal DNA in yeast cell. According to Zenklusen and Singer's protocol, we synthesized four 50 nt Cy3 labeled oligonucleotide probes in the T-DNA region. To optimise the FISH procedure for DNA detection, samples were heated at 80 ^{o}C for 30 min in hybridization mixture, under which conditions double stranded DNA should be able to denature into single stranded form and allows the hybridization with the Cy3 labeled oligonucleotide probes. More importantly, in order to see sigal moleculars of Cy3-labeled T-DNA signal under fluorescence, 8 copies of the probe-containing region containing 128 Cy3 labeling sites were designed into the T-DNA to enhance the signal of single copy of DNA molecular. Moreover, in order to maximize the hybridization between FISH probes and T-DNA, RNase was added in the digestion mixture to remove signals from mRNA. In addition, to facilitate the optimizing of FISH protocol, wild type strain

containing binary vector pHT110 was used as positive controls. This positive strain contains both DNA and mRNA of the probe-containing region, thus should be able to detect both mRNA signals as well as DNA signals. This assay is the first that single copy of DNA molecular can be detected and seen under fluorescent microscope using FISH technique.

We thus further want to detect the T-DNA molecular inside yeast cell during the the T-DNA transfer process *in vivo*. However, after doing the 24 h cocultivation and perform the FISH assay, we can hardly see yeast cells containint red dots signals (data not shown). From previous result, we can see that under the condition we used for AMT, the transformation efficiency for wild type yeast ranges from 10^{-5} to 10^{-4}, which means after 24 h co-cultivation, among 10^4 to 10^5 wild type yeast cells, only 1 cell could form the successful transformant. The transformation efficiency of ost3 mutant strain is about 30 folds lower that that. That range is too low for microscope detection. We can hardly find one positive signal amany 10^4 cells under microscope regardless of the hybridization efficieny of the FISH assay. During the T-DNA transfer process inside yeast cell, T-DNA was delivered from outside of yeast to cytoplasm then to nuclear and finally integrated to yeast genome or form a plasmid. Although there is a possibility that the number of yeast cells receiving T-DNA may far more than the number of transformant yeast, no result could support this hypothesis. In addition, the T-complex may also under degradation or digestion by inside yeast cells. Thus, the approach for tracking T-DNA inside yeast cells is still infeasible at this moment.

Secondly, probes may be not able to enter yeast nucleus so that no chromosomal DNA could be detected. Lyticase digestion enables the entry of probes into yeast cytoplasam; however, there is no way or any treatment performed to ensure the

penetration of the FISH probes to enter the yeast nuclear pore although 50 nt in is quite small for nuclear pore. Moreover, DNA degradation may be another concern for DNA detection in FISH.

However, other strategies may still be used to track T-DNA signal and analyse the spatial distribution of T-DNA inside yeast cells in the future. For example, we may track T-DNA inside plant cell use the same strategy. Plant is the natural host of *A. tumefaciens*, thus the transformation efficiency is much higher than that of *S. cerevisiae*, in accordance with the detection range by using fluorescence microscope.

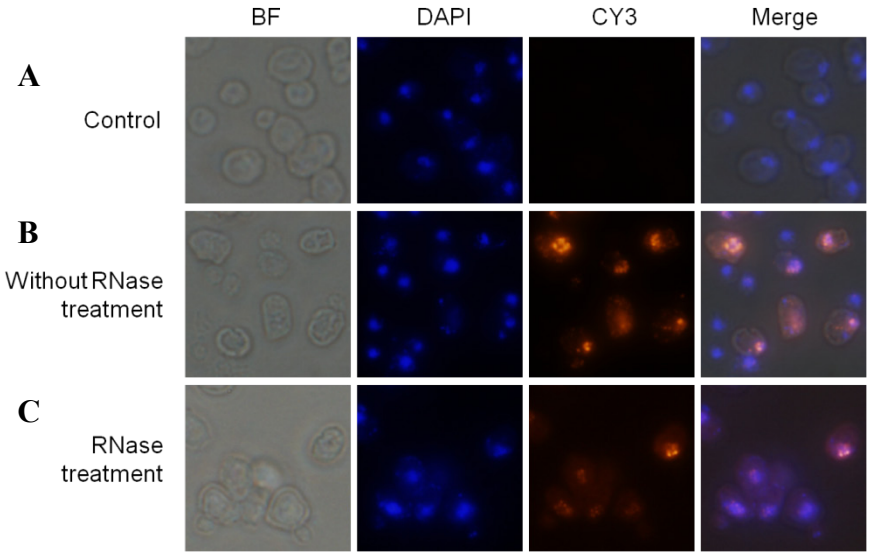


Figure 5.6. FISH results of T-DNA containing yeast. **A**: Wild type cells without RNase treatment of the cells. **B**: Wild type strain containing plasmid pHT110 were used as a control. At the same time of lyticase digestion, 100 µg/ml of RNase was used to remove mRNA in the samples. **C**: Wild type strain containing plasmid pHT110 were used as a control. No RNase treatment was conducted for this sample.

5.6. The effect of *OST3* on VirE2 import

Recently, our lab has established a novel system, which is able to quantatively monitor VirE2 protein import from *Agrobacterium* to yeast (Li X.Y. and Yang Q.H., unpublished data). This system uses a split-GFP complementation strategy to lable

Agrobacterium VirE2, thus can monitor the amount and position of VirE2 or the T-complex inside yeast cell real-timely by using fluorescence microscope. *OST3* is a component of the OST complex, which catalyses new synthesized pre-mature glycoproteins. Many *OST3* downstream glycoproteins may reside on the membranes thus may be important for VirE2 or the T-complex transportation from outside of yeast cell to cytoplasm or from cytoplasm to nucleus. Therefore, the effect of *OST3* on the VirE2 import was tested using the VirE2 split-GFP complementation assay.

5.6.1. Introduction of the VirE2 split-GFP complementation system

The purpose of developing the VirE2 split-GFP system is to label VirE2 and track the transportation of VirE2 inside yeast cell. One of the convenient ways is to fuse the GFP protein with the VirE2 protein. However, the N-terminal and C-terminal of VirE2 were reported not suitable for GFP fusion because of their important roles in nuclear targeting and T-DNA binding. Moreover, GFP has 239 bp which is too big for insertion into the internal region of the VirE2 protein. Cabantous *et al.* established a novel method to split GFP into a 16 aa small fragment (S11) and a 214 big fragment (S1-10) (Cabantous S. *et al.*, 2005). These two fragments are expressed separately and can restore the GFP activity when they meet together. Thus, the small is suitable for insertion into the internal region of VirE2. More importantly, VirE2 has self-aggragation activity. The polymer of VirE2 can be visualized under fluorescence microscope. It was proven that the VirE2 split-GFP complementation is suitatble for detecting and monitoring the trafficking of VirE2 inside yeast cell (Li X.Y. and Yang Q.H., unpublished data).

The 16 aa GFP S11 smll fragment was inserted into the internal position between 54 aa and 55 aa of VirE2. The big GFP fragment S1-10 was expressed in a yeast expression vector pQH04-S1-10 under yeast ADH1 promoter. This vector was

introduced into yeast wild type and mutant strains. During the transformation process, VirE2::S11 fusion protein was delivered into yeast cells through T4SS together with the T-DNA. Upon entering into yeast cells, the GFP S11 fragment will meet the S1-10 big fragment and aggragated to form polymer, which can be visualized under fluorescence microscope. By comparing the number and the position of the VirE2::GFP complemented fusion proteins, we can monitor the spatial distribution of the VirE2 inside yeast cell during the T-complex trafficking in yeast cell.

5.6.2. VirE2 split-GFP import analysis

Binary vector pHT110 was electroporated into the VirE2::S11 constructed *A. tumefaciens* strain EHA105 VirE2::S11, which was used in the AMT assay. The GFP S1-10 containing plasmid pQH04-S1-10 was introduced into yeast wild type strain BY4741 and mutant strain *ost3*Δ. Yeast strains were grown in SD His- medium to maintain plasmids. Normal AMT protocol was performed before VirE2 split-GFP import analysis (see section 2.4). After co-cultivation of yeast and *Agrobacteria* for different time points, the cell mixture was washed off by PBS and collected into 1.5 eppendorf tubes. The mixture was wash twice by using 1 ml of PBS using bench-top micro-centrifuge for 15 sec to remove *Agrobacterium* cells. 5 µl of anti-fade solution with 1× DAPI was added into the cell pellet. 3 µl of the resuspention was applied onto slide for observion under fluorescence microscopy. Random regions of the slide were used for photograph snap-shoting. Total yeast cells and yeast cells with GFP spots were counted for calculating importing efficiency of VirE2 GFP fusion protein.

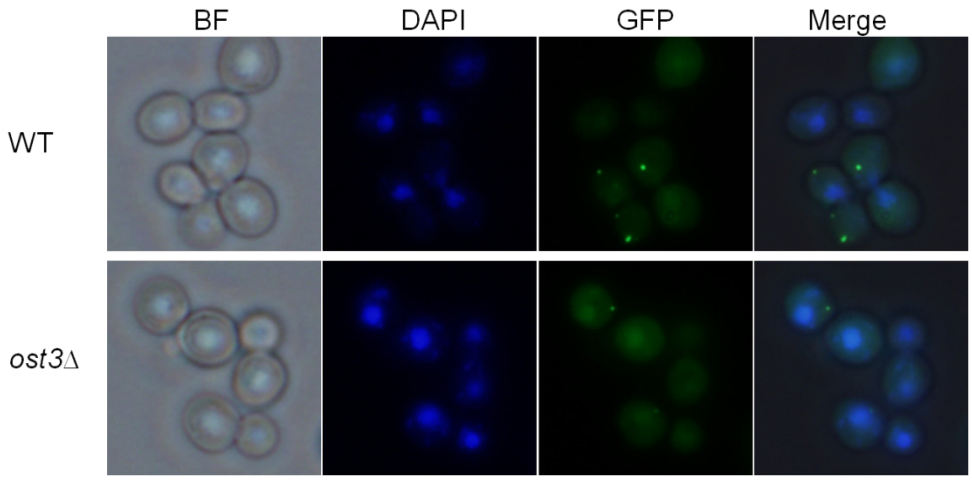
5.6.3. Result

VirE2 split-GFP complementation system enables us to track the VirE2 or T-complex inside yeast cells. By exploring this system, surprisingly, we found that the

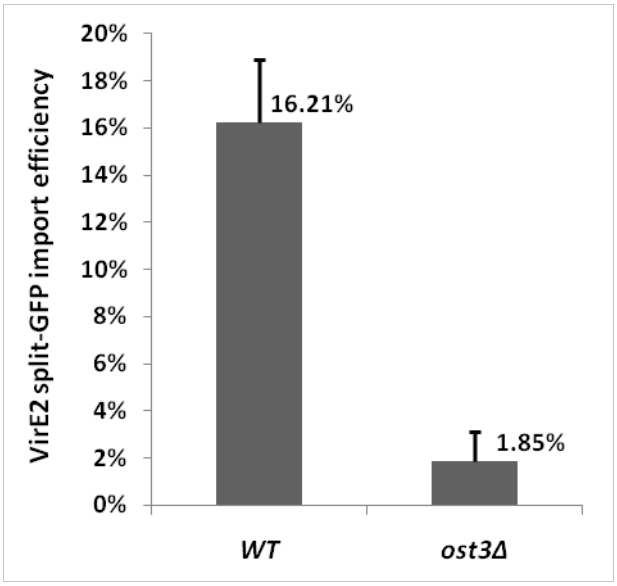
Ost3p plays a role in the VirE2 importing process. As shown in Fig. 5.7B, after 24 h co-cultivation, 16.21% of wild type yeast cells had GFP signals, which means the VirE2 importing efficiency into wild type yeast cell is 16.21%; while, the VirE2 importing efficiency into *ost3*Δ mutant strain is only 1.85%, which significantly decreased when compared with wild type. It is very interesting to notice that the AMT efficiency of wild yeast is around 10^{-5} to 10^{-4}, which is about 1000-fold lower than the VirE2 import efficiency. It implies that the VirE2 protein import into yeast by *Agrobacterium* is very efficient and the amount of imported VirE2 is abounded to coat ss T-DNA inside yeast. The time course study shows (Fig 5.7C & D) that the VirE2 import efficiency of wild type increased with the increase of co-cultivation period. The efficiency reached the maximum value within 24 h and decreased after 42 h incubation. However, for *ost3*Δ mutant strain as shown in Fig 5.7C & D, before 16 h VirE2 import efficiency was very lower than 1%. It also reached top at 24 h and decreased after 42 h. This VirE2 split-GFP import assay clearly showed that *OST3* significantly affected VirE2 import efficiency, which is consistant with the role of *OST3* on AMT process. Thus, the effect of *OST3* on T-DNA transfer process may directly or partially due to the effect of *OST3* on VirE2 import.

This assay is the first to track the VirE2 import from *Agrobacteirum* to yeast cell and showed that *OST3* significantly affect this process.

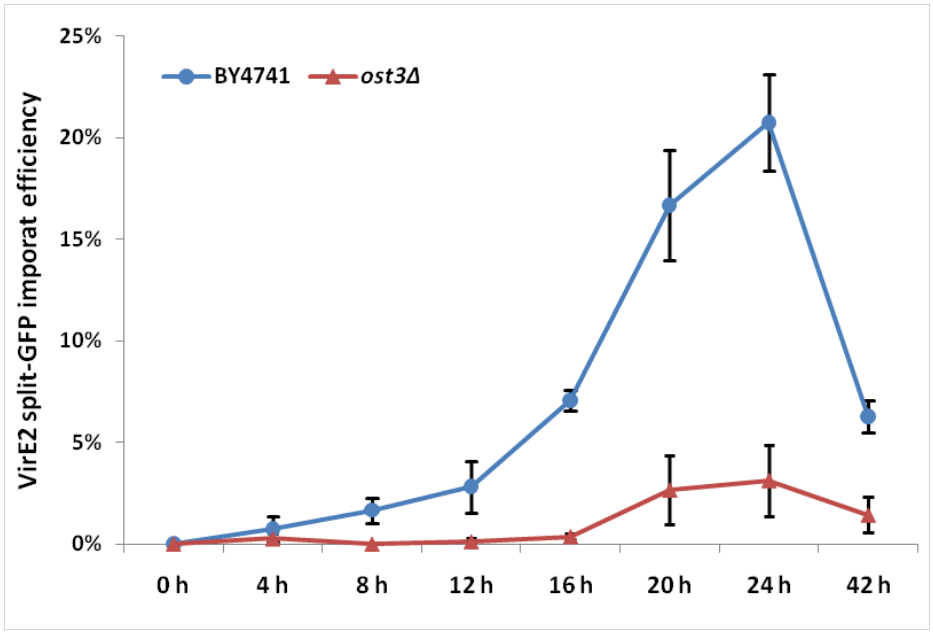
A


BF
DAPI
GFP
Merge
WT
ost3Δ

B


VirE2 split-GFP import efficiency
20%
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
16.21%
1.85%
WT
ost3Δ

C


VirE2 split-GFP imporat efficiency
25%
20%
15%
10%
5%
0%
BY4741
ost3Δ
0 h
4 h
8 h
12 h
16 h
20 h
24 h
42 h

D

	BY4741	*ost3Δ*	*Fold Change*
0 h	0	0	-
4 h	0.73%	0.27%	0.364
8 h	1.67%	0.00%	0.000
12 h	2.83%	0.11%	0.040
16 h	7.07%	0.37%	0.052
20 h	16.67%	2.66%	0.159
24 h	20.75%	3.13%	0.151
42 h	6.29%	1.43%	0.228

Figure 5.7. VirE2 split-GFP import analysis. **A**: Pictures show the import of VirE2 after 24 h cocultivation by split-GFP complementation system. Green spots are the VirE2::GFP signals. The blue area indicates the nuclear by DAPI staining. **B**: Comparision of VirE2 split-GFP import efficiency between wild type strain and *ost3*Δ after 24 h cocultivation. **C**: Time course study of VirE2 split-GFP import between wild type strain and *ost3*Δ. **D**: Data for Time course study of VirE2 split-GFP import assay.

