INVESTIGATION OF SUBCELLULAR LOCALIZATION OF A YELLOW RUST PATHOGEN EFFECTOR CANDIDATE

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ABSTRACT

INVESTIGATION OF SUBCELLULAR LOCALIZATION OF A YELLOW RUST PATHOGEN EFFECTOR CANDIDATE

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Puccinia striiformis f. sp. *tritici* is one of the fungal pathogens, which causes one of the most disruptive diseases of wheat called, stripe rust, in many parts of the world. Because of their highly destructive character, *Puccinia* species including yellow rust are responsible for considerable yield losses in worldwide wheat production. Despite its economic importance, the genomics of the pathogens and the plant-pathogen interactions have not been identified yet. For sustainable control of this plant disease, creating durable resistant crops will require accumulation of information for the long-term solution against fungal pathogens. Toward which, the main objective of this thesis is to address developing better understanding of plant-pathogen interactions. Together with, characterization of pathogenicity and secreted effector proteins can help to guide these efforts.

In this thesis, one of the candidate effector genes of *Puccinia striiformis* f. sp. *tritici* Pstha12h2 is studied. The aim is to identify its function during host infection and for future investigation of the interaction between host factors of wheat *Triticum aestivum* L. infected with yellow rust. This effector gene was synthesized with FLAG-Tag at its N-terminus with the *Pac*I-*Not*I restriction sites at the ends. This gene was cloned into pK7FWG2, which is a Gateway destination vector, and transformed into *Agrobacterium tumefaciens* for expression in *Nicotiana benthamiana* leaves. By achieving this, the candidate effector gene was expressed using a GFP

fusion protein in *N. benthamiana*. Additionally, the gene construct was cloned into pEDV6 vector, which is also a gateway destination vector for an expression in wheat leaves, by *Pseudomonas fluorescens* mediated transformation. Here, the aim will be observe hypersensitive response caused by effector gene if it happens to be an *Avr* against any resistance (R) gene. The advantage of this bacterial system is its ability to deliver genes to wheat cells by their Type III secretion systems (TTSS).

As part of a future work, wheat cultivars will be infiltrated by *P. fluorescens* suspensions and the delivery of effector genes can be observed by staining of the presence of hydrogen peroxide accumulation, if the complementary 'R' gene is present in the wheat line tested.

Also, the gene construct was cloned into pJL48-TRBO vector for an expression in *Nicotiana benthamiana via* agrobacterium-mediated gene transformation. The expressed protein with FLAG-tag will be isolated and purified as a future study. By immunoprecipitation, effector protein will be used to puldown putative host interactors. Using mass spectroscopy analysis, the interactor protein will be easily identified.

Keywords: Wheat, yellow rust, *Puccinia striiformis* f. sp. *tritici*, Gateway cloning, PstHa12h2, *Nicotiana benthamiana*, Agrobacterium, Gateway cloning.

ÖZ

SARI PAS PATOJENİ EFEKTÖR ADAYININ HÜCRE İÇİ LOKALİZASYONUNUN İNCELENMESİ

ANDAÇ, Ayşe

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Puccinia striiformis f. sp. *tritici* buğdayda çok önemli hasara neden olan, sarı pas hastalığının oluşmasını sağlayan fungal patojendir. Son derece yıkıcı özelliği olmasından dolayı, sarı pas patojenlerinin de dahil olduğu *Puccinia* türleri dünya çapındaki buğday üretiminde önemli kayıplara yol açmaktadır. Ekonomik olarak çok önemli bir hastalık olmasına rağmen patojen genomiği ve bitki-patojen etkileşimleri henüz yeterli derecede çözümlenememiştir. Bu hastalığın kontrolü için genetik olarak dayanıklı bitkiler üretmek uzun vadeli çözüm sağlayabilir. Bu tezin amacı, bitki-patojen ilişkilerinin anlaşılmasını geliştirmeye yöneliktir. Bununla birlikte, *Puccinia* patojeninin ve salgılanan efektör proteinlerin karakterizasyonu bu çabalar için yardımcı olabilir.

Bu tez çalışmasında, *Puccinia striiformis* f. sp. *tritici'*nin efektör gen adaylarından birisi olan Pstaha12h2 konakçı bitkideki enfeksiyon sırasındaki görevi ve sarı pas hastalığına sahip buğdaydaki (*Triticum aestivum* L.) konakçı faktörleriyle etkileşimi incelenecektir. Bu efektör geni 'N-terminal' bölgesinde FLAG-Tag ve *Pac*I-*Not*I uçlarıyla sentezlettirilmiştir. Bu gen pK7WGF2 'gateway' vektörüne klonlanmıştır ve daha sonra *Nicotiana benthamiana* bitkisinde anlatımı için 'agrobacterium'a aktarılmıştır. Bunu yaparak *Nicotiana benthamiana*'da GFP ekspresyonuyla aday efektör genin hücre içi lokalizasyonu belirlenebilecektir. Ek olarak, bu gen yine bir 'gateway' vektörü olan pEDV6'e klonlanmıştır. *Pseudomonas fluorescens* transformasyonu yoluyla buğday yapraklarında ekspresyon sağlanıp aday efektör geninin sağlamasıyla hiper duyarlı cevap (hypersensitive response) verilmesi incelenmesi daha sonra

yapılacaktır. *P. fluorescens* bakteri sisteminin avantajı, efektör genlerinin bakterinin tip III salgı sistemiyle direk buğday hücrelerine girmesidir. Transformasyondan sonra *P. fluorescens* süspansiyonları bitki çeşitlerine infiltre edilip, efektör genlerinin transferi hidrojen peroksit oluşumlarının boyanmasıyla test edilecektir. Ayrıca bu genin pJL48-TRBO vektörüne klonlanmış agrobacterium gen transferi yoluyla *N. benthamiana'*da ekspresyonu incelenmesi ve ekspresyonu sağlanan protein FLAG-Tag ile Kütle spektrometre yöntemi ile etkileşime giren aday efektör protein tanımlanması daha sonra yapılacaktır.

Anahtar Kelimeler: Buğday, Sarı Pas, *Puccinia striiformis* f. sp. *tritici*, Gateway klonlaması, PstHa12h2, *Nicotiana benthamiana*, Agrobakteri.

Dedicated to my family:

For always being generous to support, to love and, to encourage…

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Amp:	Ampicillin
Avr:	Avirulence
bp:	Base pair
DNA:	Deoxyribonucleic acid
dNTP:	Deooxy-nucleotidetriphosphate
dpi:	Days post-inoculation
ER:	Endoplasmic reticulum
ETI:	Effector-triggered immunity
Gen:	Gentamicin
GFP:	Green fluorescent protein
GOI:	Gene of interest
HR:	Hypersensitive response
Kan:	Kanamycin
kb:	Kilobase
LB:	Liquid broth
LRR:	Leucine rich repeat
M:	Molar
mg:	Miligram
mL:	Mililiter
NB:	Nucleotide binding
ng:	Nanogram

NT:	Non-template
PAMP:	Pathogen-associated molecular patterns
PCD:	Programmed cell death
PCR:	Polymerase chain reaction
pEDV:	Effector detector vector
pmol:	Picomole
PRR:	Pattern recognition receptor
Pst:	*Puccinia striiformis* f. sp. *tritici*
PTI:	PAMP-triggered immunity
R:	Resistance
Rif:	Rifampicin
Spec:	Spectinomycin
T-DNA:	Transfer DNA
Taq:	*Thermus aquaticus*
Ti:	Tumor-inducing
TMV:	Tobacco mosaic virus
TRBO:	TMV RNA-based overexpression
TTSS:	Type-III secretion system
u:	Unit
Vir:	Virulence

CHAPTER 1

INTRODUCTION

1.1 Wheat

Wheat is one of the most important grains that have been cultivated for thousands of years as the main source of nutrition by humans. The early wheat cultivation occurred at around 8 000 years B.C in a small area near the Tigris and Euphrates River near Karacadağ Mountain in Diyarbakır, Turkey (Lev-Yadun *et al*., 2000; Heun *et al*., 1997). This crop provides nearly two-thirds of the global human diet and supplies significant inputs for both animal feed and industrial products. It was one of the first domesticated food crops and still it is the most important staple food in the world with an increasing demand (Curtis, 2002). According to Food and Agricultural Organization, wheat is the most widely grown crop in the world and its annual production exceeds about 600 million tones. In 2013, the production of wheat reaches about 690 million tones and its production is expected to increase by 60% in 2050 to meet the demand (FAO, 2013). Wheat is the major produced cereal in Turkey and its production reaches approximately 20.100 thousand tons according TÜİK (TurkStat, 2013). Additionally, Turkey is one of the most important wheat producer countries which is ranked 9th in the world (Table 1). This is because of its ecological and climatic conditions; also its rich crop pattern and land factors.

In comparison to other cereals, wheat has the best source for nourishment because of providing essential amino acids, minerals, and vitamins, beneficial phyto-chemicals and dietary fiber components to the human diet (Shewry, 2009). Furthermore, its broad range ability to environmental adaptation, easiness of grain storage and converting grain into flour for usage as food make wheat as a major diet component (Curtis, 2002).

In spite of its relatively recent origin, wheat contains genetic diversity sufficient to enable the development of over 25,000 types which are adapted to a wide range temperate and environment (Shewry, 2009). Adaptation ability of wheat is so high because of its complex nature of its genome and this provides the crop a good plasticity. It has the ability to grow from temperate wet to dry and high rainfall areas, and from warm humid to dry cold environments. The optimum temperature for wheat growth is about 25 ^{o}C with minimum and maximum limits from 3 ^{o}C to 32 ^{o}C (Acevedo *et al*, 2008; Curtis, 2002).

Table 1.1 Wheat production: Leading producers.

Countries	Average 2010-12 (10^6 t)	2013
EU	135.2	138.0
China	117.7	121.4
India	87.5	92.3
United States	58.8	58.0
Russia	45.2	53.0
Australia	26.5	23.0
Canada	25.3	28.0
Pakistan	23.9	24.7
Turkey	20.5	20.5
Ukraine	18.3	19.5
World	672.5	690.0

Source: Food and Agriculture Organization 2013.
(http://www.fao.org/docrep/017/al998e/al998e.pdf)

1.2 Wheat stripe rust disease

Rust diseases of wheat are the oldest known plant diseases. Since their discovery, many studies have been performed on their life cycles and their diagnosis (Marsalis, 2006). There are three rust fungi, stem rust, leaf rust and stripe rust. All of them have the ability to infect wheat (*Triticum aestivum* L.), and cause severe damage (McMullen *et al*., 2005). The rust pathogens are obligate parasites, which mean that they cannot live outside their host. They can grow and multiply only on living plant tissue. They are very specialized parasites with their narrow host range (Wegulo, 2012). Rust fungi all require similar infection conditions and result in similar disease symptoms. Infection occur on any part of the plant according to the type of pathogen which enables production of pustules that contains thousands of yellow or orange, reddish-brown or black spores.

Table1.2 The rust diseases of wheat, their primary and alternate hosts and symptoms.

Disease	Pathogen	Primary hosts	Alternate hosts	Symptoms
Leaf rust	*Puccinia triticina*	Bread and durum wheats, triticale	Thalictrum, Anchusa, Isopyrum, Clematis	Isolated uredinia on upper leaf surface and rarely on leaf sheaths
Stem rust	*Puccinia graminis* f.sp. *tritici*	Bread and durum wheats, barley, triticale	Berberis vulgaris	Isolated uredinia on upper and lower leaf surfaces, stem and spikes
Stripe rust	*Puccinia striiformis* f.sp. *tritici*	Bread and durum wheats, triticale, a few barley cultivars	Unknown*	Systemic uredinia on leaves and spikes and rarely on leaf sheaths

Source: Curtis *et al*., 2002. *Recently, an alternate host was claimed (Jin, 2010).

Puccinia striiformis is the causative agent of stripe rust which is an important disease of wheat, barley, rye, and particular graminaceous hosts for many years. The most economically destructive form of these fungal pathogens is *Puccinia striiformis* f. sp. *tritici* (*Pst*) which is responsible from wheat stripe rust, also well known as yellow rust (Wellings, 2010). This disease was first described and documented by Gadd in 1777 (Eriksson and Henning, 1896). The origin for *P. striiformis* is thought to be Transcaucasia where they had grasses as the primary host, and from there the pathogen moved into Europe to China and eastern Asia along mountain ranges (Races, 1985). It has appeared in over 60 countries in the world and it is reported that it spreads all over the world except Antarctica (Evans *et al*., 2008).

Puccinia striiformis is the most sensitive pathogen to environmental conditions. *P. striiformis* does not have a sexual stage and does not require an alternate host to complete its life cycle. However, the existence of recently an alternate host was claimed (Jin, 2010). Between three rust diseases, stripe rust has lower optimum temperatures for infection, which limits development of the pathogen in many parts of the world (Singh, 2002). The optimum temperature for infection is between 9-13 ^{o}C with minimum and maximum limits from 0 ^{o}C to 23 ^{o}C (Brown and Sharp, 1969; Hogg *et al*., 1969). Also, it requires eight hours of free moisture on the leaf surface for spore germination (Wegulo, 2012). Light condition is another important factor for infection. It was studied that infection was observed highly in low light conditions. High light intensities inhibits the germination of spores (Lucas *et al*., 1975). Optimal disease development temperatures are 15-20 ^{o}C and release of spores from the plant develops within 11-15 days. At freezing temperatures the time required for infection to spore release may exceeds 180 days. Before spore formation, yellow spots appear on the leaves and chlorotic streaks can be formed

on the surface of the leaf. Urediniaspores which have a yellow color appear on the leaf axis in rows. These lesions on the whole leaf can be the result of one small urediniospore infecting the wheat leaf (Evans *et al*., 2008).

Table 1.3 Conditions of stripe rust development in nature.

Stripe Rust Stage	Temperature (^{o}C)			Light	Free Water
	Min.	Optimum	Max.		
Germination	0	9-13	23	Low	Essential
Germling	-	10-15	-	Low	Essential
Appressorium	-	-	(not formed)	None	Essential
Penetration	2	9-13	23	Low	Essential
Growth	3	12-15	20	High	None
Sporulation	5	12-15	20	High	None

Source: Conditions of stripe rust development in nature (Roelfs *et al*., 1992).

Puccinia striiformis urediniaspores have the ability to disperse by wind. Urediniaspores can be affected by ultraviolet radiation highly, so its dispersal by wind is restricted to short distances (Maddison and Manners, 1972). Interestingly, it was estimated that spores of this pathogen can spread by wind over long distances. The dispersal can be either in a single step or in many steps which increase the chance of disease formation across large areas. It was reported that spores of yellow rust were spread 800 km by the wind in viable state in Europe (Zadoks, 1961).