5.7. Isolation of *OST3* regulated downstream glycoproteins

5.7.1. Introduction

OST3 belongs to the gamma subunit of the 9-component OST complex, which catalytic the transfer of oligosaccharides to nascent peptides at the concensus sites N-X-S/T (Knauer and Lehle, 1999a). Glycoproteins are involved in many processes such as immune response (Opdenakker *et al.*, 1993), intracellular targeting (Gleeson *et al.*, 1994), signal recognition (Wagner *et al.*, 1994), and proper protein folding, stabilization, and solubility (Imperiali and Rickert, 1995; Riederer and Hinner, 1991; Alien *et al.*, 1995; Duranti *et al.*, 1995). Although disruption of either *OST3* or *OST6* only leads to a minor underglycosylation (Chavan *et al.*, 2006), Ost3 protein is important for N-linked glycosylation of many proteins (Karaoglu *et al.*, 1995).

In this study, we found that *OST3* positively regulates the *Agrobacterium*-yeast T-DNA transfer process and Ost3p does not glycosylate the VirD2 protein. Therefore, the Ost3 downstream glycoproteins may be directly or indirectly affect the AMT

transfer process. In this section, we want to compare the glycoprotein profiles between wild type yeast cells and *ost3*Δ mutant cells. By comparing the glycoprotein profiles, we may be able to find out some glycoproteins specifically changed in the *ost3*Δ mutant cells during the AMT process. Thus, we may obtain useful information of Ost3p and its downstream glycoproteins in the *Agrobacterium*-yeast T-DNA transfer process.

5.7.2. Sample preparation

To mimic the co-cultivation conditions, yeast cells need to be induced in CM liquid broth before extracting protein crude extract. In order to compare the glycoprotein profile under different conditions, yeast cells were grown in different medium such as YPD, SD, and CM.

For crude protein extract from YPD or SD medium, wild type and *ost3*Δ single colonies were inoculated from YPD plate into 2 ml of YPD broth. After grown over night at 28 ^{o}C, 50 µl of yeast culture was subcultured into 50 ml fresh YPD or SD broth and grown over night until OD_{600} equals to 1.0. Collect cells by centrifugation at 5000 rpm 1 min. Wash cells twice with 10 ml ddH_2O. After removing supernatant, samples were stored at -20 ^{o}C until use.

For crude protein extract from CM medium, wild type and *ost3*Δ single colonies were inoculated from YPD plate into 2 ml of YPD broth. After grown over night at 28 ^{o}C, 50 µl of yeast culture was subcultured into 30 ml fresh YPD and grown at 28 ^{o}C. Cells were collected until OD_{600} equals to 1.0. Wash cells twice with 10 ml ddH_2O and once with 10 ml CM broth. Then cells were induced in 50 ml CM broth with AS 100 µM Km 100 µg/ml at 28 ^{o}C for 18 h. Cells were collected by centrifugation at

5000 rpm 1 min. Wash cells twice with 10 ml ddH_2O. After removing supernatant, samples were stored at -20 ^oC until use.

5.7.3. Total protein extraction and qualification

After taking out the from -20 ^oC, cell samples wer resuspended in 1.6 ml of ice cold cell freshly-made lysis buffer A [10mM K-Hepes pH7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM Phenylmethylsulfonyl floride (PMSF), 0.5mM DTT, EDTA-free proteinase inhibitor cocktail]. The cells were then passed through French Press three times at 1,000 PSI to break the cell wall and release the total proteins. The cells were collected using 2 ml eppendorf tube then incubate on ice. 400 µl of 1M KCl was added in to reach a final concentration of 0.2 M. The protein extract was then centrifuged at 13,800 rpm for 1 h at 4˚C and the supernatant was harvested.

Protein concentration was quantified using Coomassie Plus Protein Assay (Pierce). A standard curve was prepared by a serial dilution of bovine serum albumin fraction V (Roche) according to manufacer's manual. The optical densities of protein samples were measured using 595 nm wave length. Protein concentrations were calculated according to the equation generated from the protein standard curve.

5.7.4. Glycoprotein isolation

Two kits were used to isolate glycoproteins in yeast cells. The first one is Glycoprotein Isolation Kit, ConA (Thermo Scientific, Cat. No.: 89804). Lectins are proteins that selectively bind to carbohydrate moieties. The lectin concanavalin A (ConA) preferentially recognizes N-linked glycans thus can be used for isolating N-linked glycoproteins. Firstly, equilibrate the Binding/Wash Buffer and Elution Buffer to room temperature. Gently swirl the ConA Lectin resin to obtain a homogeneous suspension and transfer 200 µl of 50% resin slurry to a column. Centrifuge 1 min at

1000 g and discard the flow-through. Wash the resin in the column 3 times with 200 µl of 1× Binding/Wash Buffer. Place a bottom cap on column and add about 1.5 mg of total protein sample diluted in 1× Binding/Wash Buffer. Close the top cap. Incubate the column for 10 min at room temperature with end-over-end mixing using a rotator. Remove top cap and then bottom cap. Place column in the collection tube and centrifuge 1 min at 1000 g and discard the flow-through. Place a bottom cap on column and wash the resin 3 times with 400 µl of 1× Binding/Wash Buffer after incubation for 5 min each time with end-over-end mixing. Place a bottom cap on column and add 200 µl Elution Buffer to resin and cap column. Incubate column for 5 min at room temperature with end-over-end mixing. Collect the elution sample in a new collection tube by centrifugation. Repeat the sample collection step once and combine the glycoprotein sample. Store eluted glycoprotein on ice for immediate use or freeze at -20 ^{o}C for later analysis.

The second glycoprotein isolation kit is the Glyco Kit MB-LAC ConA (Bruker Daltonics, Cat No.: 252665). The Glyco Kit MB-LAC Con A can specifically capture the N-glycosylated proteins and peptides derived from biological samples based on lectin affinity. Firstly, the Magnetic Beads is resuspended by vortexing. Transfer 20 µl of resuspended Magnetic Beads to a thin wall PCR tube and add 100 µl WB 1.1. Separate the beads from supernatant for 20 s using a Magnetic Separator and remove the supernatant. Wash the Magnetic beads once with 100 µl WB 1.1. Resuspend the beads in 10 µl BB 1.1 and add 10 µl of total protein sample. Incubate the magnetic beads for 1 h at RT with swirling the tube. Remove supernatant after separating beads by magnetic separator. Wash the beads once with 100 µl WB 1.1 and twice with WB2. Add 10 µl of EB and mix thoroughly. Incubate 25 min at RT with swirling. Separate the beads on the magnetic separator and transfer the elution solution

containing purified glycoproteins into a new eppendorf tube. Store eluted glycoprotein on ice for immediate use or freeze at -20 ^{o}C for later analysis.

5.7.5. Glycoprotein profile analysis

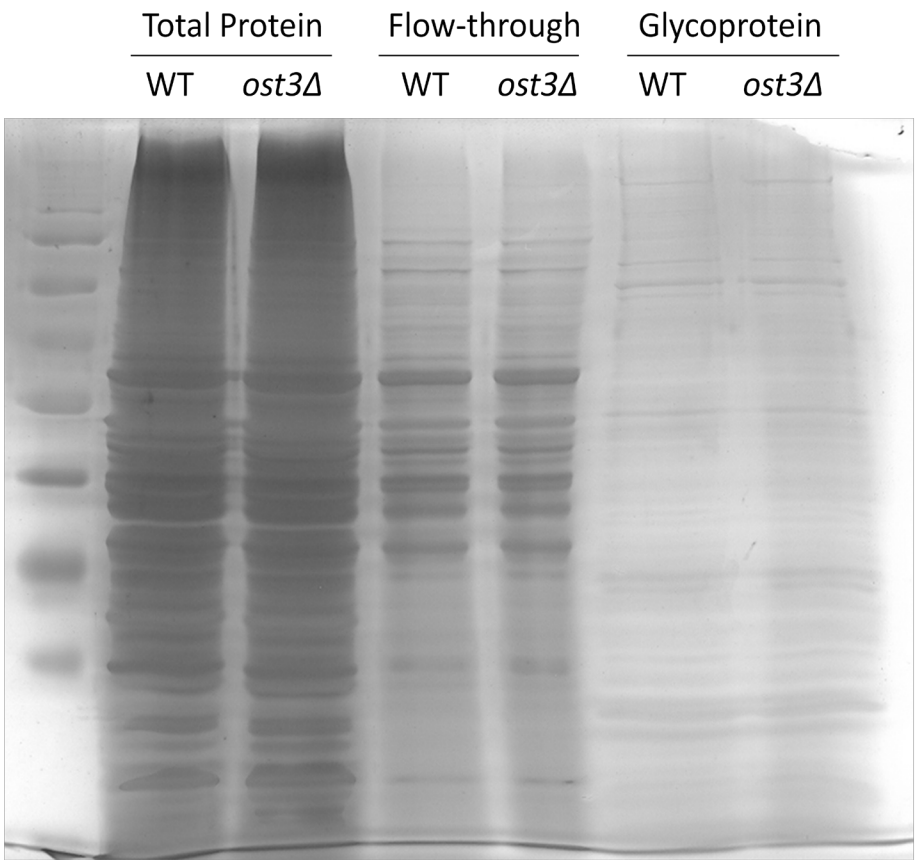
After glycoproteins are isolated from total proteins, 10% SDS PAGE was done to separate the glycoproteins (see section 2.3.2 for methods). Commassie blue staining (see section 2.3.3 for methods) and silver staining (see section 2.3.4 for methods) were performed to obtain the protein profiles of each sample.

5.7.6. Results

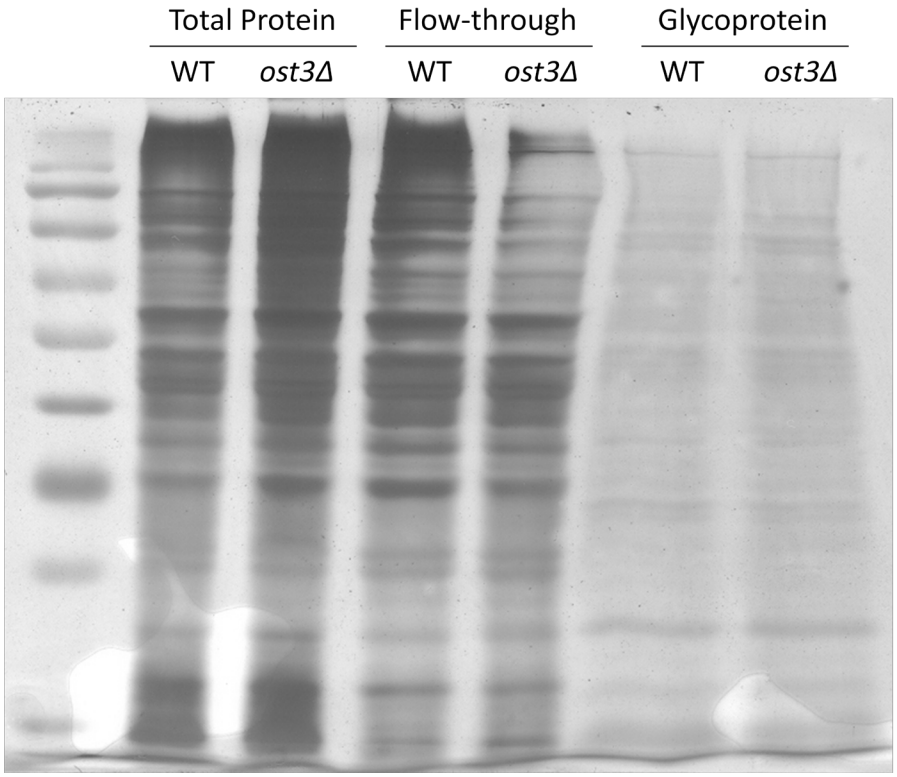
As shown in Fig 5.7A and B, for proteins extracted from YPD culture, there is no difference between wild type and *ost3*Δ in the total protein sample, flow-through sample, and glycoprotein sample. Interestingly, as can be seen in Fig 5.7C, for proteins extracted from CM culture, two glycoproteins are absent in the *ost3*Δ mutant strain. These results show that under normal rich or mimimal growth conditions the glycoprotein profiles between wild type strain and *ost3*Δ mutant strain are same. However, under the *Agrobacterium*-yeast co-cultivation CM medium, severel glycoproteins are not present in the *ost3*Δ, indicating these glycoproteins are likely glycosylated by Ost3p. Moreover, these glycoproteins are probably directly involved in the AMT process.

Unfortunately, due to the limited yield from glycoprotein isolation and the insolubility of these glycoproteins, the amounts of glycoproteins obtained from the silver staining gel are not enough for MOLDI-TOF analysis. The identification of these Ost3p specific glycoproteins is needed in the future.