Yield losses from stripe rust were reported ranging from 10% to 70% on susceptible varieties in wheat producing areas due to susceptibility of the cultivar, time of the infection, disease development rate and longevity of the disease (Chen, 2005). Even total loss can occur if the infection happens in the growing season of susceptible cultivar of wheat and in favorable conditions. For controlling the stripe rust disease, planting resistant cultivars can be the most effective, economical and environmentally friendly solution. However, stripe rust can evolve into new strains in which become more virulent and cause more damage. Thus, resistance in current cultivars can be overcome by emergence of new strains (Wegulo, 2012). One of the examples is wheat stem rust gene strain, *Ug99*, which was originated from Uganda in 1999. It was found in Kenya in 2001 and then in Ethiopia in 2003 (Shankar, 2007).

Figure 1.1 Stripe rust disease in wheat (Evans *et al*., 2008).

1.3 Plant Immune System

Plants are continuously exposed to pathogen attack like animals. Unlike animals, plants have no circulatory system, antibodies or specialized cells like macrophages and adaptive immune system. Thus, a defense mechanism different from animals has been evolved by plants (Staskawicz *et al*., 1995). Against a wide variety of pathogens including bacteria, fungi, viruses and nematodes, plants develop rapid and efficient defense responses, which are called innate immune system. In plants, disease resistance gene product interacts with pathogenic effectors to initiate defense responses. The resistance genes are reported that they have motifs similar within the pathways of immune responses of animals and they have similar downstream signaling processes (Cohn *et al*., 2001).

Plants detect pathogens and protect themselves by two modes of response mechanism: pathogen-associated molecular pattern (PAMP) -triggered immunity (PTI) and effector-triggered immunity (ETI) (Zipfel & Felix, 2005). On the cell surface of the plant, there are pattern recognition receptors (PRRs). When pathogen attacks the host plant, these receptor proteins recognize pathogen associated molecular patterns (PAMPs) which are the necessary components of entire classes of pathogens, such as bacterial flagellin, peptidoglycan, fungal chitin or lipoteichoic acid. PAMP- triggered immunity (PTI), the basal defense system of plant, is induced by stimulation of PRRs (Dodds & Rathjen, 2010).

Several pathogenic microbes have the ability to deliver their effectors which are pathogen virulence molecules into the host plant cell to initiate virulence by suppressing PTI. Thus, the second class of response mechanism is induced by detection of intracellular receptors of effectors which interacts with corresponding plant resistance genes and this recognition is called effector-triggered immunity (ETI) (Zhang *et al.*, 2010). By developing effector molecules, pathogens inhibit PTI which changes the state of the host plant cell and the first line of defense is destroyed (Deyoung & Innes, 2006). Thus, the second class of response mechanism is induced by detection of intracellular receptors of effectors which interacts with corresponding plant resistance genes and this recognition is called effector-triggered immunity (ETI) (Zhang *et al.*, 2010).

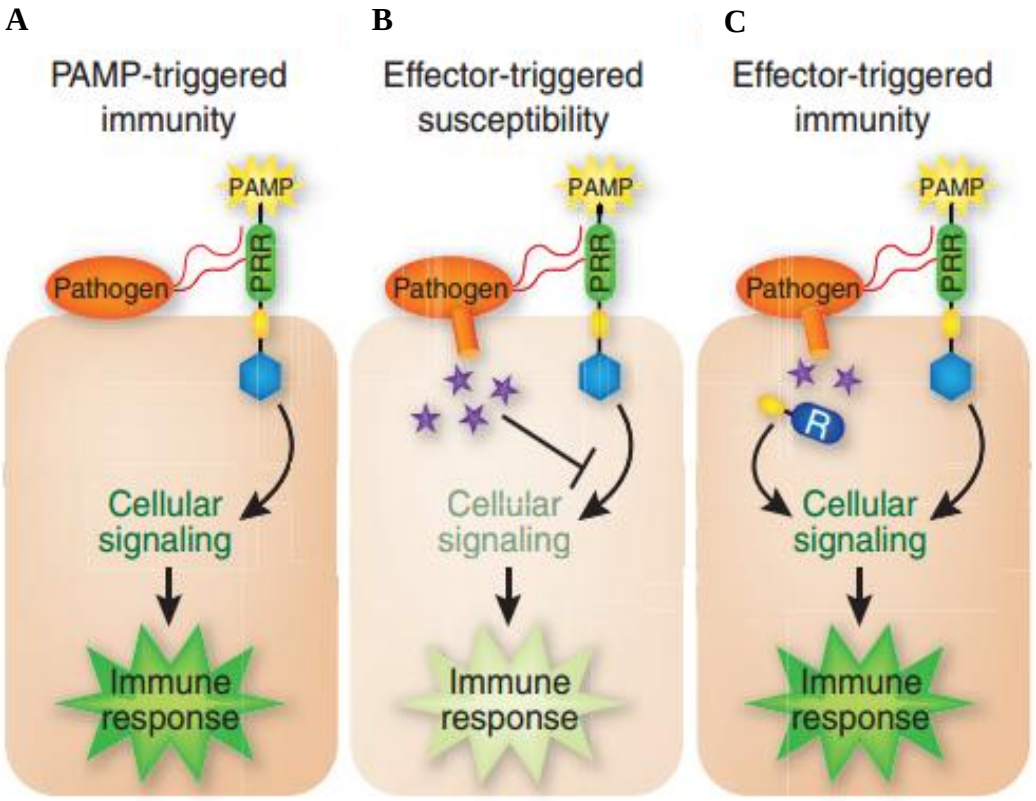

Figure 1.2 Schematic view of plant immune system. **a)** In pathogen attack, pattern recognition receptors (PRRs) in the host are activated by pathogen associated molecular patterns (PAMPs), resulted in pathogen-triggered immunity (PTI). **b)** Effectors from virulent pathogens inhibit PTI and they trigger effector-triggered susceptibility, which leads to development of disease. **c)** If the host cell has resistance proteins (R) for recognizing corresponding effector from virulent pathogen, effector triggered immunity is evoked (Pieterse *et al*., 2009).

Typically, PTI and ETI cause similar responses. PTI is more effective in protecting plants from non-adapted pathogens in a phenomenon called non-host resistance, but ETI is more effective on adapted pathogens (Dodds & Rathjen, 2010). It is more effective because ETI induces stronger and powerful defense system that results in hypersensitive response (HR). Hypersensitive response is a kind of rapid, localized programmed cell death (PCD) which happens at the infection site. This cell death prevents pathogen proliferation by limiting them to access nutrient sources of the plant and it preserves plant to prevent spreading of the infection. Also, some reports suggest that signal molecules which releases after cell death activate defense related genes in the extracellular matrix of the plant cell so that it stimulates surrounding cells for pathogen attack (Hammond-Kosack *et al*., 1996; Dangl *et al*., 1996).

In the beginning of pathogen invasion, changes in the plant physiology, such as permeability of plasma membrane, occurs which is calcium (Ca^{2+}), proton influx and potassium and chloride efflux (McDowell and Dangl, 2000). After pathogen invasion, these changes trigger production of reactive oxygen species, intracellular pH changes resulted from transient ion-flux leading, secondary signal releasing like nitric oxide (NO), strengthening of the cell wall at the lesion site and formation of antimicrobial products such as phenolics and phytoalexin (Cohn *et al*., 2001). The reason for these changes is explained by a 'gene for gene' model which is proposed by Flor in 1942. This model states that plant disease resistant develops due to interaction between *R* genes in resistant plant and pathogenic effector molecules which enable pathogens to overcome PTI. *R* gene products from resistant plant recognize effectors encoded by avirulence (*Avr*) genes from the pathogen. R genes products are found in different cellular compartments like apoplast, cytoplasm and nucleus. The interaction between *R*- and *Avr* gene results in hypersensitive response which means host resistance against pathogen. If this interaction does not happen because of the absence of one of these genes, disease development occurs (Dangl *et al*., 2001; Cohn *et al*., 2001).

Recognition in ETI is based on a class of receptor proteins produced by *R* genes. These receptor proteins are called NB-LRR proteins which are composed of nucleotide binding (NB) domains and leucine rich repeats (LRRs). These proteins are some of the largest proteins known in plants by having about 860 to 1,900 amino acids (McHale *et al*., 2006). If one of the pathogen effectors is detected by corresponding NB-LRR proteins, ETI is induced. The recognized effector is identified as avirulence *(Avr*) protein (Jones & Dangl, 2006). NBS-LRR proteins are controlled by complex interactions between NBS and LRR domains. LRR domains consist of tandem LRRs and they are found at the carboxy termini of plant NB-LRR proteins. Several evidences showed that these domains are involved in specific identification and binding of pathogen effector molecules. NB domain is composed of blocks of sequences which includes many conserved motifs. Although the functions of these domains have not been identified yet, they seem to be important; because substitutions in these domains affect NBS-LRR function (DeYoung & Innes, 2006). The simplest explanation about NB-LRR protein function is that receptor binding when detects effector molecules of pathogens. However, direct interactions

between NB-LRR and effector proteins have been identified only rarely (McHale *et al*., 2006). They can also recognize effectors indirectly through an accessory protein which is part of NB-LRR complex. The effector induces a change in the accessory protein which is recognized by NB-LRR. This indirectly recognition system proposes 'guard hypotheses'. In this hypothesis, R proteins interact with accessory protein which is the target of avirulence proteins of pathogens (Dodds & Rathjen, 2010). Thus, this system explains how slowly evolving R genes compete with faster evolving pathogens by accessory proteins. Only rapidly evolving R genes can directly interact and compete with these fast evolving pathogens (Jones & Dangl, 2006).

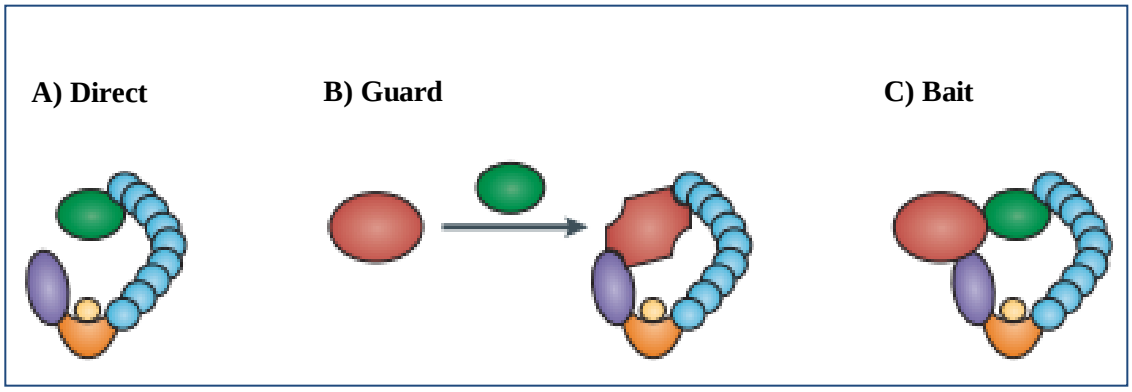

Figure 1.3 Schematic overviews of direct and indirect interactions. a) In direct interactions, effector (green) binds nucleotide binding (NB) Leucine Rich Repeat (LRR) receptors (blue, orange, purple and yellow) physically and triggers immune system. b) In indirect interactions, the effector protein form an accessory protein (red) which is the target of avirulence proteins (guard model) or structural mimic of these targets (decoy model). c) In the bait system, interaction between accessory and effector proteins induces direct identification by the NB-LRR receptor (Dodds & Rathjen, 2010).

The main role of effector protein is inhibiting plant defense system. In all bio-trophic plant pathogens, successful transfer of effector proteins need the development of a structure called haustorium. It develops within the lumen of the host cell to take nutrients for their growth and proliferation. While haustorium grows, the host cell forms a membrane from an unknown origin that surrounds the haustorium. Expression of the effector *Avr* proteins in the haustoria show that it has a role in delivering effector genes to the host cell. Some bacterial pathogens send their effectors to the host cell by using 'Type III Secretion System' (TTSS) but in general the way how pathogen transfers their effectors to the host cell is still unclear. In addition to their unidentified pathways, there are not many effectors and effector candidates identified from haustoria developing fungal pathogens due to the difficulty of isolation of them (Godfrey *et al*., 2010).

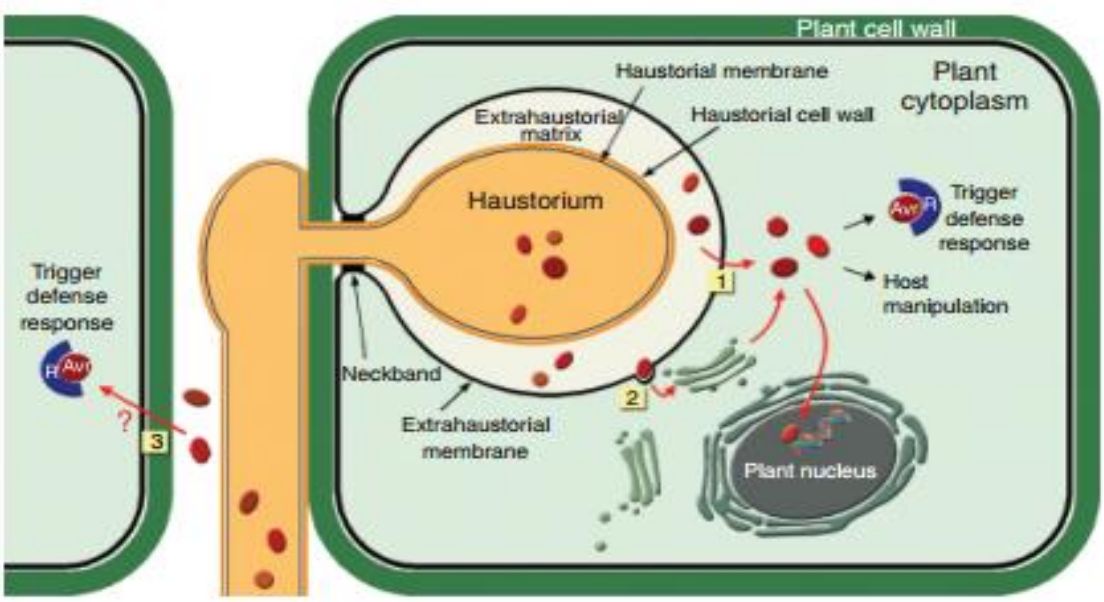

Figure 1.4 Schematic overview of haustorium and host interaction. It shows the haustorium, extrahaustorial matrix formation in the host cell. From the haustoria, effector proteins are secreted into the extrahaustorial matrix. Then, a group of proteins are sent into the host cell by directly crossing the extrahaustorial membrane (1) or by vesicles (2). Inside the host cytoplasm, *Avr* genes are recognized by *R* genes to evoke a defense response. Other effectors may be transported into host organelles like nucleus to change host transcription. The effectors which is secreted from hyphae into the apoplast can enter host cells by an unknown mechanism, if they are recognized by host cell defense response is activated (Catanzariti *et al*., 2007).

To sum up, R proteins must have two functions for evoke an immune response: detecting pathogen-derived signal and inducing a downstream signaling which leads plant defense response. The mode of actions of candidate effector genes and identified effector genes should be characterized well to help for determining host targets, and also the evolutionary changes acting both on effectors and hosts. Moreover, haustorial structure is also an important issue to understand the host-pathogen interaction. Identification and understanding the population biology of effector proteins and their co-evolving host targets will determine the evolution of plant immune system and it will guide ways to control disease in a more effective way (Jones & Dangl, 2006).

1.4 *Agrobacterium tumafeciens* mediated gene transfer

Agrobacterium-mediated gene transfer is a highly efficient method which is widely used for creating transgenic plants. Agrobacterium is a Gram negative soil bacterium and it is the only organism which has the ability of transfer genes from prokaryotes to eukaryotes. This soil pathogen causes an important crown gall disease which affects most dicotyledonous plants by developing a tumor growth on the plant tissues which is called crown gall disease. It possesses a large tumor inducing (Ti) plasmid consist of T-DNA which is transferred to the host cell and virulence (*Vir)* regions which are needed for virulence. T-DNA segment of Ti plasmid from bacterium is integrated into the nucleus of infected plant cells and integration into the host cell genome results in the crown gall disease (Mehrotra & Goyal, 2012). For tumor growth after transferring of bacterium, host cell begin to produce plant hormones and it produces novel amino acid-sugar conjugates (opines) for the bacterium growth. Opines are the carbon and nitrogen source for its growth (Pitzschke & Hirt, 2010).

T-DNA regions are identified by left and right borders sequences which are 25 bp long repeats. This region is flanked by these two borders. These two borders are the only required sequences for T-DNA transfer. These repeats are *cis-acting* sequences that transfer processes of T-DNA initiates at the right border, progresses leftwards and terminates at the left border (Gheysen *et al*., 1987). Additionally, T-DNA transfer is mediated by *vir* genes. Agrobacterium can sense phenolic compound which is produced from plants and this is the signal for initiation of *vir* genes expression. *Vir* genes also direct T-DNA processing from parental Ti plasmid and secrete it into the host genome (Lee & Gelvin, 2008).

For improvement of introducing gene of interest into Agrobacterium T-DNA region for transferring into plant cell, the T-DNA and *vir* genes are separated into different two vectors which is called binary T-DNA vector including gene of interest and helper *vir* plasmid in one Agrobacterium cell. This system can function both in *E. coli* and *Agrobacterium tumefaciens.* In this binary system, tumor induction cells and opines synthesis cells, which do not play important roles for transfer, are removed not to harm the plant cells. Cloning of gene of interest into T-DNA regions in binary vector systems becomes much easier because T-DNA region is located on smaller binary vector than Ti plasmid. For advantage of genetic manipulations, changes in Agrobacterium strains having *vir* helper plasmids have been developed (Lee & Gelvin, 2008).

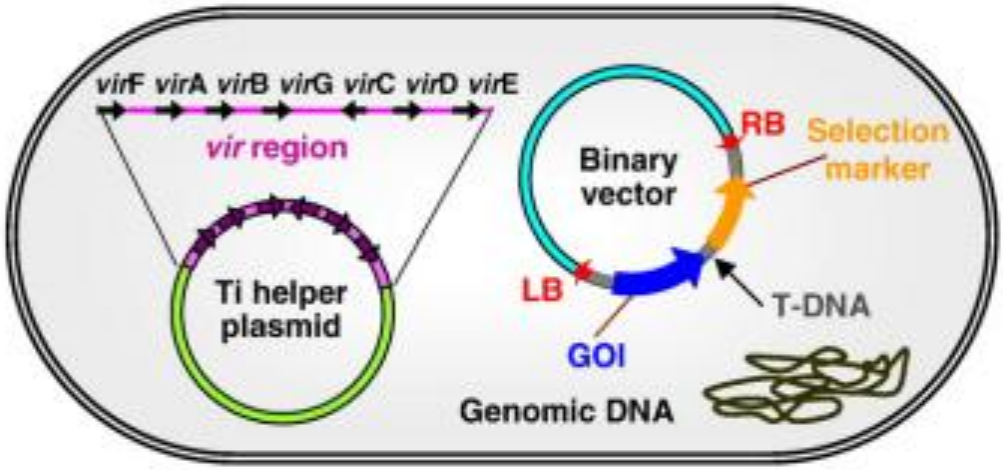

Figure 1.5 Schematic representation of genetic elements in a binary T-DNA vector. The target gene is cloned into T-DNA region of binary vector in which T-DNA is limited with right and left borders. *Vir* proteins are encoded by *vir* genes which are located on a separate replicon called Ti helper plasmid. T-DNA progressing from binary vector and transferring from bacterium into plant cell are mediated by *vir* genes on Ti helper plasmid (Meyers *et al.*, 2010).

1.4.1 pJL48-TRBO vector

Expression vectors which are based on *Tobacco mosaic virus* (TMV) have the ability to express high levels of foreign proteins in plants. The pJL48-TRBO vector is agro-delivery compatible, TMV RNA-based overexpression (TRBO) vector. Because of its efficiency and ease of use, TRBO vector is more favorable transient vector for recombinant protein expression in plants than TMV (Lindbo, 2007).

To achieve abundant levels of protein expression by using TMV as an expression vector, the level of Agrobacterium suspension for infiltration into plant must be very high. In some plant species, this high level of infiltration cause hypersensitive response in plants (Wroblewski *et al.*, 2005). However, these disadvantages were fixed by changes in TMV vector such as deletion of promoter and viral genes. It was hypothesized that viral genes had a negative effect on efficiency of agro-infiltration assay by triggering RNA silencing in plants. By these alterations the size of the pJL48-TRBO vector was kept smaller than TMV vector in which cloning and handling became easy. The agro-infiltration rate of this plasmid is very high than TMV, even very dilute Agrobacterium suspensions can produce hypersensitive response in plants. By using pJL48-TRBO vector, high expression levels of proteins can be achieved (Lindbo, 2007).

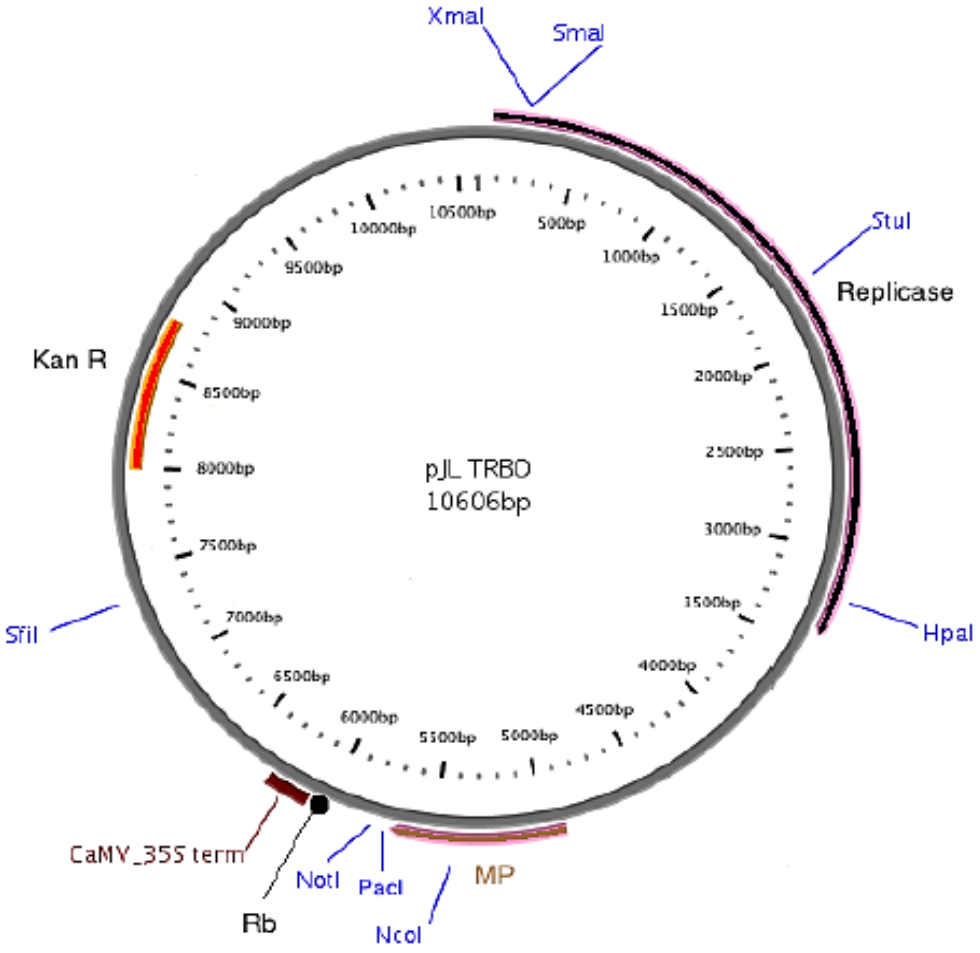

Figure 1.6 Vector map of pJL48-TRBO. It begins with replicase and movement (MP) encoding genes and continues with multiple cloning site (only *Pac*I and *Not*I sites are shown), Rb (ribozyme) genes and CaMV-35S terminator sequence. It has Kanamycin (Kan) resistance and it can replicate in Agrobacterium as well as in *E. coli*. The right and left borders of T-DNA are not represented in the vector map (Xiaoli Dong *et al*., 2004).

1.5 Co-immunoprecipitation

Co-immunoprecipitation is the widely used method to identify protein-protein interactions. For identifying the interactions *in vivo*, it is the simplest and most effective method to determine the functions of proteins and interaction between them *in vivo*. However, the antibody against that protein sometimes is not available and for this situation, this method is performed by expressing tagged proteins which is inserted into N- or C-terminus of the protein. Thus, antibodies against these tags can be found easily (Lee, 2007). After the protein is tagged which becomes the bait protein, the interacting co-member of this protein which is called prey is isolated and purified. The identification of the interacting proteins can be further analyzed by Mass Spectroscopy (Geva & Sharan, 2011).

1.6 Gateway cloning

In the process of gene discovery for determining gene functions, it generally needs multiple tasks such as gene expression, encoded protein purification, subcellular localization determination and antibodies production (Hartley et al., 2000). For example, expression of a protein in plants which is engineered to have oligopeptide epitope tag enables affinity purification of that protein and any interacting proteins for effective and easier isolation, determination and biochemical analysis of protein complexes. To achieve these goals, specialized vector constructs must be engineered for cloning target gene of interest (Earley et al., 2006). Traditional ligase dependent methods which rely on restriction digestion and ligation are both time-consuming and troublesome which produce a barrier for functional genomics or proteomics. These barriers are lowered or disabled by a highly accurate and effective method, Gateway cloning systems, for the rapid directional cloning of gene of interests. Instead of ligase, it uses the bacteriophage lambda recombination system. The advantage of this system, it uses a modified version of site specific recombination bacteriophage λ in *E. coli* which enables bacteriophage λ to integrate and excise itself in and out of a bacterial chromosome during their lysogenic and lytic states respectively (Karimi *et al.*, 2007).

Gateway cloning system has four types of vectors and each has specific recognition sites. They are entry vector (having *att*L), donor vector (having *att*P), destination vector (having *att*R) and expression vector (having *att*B). The main approach of gateway cloning is having an entry clone. Once inserted into entry vector, the gene of interest is recombined by a variety of numerous destination vectors. Polymerase chain reaction (PCR) of gene of interest is performed to amplify the target gene and add CACC sequence at its 5' end. By this sequence, gene of interest is inserted into entry vector by topoisomerase mediated cloning (Earley *et al*., 2006). After inserted

into entry vector, the resulted plasmid has the target gene with *att*L recombination site. The target gene is transferred from entry to destination vector by LR clonase reaction. In this cloning system, addition to antibiotic resistance, there is a second selection marker. Destination vector include *ccdB* gene between *att*R sites which activates gyrase-mediated cleavage of double-stranded DNA and protein encoded by this gene prevents survival of most *E. coli* strains unless they have *ccdB* resistance. Thus, only desired transformants having gene of interest (GOI) can survive in which colonies with entry vector cannot survive due to two selection systems (Hartley *et al*., 2000; Bernard *et al*., 1992).

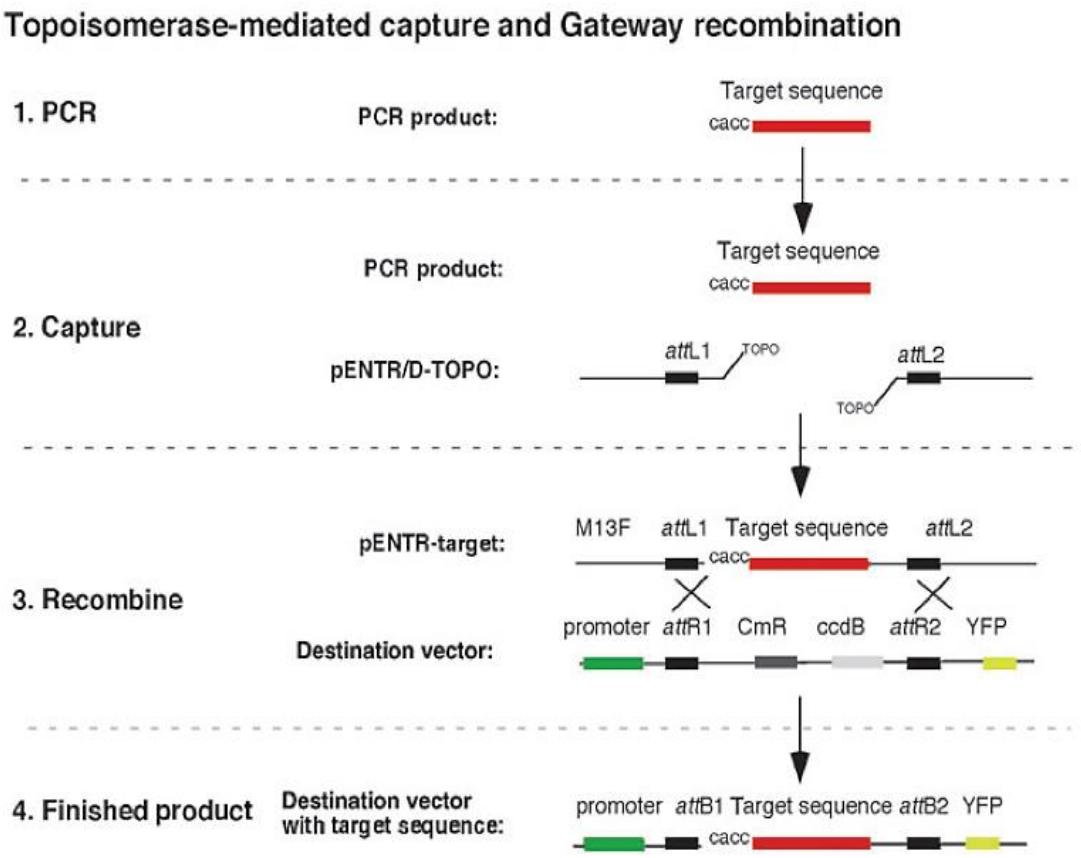

Figure 1.7 Schematic overview of Gateway recombination system. Firstly, CACC sequence is added to 5'end of the target gene for directional topoisomerase mediated cloning (1). Obtained PCR product is inserted into pENTR/D-TOPO vector with *att*L sites by topoisomerases found at its both ends (2). By LR clonase reaction, the gene of interest is transferred from pENTR/D-TOPO vector to destination vector between *att*L and *att*R sites (3). In transformants, only destination vector with target sequence can survive because of having a lethal *ccdB* gene between *att*R sites (4) (Earley *et al*., 2006).