A


Total Protein
Flow-through
Glycoprotein
WT
ost3Δ
WT
ost3Δ
WT
ost3Δ

B


Total Protein
Flow-through
Glycoprotein
WT
ost3Δ
WT
ost3Δ
WT
ost3Δ

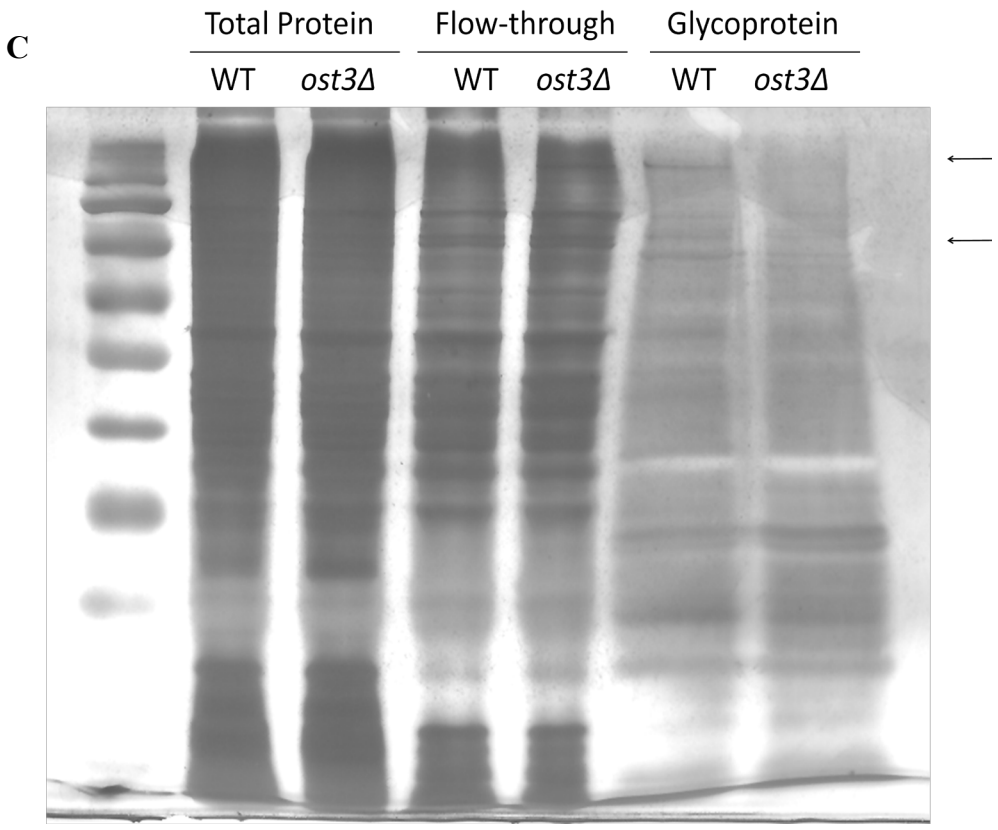


Figure 5.8. Glycoprotein profiles analysis of *ost3*Δ. Silver stain was performed after SDS-PAGE electrophoresis. A: Yeast cells were grown in YPD broth before glycoprotein extraction. B: Yeast cells were grown in SD broth before glycoprotein extraction. C: Yeast cells were induced in CM broth with AS 100 μM Km 100 μg/ml before glycoprotein extraction.

CHAPTER 6. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Agrobacterium-mediated transformation (AMT) of yeast is a very good model to study gene transfer process, particularly between two different organisms. The AMT of yeast model has been used to study the host factors involved in the *Agrobacterium*-yeast gene transfer (AYGT) process. In order to find host gene involved in the AYGT process, this project screened the whole genome of *Saccharomyces cerevisiae* using a novel screening system. This chapter summarizes the general findings of this project and proposes several suggestions for future consideration. The scope of this project is discussed in this chapter as well.

6.1. General conclusions and discussions

The first objective of this project was to develop a liquid based protocol for *Agrobacterium*-mediated transformation of yeast, which should be different from existing AMT protocols. It was found that the newly developed protocol can be used for the AMT of yeast. The transformation efficiency of the new protocol had similar, if not higher, transformation efficiency when compared with existing ones (Bundock et al, 1995; Piers et al, 1996). The difference between the newly developed method and previous co-cultivation methods (Bundock et al, 1995; Piers et al, 1996) lies in the co-cultivation environment: the newly developed one is in a liquid environment but other methods are on solid CM agar plates. Several modifications may attribute to the success of the liquid-based AMT. Firstly, a small amount of YPD broth coming from the yeast culture is added to the IBPO4 induction broth, which maintains the minimum growth of yeast cells during the co-cultivation. Yeast cells may not be transformed without this step. Secondly, the co-cultivation is carried out without

shaking, which is important for the attachment of *Agrobacterium* cells to yeast cells because cells precipitate down after few hours of co-cultivation. To our knowledge, this protocol is the first liquid-based AMT, which is easier to handle and can reduce cell lost during the cell collection process. In particular, this protocol is the first liquid based AMT protocol and provides a crucial approach for large-scale screenings because the liquid based AMT system is applicable for 96-well micro-plates and robot-aided ejection and collection systems.

Secondly, a liquid based large-scale screening of yeast genes involved in AMT was performed and a global profile of yeast genes involved in the *Agrobacterium*-yeast gene transfer (AYGT) was obtained. Two hundred and nine mutants including reduced and enhanced AYGT efficiency mutants were found to be involved in the T-DNA transfer process. Moreover, it was found that many mutants related to endocytosis, ion channel, TCA cycle, mitochondrion, and glycosylation showed enhanced or decreased T-DNA AYGT efficiency, which implies that those intracellular processes may play important roles in the AYGT process. Compared with other host factors screening systems (Robberts et al, 2003; Zhu et al, 2003a; Crane and Gelvin, 2007), this new screening system is efficient and holistic because it can detect both enhanced mutants and reduced mutants.

This screening system is the first one to examine the effect of almost all of the yeast genes on the AYGT process. This assay may not identify all the yeast genes involved in the AMT process, such as other components of the OST complex were not identified in the large-scale screening. Moreover, the genes identified in the liquid based co-cultivation process may not be same as the ones identified in the tranditional plate based co-cultivation, because the conditions for the two processes are different

and different metabolic pathways are involved in the two processes. However, this assay still identified 209 novel factors. Most of the genes identified in this screening system are novel host factors involved in the AYGT, which will provide important clues in the dissecting of the T-DNA transfer pathway. The global profile of AYGT obtained in this study will also shade light on the understanding of the overall picture of the gene transfer process.

The effects of the oligosaccharyltransferase (OST) complex on the AYGT were examined. It was found that besides *ost3*Δ, which was identified from the large-scale screening, the AYGT efficiencies of other components of the OST complex including *ost2*Δ, *ost4*Δ, and *ost5*Δ were dramatically reduced, which strongly suggests that the whole OST complex or the glycosylation process is important in the T-DNA transfer process. The involvement of the *OST3* gene in the AYGT was also supported by the complementation assay, which fully restored T-DNA transfer efficiency by introducing yeast *OST3* gene into *ost3*Δ. However, after the lithium acetate-mediated DNA transfer treatment the linearized and circular plasmid DNA transformation efficiencies in *ost3*Δ were not decreased. Thus, we can conclude that *OST3* gene or maybe the whole OST complex specifically influences AYGT process but not the LiAc-mediated DNA transfer. It is possible that AYGT and the lithium acetate-mediated DNA transfer shares different gene trafficking pathways since chemical and physical-mediated DNA transfer process is different from the natural *Agrobacterium*-mediated T-DNA transfer system.

This study is the first to show that *OST3* gene as well as the OST complex or maybe glycosylation process is important for gene transfer, and it will provide novel insight in the understanding of the gene transfer process.

The bindings between OST components and VirD2 protein were examined. It was found that several OST components, including Ost1p, Ost3p, Ost5p, and Swp1p, could bind to VirD2 protein in the TAP assay. The physical interactions between OST components and VirD2 protein further confirm the involvment of *OST3* gene and OST complex in the AMT process. The GFPVirD2 co-localization assay showed that *OST3* did not function in regulating the VirD2 nuclear targeting. In addition, it was also proved that VirD2 was not glycosylated by *OST3*. These results indicate that the effect of Ost3p on AMT process is not through direct glycosylation of VirD2 or on the VirD2 nuclear targeting, although it physically associates with VirD2 protein. This finding provides more clues for the mechanism of the effect of Ost3p on AYGT process.

In order to track the T-DNA transfer process inside yeast cell and monitor the spatial distribution of the T-DNA in wild type yeast or *ost3*Δ strain, fluorescence *in situ* hybridization assay was performed. DNA signals and mRNA signals were successfully detected in yeast strain containing T-DNA. This is the first time to show that single molecular of T-DNA can be visualized by using FISH technique. However, the detection of T-DNA during the transfer process was not successful. It may be due to the limitation of detection range by counting the positive yeast cells under fluorescent microscope when compared with the relatively low AMT efficiency (about 10^{-5} to 10^{-4}). Under this AMT efficiency, we can hardly find cells with positive signals. However, the similar strategy may be performed in plant system, because plant is the natural host of *Agrobacterium* thus has higher AMT efficiency than yeast system. The detection of spatial distribution of T-DNA inside plant cell by using FISH assay may reveal the T-DNA trafficking process in host cells.

A novel system was explored to examine VirE2 import efficieny by using the split-GFP complementation assay. This assay is the first system that can detect the VirE2 import. Interestingly, results show that the VirE2 import efficiency was significantly decreased in *ost3*Δ mutant strain, implying the importance of *OST3* on VirE2 transport. Moreover, results also revealed that the VirE2 impor efficiency is far more higher than the T-DNA deliver efficiency, which is also very interesting. The examing of VirE2 import provides another useful tool to understand the T-DNA trafficking and the role of yeast genes in this process.

The glycoprotein profile of Ost3p was examined. Two downstream glycoproteins were found to be absent in *ost3*Δ mutant. Although disruption of either *OST3* or *OST6* has minor effect on glycosylation (Chavan *et al.*, 2006), Ost3 protein is important for N-linked glycosylation of many specific proteins (Karaoglu *et al.*, 1995). These Ost3p specific glycoproteins may play important roles in the AYGT process. This finding will also shade light on the understanding of the Ost3p function and the gene transfer process pathway.

Sequence analysis revealed that Ost3 protein family is highly conserved among many species including human beings (Kelleher and Gilmore, 2006). It shares 20% sequenece identity to human *N33*, which is a candidate tumour suppressor gene (MacGrogan *et al.*, 1996). Moreover, human *N33* has been found to be involved in many important cellular biological processes and disorders, such as nonsyndromic autosomal recessive mental retardation (Molinari *et al.*, 2008; Garshasbi *et al.*, 2008), development of lymph node metastasis in larynx and pharynx carcinomas (Guervós *et al.*, 2007), and in ovarian carcinoma and survival (Pils *et al.*, 2005). All these evidence seems to imply that *OST3* plays important roles other than N-linked

glycosylation. Thus, the findings of the effect of *OST3* on gene transfer process will also shade light on the understanding of the *N33* on the cancerology and may provide additional clues to cancer therapy.

6.2. Future directions

This study is the first to develop a new liquid based AMT protocol and a high throughput host factor screening system. Although this liquid assay and screening system are efficient and holistic, some points still need to be mentioned. Firstly, this new protocol is based on a liquid co-cultivation environment without shaking, which is different from the traditional CM plate based co-cultivation environment. In a static liquid medium, where the air and nutrition supply are different, yeast cells as well as the *Agrobacterium* cells may not follow the normal metabolic pathway so that the cell stage may be different. Thus, what is found in a liquid based screening system may not be applicable to a solid plate based screening system. Therefore, the genes found in the liquid screening need to be confirmed by a traditional solid assay. Secondly, although the yeast cells can be transformed by *Agrobacteria* via a liquid assay, the ideal conditions for the liquid assay have not been studied. Hence, conditions like nutrition, *Agrobacteria*-yeast ratio, and co-cultivation duration need to be optimized in the future.

Our data showed that the *OST3* gene and the OST complex process were involved in the *Agrobacterium*-yeast T-DNA transfer process, which strongly suggests the involvement of the glycosylation in the gene transfer process. However, additional direct evidence is needed to verify and support this hypothesis. The finding of the Ost3p downstream glycoproteins will provide strong support for the

understanding the relationship between glycosylstion process and the T-DNA transfer process. Future work needs to be done to identify the downstream glycoproteins.

Spatial distribution of the T-DNA in the whole T-DNA transfer process is crucial for us to understanding the whole picture of the gene transfer process and factors involved in the process. Although the FISH assay to monitor the T-DNA tranfficking process inside yeast cells was not successful, this assay can be explored in the plant system due to its higher T-DNA transfer efficiency.

REFERENCES

Agrios G.N. (1988) Plant Pathology, 3rd Ed. Academic Press Inc., London. 558-65.

Albright L.M., Yanofsky M.F., Leroux B., Ma D., and Nester E.W. (1987) Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. *J Bacteriol* **169**: 1046-55.

Alien S. Naim H.Y. and Bulleid N.J. (1995) Intracellular folding of tissue-type plasminogen activator: Effects of disulfide bond formation on N-linked glycosylation and secretion. *J Biol Chem* **270** (9): 4797-804.

Allardet-Servent A., Michaux-Charachon S., Jumas-Bilak E., Karayan L., and Ramuz M. (1993) Presence of one linear and one circular chromosome in the *Agrobacterium tumefaciens* C58 genome. *J Bacteriol* **175**: 7869-74.

Allen O.N. and Holding A.J. (1974) Genus II. Agrobacterium Conn. 1942. 359. In Buchanan R.E., Gibbons N.E., eds, Bergey's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore 264-67.

Alonso J.M., Stepanova A.N., Leisse T.J., Kim C.J., Chen H., Shinn P., et al. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653-57.

Aly K.A. and Baron C. (2007) The VirB5 protein localizes to the T-pilus tips in *Agrobacterium* tumefaciens. *Microbiology* **153**: 3766–75.

Anand A., Krichevsky A., Schornack S., Lahaye T., Tzfira T., Tang Y., Citovsky V., and Mysore K.S. (2007) *Arabidopsis* VirE2 Interacting Protein2 is required for *Agrobacterium* T-DNA integration in plants. *Plant Cell* **19**: 1695–708.

Anderson L.B., Hertzel A.V., and Das A. (1996) *Agrobacterium tumefaciens* VirB7 and VirB9 form a disulfide-linked protein complex. *Proc Natl Acad Sci USA* **93:** 8889-94.

Atmakuri K., Ding Z., and Christie P.J. (2003) VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium* tumefaciens. *Mol Microbiol* **49**: 1699-713.

Azpiroz-Leehan R. and Feldmann K.A. (1997) T-DNA insertion mutagenesis in Arabidopsis: going back and forth. *Trends Genet* **13** (4): 152-26.

Backert S., Fronzes R., and Waksman G. (2008) VirB2 and VirB5 proteins: specialized adhesins in bacterial type-IV secretion systems? *Trends Microbiol* **16**: 409–13

Baenziger J.U. (1994) Protein-specific glycosyltransferases: how and why they do it! *FASEB J* **8** (13): 1019-25.

Bakó L., Umeda M., Tiburcio A.F., Schell J., and Koncz C. (2003) The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. *Proc Natl Acad Sci USA* **100**: 10108-13.