1.6.1 pK7FWG2 vector

Destination vectors of Gateway cloning system provide to utilize benefits of this cloning method and Agrobacterium mediated gene transformation for plant functional genomics studies. pK7FWG2 is a destination vector which has a T-DNA region with left and right borders. Because it is a Gateway destination vector, the transfer of gene of interest is fast and reliable. The target gene transfer from entry vector to pK7FWG2 happens between *att*L and *att*R sites by LR clonase enzyme system of Gateway cloning (Karimi *et al*., 2002).

Binary T-DNA vector, pK7FWG2, is generally used in subcellular localization of a candidate protein according to GFP fusion to C-terminus of a target protein (Karimi *et al*., 2002). GFP fusion to C-terminus is a certain way for analyzing the expression of the protein. If GFP fusion is located on the N-terminus, the expression of the protein may not be completed but GFP expression can be seen anyway. Thus, GFP fusion at C terminus is better for subcellular protein localization (Palmer & Freeman, 2004). Additionally, *Avr* genes of pathogens encode small proteins with N-terminal signal peptides which deliver them to the endoplasmic reticulum (ER) secretory pathway (Catanzariti *et al*., 2007). So, signaling pathway does not affected by negatively from C-terminus fusion of GFP. Moreover, it possesses Spectinomycin (Spec) and Streptomycin (Sp) resistances for plasmid selection in bacterium and Kanamycin (Kan) resistance in plants. There is *ccdB* gene between *att*R sites, so colonies can grow only if they have target sequence inserted in pK7FWG2 vector (Karimi *et al*., 2002). The map of the vector is shown in Figure 1.6.

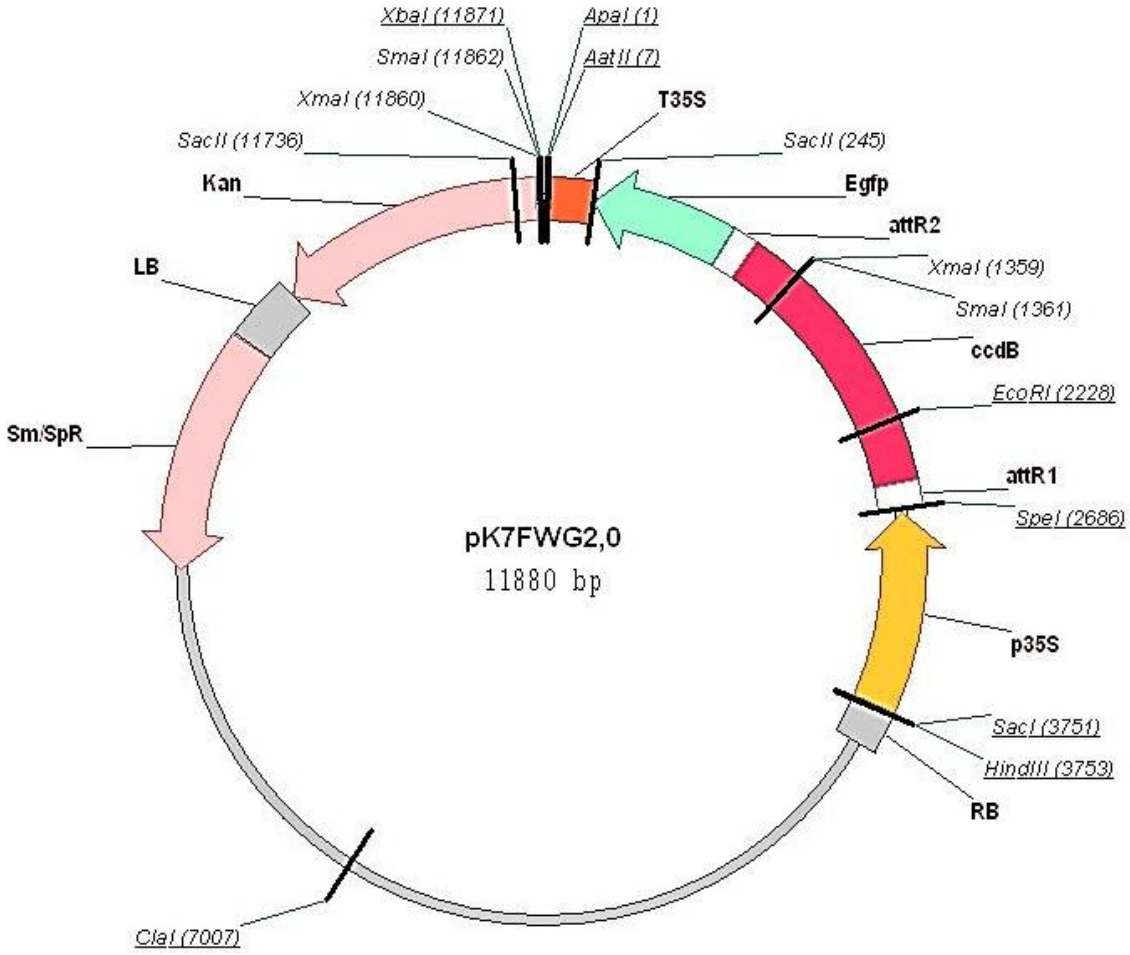

Figure 1.8 pK7FWG2 vector map (Karimi *et al*., 2002).

1.7 *Pseudomonas fluorescens* mediated wheat infiltration

Many biological assays are conducted to predict effector proteins and determine their specific functions like avirulence from cereal rusts. Agrobacterium mediated gene transfer is one of these assays and it has some limitations such as affecting small numbers of cells in grasses and difficulty in studying individual cells. For this approach, several bacterial systems were used to test delivery of effectors to wheat by their type III secretion system (TTSS). *Pseudomonas fluorescens* were determined that it was unable to produce hypersensitive response when it was delivered into wheat; however it can deliver bacterial effectors and fungal toxin *Tox*A into wheat

and cause hypersensitive response. Thus, for predicting effector proteins, they can be infiltrated into wheat by *P. fluorescens* and hypersensitive response can be detected by staining the accumulation of hydrogen peroxide or callose deposits (Yin & Hulbert, 2010).

Many Gram negative bacteria use type III secretion system which behaves as syringes. They can infiltrate their bacterial proteins named as type III effectors into cytoplasm of eukaryotic host cells. Then, the injected effectors are able to convert the functions of the host cell for the benefit of the infecting bacteria. TTSS are encoded by *hrp* genes which is responsible from disease initiation and hypersensitive response development (Mavrodi *et al*., 2011). There are three strategies for changing host responses by pathogenic bacteria. It can be directly cleavage of ubiquitination, modulation of RNA metabolism or suppression of kinases found in plant defense signaling. In order to be successful in causing disease, bacterial type III effectors must evade both PTI and ETI (Block *et al*., 2008). The injection system of type III effectors to the plant and their targets in the host cell are the most studied areas of research in plant microbe interactions (Mavrodi *et al*., 2011).

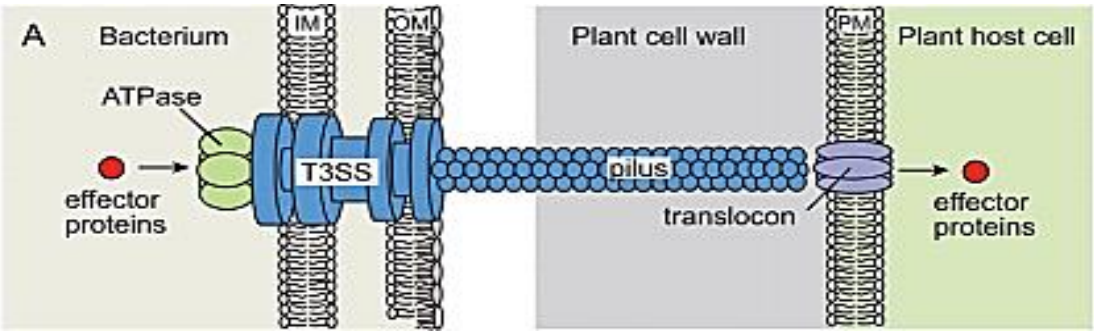

Figure 1.9 Overview of type three secretion system. Inner and outer membranes of bacterium are connected by secretion apparatus and this apparatus interacts with cytoplasmic ATPase. This system is connected to the plant cell wall by its pilus and effector proteins are delivered from bacteria to the plant host cell (Büttner & He, 2009).

1.7.1 pEDV6 vector

pEDV6 (Effector Detector Vector) is a Gateway destination vector which is designed for delivering bacterial effectors to the wheat by using TTSS of *Pseudomonas* to activate plant defense system. It is developed to fuse candidate pathogen effectors to its N-terminus of the

*avr*RPS4 protein to direct them into the wheat cells. Effector proteins of most pathogenic bacteria can deliver directly into plant cells by their specialized TTSS (Yin & Hulbert, 2010).

pEDV6 has a *avr*RPS4 promoter which regulates *avr*RPS4 protein to guide effector proteins to deliver into wheat cells by *Pseudomonas* TTSS. It has Gentamycin (Gen) resistance for plasmid selection in bacterium. The gene of interest from entry vector is inserted into pEDV6 destination vector between *att*L and *att*R sites by LR clonase enzyme system. Like other destination vectors, it has *ccdB* lethal gene between *att*R sites for selection of successful transformants (Remigi et al., 2011). The vector map is represented in Figure 1.10.

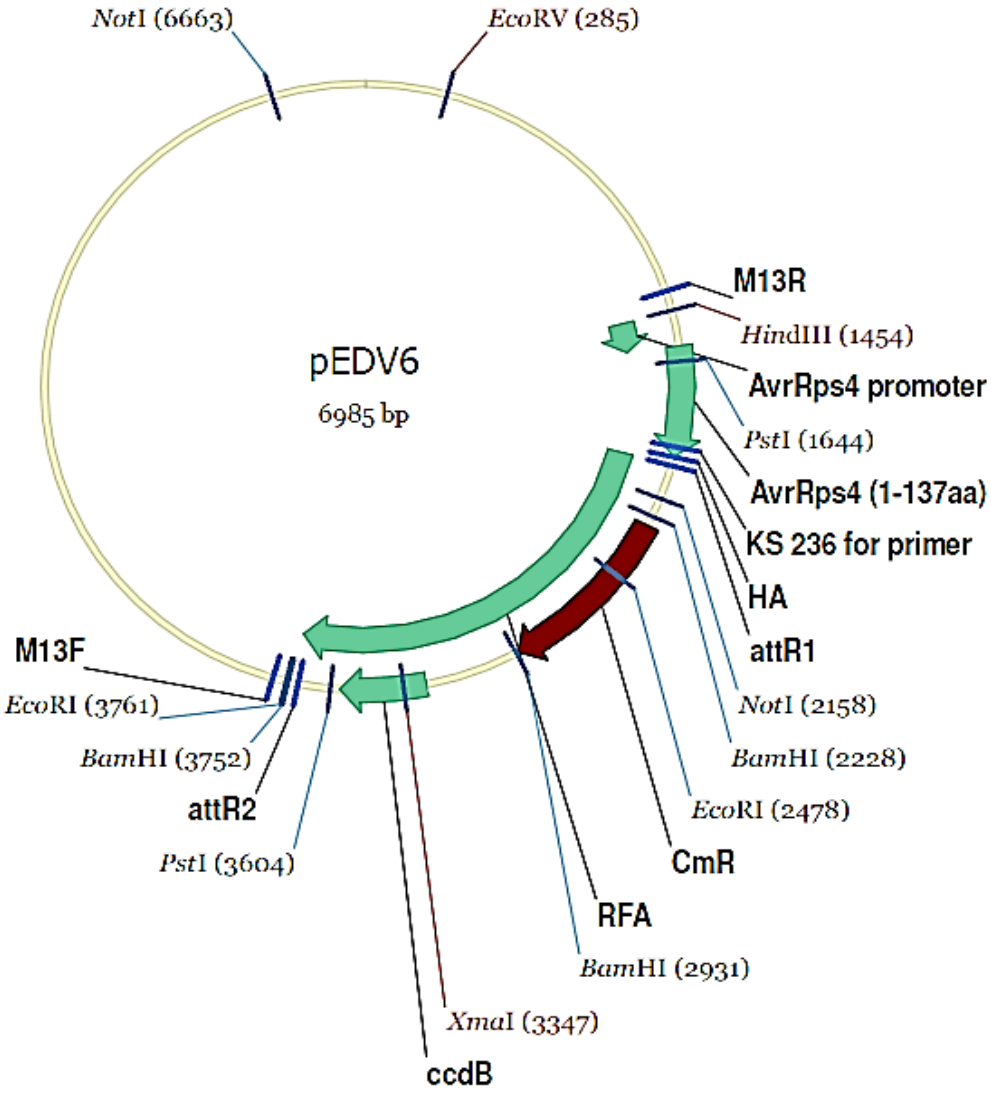

Figure 1.10 pEDV6 vector map.

1.8 PstHa12h2 effector gene

Effector genes which encode secreted proteins were predicted from haustorial cDNA library of infected wheat leaves. The full-length unisequences, open reading frames that have start and stop codons, were selected to determine putative secreted proteins from haustorial cDNA. Moreover, the signal peptide at the N-terminus was detected by the signal P 3.0 algorithm and iPSORT programs and they found 15 unisequences which have signal peptide at the N-terminus (Yin *et al.*, 2009). PstHa12h2 is one of these effector genes which are accepted putative secreted protein. Expression analysis of this gene revealed that it is expressed in all stages of development such as in urediniospore, germination of urediniospore and leaves with *Puccinia striiformis* f. sp. *tritici* (*Pst*) infection; however, the highest expression were seen in *Pst*-infected leaves. The ratio of expression level in infected leaves is ten-fold higher than expression level in germinated urediniospores and it is approximately eight-fold higher than urediniospores. It shows no homology sequence with the studied protein sequences. Because of high expression level in infected leaves, PstHa12h2 effector gene should play an important function in developing yellow rust disease by *Pst*. Moreover, it has five cysteine residues which have an important role in protein folding. It was reported that candidate effector genes have an increased number of cysteine residues (Yin *et al*., 2009).