Ballas N. and Citovsky V. (1997) Nuclear localization signal binding protein from *Arabidopsis*mediates nuclear import of *Agrobacterium* VirD2 protein. *Proc Natl Acad Sci USA* **94**: 10723-28.

Banta L.M., Bohne J., Lovejoy S.D., and Dostal K. (1998) Stability of the *Agrobacterium tumefaciens* VirB10 protein is modulated by growth temperature and periplasmic osmoadaption. *J Bacteriol* **180**: 6597-606.

Barker R.F., Idler K.B., Thompson D.V., and Kemp J.D. (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti-plasmid pTi15955. *Plant Mol Biol* **2:** 335-50.

Bause E., Wesemann M., Bartoschek A., and Breuer W. (1997) Epoxyethylglycyl peptides as inhibitors of oligosaccharyltransferase: Double-labelling of the active site. *Biochem J* **322**: 95-102.

Beaupre, C.E., Bohne, J., Dale, E.M., and Binns, A.N. (1997) Interactions between VirB9 and VirB10 membrane proteins involved in movement of DNA from *Agrobacterium tumefaciens* into plant cells. *J Bacteriol* **179**: 78-89.

Bell C.R. (1990) Growth of *Agrobacterium tumefaciens* under octopine limitation in chemostats. Appl. Environ. *Microbiol* **56** (6): 1775-81.

Bevan M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res* **12**: 8711-21.

Bhattacharjee S., Lee L.Y., Oltmanns H., Cao H., Veena, Cuperus J., and Gelvin S.B. (2008) IMPa-4, an *Arabidopsis* importin α isoform, is preferentially involved in *Agrobacterium*-mediated plant transformation. *Plant Cell* **20**: 2661–80

Binns A.N., Beaupre C.E., and Dale E.M. (1995) Inhibition of VirB-mediated transfer of diverse substrates from *Agrobacterium tumefaciens* by the InQ plasmid RSF1010. *J Bacteriol* **177**: 4890-99.

Bolton G.W., Nester E.W., and Gordon M.P. (1986) Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science* **232**: 983-85.

Bourret R., Borkovich K., and Simon M. (1991) Signal transduction pathways involving protein phosphorylation in prokaryotes. *Annu Rev Biochem* **60**: 401-41.

Bradbury J.F. (1986). Guide to Plant Pathogenic Bacteria. CAB International Mycological Institute, London.

Braun, A.C. (1947) Thermal studies on the factors responsible for tumor initiation in crown-gall. *Am J Bot* **34**: 234–240.

Braun, A.C. and Mandle, R.J. (1948) Studies on the inactivation of the tumor-inducing principle in crown gall. *Growth* **12**: 255-269.

Bundock P, Dulk-Ras AD, Beijersbergen A, Hooykaas P.J.J. (1995) Trans-Kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J* **14** (13): 3206-14.

Bundock P. and Hooykaas P.J.J. (1996) Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. *Proc Natl Acad Sci USA* **93**: 15272-75.

Bundock P., van Attikum H., den Dulk-Ras A., and Hooykaas P.J.J. (2002) Insertional 13 mutagenesis in yeasts using T-DNA from *Agrobacterium tumefaciens*. *Yeast* **19**: 529-36.

Burns D.L. (2003) Type IV transporters of pathogenic bacteria. *Curr Opin Microbiol* **6** (1): 29-34.

Burridge K., Fath K., Kelly T., Nuckolls G., and Turner C. (1988) Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* **4**: 487-525.

Cabantous S., Terwilliger T.C., and Waldo G.S. (2005) Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat Biotechnol* **23**(1): 102-7.

Cangelosi G.A., Ankenbauer R.G., and Nester E.W. (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc Natl Acad Sci USA* **87**: 6708-12.

Cangelosi G.A., Best E.A., Martinetti G., and Nester E.W. (1991) Genetic analysis of *Agrobacterium*. *Methods Enzymol* **204**: 384-97.

Cangelosi G.A., Hung L., Puvanesarajah V., Stacey G., Ozga D.A., Leigh J.A., and Nester E.W. (1987) Common loci for *Agrobacterium tumefaciens* and Rhizobium meliloti exopolysaccharide synthesis and their roles in plant interactions. *J Bacteriol* **169**: 2086 - 91.

Cangelosi G.A., Martinetti A.G., Leigh J.A., Lee C.C., Theines C., and Nester E.W. (1989) Role of *Agrobacterium tumefaciens* ChvA protein in export of β-1,2-glucan. *J Bacteriol* **171**: 1609-15.

Cascales E. and Christie P.J. (2003) The versatile bacterial type IV secretion systems. *Nature Rev Microbiol* **1**: 137-49.

Cascales E. and Christie P.J. (2004) Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* **304**: 1170-73.

Cavara, F. (1897) Tubercolosi della vite. Intorno alla eziologia de alcune malattie di piante coltivate. *Stazoni Sperimentale Agrarie Italiane* **30**: 483-87.

Celis J.E., Carter N., Hunter T., Simons K., Small J.V., and Shotton D. (2006) Protein detectin in gels by silver staining: a procedure compatible with mass-spectrometry. *Cell Biology: A Laboratory Handbook*. 3rd Edition. Elsevier. Academic Press.

Chan, M.T., Chang H.H., Ho S.L., Tong W.F., and Yu S.M. (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric alpha-amylase promoter/beta-glucuronidase gene. *Plant Mol Biol* **22**: 491–506.

Chan, M.T., Lee T.M., and Chang H.H. (1992) Transformation of Indica rice (Oryza sativa L.) mediated by *Agrobacterium* tumefaciens. *Plant Cell Physiol*. **33**: 577.

Charest P.J., Holbrook L.A., Gabard J., Iyer V.N., and Miki B.L. (1988) *Agrobacterium* mediated transformation of thin cell layer explants from Brassica napus L. *Theor Appl Genet* **75** (3): 438-45.

Charles T.C. and Nester E.W. (1993) A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium* tumefaciens. *J Bacteriol* **175**: 6614-25.

Chavan M., Chen Z., Li G., Schindelin H., Lennarz W.J., and Li H. (2006) Dimeric organization of the yeast oligosaccharyl transferase complex. *Proc Natl Acad Sci USA* **103**: 8947–52.

Chavan M., Yan A., and Lennarz W.J. (2005) Subunits of the translocon interact with components of the oligosaccharyl transferase complex. *J Biol Chem* **280**: 22917–24.

Chee P.P. and Slightom J.L. (1995) Transformation of soybean (Glycine max) via *Agrobacterium tumefaciens* and analysis of transformed plants. *Methods Mol Biol* **44**: 101-19.

Chen I., Christie P.J., and Dubnau D. (2005) The ins and outs of DNA transfer in bacteria. *Science* **310**: 1456-60.

Chen L., Li C.M., and Nester E.W. (2000) Transferred DNA (T-DNA)-associated proteins of *Agrobacterium tumefaciens* are exported independently of virB. *Proc Natl Acad Sci USA* **97**: 7545-50.

Cheng M. Fry J.E., Pang S., Zhou H., Hironaka C.M., Duncan D.R., Conner T.W., and Wan Y. (1997) Genetic transformation of wheat mediated by *Agrobacterium* tumefaciens. *Plant Physiol* **115**: 971-80.

Chi J.H., Roos J., and Dean N. (1996) The *OST4* gene of *Saccharomyces cerevisiae* encodes an unusually small protein required for normal levels of oligosaccharyltransferase activity. *J Biol Chem* **271**: 3132–40.

Chilton, M.D. (1993) *Agrobacterium* gene transfer: Progress on a “poor man’s vector” for maize. *Proc Natl Acad Sci USA* **90**: 3119-20.

Chilton, M.-D., Drummond, M.H., Merlo, D.J., and Sciaky, D. (1978) Highly conserved DNA of Ti plasmids overlaps T-DNA maintained in plant tumors. *Nature* **275**: 147-49.

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P., and Nester, E.W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11**: 263-71.

Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119-22.

Christie P.J. (1997) *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J Bacteriol* **179**: 3085-94.

Christie P.J. (2001) Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol Microbiol* **40**: 294-305.

Christie P.J. and Cascales E. (2005) Structural and dynamic properties of bacterial type IV secretion systems. *Mol Membr Biol* **22**: 51-61.

Christie P.J. and Vogel J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol*. **8**: 354-60.

Christie P.J., Ward J.E., Winans S.C., and Nester E.W. (1988) The *Agrobacterium tumefaciens* virE2 gene product is a single-stranded-DNA-binding protein that associates with T-DNA. *J Bacteriol* **170**: 2659-67.

Citovsky V., De Vos G., and Zambryski P. (1988) Single-stranded DNA binding protein encoded by the virE locus of *Agrobacterium* tumefaciens. *Science* **240**: 501-04.

Citovsky V., Guralnick B., Simon M.N., and Wall J.S. (1997) The molecular structure of *Agrobacterium* VirE2-single stranded DNA complexes involved in nuclear import. *J Mol Biol* **271** (5): 718-27.

Citovsky V., Kozlovsky S.V., Lacroix B., Zaltsman A., Dafny-Yelin M., Vyas S., Tovkach A. and Tzfira T. (2007) Biological systems of the host cell involved in *Agrobacterium* infection. *Cell Microbio* **9** (1): 9-20.

Citovsky V., Lee L.Y., Vyas S., Glick E., Chen M.H., Vainstein A., Gafni Y., Gelvin S.B., and Tzfira T. (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. *J Mol Biol* **362** (5): 1120-31.

Citovsky V., Wong M.L., and Zambryski P. (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single stranded DNA: implications for the T-DNA transfer process. *Proc Natl Acad Sci USA* **86**: 1193-97.

Citovsky V., Zupan J., Warnick D., and Zambryski P. (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. *Science* **256**: 1802-05.

Clauce-Coupel H., Chateau S., Ducrocq C., Niot V., Kaveri S., Dubois F., Sangwan-Norreel B., Sangwan R.S. (2008) Role of vitronectin-like protein in *Agrobacterium* attachment and transformation of *Arabidopsis* cells. *Protoplasma* **234** (1-4): 65-75.

Clough S.J. and Bent A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16** (6): 735-43.

Conn H.J. (1942) Validity of the genus Alcaligenes. *J Bacteriol* **44**: 353-360.

Costantino, P., Hooykaas P. J. J., den Dulk-Ras H., and Schilperoort R. A. (1980) Tumor formation and rhizogenicity of *Agrobacterium* rhizogenes carrying Ti plasmids. *Gene* **11**: 79–87.

Crane Y.M. and Gelvin S.B. (2007) RNAi-mediated gene silencing reveals involvement of *Arabidopsis* chromatin-related genes in *Agrobacterium*-mediated root transformation. *Proc Natl Acad Sci USA* **104**: 15156–61.

Das A. (1998) DNA transfer from *Agrobacterium* to plant cells in crown gall tumor disease. *Subcell Biochem* **29**: 343-63.

Das A. and Xie Y.H. (1998) Construction of transposon Tn3phoA: Its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. *Mol Microbiol* **27**: 405-14.

David C.A and Daniel J.B. (2005) Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press 107.

de Buck S., Jacobs A., Van Montagu M., and Depicker A. (1999) TheDNA sequences ofT-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. *Plant J* **20**: 295–304.

de Groot M.J., Bundock P., Hooykaas P.J., and Beijersbergen A.G. (1998) *Agrobacterium* tumefaciens-mediated transformation of filamentous fungi. *Nat Biotechnol* **16**: 839-42.

de Iannino N.I. and Ugalde R.A. (1989) Biochemical characterization of avirulent *Agrobacterium tumefaciens* chvA mutants: synthesis and excretion of β-(1, 2)glucan. *J Bacteriol* **171**: 2842-49.

de Neve M., De Buck S., Jacobs A., Van Montagu M., and Depicker A. (1997) T-DNA integration patterns in cotransformed plant cells suggest thatT-DNA repeats originate fromcointegration of separateT-DNAs. *Plant J* **11**: 15–29.

Deeken R., Engelmann J.C., Efetova M., Czirjak T., Müller T., Kaiser W.M., Tietz O., Krischke M., Mueller M.J., Palme K., Dandekar T., and Hedrich R. (2006) An integrated view of gene expression and solute profiles of *Arabidopsis* tumors: a genome-wide approach. *Plant Cell* **18** (12): 3617-34.

Dempski Jr R.E. and Imperiali B. (2002) Oligosaccharyl transferase: gatekeeper to the secretory pathway. *Curr Opin Chem Biol* **6** (6): 844-50.

Dempski Jr R.E. and Imperiali B. (2004) Heterologous expression and biophysical characterization of soluble oligosaccharyl transferase subunits. *Arch Biochem Biophys* **431**: 63–70.

Deng W., Chen L., Wood D.W., Metcalfe T., Liang X., Gordon M.P., Comai L., and Nester E.W. (1998) *Agrobacterium* VirD2 protein interacts with plant host cyclophilins. *Proc Natl Acad Sci USA* **95**: 7040-45.

Depicker, A., Van Montagu, M., and Schell, J. (1978) Homologous sequences in different Ti plasmids are essential for oncogenicity. *Nature* **275**: 150-52.

Dong, J.J., Teng, W.M., Buchholz, W.G. and Hall, T.C. (1996) *Agrobacterium*-mediated transformation of javanica rice. *Mol Breeding* **2**: 267-76.

Douglas C.J., Halperin W., and Nester E.W. (1982) *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. *J Bacteriol* **152**: 1265-75.

Douglas C.J., Staneloni R.J., Rubin R.A., and Nester E.W. (1985) Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence gene. *J Bacteriol* **161**: 850-60

Dumas F., Duckely M., Pelczar P., van Gelder P., and Hohn B. (2001) An *Agrobacterium* VirE2 channel for transferred-DNA transport into plant cells. *Proc Natl Acad Sci USA* **98**: 485-90.

Duranti M., Gius C., Sessa F., and Vecchio G. (1995) The saccharide chain of lupin seed conglutin gamma is not responsible for the protection of the native protein from degradation by trypsin, but facilitates the refolding of the acid-treated protein to the resistant conformation. *Eur J Biochem* **230** (3): 886-91.

Durrenberger F., Crameri A., Hohn B., and Koukolikova-Nicola Z. (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. *Proc Natl Acad Sci USA* **86**: 9154-58.

Dwek R.A. (1995) Glycobiology: Towards understanding the function of sugars. *Biochem Soc Trans* **23** (1): 1-25.

Eisenbrandt R., Kalkum M., Lai E.M., Lurz R., Kado C.I., and Lanka E. (1999) Conjugative pili of IncP plasmids and the Ti plasmid T pilus are composed of cyclic subunits. *J Biol Chem* **274**: 22548-55.

Enenkel C., Blobel G., and Rexach M. (1995) Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J Biol Chem* **270** (28): 16499-502.

Englund P.T. (1993) The Structure and Biosynthesis of Glycosyl Phosphatidylinositol Protein Anchors. *Ann Rev Biochem* **62**: 121-38.