1.9 Aim of the study

Domesticated cereals are an important food source for humans. Controlling the biotic (pathogens, viruses, nematodes) and abiotic (environmental, nutritional) stresses to grow cereals with high quality and high yield is a very important issue. Because of these stresses, huge yield losses occur every year in the world, also in Turkey. Thus, preventing yield losses, these stresses should be minimized. The chemicals for inhibition disease development are widely used in agriculture against biotic stress; however, this method is not the best solution and it gives much harm to the environment.

Wheat is the most important cereal in the world and yellow rust is a common and destructive biotic stress. In order to eliminate wheat yellow rust disease, the plant pathogen interactions in the molecular level should be understood well to develop more effective scientific solutions. By achieving this, genetically resistant crops can be developed against this disease for preventing yield losses and it can be environmentally safe solution.

Our aim is to identify the function of one of the candidate effector genes of *Puccinia striiformis* f. sp. *tritici* (PstHa12h2) and identify its interaction between the factors of wheat *Triticum*

aestivum L during infection. Thus, the gene construct was cloned into pK7FWG2 vector to analyze subcellular localization in *Nicotiana benthamiana* by GFP expression. For direct delivery of effector into wheat, this candidate gene was cloned into pEDV6 vector and hypersensitive response is expected to be observed if it interacts with R genes in the host wheat cell. In order to find the interactor proteins of this effector candidate in the host cell, the gene construct was cloned into pJL48-TRBO vector for the protein expression in *Nicotiana benthamiana via* agrobacterium-mediated gene transformation. The gene construct was synthesized with FLAG-Tag at its N-terminus, so the expressed protein can be purified by its tag. This purified protein can be immunoprecipitated to capture its interactor protein in *N. benthamiana* for identification by mass spectroscopy analysis in the future.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth conditions of plant material and pathogen maintenance

2.1.1 Wheat growth conditions and pathogen inoculation

Pst inoculation protocol was taken from Laboratoire de Pathologie Végétale, INRA BIOGER, Thiverval Grignon, France in April 2004. Seeds of the susceptible plant, Avocet-S, were used for *Pst* inoculation. Approximately, 40-50 seeds per pot (650 cm^3) each were sowed in soil. They were grown for ten days under 16-hour light at 18 ^oC and 8-hour dark at 15 ^oC in growth chamber. After ten days growth, the seedlings were ready for *Pst* inoculation with yellow rust spores. Spores that were kept at -80° C were placed into 42 ^oC for 10 minutes. If they were kept at +4 °C, they were incubated for 5 minutes at 42 ^oC. Then, the spores were sprayed on leaves by using equipment similar to atomizer in the sterile hood. The pathogen infected plants were kept at overnight dark at 10 °C with steam engine. After 16-18 hours, steam engine was taken out and the lights were turned out. About 10-15 days after infection, the spores were observed on leaves of the plants and they were collected.

Figure 2.1 *Pst* inoculation. The spores of *Pst* are sprayed on leaves by an atomizer equipment (by Ayşe Andaç, in the lab).

Figure 2.2 10 dpi incubation of infected wheat plants in growth chamber (by Ayşe Andaç, in the lab).

2.1.2 *Nicotiana benthamiana* growth

Nicotiana benthamiana seeds were planted and grown in pots which have 650 cm^3 volumes at 16-hour light and 8-hour dark cycle at 24 ^oC. After 1 week, they were sub-cultured that only one plant was planted in each pot (Gopinath and Kao, 2007). They became ready for agro-infiltration after 4-6 weeks.

2.2 PstHa12h2 gene construct design

PstHa12h2 sequence GenBank Accession number is GH737173 and it was obtained from express tag sequences (EST) of National Center of Biotechnology Information (NCBI) databases. The signal peptide encoding sequence of this sequence was predicted by using 'SignalP 4.0 Server' tool (http://www.cbs.dtu.dk/services/SignalP/). Then, the sequence which encodes signal peptide was removed because signal peptide directs delivery of the effectors and in our experiments it can send the effector gene outside of the host such as apoplastic fluid.

The gene was synthesized with additional restriction sites, *Pac*I at the 5'end and *Not*I at the 3'end, for directional cloning into pJL48-TRBO vector and FLAG-Tag region was added at the 5'end for co-immunoprecipitation after cloning into this vector (Figure 2.2). The synthesized gene was arrived as clone in pUC57 vector (Figure 2.3).

***Pac*I** **FLAG-Tag** **Linker**

TTAATTAAATG**GACTACAAGGACGACGATGACAAA**GTCAAGCTTCTCGAGAATTCC**GGC**
CTTGTCACGGAGGCAGAATCTGCGGCAGCGACGTCAGTCAAAGATGTCGGCGAAGGCGC
TCCCGAGTTTGGTGGCGACGATAAGTCCTGCGCTGAGTGTCATAGGTTGGGGGTGCAGT
GTATGTGGTGCTCCCGCGCTAAT**GCGGCCGC**

***Not*I**

Figure 2.3 PstHa12h2 gene construct (Accession No: GH737173). The gene has *Pac*I and *Not*I restriction sites at the 5'- and 3'ends respectively. FLAG-Tag was added at the 5'end of the gene and it is followed by linker sequence for independence of expressed protein and conserving its biological activity.

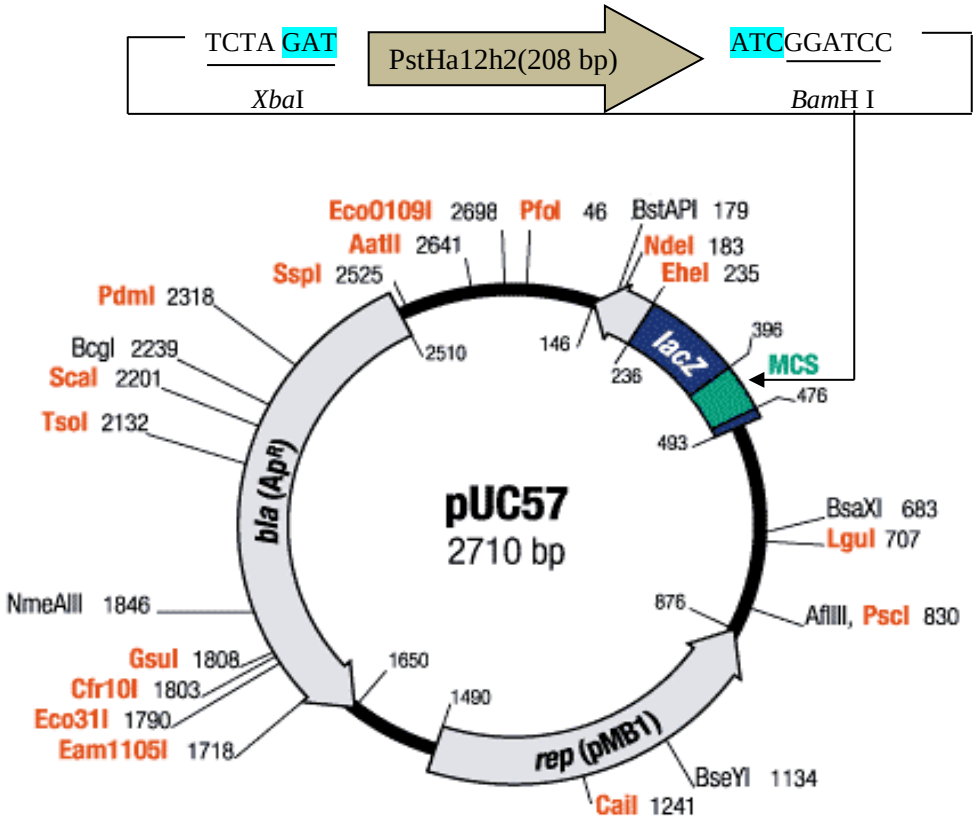

Figure 2.4 PstHa12h2 gene construct in pUC57 vector It was cloned in pUC57 (Amp^R) by *EcoR*V restriction sites which is represented in blue (The vector is retrieved from the website: http://www.thermoscientificbio.com/molecular-cloning/puc57-dna/).

2.2.1 PstHa12h2 primers

For cloning gene of interest into related vectors, the following primer sets were designed (Table 2.1). Pst12h2-CACC-Fwd primer was used to add CACC sequence to the gene of interest for cloning into pENTR/D-TOPO vector and then into pK7FWG2 destination vector by Gateway cloning. Pst12h2-Rev is the reverse primer for this cloning and it has no stop codon. For cloning into pEDV6 destination vector, the same forward primer Pst12h2-CACC-Fwd was used because of adding CACC site; but Pst12h2-Stp-Rev primer was used as reverse primer and it has stop codon. Pst12h2-Flag-Fwd and Pst12h2-Rev primers were used to clone into pJL48-TRBO

vector. In forward primer, there is FLAG-Tag sequence so that co-immunoprecipitation can be performed later.

Table 2.1 PstHa12h2 primer list.

Primer Names	**Primer Sequences (5'to 3')**
Pst12h2-CACC-Fwd	caccatgggccttgtcacggag
Pst12h2-NoSTP-Rev	attagcgcgggagcaccacata
Pst12h2-Stp-Rev	ctaattagcgcgggagcaccaca
Pst12h2-Flag-Fwd	ggattaattaaatggactacaaggacgacgatgacaaagtcaagcttct
Pst12h2-Rev	ggggaattcttaattagcgcgggagcaccac

2.3 Polymerase chain reaction (PCR)

For amplification of the gene of interest, PCR reaction was conducted with each set of primers. PCR reagents and their amounts for one reaction are listed at Table 2.2. PCR amplifications were conducted with Q5 High Fidelity DNA Polymerase (New England Biolabs).

Table 2.2 PCR components and their amounts per reaction.

PCR reagents (in 200µL PCR tube)	Amount	Final concentration
Template DNA	Variable (50pg-150ng)	~150 ng /rxn
5X Q5 Reaction Buffer (Cat # B9027S, Lot # 0021207)	5 µL	1X
10mM dNTPs (Cat # N0447S, Lot # 0711209)	0.5 µL	200 µM
10µM Forward Primer (10pmol/ µL)	1.25 µL	0.5 µM
10µM Reverse Primer (10pmol/ µL)	1.25 µL	0.5 µM
Q5 High Fidelity DNA Polymerase (2000U/ml) (Cat # M0491S, Lot # 0041209)	0.25 µL	0.05 U/µL
5X Q5 High GC Enhancer (Cat # B9028A, Lot # 0021207)	5 µL	(1X)
Nuclease Free Water	Variable (µL)	
Total PCR volume	25 µL	

After mixing of each tube, PCR reaction was performed at the conditions which are listed in Table 2.3 in thermocycler (Eppendorf Mastercycler Gradient). After, the PCR products were loaded on 1% agarose gel (AppliChem, Lot# 002306) in 1X TAE buffer (AppliChem, Lot# 7B010350).

Table 2.3 PCR conditions in thermocycler**.**

Step	Temperature, ^{o}C	Time	
Initial Denaturation	98	30 sec	
Denaturation	98	10 sec	35 cycle
Annealing	55	30 sec	
Extension	72	20 sec	
Final Extension	72	2 min	

2.4 Plasmid Isolations

QIAprep® Spin Miniprep Kit (Lot# 142349895) was used for plasmid isolation and its protocol with minor changes was applied. Overnight grown cells in 4 mL LB medium having appropriate antibiotic at 37 ^{o}C, 250 rpm were harvested by centrifuging (CLP Model 3410 Microcentrifuge) at 5000 rpm for 2 minutes in a clean 2.0 mL centrifuge tube. Supernatant was poured and pelleted bacterial cells which have plasmids were resuspended in 250 µL Buffer P1 containing RNAse A by vortexing. From this step, no more vortexing is applied because it causes the genomic DNA to shear. Next, 250 µL Buffer P2 was added and the tubes were inverted gently for 4-6 times until the solution appear as viscous, slightly clear and in blue color if the LyseBlue reagent was added in Buffer P1. Then, 350 µL Buffer N3 was added and mixed immediately by inverting the tubes 4-6 times until the blue color of the solution disappeared and becomes colorless and cloudy. After totally mixing the solution, the tubes were centrifuged for 10 min at 13,000 rpm in a micro centrifuge. At the end of centrifuging, a dense white pellet was formed. The supernatant was collected by pipetting in a QIAprep spin column and the spin columns were centrifuged for 1 min at 13,000 rpm. After discarding the flow through in the collection tubes, the spin columns were washed by adding 750 µL PE Buffer and it was centrifuged for 1 min at 13,000 rpm. The flow through collected in tubes was discarded again and the spin columns were centrifuged additional 2 min to remove the remaining PE Buffer. Then, the spin columns were placed in new 1.5 mL centrifuge tubes and the plasmids were eluted by adding 30-50 µL water. After adding, the spin columns were kept for 3-5 min for absorption of water in room temperature. Then, the tubes were centrifuged for 3-5 min at 13,000 rpm. Lastly, the spin columns were removed from centrifuge tubes and the concentrations of plasmids were measured by NanoDrop (ND-1000 spectrophotometer).

2.5 Gateway cloning

PstHa12h2 gene was inserted into entry vector named as pENTR^{TM}/D-TOPO (Kan^{R}) by topoisomerase mediated cloning. Then, this gene was transferred into pK7FWG2 (Spec^{R}) and pEDV6 (Gen^{R}) destination vectors by LR Clonase enzyme mix.

2.5.1 Cloning of PstHa12h2 gene into pENTR/D-TOPO vector

Before performing ligation reaction, PstHa12h2 gene was amplified with Pst12h2-CACC-Fwd and Pst12h2-NoSTP-Rev primers for pK7FWG2 vector and with Pst12h2-CACC-Fwd and Pst12h2-Stp-Rev primers for pEDV6 vector (Table 2.1). The PCR product was first cloned into pENTR vector. The PCR reaction and conditions are listed in Table 2.2 and Table 2.3, respectively. Invitrogen pENTR^{TM} Directional TOPO^{R} cloning kit (Lot # 1300973A, Invitrogen) was used and its protocol with minor changes which is listed in Table 2.4 was applied.

Table 2.4 The reagents and their amounts for insertion in pENTR/D-TOPO vector.

Reagents (in 200 µL PCR tube)	**Amounts (µL)**
PCR product	0.5
Salt solution	0.5
pENTR/D-TOPO vector	0.25
Nuclease free water	1.75
Total volume	3

In preparation this mix, vortexing or pipetting vigorously harm the pENTR/D-TOPO vector and it was mixed gently. The reaction was incubated for 30 min at 22-23 ^{o}C in thermocycler. Then, the tubes were transferred on ice for transformation.