Engstrom P., Zambryski P., Montagu M.V., and Stachel S. (1987) Charaterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. *J Mol Biol* **197**: 635-45.

Fabre, E. and Dunal, F. (1853) Observations sur les maladies regantes de la vigne. *Bull Soc Cent Agric Dep Herault* **40**: 46.

Feldmann K.A. (1991) T-DNA insertion mutagenesis in *Arabidopsis*: Mutational spectrum. *Plant J* **1**: 71- 82.

Femino A.M., Fay F.S., Fogarty K., and Singer R.H. (1998). Visualization of Single RNA Transcripts *in situ*. *Science* **280**: 585-90

Fernandez D., Dang T.A., Spudich G., Zhou X.R., Berger B.R, and Christie P.J. (1996a) The *Agrobacterium tumefaciens* virB7 gene product, a proposed component of the T-complex transport apparatus, is a membrane-associated lipoprotein exposed at the periplasmic surface. *J Bacteriol* **178** (11): 3156-67.

Fernandez D., Spudich G., Zhou X.-R., and Christie P. (1996b) The *Agrobacterium tumefaciens* VirB7 lipoprotein is required for stabilization of VirB proteins during assembly of the T-complex transport apparatus. *J Bacteriol* **178** (11): 3168-76.

Fetrow J.S., Siew N., Di Gennaro J.A., Martinez-Yamout M., Dyson H.J., and Skolnick J. (2001) Genomic-scale comparison of sequence- and structure-based methods of function prediction: does structure provide additional insight? *Protein Sci* **10**: 1005–14.

Filichkin S.A., and Gelvin S.B. (1993) Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1 and VirD2 endonuclease. *Mol Microbiol* **8**: 915-26.

Friesner J. and Britt A.B. (2003) Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration. *Plant J* **34**: 427-40.

Fu J. and Kreibich G. (2000) Retention of subunits of the oligosaccharyltransferase complex in the endoplasmic reticulum. *J Biol Chem* **275**: 3984–90.

Gallego M.E., Bleuyard J.Y., Daoudal-Cotterell S., Jallut N., and White C.I. (2003) Ku80 plays a role in non-homologous recombination but is not required for T-DNA integration in *Arabidopsis*. *Plant J* **35**: 557–65.

Galloway B.T. (1902) Report of the chief Bureau of Plant Industry. *Annual Reports of the Department of Agriculture* 47-108.

Garcia-Rodriguez F.M., Schrammeijer B., and Hooykaas P.J.J. (2006) The *Agrobacterium* VirE3 effector protein: a potential plant transcriptional activator. *Nucl Acids Res* **34**: 6496–504.

Garshasbi M., Hadavi V., Habibi H., Kahrizi K., Kariminejad R., Behjati F., Tzschach A., Najmabadi H., Ropers H.H., and Kuss A.W. (2008) A defect in the *TUSC3* gene is associated with autosomal recessive mental retardation. *Am J Hum Genet* **82** (5): 1158-64.

Gaspar Y.M., Nam J., Schultz C.J., Lee L.Y., Gilson P.R., Gelvin S.B. and Bacic A. (2004) Characterization of the *Arabidopsis* lysine-rich arabinogalactan-protein AtAGP17 mutant (*rat*1) that results in a decreased efficiency of *Agrobacterium* transformation. *Plant Physiol* **135**: 2162–71.

Gavel Y. and von Heijne G. (1990) Sequence differences between glycosylated and nonglycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Protein Eng* **3**: 433-42.

Gaynor E.C., te Heesen S., Graham T.R., Aebi M., and Emr S.D. (1994) Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J Cell Biol* **127**: 653–65.

Gelvin S.B. (1998) *Agrobacterium*VirE2 proteins can forma complex withTstrands in the plant cytoplasm. *J Bacteriol* **180**: 4300–02.

Gelvin S.B. (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and interaction. *Annu Rev Plant Physiol Plant Mol Biol* **51**: 223-56.

Gelvin S.B. (2009). *Agrobacterium* in the genomics age. *Plant Physiol* **150** (4): 1665-76.

Gelvin S.B. (2010) Plant proteins involved in *Agrobacterium*-mediated genetiic transformation. *Annu Rev Phytopathol* **48**: 3.1-3.24.

Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol Mol Biol Rev* **67**: 16-37.

Ghai J. and Das A. (1989) The virD operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. *Proc Natl Acad Sci USA* **86**: 3109-13.

Gheysen G., Van Montagu M., and Zambryski P. (1987) Integration of *Agrobacterium tumefaciens* transfer DNA (T-DNA) involves rearrangements of target plant DNA sequences. *Proc Natl Acad Sci USA* **84**: 6169-73.

Giaever G., Chu A.M., Ni L., Connelly C., Riles L., *et al.* (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**: 387–91.

Gietl C., Koukolikova-Nicola Z., and Hohn B. (1987) Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA. *Proc Natl Acad Sci USA* **84**: 9006-10.

Gietz R.D. and Schiestl R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* **2** (1): 31-34.

Gleeson P.A., Teasdale R.D., and Burke J. (1994) Targeting of proteins to the Golgi apparatus. *Glycoconj J* **11** (5): 381–94.

Gonczy P., Echeverri C., Oegema K., Coulson A., Jones S.J., Copley R.R., *et al.* (2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **408**: 331–36.

Goodner B., Hinkle G., Gattung S., Miller N., Blanchard M., Qurollo B., *et al.*, (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**: 2323–28.

Goodner, B.W., Markelz B. P., Flanagan M. C., Crowell C. B., Racette J. L., Schilling A., Halfon L. M., Mellors J. S., and Grabowski G. (1999) Combined genetic and physical map of the complex genome of *Agrobacterium* tumefaciens. *J Bacteriol* **181**: 5160–66.

Gouka R.J., Gerk C., Hooykaas P.J.J., Bundock P., Musters W., Verrips C.T., and de Groot M.J. (1999) Transformation of Aspergillus awamori by *Agrobacterium* tumefaciens-mediated homologous recombination. *Nat Biotechnol* **17**: 598-601.

Guervós M.A., Marcos C.A., Hermsen M., Nuño A.S., Suárez C., and Llorente J.L. (2007) Deletions of *vir* , STK11 and TP53 are involved in the development of lymph node metastasis in larynx and pharynx carcinomas. *Cell Oncol* **29** (4): 327-34.

Guo M, Hou Q, Hew CL, Pan SQ. (2007a). *Agrobacterium* VirD2-binding protein is involved in tumorigenesis and redundantly encoded in conjugative transfer gene clusters. *Mol Plant Microbe Interact* **20** (10): 1201-12.

Guo M., Jin S., Sun D., Hew C.L., Pan S.Q. (2007b) Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type IV secretion apparatus. *Proc Natl Acad Sci USA* **104** (50): 20019-24.

Guralnick B., Thomsen G., and Citovsky V. (1996) Transport of DNA into the nuclei of Xenopus oocytes by a modified VirE2 protein of *Agrobacterium*. *Plant Cell* **8**: 363–73.

Gutiérrez A., Sánchez-López R., Ramos M., and Alagón A. (2000) Cloning of the entamoeba histolytica *STT3* gene, a subunit of the oligosaccharyltransferase complex. *Arch Med Res* **31**: S162-S164.

Guyon P., Chilton M.D., Petit A., Tempé J. (1980) Agropine in "null-type" crown gall tumors: Evidence for generality of the opine concept. *Proc Natl Acad Sci USA* **77** (5): 2693-97.

Guzzo F., Campagnari E., and Levi M. (2000) A new FISH protocol with increased sensitivity for physical mapping with short probes in plants. *J Exp Bot* **51** (346): 965-70.

Hamilton R.C., and Fall M.Z. (1971) The loss of tumor initiating ability in *Agrobacterium tumefaciens* by incubation at high temperature. *Experientia* **27**: 229-30.

Hawes M.C. and Smith L.Y. (1989) Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. *J Bacteriol* **171**: 5668-71.

Hennet T. (2009) From glycosylation disorders back to glycosylation: What have we learned? *Biochim Biophys Acta* **1792** (9): 921-24.

Hiei Y., Ohta S., Komari T., and Kumashiro T. (1994) Efficient transformation of rice (Oryza sativa L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* **6**: 271-82.

Holmes B. (1988) The taxonomy of *Agrobacterium*. *Acta Hort* **225**: 47–52.

Holmes B. and Roberts P. (1981) The classification, identification and nomenclature of agrobacteria. *J Appl Bacteriol* **50**: 443-67

Hood EE, Gelvin SB, Melchers S and Hoekema A. (1993) New *Agrobacterium* helper plasmids for gene transfer to plants (EHA105). *Trans Res* **2**: 208-18

Howard E. and Citovsky V. (1990) The emerging structure of the *Agrobacterium* T-DNA tansfer complex. *Bioessays* **12**: 103-08.

Howard E.A., Zupan J.R., Citovsky V., and Zambryski P.C. (1992) The VirD2 protein of *Agrobacterium tumefaciens* contains a C-terminal bipartite nuclear localization signal: Implications for nuclear uptake of DNA in plant cells. *Cell* **68**: 109-18.

Hughes T.R., Marton M.J., Jones A.R., Roberts C.J., Stoughton R., *et al.* (2000) Functional discovery via a compendium of expression profiles. *Cell* **102** (1): 109-26.

Hwang H.H and Gelvin S.B. (2004) Plant proteins that interact with VirB2, the *Agrobacterium tumefaciens* pilin protein, mediate plant transformation. *Plant Cell* **16**: 3148–67.

Ilyina T., and Koonin E. (1992) Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucleic Acids Res* **20**: 3279-85.

Imperiali B. and Hendrickson T.L. (1995) Asparagine-linked glycosylation: specificity and function of oligosaccharyl transferase. *Bioorg Med Chem* **3** (12): 1565-78.

Imperiali B. and Riekert K.W. (1995) Conformational implications of asparagine-linked glycosylation. *Proc Natl Acad Sci USA* **92** (1): 97-101.

Inoue H, Nojima H, Okayama H. (1990) High efficiency transformation of Escherichia coli with plasmids. *Gene* **96**: 23-26.

Ishida, Y., Saito H., Ohta S., Hiei Y., Komari T., and Kumashiro T. (1996) High efficiency transformation of maize (Zea mays L.) mediated by *Agrobacterium* tumefaciens. *Nat Biotechnol* **14**: 745–50.

Jarchow E., Grimsley N.H. and Hohn B. (1991) *virF*, the host-range-determining virulence gene of *Agrobacterium tumefaciens*, affects T-DNA transfer to *Zea mays*. *Proc Natl Acad Sci USA* **88** (23): 10426-30.

Jasper F., Koncz C., Schell J., and Steinbiss H-H. (1994) *Agrobacterium* T-strand production *in vitro*: sequence-specific cleavage and 5' protection of single-stranded DNA templates by purified VirD2 protein. *Proc Natl Acad Sci USA* **91**: 694-98.

Jayaswal R.K., Veluthambi K., Gelvin S.B., and Slightom J.L. (1987) Double stranded T-DNA cleavage and the generation of single stranded T-DNA molecules in *E. coli* by a virD-encoded border-specific endonuclease from *A. tumefaciens*. *J Bacteriol* **169**: 5035-45.

Jin S., Song Y., Pan S.Q., and Nester E.W. (1993) Characterization of a virG mutation that confers constitutive virulence gene expression in *Agrobacterium* tumefaciens. *Mol Microbiol* **7**: 555-62.

Jones A.L., Lai E.M., Shirasu K., and Kado C.I. (1996) VirB2 is a processed pilin-like protein encoded by the *Agrobacterium tumefaciens* Ti plasmid. *J Bacteriol* **178**: 5706-11.

Jones A.L., Shirasu K., and Kado C.I. (1994) The product of the virB4 gene of *Agrobacterium tumefaciens* promotes accumulation of VirB3 protein. *J Bacteriol* **176**: 5255-61.

Joos H., Inze D., Caplan A., Sormann M., Van Montagu M., and Schell J. (1983) Genetic analysis of T-DNA transcripts in nopaline crown gall. *Cell* **32**: 1057-67.

Kado C.I. (2000) The role of the T-pilus in horizontal gene transfer and tumorigenesis. *Curr Opin Microbiol* **3**: 643–48.

Kamath R.S., Fraser A.G., Dong Y., Poulin G., Durbin R., Gotta M., Kanapin A., *et al.* (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**: 231–37.

Kamoun S., Cooley M.B., Rogowsky U.M., and Kado C.I. (1989) Two chromosomal loci involved in production of exopolysaccharide in *Agrobacterium* tumefaciens. *J Bacteriol* **171**: 1755-59.

Karaoglu D., Kelleher D.J., and Gilmore R. (1995) Functional characterization of Ost3p. Loss of the 34-kD subunit of the *Saccharomyces cerevisiae* oligosaccharyltransferase results in biased underglycosylation of acceptor substrates. *J Cell Biol* **130** (3): 567-77.

Karaoglu D., Kelleher D.J., and Gilmore R. (1997) The highly conserved Stt3 protein is a subunit of the yeast oligosaccharyltransferase and forms a subcomplex with Ost3p and Ost4p. *J Biol Chem* **272**: 32513–20.

Kawai S., Pham T.A., Nguyen H.T., Nankai H., Utsumi T., Fukuda Y., and Murata K. (2004) Molecular insights on DNA delivery into *Saccharomyces cerevisiae*. *Biochem Biophys Res Commu* **317** (1): 100-07.

Keane, P. J., Kerr A., and New R. B. (1970) Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Aust J Biol Sci* **23**: 585-95.

Kelleher D.J. and Gilmore R. (2006) An evolving view of the eukaryotic oligosaccharyltransferase. *Glycobiology* **16** (4): 47R-62R.

Kelleher D.J., Karaoglu D., Mandon E.C., and Gilmore R. (2003) Oligosaccharyl-transferase isoforms that contain different catalytic *STT3* subunits have distinct enzymatic properties. *Mol Cell* **12**: 101-11.

Kennedy B. (1980) Estimates of U.S. crop losses to prokaryote plant pathogens. *Plant Dis* 674-76.

Kerr A. and Panagopoulos C.G. (1977) Biotypes of *Agrobacterium* radiobacter var. tumefaciens and their biological control. *Phytopath Z* **90**: 172-79.

Kerr A., Young J.M., and Panagopoulos C.G. (1978) Genus II. *Agrobacterium* Conn 1942. In Young J.M., Dye D.W., Bradbury J.F., Panagopoulos C.G., Robbs C.F., eds, A proposed nomenclature and classification for plant pathogenic bacteria. *NZ J Agric Res* **21**: 153-77.