2.5.2 *E. coli* TOP10 competent cells preparation

A single colony of *E. coli* TOP10 was inoculated in 4 mL LB (5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 1.6 mL NaOH (0.5 M) in 0.5 L ddH_2O, sterilized by autoclave) and grown overnight at 37 ^{o}C with shaking at 250 rpm with no antibiotics. From overnight culture, 1 mL cell was inoculated into 100 mL sterile LB medium and it was shaken approximately two hours at 250 rpm until $A_{600\ nm}$ reached to 0.375. Then, the culture was divided into pre-chilled two 50 mL falcon tubes and they were let sit on ice for 10 min. The cells were centrifuged for 5 min at 5000 rpm, at 4 ^{o}C. After centrifuging, the supernatant was discarded and pelleted cells in each tube were resuspended in filter sterilized 10 mL cold 100 mM $CaCl_2$ by gentle mixing or inverting.

The cells were let sit for 10 min on ice and they were centrifuged again for 5 min at 5000 rpm, 4 ^{o}C. Supernatant was discarded again and the cells in each tube were washed again as previously. The tubes were waited 30 min on ice and after that they were centrifuged again for 5 min at 5000 rpm, 4 ^{o}C. The supernatant was poured off and the pelleted cells were resuspended by 2 mL cold 100 mM $CaCl_2$. They were stored at +4 ^{o}C and they can keep their competence for 1-1.5 week.

2.5.3 Transformation of ligation product into *E. coli* competent cells

3 µL ligation product of pENTR/D-TOPO with PstHa12h2 gene was mixed gently with 100 µL *E. coli* TOP 10 competent cell, in a 1.5 mL microfuge tube. Then the mixture was incubated on ice for 10 min. After that, heat shock was applied at 42 ^{o}C for 45 sec in water bath. The tube was transferred back into ice immediately and incubated on ice for 5 min. Next, 500 µL LB medium was added to transformation product and it was incubated at 37 ^{o}C between 1-2 hours with shaking at 200 rpm. From which 50, 100 and 200 µL of cells were spread on LB agar plates containing 50 µg/mL kanamycin (LB agar plates are prepared by dissolving 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g agar, 1.6 mL NaOH (0.5 M) in 0.5 L ddH_2O *via* autoclave and after cooling of LB agar to about 50 ^{o}C, 50 µg/mL kanamycin (Kan) to final concentration was added). The cells were incubated at 37 ^{o}C overnight. From the plates, 4 or 5 colonies were selected randomly and for confirming successful transformed cells, colony PCRs were performed.

2.5.4 Colony PCR reaction

The PCR reaction was carried out by *Taq* DNA Polymerase with Standard *Taq* (Mg free) Buffer (NEB). Master mix was prepared for 5 samples, one of them was negative control that has no template and the mixture was divided into five 0.2 mL PCR tubes (Table 2.5).

Table 2.5 Master mix for verification of positive colonies.

Reagents	**Amount (µL) (for 1 rxns)**	**Final Concentration**
10X Standard *Taq* Reaction Buffer (Cat # B9015S, Lot # 0011112)	2.5	1X
10 mM $MgCl_2$ (Cat # B9021S, Lot # 0011107)	1.5	1.5 mM
25 mM dNTPs (Cat # N0447S, Lot # 0711209)	0.5	200 µM
10 µM forward primer	0.5	0.2 µM
10 µM reverse primer	0.5	0.2 µM
Template DNA	1	<1,000 ng
Taq Polymerase (Cat # M0320S, Lot #0141203)	0.125	0.025 U/µL
Nuclease free water	18.375	
Total volume	25	

As in the Table 2.1, Pst12h2-CACC-Fwd with Pst12h2-NoSTP-Rev primers were used for amplifications of the insert in the recombinant pENTR/D-TOPO vector which later transferred into pK7WGF2 vector and Pst12h2-CACC-Fwd with Pst12h2-Stp-Rev primers were used for verification of transformed cells which later transferred into pEDV6 vector. By using the combination in Table 2.5 for master mix for PCR reaction, the tubes were placed in thermocycler in the conditions which are listed on Table 2.6.

Table 2.6 PCR conditions for colony PCR by *Taq* DNA polymerase.

PCR steps	Temparature, ^{o}C	Time	
Initial denaturation	95	30 sec	
Denaturation	95	30 sec	30 cycle
Annealing	55	1 min	
Extension	68	1 min	
Final extension	68	5 min	

After PCR reaction, the products were observed on 1% agarose gel (0.5 mg agarose was dissolved in 50 mL 1X TAE buffer). The plasmid isolations were conducted from positive clones as in mentioned in Section 2.4 to use in LR clonase reaction to clone into destination vector.

2.5.6 LR clonase reaction

For transferring PstHa12h2 from pENTR/D-TOPO vector to destination vector, LR Clonase reaction was applied. This reaction was performed by using Invitrogen Gateway^{R} LR Clonase^{TM} II Enzyme Mix kit. For positive control, there is pENTR-gus provided by the kit. In negative control, the reaction should not work; because the destination vectors have *ccdB* lethal gene. In Table 2.7, the reaction content is represented. For cloning into pK7FWG2 and pEDV6 vectors from entry vector, the same amount of reagents was used; because their concentrations (in ng/µL) were approximately the same.

Table 2.7 LR clonase enzyme mix reagents and their amounts.

Reagent (in 200 µL PCR tube)	Sample (µL)	Negative Control (µL)	Positive Control (µL)
pENTR-gus (50ng/µL)	-	-	1
pENTR-Pst12h2(100ng/µL)	1	-	-
Destination vector (200ng/µL) (pK7FWG2 or pEDV6)	1	1	1
TE buffer (2X)	2.5	2.5	2.5
LR Clonase enzyme mix	0.5	0.5	0.5
ddH_2O	-	1	-
Total volume	5	5	5

The reagents listed in above table were mixed in 200 µL PCR tubes. LR clonase enzyme was added lastly and before adding the enzyme was let sit on ice for 2 minutes to thaw. The reaction components were mixed by gentle pipetting. The tubes were incubated at 25 ^{o}C for 1 hour. After incubation, 1 µL Proteinase K solution supplied with kit was added into each tube and they were mixed briefly by spinning. They were incubated at 37 ^{o}C for 10 minutes in order to end the reaction. At the end of the reaction, they were transferred on ice for transformation. Transformation procedure was followed which is mentioned in Section 2.5.3. However, this time 2.5 µL ligation product was mixed with 100 µL of *E. coli* TOP10 competent cells. The grown transformants in *E. coli* TOP10 cells as 50, 100 and 200 µL were spread on agar plates which have 100 µg/mL Spectinomycin. The plates were incubated at 37 ^{o}C for overnight. Then, 4-5 colonies were picked from plates and colony PCR was performed to verify the positive clones. The PCR protocol and conditions were followed which is mentioned in Table 2.5 and Table 2.6, respectively. From the positive clones, plasmid isolation was performed according to Section 2.4. After plasmid isolation, it was double digested by *Spe*I and *Ecor*I to verify the ligation reaction secondly. The reagents and their amounts are listed in Table 2.8.

Table 2.8 Double digestion of pK7FWG2-PstHa12h2 for verification.

Components (in 200 µL PCR tube)	Amounts	Final concentration
Plasmid DNA (~ 150 ng)	11.80 ng	~1700 ng/rxn
*Spe*I (10,000 U/ml) (Cat# R0145S, Lot # 0401105)	1 µL	10 U
*Xba*I (20,000 units/ml) (Cat# R3101S, Lot # 0031106)	1 µL	20 U
NEB buffer#4 (10x) (Cat # R0547s,Lot# 0641206,NEB)	1.5 µL	1X
BSA (100X) (Cat # R0547s,Lot# 0641206,NEB)	0.15 µL	1X
Total volume	15 µL	

The reaction was incubated for 2 hours at 37 ^{o}C and it was loaded on 1% agarose gel in 1X TAE buffer. Then, the digested inserts were detected on the gel.

2.6 *Agrobacterium*-mediated gene transfer

2.6.1 Preparation of GV3101 (pMP90) competent cells

Agrobacterium cells GV3101 (pMP90) strain were received from Dr. Csaba Koncz, Max-Planck Institute Plant Breeding Research Department, Cologne, Germany. This strain has the ability to transform with all binary vectors, if the vector has an autonomous replicon for *Agrobacterium*. Moreover, GV3101 pMP90 is resistant to 100 mg/mL Rifampicin (GV3101 chromosomal marker) and 25 mg/mL Gentamycin (marker of the pMP90 helper Ti plasmid) (Konczl & Schell, 1986).

First, streak plate (containing 2.5 µg/mL Tetracycline (Tet)) was performed from GV3101 (pMP90) -80 ^{o}C stock cells and they were incubated for 2 days at 28 ^{o}C. Then from grown colonies, a single colony was picked and inoculated in 4 mL LB medium containing 2.5 µg/mL Tet. It was grown overnight at 28 ^{o}C with shaking at 250 rpm. Inoculate of 1 mL was used for

the overnight culture and inoculated again into 100 mL LB medium which has Tet (5 µg/mL) for grown overnight at 28 ^{o}C with shaking. Optical density (A_{600nm}) was measured in the following morning and the desired range was between 0.5-0.7. After measuring the optical density, the grown culture was divided into two 50 mL falcon tubes and they were let sit on ice for 30 min. Then, centrifugation was performed for 15 minutes at 3500 rpm, 4 ^{o}C and the supernatant was poured off. The pelleted cells were washed by filter-sterilized 50 mL ice cold 10 % glycerol. Centrifugation was conducted for 15 minutes at 3500 rpm, 4 ^{o}C and the supernatant discarded again. The pelleted cells were re-suspended by filter-sterilized 50 mL ice cold 10 % glycerol one more time and centrifugation performed at the same conditions. Supernatant was discarded and this time, the pelleted cells were re-suspended in 200 µL GYT medium which is composed of 10 % glycerol, 0.125 % yeast extract, and 0.25 % tryptone. Lastly, the competent cells were aliquoted in 1.5 mL micro centrifuge tubes, containing 50 µL competent cells in each one and the tubes were stored at -80 ^{o}C.

2.6.2 Electroporation by *Agrobacterium* competent cells

2 µL of pK7FWG2-PstHa12h2 plasmid was mixed with 50 µL GV3101 (pMP90) competent electroporation cells and the sample was nested on ice approximately 10 min in a 1.5 mL microcentrifuge tube. Next, the sample was transferred into pre-chilled pulser cuvette which has 1mm gap. The cuvette was placed in electroporator (Celljject duo, Therma Corp.) and 2.2 kV electro-shock was applied to the cells. After, 1 mL SOC medium was added to the cuvette and it was transferred to 1.5 mL microcentrifuge tube. The cells were grown for 1.5 hours at 28 ^{o}C. The grown cells were spread as 50,100,200 µL cells on agar plates contain 100µg/mL Spectinomycin. The plates were incubated at 28 ^{o}C approximately for 2 days. After colonies were grown on plates, 5 of them were selected and they were inoculated into falcon tubes containing 4 mL LB with 100 µg/mL Spectinomycin. The falcon tubes were incubated 28 ^{o}C for 2 days. For verification of the clones, colony PCR was conducted for each colony. Colony PCR procedure and conditions were followed in Table 2.5 and Table 2.6, respectively. From the positive clones, the grown cultures were stocked at -80 ^{o}C with 50% glycerol.

2.6.3 Agro-infiltration into *Nicotiana benthamiana*

Before agro-infiltration, preparations of mediums and solutions were performed. First, LB media which includes 10 g tryptone, 5 g yeast extract, 5 g NaCl, 3.2 mL NaOH per L, 0.1 M acetosyringone in DMSO, 0.5 M MES (pH 5.7), 1M $MgCl_2$ were prepared. Then, L-MESA medium which was composed by 10 mM MES pH 5.7, 20 µM acetosyringone in LB media, and agroinduction medium which was composed of 10 mM MES pH 5.7, 10 mM $MgCl_2$ 150 µM Acetosyringone in ddH_2O were prepared (Table 2.8). Also, preparations of 50 µg/mL Kanamycin (Kan), 10 µg/mL Rifampicin (Rif) and 25 µg/mL Gentamicin (Gen) were performed for adding the mediums.

Table 2.9 Agro-infiltration media content.

Media	Amounts and contents
LB media	10 g tryptone, 5 g yeast extract, 5 g NaCl, 3.2 mL NaOH per L
L-MESA	10 mM MES pH 5.7, 20 µM Acetosyringone in LB media
Agroinduction media	10 mM MES pH 5.7, 10 mM MgCl2, 150 µM Acetosyringone in ddH2O

Acetosyringone was added in agroinduction medium and L-MESA just before use. Concentrations of antibiotics were the final concentrations in L-MES and L-MESA.

From the stock Agrobacterium cells which have pK7FWG2-PstHa12h2, streak plate containing Spec was performed and they were incubated at 28 ^{o}C for two days. After growing of the colonies, single colonies were selected and each one was inoculated into 3 mL L-MESA (with Kan, Rif, and Gen) medium in 15 mL falcon tubes. The tubes were incubated at 28 ^{o}C for 24 to 30 hours with shaking at 250 rpm. The cell cultures must be very dense that they should be in log or late stationary phase. 500 µL Agrobacterium culture was taken from the grown cells and they were transferred into 5mL L-MESA (with Kan, Rif and Gen). They were grown at 28 °C, with shaking at 250 rpm for 6-8 hours. Optical density (OD) at 600 nm was measured and the desired absorbance was between 0.8 and 1.0. After reaching this optical density, the Agrobacterium cells were centrifuged at 3500X g for 10 minutes in microcentrifuge. The supernatant was poured off without destroying the pelleted cell. The pellet was resuspended by adding agro-induction media until A_{600nm} reached 1.0. This Agrobacterium cells in agro-induction media were waited overnight at room temperature. The leaves of *Nicotiana benthamiana* plants which were 5 weeks old were labeled properly. From the overnight media, the Agrobacterium cell cultures was taken by 0.5 mL syringe without needle and it was

infiltrated at the center of the one half of the leaf underside. At the same time, opposite side of the leaf which syringe infiltrates was covered by a gloved finger.

Figure 2.5 Infiltration of Agrobacterium cell cultures to *N. benthamiana* leaves (by Ayşe Andaç).

2.7 Visualization of subcellular localization in *Nicotiana benthamiana* leaves

After two days post inoculation, infiltrated leaves cut randomly around the sites of infiltration. They were placed on glass slides and after pouring some water drops on them, they were closed with cover slip. Imaging was performed by both light microscope (Leica, DFC 280) and confocal microscope (Zeiss, LSM 500). In light microscope, 40X and 100X magnifications with GFP filter was used and in confocal microscope, 40 X oil-immersion objective was used. More detailed and higher resolution analyses can be observed with confocal microscope than the light microscope.