Kersters K. and De Ley J. (1984) Genus III. *Agrobacterium* Conn. In Krieg N.R., Holt J.G., eds, Bergey's Manual of Systematic Bacteriology, Vol. 1, 8th ed. The Williams and Wilkins Co., Baltimore-London, 244-54

Klee H, Montoya A, Horodyski F, Lichtenstein C, Garfinkel D, Fuller S, Flores C, Peschon J, Nester E, Gordon M. (1984) Nucleotide sequence of the tms genes of the pTiA6NC octopine Ti plasmid: two gene products involved in plant tumorigenesis. *Proc Natl Acad Sci USA* **81**: 1728–32.

Knauer R and Lehle L. (1999a) The oligosaccharyltransferase complex from yeast. *Biochim Biophys Acta* **1426** (2): 259-73.

Knauer R. and Lehle L. (1999b) The oligosaccharyltransferase complex from *Saccharomyces cerevisiae*: isolation of the Ost6p gene, its synthetic interaction with Ost3p, and analysis of the native complex. *J Biol Chem* **274**: 17249-56.

Krizkova L. and Hrouda M. (1998) Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. *Plant J* **16**: 673–80.

Kumar R.B. and Das A. (2002) Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein VirD4. *Mol Microbiol* **43**: 1523-32.

Kunik T., Tzfira T., Kapulnik Y., Gafni Y., Dingwall C., and Citovsky V. (2001) Genetic transformation of HeLa cells by *Agrobacterium*. *Proc Natl Acad Sci USA* **98**: 1871-76.

Lacroix B, Tzfira T, Vainstein A, Citovsky V. (2006) A case of promiscuity: *Agrobacterium*'s endless hunt for new partners. *Trends Genet* **22**: 29–37.

Lacroix B., Vaidya M., Tzfira T., and Citovsky V. (2005) The VirE3 protein of *Agrobacterium* mimics a host cell function required for plant genetic transformation. *EMBO J* **24**: 428–37.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**: 680-85.

Lai E.M, Kado C.I. (1998) Processed VirB2 is themajor subunit of the promiscuous pilus of *Agrobacterium* tumefaciens. *J Bacteriol* **180**: 2711–17.

Lai E.M. and Kado C.I. (2000) The T-pillus of *Agrobacterium* tumefaicens. *Trends Mocrobiol* **8**: 361-69.

Lamppa G., Nagy F., and Chua N.H. (1985) Light-regulated and organ-specific expression of a wheat Cab gene in transgenic tobacco. *Nature* **316**: 750-52.

Lee C.W., Efetova M., Engelmann J.C., Kramell R., Wasternack C., *et al.* (2009) *Agrobacterium tumefaciens* promotes tumor induction bymodulating pathogen defense in *Arabidopsis thaliana*. *Plant Cell* **21**: 2948–62.

Lee L.Y., Fang M.J., Kuang L.Y., and Gelvin S.B. (2008) Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. *Plant Methods* **4**: 24.

Lennarz W.J. (2007) Studies on oligosaccharyl transferase in yeast. *Acta Biochim Pol* **54** (4): 673-7.

Levsky J.M., Shenoy S.M., Pezo R.C., Singer R.H. (2002) Single-cell gene expression profiling. *Science* **297**: 836-40.

Li G., Yan Q., Oen H.O., and Lennarz W.J. (2003) A specific segment of the transmembrane domain of Wbp1p is essential for its incorporation into the oligosaccharyl transferase complex. *Biochemistry* **42**: 11032–39.

Li J., Krichevsky A., Vaidya M., Tzfira T., and Citovsky V. (2005a) Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by *Agrobacterium*. *Proc Natl Acad Sci USA* **102**: 5733–38.

Li J., Vaidya M., White C., Vainstein A., Citovsky V., and Tzfira T. (2005b) Involvement of KU80 in T-DNA integration in plant cells. *Proc Natl Acad Sci USA* **102**: 19231–36.

Li L., Jia Y., Hou Q., Charles T.C., Nester E.W., and Pan S.Q. (2002) A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid. *Proc Natl Acad Sci USA* **99** (19): 12369-74.

Lichtenstein C, Klee H, Montoya A, Garfinkel D, Fuller S, Flores C, Nester E, Gordon M. (1984). Nucleotide sequence and transcript mapping of the tmr gene of the pTiA6NC octopine Ti plasmid: a bacterial gene involved in plant tumorigenesis. *J Mol Appl Genet* **2**: 354–62.

Lioret C. (1956) Sur la mise en evidence d'un acide amine non identifie particulier aux tissus de 'crown-gall'. *Bull Soc Fr Physiol Veg* **2**: 76.

Lippincott B.B. and Lippincott J.A. (1969) Bacterial attachment to a specific wound site as an essential stage in tumor initiation by *Agrobacterium* tumefaciens. *J Bacteriol* **97**: 620-28.

Llosa M., Zunzunegui S., and Cruz F. (2003) Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc Natl Acad Sci USA* **100**: 10465-70.

Loake G.J., Ashby A.M., and Shaw C.M. (1988) Attraction of *Agrobacterium tumefaciens* C58C1 towards sugars involves a highly sensitive chemotaxis system. *J Gen Microbiol* **134**: 1427-32.

Long R.M., Elliott D.J., Stutz F., Rosbash M., Singer R.H. (1995) Spatial consequences of defective processing of specific yeast mRNAs revealed by fluorescent *in situ* hybridization. *RNA* **1** (10): 1071-78.

Lopatin M.I. (1939) Influence of bacterial root canter on the development of the cherry tree in the orchard. *Plant Prot* **18**: 167-73.

Loyter A., Rosenbluh J., Zakai N., Li J., Kozlovsky S.V., Tzfira T., and Citovsky V. (2005) The plant VirE2 interacting protein 1. A molecular link between the *Agrobacterium* T-complex and the host cell chromatin? *Plant Physiol* **138**: 1318–21.

MacGrogan D., Levy A., Bova G.S., Isaacs W.B., and Bookstein R. (1996) Structure and methylation-associated silencing of a gene within a homozygously deleted region of human chromosome band 8p22. *Genomics* **35** (1): 55-65.

Makishima T., Nakashima T., Nagata-Kuno K., Fukushima K., Iida H., Sakaguchi M., Ikehara Y., Komiyama S., and Nishimoto T. (1997) The highly conserved DAD1 protein involved in apoptosis is required for N-linked glycosylation. *Genes Cells* **2**: 129-41.

Marek K., Vijay I., and Marth J. (1999) A recessive deletion in the GlcNAc-1-phosphotransferase gene results in periimplantation embryonic lethality. *Glycobiology* **9**: 1263-71.

Marmagne A., Rouet M.A., Ferro M., Rolland N., Alcon C., Joyard J., Garin J., Barbier-Brygoo H., and Ephritikhine G. (2004) Identification of new intrinsic proteins in the *Arabidopsis* plasma membrane proteome. Mol. Cell. *Proteomics* **3**: 675–91.

Matthijs G. and Jaeken J. (2001) Congenital disorders of glycosylation. *Annu Rev Genom Hum Genet* **2**: 129-51.

Matthysse A.G. and McMahan S. (1998) Root colonization by *Agrobacterium tumefaciens* is reduced in cel, attB, attD, and attR mutants. *Applied and Environmental Microbiology* 64: 2341-45.

Matthysse A.G., Yarnall H., Boles S.B., and McMahan S. (2000) A region of the *Agrobacterium tumefaciens* chromosome containing genes required for virulence and attachment to host cells. *Biochimica et Biophysica Acta* **1490**: 208-12.

Michielse C.B., Hooykaas P.J.J., van den Hondel C.A.M.J.J., and Ram A.F.J. (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Curr Genet* **48**: 1-17.

Minnemeyer S.L., Lightfoot L.R., and Matthysse A.G. (1991) A semi-quantitative bioassay for relative virulence of *Agrobacterium tumefaciens* strains on *Bryophyllum daigremontiana*. *J Bacteriol* **173**: 7723-24.

Mnaimneh S., Davierwala A.P., Haynes J., Moffat J., Peng WT., Zhang W., *et al.* (2004) Exploration of essential gene functions via titratable promoter alleles. *Cell* **118** (1): 31-44.

Molinari F., Foulquier F., Tarpey P.S., Morelle W., Boissel S., Teague J., Edkins S., Futreal P.A., Stratton M.R., Turner G., Matthijs G., Gecz J., Munnich A., and Colleaux L. (2008) Oligosaccharyltransferase-subunit mutations in nonsyndromic mental retardation. *Am J Hum Genet* **82** (5): 1150-57.

Mononen I. and Karjalainen E. (1999) Structural comparison of protein sequences around potential N-glycosylation sites. *Biochim Biophys Acta* **788**: 364-67.

Moore L.W., Bouzar H., and Burr T.J. (2001) *Agrobacterium*. In Schaad N.W., Jones J.B., Chun W., eds. Laboratory Guide for Identification of Plant Pathogenic Bacteria. American Phytopathological Society Press, St. Paul, Minnesota. 17-33

Mushegian A.R., Fullner K.J., Koonin E.V., and Nester E.W. (1996) A family of lysozyme-like virulence factors in bacterial pathogens of plants and animals. *Proc Natl Acad Sci USA* **93**: 7321-26.

Mysore K.S., Nam J., and Gelvin S.B. (2000) An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. *Proc Natl Acad Sci USA* **97**: 948-53.

Nagy Z. and Tora L. (2007) Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. *Oncogene* **26**: 5341–57.

Nakashima T., Sekiguchi T., Kuraoka A., Fukushima K., Shibata Y., Komiyama S., and Nishimoto T. (1993) Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. *Mol Cell Biol* **13**: 6367-74.

Nam J., Matthysse A.G., and Gelvin S.B. (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T -DNA integration. *Plant Cell* **9**: 317–33.

Nam, J., Mysore, K.S., Zheng, C., Knue, M.K., and Matthysse, A.G., and Gelvin S.B. (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. *Mol Gen Genet* **261**: 429-38.

Narasimhuhu S.B., Deng X.-B., Sarria R., and Gelvin S.B. (1996) Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. *Plant Cell* **8**: 873-86.

Nasab F.P., Schulz B.L., Gamarro F., Parodi A.J., and Aebi M. (2008) All in one: *Leishmania major STT3* proteins substitute for the whole oligosaccharyltransferase complex in *Saccharomyces cerevisiae*. *Mol Biol Cell* **19** (9): 3758-68.

Neff N.T and Binns A.N. (1985) *Agrobacterium tumefaciens* interaction with suspension-cultured tomato cells. *Plant Physiol* **77**: 35–42.

Neff N.T., Binns A.N, and Brandt C. (1987) Inhibitory effects of a pectin-enriched tomato cell wall fraction on *Agrobacterium tumefaciens* binding and tumor formation. *Plant Physiol* **83**: 525–28.

Newton J.A. and Fray R.G. (2004) Integration of environmental and host-derived signals with quorum sensing during plant-microbe interactions. *Cell Microbiol* **6**: 213-24.

Nilsson I., Kelleher D.J., Miao Y., Shao Y., Kreibich G., Gilmore R., von Heijne G., and Johnson A.E. (2003) Photocross-linking of nascent chains to the *STT3* subunit of the oligosaccharyltransferase complex. *J Cell Biol* **161**: 715–25.

Nixon B.T., Ronson C.W., and Ausubel F.M. (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes ntrB and ntrC. *Proc Natl Acad Sci USA* **83** (20): 7850-54.

Nziengui H., Bouhidel K., Pillon D., Der C., Marty F., and Schoefs B. (2007) Reticulon-like proteins in *Arabidopsis* thaliana: structure, organization, and ER localization. *FEBS Lett* **581**: 3356–62.

O'Connell K.P. and Handelsman J. (1989) *chvA* locus may be involved in export of neutral cyclic β-1, 2 linked D-glucan from *Agrobacterium* tumefaciens. *Mol Plant-Microbe Interact* **2**: 11-16.

Offringa R., de Groot M.J.A., Haagsman H.J., Does M.P., van den Elzen P.J.M. and Hooykaas P.J.J. (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation. *EMBO J* **9**: 3077-84.

Opdenakker G., Rudd P.M., Ponting C.P., and Dwek R.A. (1993) Concepts and principles of glycobiology. *FASEB J* **7** (14): 1330–37.

Otten L., DeGreve H., Leemans J., Hain R., Hooykaas P., and Schell J. (1984) Restoration of virulence of *vir* region mutants of *Agrobacterium tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacterium* strains. *Mol Gen Genet* **195**: 159–63.

Panagopoulos C.G., Psallidas P.G., and Alivizatos A.S. (1978) Studies on biotype 3 of *Agrobacterium radiobacter var. tumefaciens*. In Station de Pathologie Végétale et

Phytobactériologie, ed., Plant Pathogenic Bacteria Proc 4th Internat Conf Plant Path Bact, Angers, France, 221-28.

Pansegrau W., Schoumacher F., Hohn B., and Lanka E. (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. *Proc Natl Acad Sci USA* **90**: 11538-42.

Park H. and Lennarz W. (2000) Evidence for interaction of yeast protein kinase C with several subunits of oligosaccharyl transferase. *Glycobiology* **10**: 737-44.

Parke, D., Ornston, L.N., and Nester, E.W. (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *J Bacteriol* **169**: 5336-38.

Pathak R., Hendrickson T.L., and Imperiali B. (1995) Sulfhydryl modification of the yeast Wbp1p inhibits oligosaccharyl transferase activity. *Biochemistry* **34**: 4179–85.

Peralta E.G. and Ream L.W. (1985) T-DNA border sequence required for crown gall tumorigenesis. *Proc Natl Acad Sci USA* **82**: 5112-16.

Peralta E.G., Hellmiss R., and Ream L.W. (1986) Overdrive, a T-DNA transmission enhancer on the *A. tumefaciens* tumor-inducing plasmid. *EMBO J* **5**: 1137-42.

Piers K.L., Heath J.D., Liang X.Y., Stephens K.M., and Nester E.W. (1996) *Agrobacterium tumefaciens*-meidated transformation of yeast. *Proc Natl Acad Sci USA* **93**: 1613-18.

Pils D., Horak P., Gleiss A., Sax C., Fabjani G., Moebus V.J., Zielinski C., Reinthaller A., Zeillinger R., and Krainer M. (2005) Five genes from chromosomal band 8p22 are significantly down-regulated in ovarian carcinoma: *N33* and *EFA6R* have a potential impact on overall survival. *Cancer* **104** (11): 2417-29.

Puig O., Caspary F., Rigaut G., Rutz B., Bouverete E., Braggado-Nelson E., Wilm M., Seraphin B. (2001) The Tandem Affinity Purification (TAP) Method: A general procedure of complex protein purification. *Methods* **24**: 218-29.

Rashid, H., Yokoi S., Toriyama K., and Hinata K. (1996) Transgenic plant production mediated by *Agrobacterium* in Indica rice. *Plant Cell Rep* **15**: 727–30.