2.8 Ligation of PstHa12h2 into pJL48-TRBO vector

In order to ligate PstHa12h2 into pJL48-TRBO vector, amplification of the GOI was performed by the procedure mentioned in Table 2.2 and the conditions mentioned in Table 2.3. For PCR reaction, Pst12h2-Flag-Fwd with Pst12h2-Rev primers were used which are listed in Table 2.1. pJL48-TRBO vector was inoculated in four falcon tubes contain 4 mL LB medium each from -80 ^{o}C stock and it was grown for overnight at 37 ^{o}C. From the overnight cultures, plasmid isolation was performed by the same procedure in Section 2.4 with small changes. The first one was in collection of the cells by microcentrifuge. The cells in two falcon tubes were collected in one 1.5 mL microcentrifuge tubes and the same was performed for the other two falcon tubes. Another change was when the dense white pellet was formed after centrifuge; the supernatant was collected in one spin column to increase the concentration of the plasmid.

Normally, for inserting the GOI into pJL48-TRBO vector, first PCR amplification was performed and the entire PCR product was loaded on gel. Then, it was purified by gel extraction method and from this eluted DNA, insert was double digested. However, because of the small size of the insert, it was difficult to be sure of digestion of it. Thus, after PCR reaction which is explained in Section 2.7, TA cloning was performed by pGEM-T-Easy vector (Promega). The components and their amounts are listed in Table 2.9.

Table 2.10 The reagents and their amounts for TA cloning.

Reagents (in 200 µL PCR tube)	Amounts (µL)	Final Concentration
PCR product	5	
pGEM®-T or pGEM®-T Easy Vector (50ng/µL) (Cat # A137A, Lot # 20635703)	1	50ng
10X T4 Ligase Buffer (Cat # B0202S, Lot # 0021105)	1	1X
T4 DNA ligase with 10 mM ATP (5 unit/µL) (Cat # M0203S, Lot # 0391106)	0.1	0.5U
ddH_2O	2.9	
Total Volume	10	

The mixture was incubated at 1 hour at 22 ^{o}C. Then, it was waited overnight at +4 ^{o}C. Next, it was transformed to *E. coli* TOP10 competent cells according to Section 2.5.3. Then, it was spread as 50, 100, 200 µL on the agar plates which has Ampicillin (Amp) and the plates were incubated overnight at 37 ^{o}C. In the following day, single colonies were selected and they were inoculated into falcon tubes containing 4 mL LB medium with Amp and again they were grown overnight at 37 ^{o}C. The colony PCR was conducted from the grown cultures to verify the positive clones. PCR protocol and conditions was the same as in Table 2.5 and Table 2.6, respectively. After verification, the plasmid isolation was performed from grown cultures according to Section 2.4.

2.8.2 Double digestion of pJL48-TRBO vector and gene of interest

pJL48-TRBO vector was double digested by *Pac*I (New England Biolabs (NEB)) and *Not*I HF (NEB) restriction enzymes to produce sticky ends. With the same restriction enzymes Pstha12h2 was double digested from pGEM-T-Easy vector. The digestion components and their amounts are shown in Table 2.9. In the Table 2.9, vector refers to both pJL48-TRBO and pGEM-T-Easy vector ligated with PstHa12h2. Approximately 1000 or 1500 ng vector or insert concentration was sufficient for double digestion.

Table 2.11 Digestion mixture contents and their amounts.

Reagents (in 200 µL PCR tube)	Amounts (µL)	Final Concentration
Vector	10	(~ 100-150 ng/rxn)
*Pac*I (10,000unit/mL) (Cat # R0547s,Lot# 0641206,NEB)	0.5	5U
*Not*I HF (20,000unit/mL) (Cat# R31895,Lot#0031112,NEB)	0.25	5U
NEB buffer 4 (10x) (Cat # R0547s,Lot# 0641206,NEB)	2	1X
BSA (100X) (Cat # R0547s,Lot# 0641206,NEB)	0.2	1X
ddH_2O	7.05	
Total	20	

The double digestion reaction was incubated for 3 hours at 37 ^oC. The products were loaded on 1% agarose gel in 1X TAE buffer.

2.8.3 Gel extraction of linearized pJL48-TRBO vector and PstHa12h2 gene fragment

After double digested products were analyzed on agarose gel about 1 hour, the bands were cut from gel. QIAquick Gel Extraction Kit (Qiagen) (Cat # 28706, Lot # 139312248) was used and manufacturer's protocol was applied.

The cut gel slices were placed in 1.5 mL microcentrifuge tubes and their weights were measured. In this step, DNA bands should be taken from gel very quickly under UV. 3 volumes of Buffer QG was added to 1 volume of gel (1 mg = ~1 µL). The samples were heated to 50 ^oC about 10 min in water bath for dissolving of the gel. Every 2-3 min, the tubes were mixed by spinning. After completely dissolving of the gel, 1 volume of isopropanol was added to each tube and mixed. The mixture was transferred into QIAquick spin column in a provided 2 mL collection tube to bind DNA. The spin column was centrifuged for 1 min at 13,000 rpm in microcentrifuge. The flow through in the collection tube was poured off and 0.5 mL of Buffer QG was added again to remove remaining agarose. The spin column was centrifuged again for 1 minute at

13,000 rpm. The flow through was discarded and the column was washed by 0.75 mL Buffer PE. It was waited for 3-5 min at room temperature to remove salts. Then, it was centrifuged for 1 min at 13,000 rpm. The flow through was poured off and the spin column was centrifuged again about 2 min to remove all wash buffer from the column. The spin column was removed from collection tube and it was transferred to a new 1.5 mL microcentrifuge tube. 30-50 µL nuclease free water was added and it was waited for 3-5 min at room temperature. After waiting, it was centrifuged for 2 min at 13,000 rpm. Finally, the eluted DNAs were analyzed on 1% agarose gel in 1X TAE buffer to see whether DNAs were extracted from bands.

2.8.4 Ligation reaction of pJL48-TRBO vector and PstHa12h2 insert

From eluted DNAs by gel purification, PstHa12h2 insert was ligated into pJL48-TRBO vector by their *Pac*I and *Not*I sites. The ligation reaction was performed according to reagents and amounts which are listed in Table 2.12.

Table 2.12 The reagents and amounts for ligation.

Reagents (in 200 µL PCR tube)	**Amounts (µL)**	**Final Concentration**
10X T4 ligase buffer (Cat # B0202S, Lot # 0021105)	1	1X
pJL48-TRBO vector	1	~300ng
PstHa12h2 insert	2	~1000ng
T4 DNA ligase with 10 mM ATP (5 unit/µL) (Cat # M0203S, Lot # 0391106)	0.2	1U
ddH_2O	6.4	
Total Volume	10	

The reaction mix was incubated at 22 ^{o}C for 1 hour at thermocycler. After 1 hour, the ligation reaction was waited at +4 ^{o}C for overnight. The day after, 5 µL of the ligation product was transferred into 100 µL *E. coli* TOP10 competent cell and the same transformation protocol was performed in Section 2.5.3. The transformant cells were grown in 500 µL LB medium for 1-1.5

hour at 37 ^{o}C with shaking at 250 rpm. The grown cells were spread as 50, 100 and 200 µL on agar plates containing 50 µg/mL Kanamycin. The plates were incubated overnight at 37 ^{o}C. In the following day, the single colonies were selected from plates and they were inoculated in falcon tubes which have 4 mL LB with 50 µg/mL Kanamycin. Colony PCR was performed from grown cultures to verify positive clones. The procedure and conditions of colony PCR was followed in Table 2.5 and Table 2.6, respectively. Pst12h2-Flag-Fwd with Pst12h2-Rev primers were used in PCR reaction which is mentioned in Table 2.1. Next, plasmid isolation which is mentioned in Section 2.4 was conducted for second verification of the positive clones. The isolated plasmid was double digested with *Pac*I and *Not*I HF restriction enzymes. The same procedure was applied as in Table 2.10 and the mixture was incubated at 37 ^{o}C for 3 hours. The double digest product was analyzed on 1% agarose gel in 1X TAE buffer to see the insert which was digested from the vector. Thus, clones were determined as definitely positive.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Subcellular localization of PstHa12h2 effector gene

3.1.1 PCR reaction of PstHa12h2

For cloning into pK7FWG2 vector, PCR was performed with Pst12h2-CACC-Fwd and Pst12h2-NoSTP-Rev primers with PstHa12h2 template as in Section 2.3. Because GFP is located at the end of gene of interest and it is expressed after the gene of interest, stop codon was not included in the amplification of PstHa12h2 gene by this reverse primer. Then, the PCR products were loaded on 1% agarose gel which is shown in Figure 3.1.

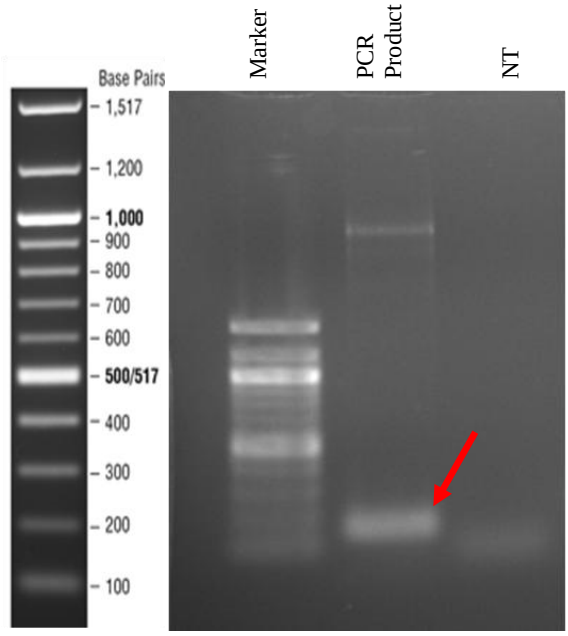

Figure 3.1 Agarose gel of PstHa12h2 PCR amplification with Pst12h2-CACC-Fwd and Pst12h2-NoSTP-Rev primers. First lane: 1 µL of 0.5 µg/µL 100 bp Ladder (Cat # N3231S, Lot # 0831006, NEB) (Marker) was loaded. Second lane: 2 µL PCR product was loaded (indicated with an arrow). Third lane: No template PCR product (indicated with NT) was loaded. The PCR product was in the expected size (145 bp). All three samples were loaded with 1X loading dye (Cat # R0611, Fermentas).

3.1.2 Cloning of PstHa12h2 into pENTR/D-TOPO vector

The fresh PCR product was used for pENTR/D-TOPO vector cloning mentioned in Section 2.5.1. The kit procedure was performed with some changes. To use the enzyme amount in minimum without decrease its effectiveness, the total volume of reaction was reduced to its half amount. By reducing the total volume led to decrease in the PCR product amount. This was a favorable change; because high amounts of PCR product decreases the chance of pENTR/D-TOPO cloning. In the first trial, high amount of PCR product decreased the efficiency of cloning. After transformation and spreading of the cell on agar plates, single colonies were selected in the following day. They were grown in LB medium for overnight and then PCR was conducted from grown cultures as in Section 2.5.4. From the PCR products, 4 of them (one of them is a negative control) were loaded with on 1% agarose gel in 1X TAE buffer (Figure 3.2.A). After verification of the clones, one positive clone was selected and plasmid isolation, as in Section 2.4, was performed Figure (3.2.B). The plasmids were stocked at – 80 ^{o}C for the later usage.

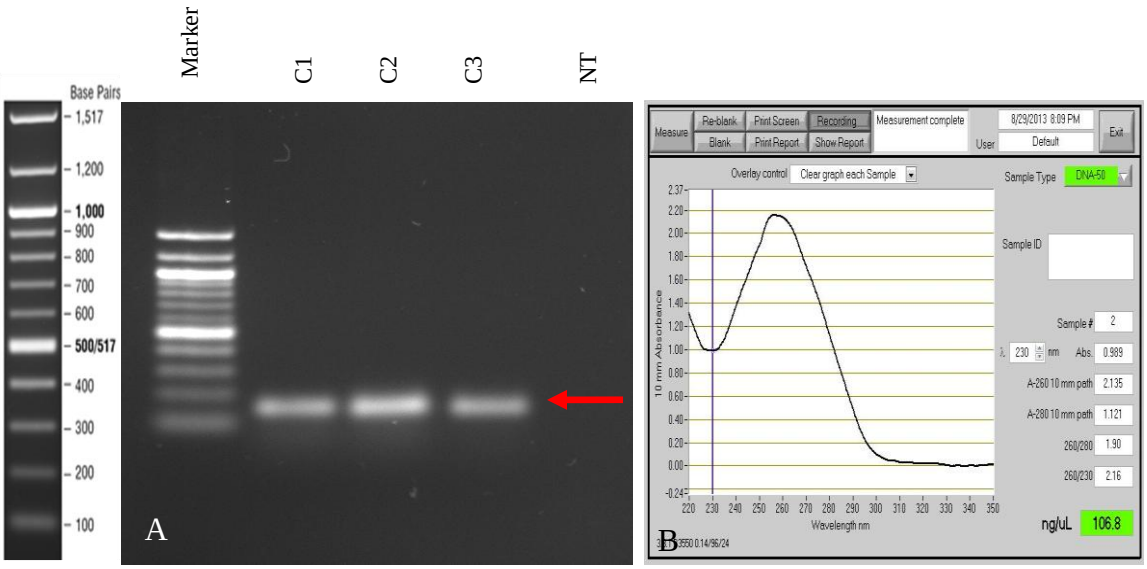

Figure 3.2 Verification of positive colonies for pENTR/D-TOPO cloning and concentration profile of one of the clones. **A)** In the 1st lane, there was 100 bp ladder (Marker). In the second 2nd, 3rd, and 4th lanes 4 µL colony PCR products were loaded. NT represents no template PCR product. The clones represented bands in the expected size (145 bp) which are shown by an arrow. **B)** pENTR-PstHa12h2 plasmid isolation result was shown, and the concentration (106.8 ng/µL) was well enough for LR reaction.

3.1.3 LR reaction

LR reaction for pK7FWG2 vector was performed as in Section 2.5.6. Minor changes were applied to the kit protocol. Total volume of the reaction was reduced to half, so enzyme amount decreased in half too. After transformation into *E. coli* TOP 10 competent cell, they were spread on agar plates. In the following day, no colony was observed in negative control plates. This was an expected result; because pK7FWG2 vector has *ccdB* lethal gene between *att*R sites expressed and function properly for negative selection. Thus, transformants did not grow. In positive control plates, many colonies were observed on the plates. This was also an expected result, because pENTR-GUS entry vector was used which was provided in the kit. For GOI plates, there were many colonies which had pK7FWG2 vector ligate with Pstha12h2 insert transformants. Single colonies were taken and grown as mentioned in the Section 2.5.6. PCR reaction was performed from the grown cultures for verification by the procedure and conditions in Table 2.5 and Table 2.6 with Pst12h2-CACC-Fwd and Pst12h2-NoSTP-Rev primers (Table

2.1). Next, four of them (one of them is negative control) were loaded on 1% agarose gel in 1X TAE buffer which is shown in Figure 3.3 A. From the gel picture, it was verified that the selected colonies were positive. Plasmid isolation was conducted from the colony 2 grown culture as in Section 2.4 (Figure 3.3 B). For the second verification, isolated DNA of clone 2 was double digested by *Spe*I and *Ecor*I HF (Cat# R3101s, Lot# 0001106) restricion enzymes mentioned in the Section 2.8. Also, they were stocked at -80 ^{o}C for further use.