Regensburg-Tuïnk A.J. and Hooykaas P.J.J. (1993) Transgenic *N. glauca* plants expressing bacterial virulence gene virF are converted into hosts for nopaline strains of *A. tumefaciens*. *Nature* **363** (6424): 69-71.

Reiss G., te Heesen S., Gilmore R., Zufferey R., and Aebi M. (1997) A specific screen for oligosaccharyltransferase mutations identifies the 9 kDa *OST5* protein required for optimal activity *in vivo* and *in vitro*. *EMBO J* **16**: 1164–72.

Reuhs B.L., Kim J.S., and Matthysse A.G. (1997) Attachment of *Agrobacterium* tumefacins to carrot cells and *Arabidopsis* wound sites is correlated with the presence of a cell-associated, acidic polysaccharide. *J Bacteriol* **179**: 5372-79.

Ricker A.J., Berbee J.G., Smalley E.B. (1959) Effects of crown gall and hairy root on the growth of apple trees. *Phytopathology* **49**: 88-90.

Riederer M.A. and Hinnen A.Z. (1991) Removal of N-glycosylation sites of the yeast acid phosphatase severely affects protein folding. *J Bacteriol* **173** (11): 3539-46.

Rigaut G., Shevchenko A., Rutz B., Wilm M., Mann M., and Séraphin B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotech* **17**: 1030 - 32.

Roberts R.L., Metz M., Monks D.E., Mullaney M.L., Hall T., Nester E.W. (2003) Purine synthesis and increased *Agrobacterium tumefaciens* transformation of yeast and plants. *Proc Natl Acad Sci USA* **100** (11): 6634-39.

Robertson J.L., Holliday T., and Matthysse A.G. (1988) Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment to host cells. *J Bacteriol* **170**: 1408-11.

Rodriguez-Lanetty M., Krupp D.A., Weis V.M. (2004) Distinct ITS types of Symbiodinium in clade C correlate with cnidarian/dinoflagellate specificity during onset of symbiosis. *Mar Ecol Prog Ser* **275**: 97-102.

Rogowsky P.M., Powell B.S., Shirasu K., Lin T.S., Morel P., Zyprian E.M., Steck T.R., and Kado C.I. (1990) Molecular characterization of the *vir* regulon of *Agrobacterium* tumefaciens: complete nucleotide sequence and gene organization of the 28.63-kbp regulon clones as a single unit. *Plasmid* **23**: 85–106.

Rossi L., Hohn B., and Tinland B. (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. *Mol Gen Genet* **239**: 345-53.

Rossi L., Hohn B., and Tinland B. (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium* tumefaciens. *Proc Natl Acad Sci USA* **93**: 126-30.

Rudd P.M. and Dwek R.A. (1991) Glycobiology. A coming of age. *Chem Ind* **18**: 660-63.

Sahi S.V., Chilton M.D., and Chilton W.S. (1990) Corn metabolites affect growth and virulence of *Agrobacterium* tumefaciens. *Proc Natl Acad Sci USA* **87**: 3879–83.

Sambrook J.F. and Russell T. (2001) Molecular Cloning: A Laboratory Manual (3rd edition). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. A2.7, A8.5, A8.40-A8.52.

Sambrook JF, Fritsch EF, and Maniatis T. (1989) Molecular Cloning: A Laboratory Mannual. Cold Spring Harbor, NY: Cold Spring Harbor Laborarotory Press. A2.2, A2.7, A8.5.

Sassel J. and Gallagher S.R. (2004) Staining proteins in gels. *Curr Protoc Immunol* Chapter 8: Unite 8.9.

Savvides S., Yeo H.-J., Beck M.R., Blaesing F., Lurz R., Lanka E., Buhrdorf R., Fischer W., Haas R., and Waksman G. (2003) VirB11 ATPases are dynamic hexameric assemblies: new insights into bacterial type IV secretion. *EMBO J* **22**: 1969-80.

Sawada H., Ieki H., Oyaizu H., and Matsumoto S. (1993) Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *Int J Syst Bacteriol* **43**: 694-702.

Sawasaki Y., Inomata K., and Yoshida K. (1996) Trans-Kingdom conjugation between *Agrobacterium* tumfaciens and *Saccharomyces cerevisiae*, a bacterium and a yeast. *Plant Cell Physiol* **37**: 103-06.

Scheiffele P., Pansegrau W., and Lanka E. (1995) Initiation of *Agrobacterium tumefaciens* T-DNA processing: purified protein VirD1 and VirD2 catalyze site- and strand-specific cleavage of superhelical T-border DNA *in vitro*. *J Biol Chem* **270** (3): 1269-76.

Schell J. and Van Montagu M. (1977) The Ti-plasmid of *Agrobacterium* tumefaciens, a natural vector for the introduction of nif genes in plants? *Basic Life Sci* **9**: 159–79.

Schilperoort, R.A., Veldstra, H., Warnaar, S.Q. Mulder, G., and Cohen, J.A. (1967) Formation of complexes between DNA isolated from tobacco crown gall tumours and complementary to *Agrobacterium tumefaciens* DNA. *Biochim Biophys Acta* **145**: 523-25.

Schmidt-Eisenlohr H., Domke N., Angerer C., Wanner G., Zambryski P.C., and Baron C. (1999) Vir proteins stabilize VirB5 and mediate its association with the T pilus of *Agrobacterium* tumefaciens. *J Bacteriol* **181**: 7485-92.

Schrammeijer B., Dulk-Ras A., Vergunst A.C., Jacome E.J., and Hooykaas P.J.J. (2003) Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3. *Nucleic Acids Research* **31**: 860-68.

Schroeder H.E., Gollasch S., Moore A., Tabe L.M., Craig S., Hardie D.C., Chrispeels M.J., Spencer D., and Higgins T. (1995) Bean [alpha]-amylase inhibitor confers resistance to the pea weevil (*Bruchus posorum*) in transgenic peas (*Pisum sativum L.*). *Plant Physiol* **107**: 1233-39.

Schroth M.N., Mccain A.H., Foott J.H., Huisman O.C. (1988) Reduction in yield and vigor of grapevine caused by crown gall disease. *Plant Dis* **72**: 241-46.

Schwarz M., Knauer R., and Lehle L. (2005) Yeast oligosaccharyltransferase consists of two functionally distinct sub-complexes, specified by either the Ost3p or Ost6p subunit. *FEBS Lett* **579** (29): 6564-68.

Sen P., Pazour G.J., Anderson D., and Das A. (1989) Cooperative binding of *Agrobacterium tumefaciens* VirD2 protein to single-stranded DNA. *J Bacteriol* **171**: 2573-80.

Setubal J.C., Wood D., Burr T., Farrand S.K., Goldman B.S., Goodner B., Otten L., and Slater S. (2009) The Genomics of *Agrobacterium*: Insights into its Pathogenicity, Biocontrol, and Evolution. In Jackson R.W., eds, Plant Pathogenic Bacteria: Genomics and Molecular Biology. Caister Academic Press, Chapter 4.

Shaked H., Melamed-Bessudo C., and Levy A.A. (2005) High-frequency gene targeting in *Arabidopsis* plants expressing the yeast RAD54 gene. *Proc Natl Acad Sci USA* **102** (34): 12265-69.

Shaw C.H.J., Watson M.D., Carter G.H., and Shaw C.H. (1984) The right hand copy of the nopaline Ti plasmid 25 bp repeat is required for tumor formation. *Nucleic Acids Res* **12**: 6031-41.

Sheng J. and Citovsky V. (1996) *Agrobacterium*-plant cell DNA transport: Have virulence proteins, will travel. *The Plant Cell* **8**: 1699-710.

Shibatani T., David L.L., McCormack A.L., Frueh K., and Skach W.R. (2005) Proteomic analysis of mammalian oligosaccharyltransferase reveals multiple subcomplexes that contain Sec61, TRAP, and two potential new subunits. *Biochemistry* **44**: 5982–92.

Shurvinton C.E., Hodge L., and Ream W. (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proc Natl Acad Sci USA* **89**: 11837-41.

Silberstein S. and Gilmore R. (1996) Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *FASEB J* **10** (8): 849–58.

Silberstein S., Collins P., Kelleher D., and Gilmore R. (1995) The essential *OST2* gene encodes the 16-KD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms. *J Cell Biol* **131**: 371-83.

Silberstein S., Kelleher D.J., and Gilmore R. (1992) The 48 kDa subunit of the mammalian oligosaccharyltransferase complex is homologous to the essential yeast protein *WBP1*. *J Biol Chem* **267**: 23658–63.

Silver P.A. (1991) How proteins enter the nucleus. *Cell* **64**: 489-97.

Simone M., McCullen C.A., Stahl L.E., and Binns A.N. (2001) The carboxy-terminus of VirE2 from *Agrobacterium tumefaciens* is required for its transport to host cells by the virB-encoded type IV transport system. *Mol Microbiol* **41**: 1283-93.

Skerman V.B.D., McGowan V., Sneath P.H.A. (1980) Approved lists of bacterial names. *Int J Syst Bacteriol* **30**: 225-420.

Slater S.C., Goldman B.S., Goodner B., Setubal J.C., Farrand S.K., Nester E.W., *et al.* (2009) Genome sequences of three *Agrobacterium* biovars help elucidate the evolution of multichromosome genomes in bacteria. *J Bacteriol* **191**: 2501–11.

Slightom J.L., Durand-Tardif M., Jouanin L., and Tepfer D. (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium* rhizogenes agropine type plasmid. *J Biol Chem* **261**: 108–12.

Smith E.F. and Townsend C.O. (1907) A plant-tumor of bacterium origin. *Science* **25**: 671-73.

Smith E.F. and Townsend C.O. (1911) Crown gall of plants; Its cause and Remedy. *USA Bureau of Plant Industry Bulletin No*. 214.

Soltani J., van Heusden G.P., and Hooykaas P.J.J. (2009) Deletion of host histone acetyltransferases and deacetylases strongly affects *Agrobacterium*-mediated transformation of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **298** (2): 228-33.

Spirig U., Bodmer D., Wacker M., Burda P., and Aebi M. (2005) The 3.4 kDa Ost4 protein is required for the assembly of two distinct oligosaccharyltransferase complexes in yeast. *Glycobiology* **15**: 1396–406.

Spirig U., Glaras M., Bodmer D., Reiss G., Burda P., Lippuner V., te Hessen S., and Aebi M. (1997) The Stt3 protein is a component of the yeast oligosaccharyltransferase complex. *Mol Gen Genet* **256**: 628-37.

Stachel S.E. and Nester E.W. (1986) The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium* tumefacins. *EMBO J* **5**: 1445-54.

Stachel S.E. and Zambryski P.C. (1986). virA and virG control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* **46**: 325-33.

Stachel S.E., Messens E., Van Montagu M., and Zambryski P. (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium* tumefaciens. *Nature* **318**: 624-29.

Stachel S.E., Timmerman B., and Zambryski P. (1986) Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer for *Agrobacterium tumefaciens* to plant cells. *Nature* **322**: 706-12.

Stachel S.E., Timmerman B., Zambryski P. (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5' virD gene products. *EMBO J* **6**: 857-63.

Stagljar I., Korostensky C., Johnsson N., and te Hessen S. (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*. *Proc Natl Acad Sci USA* **95**: 5187-92.

Starr M.P. and Weiss J.E. (1943) Growth of phytopathogenic bacteria in a synthetic asparagine medium. *Phytopathol* **33**: 313-18.

Steinberg T.H. (2009) Protein gel staining methods: an introduction and overview. *Methods Enzymol* **463**: 541 – 63.

Stiekema W.J., Heidekamp F., Louwerse J.D., Verhoeven H.A., and Dijkhuis P. (1988) Introduction of foreign genes into potato cultivars Bintje and Désirée using an *Agrobacterium tumefaciens* binary vector. *Plant Cell Rep* **7**: 47-50.

Sundberg C., Meek L., Carroll K., Das A., and Ream W. (1996) VirE1 protein mediates export of the single-stranded DNA binding protein VirE2 from *Agrobacterium tumefaciens* into plant cells. *J Bacteriol* **178**: 1207-12.

Sundberg C.D. and Ream W. (1999) The *Agrobacterium tumefaciens* chaperone-like protein, VirE1, interacts with VirE2 at domains required for single-stranded DNA binding and cooperative interaction. *J Bacteriol* **181**: 6850-55.

Suzuki K., Hattori Y., Uraji M., Ohta N., Iwata K., Murata K., Kato A., and Yoshida K. (2000) Complete nucleotide sequence of a plant tumor-inducing Ti plasmid. *Gene* **242**: 331–36.

Swart S., LogmanT.J.J, Smit G., Lugtenberg B.J.J., and Kijne J.W. (1994) Purification and partial characterization of a glycoprotein from pea (Pisum sativum) with receptor activity for rhicadhesin, an attachment protein of *Rhizobiaceae*. *Plant Mol Biol* **24**: 171–83.

Switzer R.C., Merril C.R., Shifrin S. (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem* **98** (1): 231-37.

Tabb M.M., Tongaonkar P., Vu L., and Nomura M. (2000) Evidence for separable functions of Srp1p, the yeast homolog of importin alpha (Karyopherin alpha): role for Srp1p and Sts1p in protein degradation. *Mol Cell Biol* **2**(16): 6062-73.

Tao Y., Rao P.K., Bhattacharjee S., and Gelvin S.B. (2004) Expression of plant protein phosphatase 2C interferes with nuclear import of the *Agrobacterium* T-complex protein VirD2. *Proc Natl Acad Sci USA* **101**: 5164–69.

te Heesen S., Janetzky B., Lehle L., and Aebi M. (1992) The yeast *WBP1* is essential for oligosaccharyltransferase activity *in vivo* and *in vitro*. *EMBO J* **11**: 2071–75.

te Heesen S., Rauhut R., Aebersold R., Abelson J., Aebi M., Clark M. (1991) An essential 45 kDa yeast transmembrane protein reacts with anti-nuclear pore antibodies: purification of the protein, immunolocalization and cloning of the gene. *Eur J Cell Biol* **56**: 8-18.

te Hessen S., Knauer R., Lehle L., and Aebi M. (1993) Yeast Wbp1p and Swp1p form a protein complex essential for oligosaccharyl transferase activity. *EMBO J* **12**: 279-84.

Tepfer D. (1984) Transformation of several species of higher plants by *Agrobacterium* rhizogenes: sexual transmission of the transformed genotype and phenotype. *Cell* **37**: 959-67.

Thomashow M.F., Karlinsey J.E., Marks J.R., and Hurlbert R.E. (1987) Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. *J Bacteriol* **169**: 3209-16.

Thompson D.V., Melchers L.S., Idler K.B., Schilperoort R.A., and Hooykaas P.J.J. (1988) Analysis of the complete nucleotide sequence of the *Agrobacterium tumefaciens* virB operon. *Nucleic Acids Res* **16**: 4621–36.

Tingay, S., McElroy D., Kalla R., Fieg S., Wang M., Thornton S., and Brettell R.. (1997) *Agrobacterium* tumefaciens-mediated barley transformation. *Plant J* **11**: 1369–76.

Tinland B. and Hohn B. (1995) Recombination between prokaryotic and eukaryotic DNA: integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. *Genet Eng* **17**: 209–29.

Tinland B., Koukolikova-Nicola Z., Hall M.N., and Hohn B. (1992) The T-DNA-linked VirD2 protein contains two distinct functional nuclear localization signals. *Proc Natl Acad Sci USA* **89**: 7442-46.

Tinland B., Schoumacher F., Gloeckler V., Bravo-Angel A.M., and Hohn B. (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. *EMBO J* **14**: 3585-95.

Toki S. (1997) Rapid and efficient *Agrobacterium*-mediated transformation in rice. *Plant Mol Biol Rep* **15**: 16–21.

Toro N., Datta A., Carmi O.A., Young C., Prusti R.K., and Nester E.W. (1989) The *Agrobacterium tumefaciens* virC1 gene product binds to overdrive, a T-DNA transfer enhancer. *J Bacteriol* **171**: 6845-49.

Tzfira T., Vaidya M., and Citovsky V. (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. *EMBO J* **20**: 3596–607.

Tzfira T., Vaidya M., and Citovsky V. (2004) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium. Nature* **431**: 87-92.

Tzfira T. and Citovsky, V. (2002) Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends Cell Biol* **12**: 121–28.

Usami S., Morikawa S., Takebe I., Machida Y. (1987) Absence in monocotyledonous plants of the diffusible plant factors inducing T-DNA circularization and *vir* gene expression in *Agrobacterium*. *Mol Gen Genet* **209**: 221–26.

Valentine L. (2003) *Agrobacterium tumefaciens* and the plant: the David and Goliath of modern genetics. *Plant Physiol* **133**: 948-55.

Valvekens D., Montagu M.V., and Van Lijsebettens M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc Natl Acad Sci USA* **85** (15): 5536-40.

van Attikum H. and Hooykaas P.J.J. (2003) Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **31** (3): 826-32.

van Attikum H., Bundock P. and Hooykaas P.J.J. (2001) Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. *EMBO J* **20**: 6550-58.

van Attikum H., Bundock P., Overmeer R.M., Lee L.Y., Gelvin, S.B., and Hooykaas P.J.J. (2003) The *Arabidopsis* AtLIG4 gene is required for the repair of DNA damage, but not for the integration of *Agrobacterium* T-DNA. *Nucleic Acids Res* **31**: 4247–55.

van Larebeke N., Enbler, G., Holsters, M., Van Den Elsacker, S., Zaenen, I., Schilperoort, R.A., and Schell. J. (1974) Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. *Nature* **252**: 169-70.

van Larebeke N., Genetello C., Schell J., Schilperoort R.A., Hermans A.K., van Montagu M., Hernalsteens J.P. (1975) Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. *Nature* **255**: 742-43.

Vaudequin-Dransart V., Petit A., Chilton W. S., and Dessaux Y. (1998) The Cryptic Plasmid of *Agrobacterium tumefaciens* Cointegrates with the Ti Plasmid and Cooperates for Opine Degradation. *Mol Plant Microbe Interact* **11** (7): 583–91.

Veena, Jiang H., Doerge R.W., and Gelvin S.B. (2003) Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes

involved in mediating transformation and suppresses host defense gene expression. *Plant J* **35** (2): 219-36.

Vergunst A.C., Lier M.C.M., Dulk-Ras A.D., and Hooykaas P.J.J. (2003) Recognition of the *Agrobacterium tumefaciens* VirE2 translocation signal by the VirB/D4 transport system does not require VirE1. *Plant Physiol* **133**: 978-88.

Vogel A.M. and Das A. (1992) Mutational analysis of *Agrobacterium tumefaciens* VirD2: tyrosine 29 is essential for endonuclease activity. *J Bacteriol* **174**: 303-08.

Vogel A.M. and Das A. (1994) Mutational analysis of *Agrobacterium tumefaciens* pTiA6 virD1: identification of functionally important residues. *Mol Microbiol* **12**: 811-17.

Wach A., Brachat A., Poehlmann R., and Philippsen P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793-808.

Wacker M., Linton D., Hitchen P.G., Nita-Lazar M., Haslam S.M., North S.J., Panico M., Morris H.R., Dell A., Wren B.W., and Aebi M. (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science* **298**: 1790–93.

Wagner G. and Wyss D.F. (1994) Cell surface adhesion receptors. *Curr Opin Struct Biol* **4**: 841-51.

Wagner V.T and Matthysse A.G. (1992) Involvement of a vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells. *J Bacteriol* **174**: 5999–6003.

Wang K., Herrera-Estrella L., Van Montagu M., and Zambryski P. (1984) Right 25 bp terminus sequences of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* **38**: 35-41.

Wang K., Stachel S.E., Timmerman B., van Montagu M., Zambryski P.C. (1987) Site-specific nick in the T-DNA border sequence as a result of *Agrobacterium vir* gene expression. *Science* **235**: 587-91.

Wang, K., Herrera-Estrella, A., and Van Montagu, M. (1990) Overexpression of virD1 and virD2 genes in *Agrobacterium tumefaciens* enhances T-complex formation and plant transformation. *J Bacteriol* **172**: 4432-40.

Watson B., Currier T.C., Gordon M.P., Chilton M.D., Nester E.W. (1975) Plasmid required for virulence of *Agrobacterium* tumefaciens. *J Bacteriol* **123**: 255-64.

Weigel D., Ahn J.H., Blázquez M.A., Borevitz J.O., Christensen S.K., *et al.* (2000) Activation tagging in *Arabidopsis*. *Plant Physiol* **122** (4): 1003-13.

Welply J.K., Shenbagamurthi P., Lennarz W.J., and Naider F. (1983) Substrate recognition by oligosaccharyltransferase. Studies on glycosylation of modified Asn-X-Thr/Ser tripeptides. *J Biol Chem* **258**: 11856-63.

Willmitzer L., De Beuckeleer M., Lemmers M., van Montagu M., Schell J. (1980) DNA from Ti plasmid present in nucleus and absent from plastids of crown gall plants. *Nature* **287**: 359-61.

Wilson I.B.H., Gavel Y., yon Heijne G. (1991) Amino acid distributions around O-linked glycosylation sites. *Biochem J* **275**: 529–34.

Winans S.P. (1992) Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiol Rev* **56**: 12-31.

Winzeler E.A., Shoemaker D.D., Astromoff A., Liang H., Anderson K., Andre B., *et al.* (1999) Functional characterization of the *Saccharomyces cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**: 901-06.

Wood D.W., Setubal J.C., Kaul R., Monks D.E., Kitajima J.P., Okura V.K., *et al.* (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**: 2317–23.

Wu J., Carmen A.A., Kobayashi R., Suka N. and Grunstein M. (2001) Hda2 and Hda3 are related proteins that interact with and are essential for the activity of the yeast histone deacetylase Hda1. *Proc Natl Acad Sci USA* **98**: 4391–96.

Xiang C., Han P., Lutziger I., Wang K., Oliver D.J. (1999) A mini binary vector series for plant transformation. *Plant Mol Biol* **40** (3): 711-17.

Yan A. and Lennarz W.J. (2005a) Two oligosaccharyl transferase complexes exist in yeast and associate with two different translocons. *Glycobiology* **15**: 1407–15.

Yan A. and Lennarz W.J. (2005b) Unraveling the mechanism of protein N-glycosylation. *J Biol Chem* **280** (5): 3121-4.

Yan Q. and Lennarz W. (1999) Oligosaccharyltransferase: a complex multisubunit enzyme of the endoplasmic reticulum. *Biochem Biophys Res Commun* **266**: 684-89.

Yan Q. and Lennarz W.J. (2002) Studies on the function of oligosaccharyl transferase subunits. Stt3p is directly involved in the glycosylation process. *J Biol Chem* **277**: 47692–700.

Yan Q., Prestwich G.D., and Lennarz W.J. (1999) The Ost1p subunit of yeast oligosaccharyl transferase recognizes the peptide glycosylation site sequence, -Asn-X-Ser/Thr-. *J Biol Chem* **274**: 5021–25.

Yanofsky M.F. and Nester E.W. (1986) Molecular characterization of a host-range-determining locus from *Agrobacterium tumefaciens*. *J Bacteriol* **168** (1): 244-50.

Yanofsky M.F., Porter S.G., Young C., Albright L.M., Gordon M.P., and Nester E.W. (1986) The virD operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. *Cell* **47**: 471-77.

Ye G.N., Stone D., Pang S.Z., Creely W., Gonzalez K., and Hinchee M. (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum filtration transformation. *Plant J* **19**: 249–57.

Yi H., Mysore K.S., and Gelvin S.B. (2002) Expression of the *Arabidopsis* histone H2A-1 gene correlates with susceptibility to *Agrobacterium* transformation. *Plant J* **32**: 285–98.

Yi H., Mysore K.S., and Gelvin S.B. (2002) Expression of the *Arabidopsis* histone H2A-1 gene correlates with susceptibility to *Agrobacterium* transformation. *Plant J* **32**: 285–98

Yi H., Sardesai N., Fujinuma T., Chan C.W., Veena, and Gelvin S.B. (2006) Constitutive expression exposes functional redundancy between the *Arabidopsis* histone H2A gene HTA1 and other H2A gene family members. *Plant Cell* **18**: 1575-89.

Yi H., Sardesai N., Fujinuma T., Chan C.W., Veena, Gelvin S.B. (2006) Constitutive expression exposes functional redundancy between the *Arabidopsis* histone H2A gene HTA1 and other H2A gene family members. *Plant Cell* **18**: 1575–89.

Yoshida S., Ikeda E., Uno I., and Mitsuzawa H. (1992) Characterization of a staurosporine- and temperature-sensitive mutant, stt1, of *Saccharomyces cerevisiae*: STT1 is alleleic to PKC1. *Mol Gen Genet* **231**: 337–44.

Young J. M., Kuykendall L. D., Martinez-Romero E., Kerr A., and Sawada H.. (2001) A revision of Rhizobium Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and Allorhizobium undicola de Lajudie *et al.* 1998 as new combinations: Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola and R. vitis. *Int J Sys Evol Microbiol* **51**: 89–103.

Young J.M. (2001) Implications of alternative classifications and horizontal gene transfer for bacterial taxonomy. *Int J Syst Evol Microbiol* **51**: 945-53.

Young J.M. (2008) *Agrobacterium*: taxonomy of plant-pathogenic Rhizobium species, p. 184-220. In T. Tzfira and V. Citovsky (ed.), *Agrobacterium*: from biology to biotechnology. Springer, New York, NY.

Young J.M., Pennycook S.R., and Watson D.R.W. (2006) Proposal that *Agrobacterium radiobacter* has priority over *Agrobacterium tumefaciens*. Request for an opinion. *Int J Syst Evol Microbiol* **56**: 491-93.

Yusibov, V.M., Steck, T.R., Gupta, V., and Gelvin, S.B. (1994) Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells. *Proc Natl Acad Sci USA* **91**: 2994-98.

Zaenen I., Van Larebeke N., Teuchy H., Van Montagu M., and Schell J. (1974) Supercoiled circular DNA in crown gallinducing *Agrobacterium* strains. *J Mol Biol* **86**: 109–27.

Zambryski P, Joos H, Genetello C, Leemans J, Montagu MV, Schell J. (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J* **2** (12): 2143-50.

Zambryski P. (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu Rev Plant Phys Plant Mol Biol* **43**: 465-90.

Zenklusen D. and Singer R.H. (2010) Analyzing mRNA expression using single mRNA resolution fluorescent *in situ* hybridization. *Methods Enzymol* **470**: 641-59.

Zenklusen D., Larson D.R. and Singer R.H. (2008) Single-RNA counting reveals alternative modes of gene expression in yeast. *Nat Struct Mol Biol* **15** (12): 1263-71.

Zhao Z.M., Sagulenko E., Ding Z.Y., and Christie P.J. (2001) Activities of virE1 and the VirE1 secretion chaperone in export of the multifunctional VirE2 effector via an *Agrobacterium* type IV secretion pathway. *J Bacteriol* **183**: 3855-65.

Zhou H. and Clapham D.E. (2009) Mammalian MagT1 and *TUSC3* are required for cellular magnesium uptake and vertebrate embryonic development. *Proc Natl Acad Sci USA* **106** (37): 15750-55.

Zhu J., Oger P.M., Schrammeijer B., Hooykaas P.J.J., Farrand S.K., and Winans S.C. (2000) The bases of crown gall tumorigenesis. *J Bacteriol* **182**: 3885-95.

Zhu Y., Nam J., Humara J.M., Mysore K.S., Lee L.Y, *et al.* (2003a) Identification of *Arabidopsis* rat mutants. *Plant Physiol* **132**: 494–505.

Zhu Y., Nam J., Carpita N.C., Matthysse A.G., and Gelvin S.B. (2003b) *Agrobacterium*-mediated root transformation is inhibited by mutation of an *Arabidopsis* cellulose synthase-like gene. *Plant Physiol* **133**: 1000–10.

Ziemienowicz A. (2001) Odyssey of *Agrobacterium* T-DNA. *Acta Biochimica Polonica* **48**: 623-35.

Ziemienowicz A., Gorlich D., Lanka E., Hohn B., and Rossi L. (1999) Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. *Proc Natl Acad Sci USA* **96**: 3729-33.

Ziemienowicz A., Merkle T., Schoumacher F., Hohn B., and Rossi L. (2001) Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. *Plant Cell* **13**: 369-83.

Ziemienowicz A., Tinland B., Bryant J., Gloeckler V., Hohn B.. (2000) Plant enzymes but not *Agrobacterium* VirD2 mediate T-DNA ligation *in vitro*. *Mol Cell Biol* **20**: 6317–22

Zorreguieta A. and Ugalde R.A. (1986) Formation in Rhizobium and *Agrobacterium* spp. of a 235-kilodalton protein intermediate in β-D-(1-2) glucan synthesis. *J Bacteriol* **167**: 947-51.

Zufferey R., Knauer R., Burda P., Stagljar I., te Hessen S., Lehle L., and Aebi M. (1995) Stt3p, a highly conserved protein required for yeast oligosaccharyl transferase activity *in vivo*. *EMBO J* **14**: 4949-60.

Zupan J. and Zambryski P. (1995) Transfer of T-DNA from *Agrobacterium* to plant cell. *Plant Physiol* **107**: 1041-47.

Zupan J. and Zambryski P. (1997) The *Agrobacterium* DNA transfer complex. *Crit Rev Plant Sci* **16**: 279-95.

Zupan J., Muth T.R., Draper O., and Zambryski P. (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant J* **23**: 11-28.