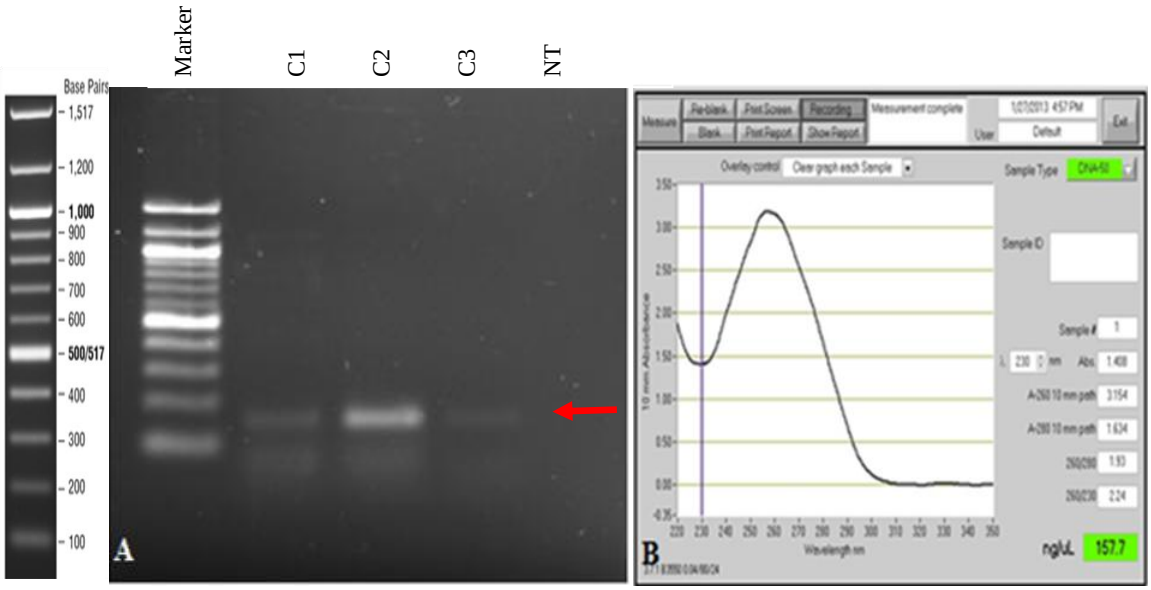

Figure 3.3 Colony PCR of pK7FWG2-PstHa12h2 and Nanodrop result after plasmid isolation. **A)** 100 bp Ladder (Marker) was loaded on the first lane. In the 2nd, 3rd, and 4th lane, 4 µL PCR products were loaded. NT was the no template PCR product. The bands resulting from PCR amplification of the clones were in expected size (145 bp). **B)** Absorbance of pK7FWG2-PstHa12h2 after plasmid isolation, showing that isolation was succesfull and concentration which is 157 ng/ µL is suitable for verification by restriction enzymes and then for transformation into *Agrobacterium.*

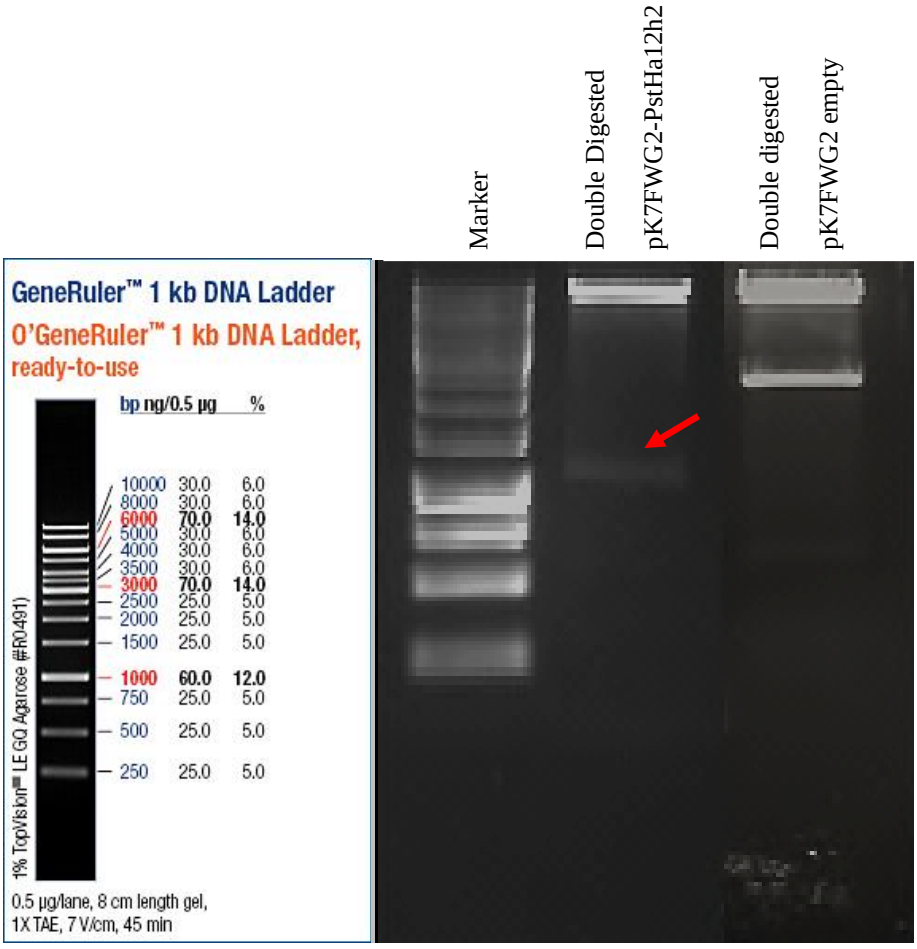

Figure 3.4 Double digestion (with *Spe*I and *Xba*I) of pK7FWG2-PstHa12h2 and pK7FWG2 empty vector. 0.5 µg/µL 1kb ladder (Marker) (Cat # SM0311, Lot # 00093172, Fermentas) was loaded in the first lane. In the other wells, digestion products were loaded.

In the Figure 3.4, two bands were observed in the empty vector lane when it was digested by these restriction enzymes. When the PstHa12h2 was inserted into vector,the vector losed its *att*R site and it produced two bands again after digestion but this time it was shorter then the empty vector band. *Spe*I restriction site is found at the end of the *att*R site and after digestion from this site, it produces a band (shown by an arrow) between 1000 and 1500 bp due to the size of the insert which comes instead of the *att*R site. After second verification of the clones, they were ready for transformation into *Agrobacterium tumefaciens*.

3.1.4 Gene transfer by *Agrobacterium* and Agro-infiltration

From the grown cultures of Agrobacterium which had pK7FWG2-PstHa12h2, colony PCR reaction was performed for verification. PCR reaction was performed by the same protocol as in Table 2.5 and Table 2.6, respectively. The Pst12h2-CACC-Fwd and Pst12h2-NoSTP-Rev primers were used from the Table 2.1. PCR products of 3 clones were loaded 1% agarose gel in 1X TAE buffer.

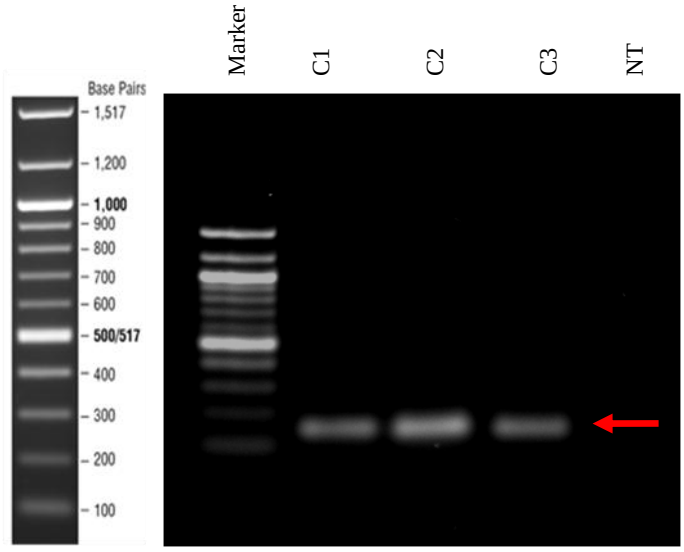

Figure 3.5 Verification of pK7FWG2-PstHa12h2 in *Agrobacterium* by PCR amplification. 100 bp ladder (Marker) was loaded in the 1st lane. In the 2nd ,3rd and 4th lanes, 4lanes, 4µL PCR products were loaded indicated by an arrow. They were in the expected size (145 bp). NT represents no template PCR product.

After verification of the clones, they were stocked at -80 ^{0}C with 50 % glycerol. For agro-infiltration, streak plate was performed from one of these stocks as in Section 2.6.3. After it was grown, it was inoculated into L-MESA and agro-induction medias. Infiltration was conducted on 5 weeks old *Nicotiana benthamiana* plants and it was performed on randomly selected leaves. Leaves which were located at upper parts had young leaves and infiltration was conducted easily on them. Young leaves have thin cuticle and more hairs than older leaves which make infiltration process easy but because of having limited plant samples, infiltration was performed both upper and below leaves for having more infiltrated samples. We had limited number of *Nicotiana* plants, because temperature and humidity requirements of this plant are very critical

for their growth and optimization of these conditions is very hard. For visualizaton, two days post inoculation leaves were cut from infiltration sites and they were analyzed with light microscope and confocal microscope which is shown in Figure 3.6.

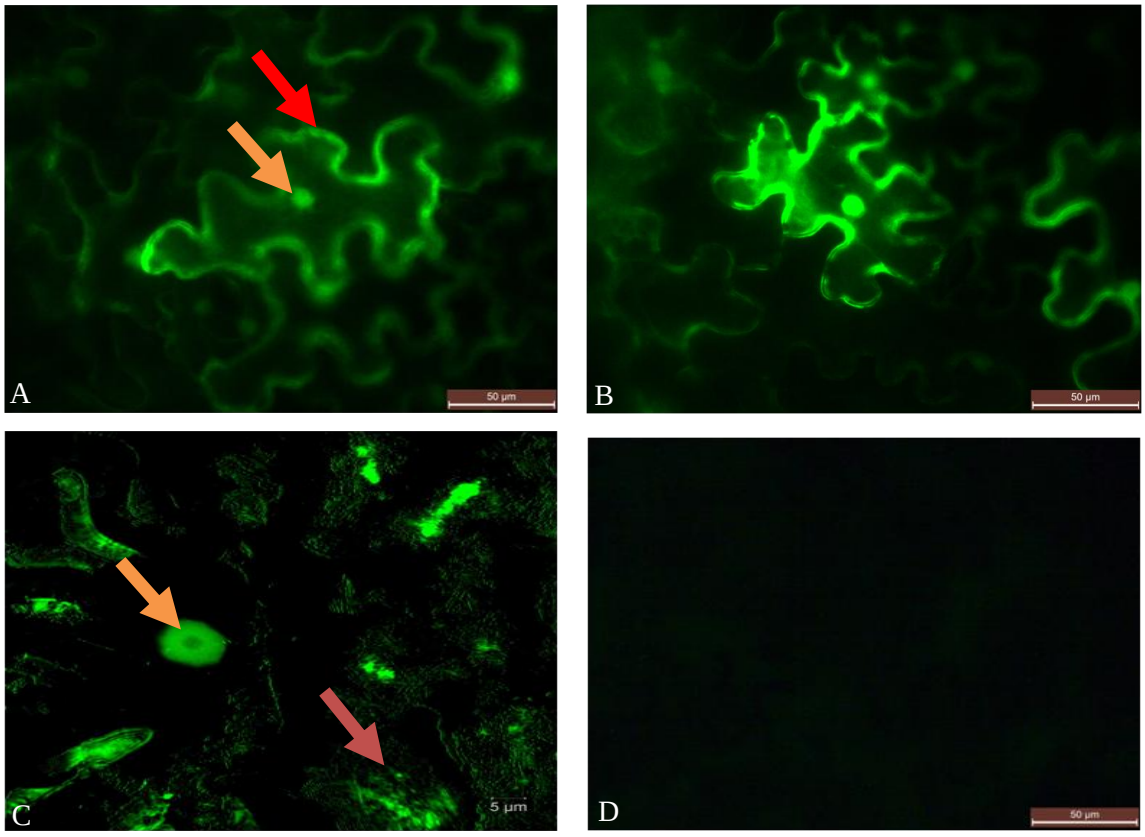

Figure 3.6 Imaging of Pstha12h2 effector protein subcellular localization in *Nicotiana benthamiana*. In this part, imaging was performed with 2 dpi leaves samples magnification. Subcellular localization was detected by GFP tagged transiently protein expression encoded by PstHa12h2. **A)** and **B)** Observation was conducted by light microscope (Leica, DFC 280) with GFP filter in 40X magnification. **C)** 40 X magnification with GFP filter of confocal microscope (Zeiss, LSM 500) was used for imaging. **D)** Non-infiltrated leaf was analyzed at 40 X magnification of light microscope with GFP filter.

Subcellular localization studies can assist in determining the classification of a particular protein and it can give more information about its possible function (Kumar & Kirti, 2011). Thus, PstHa12h2 effector candidate gene was analyzed by generating a GFP fusion in its C-terminus. Generally, most effector proteins show nucleus and cytosol localization pattern because they are localized mostly in those areas (Kumar & Kirti, 2011). In the A and B part in Figure 3.6, we can

conclude that the effector gene localization is found both in nucleus (orange arrow) and cytosol (red arrow). From this information (Kumar & Kirti, 2011), this protein may be a true effector protein. However, if it possesses an avirulence activity to any YR-gene is yet to be tested. The part C of the figure, which was obtained using a confocal microscope, the effector with tagged GFP expression was detected in the nucleus but the almost no expression was observed in nucleolus. Based on the motif search, we did not detected any Nucleolar localization Signal (NoLS), which is in accordance with our observation, nevertheless, not all the proteins localized in the nucleolus may not require NoLS (Leonelli L. *et al*., 2011; Caillaud *et al*.,2011). The absence of this effector in the nucleolus may suggest that the effector it is not involved in any of the nucleoli activities, such as ribosome biosynthesis and cell cycle regulation, DNA replication and repair (Leonelli L. *et al*., 2011). However, its presence in nucleus and in cytoplasm suggests multiple roles for pathogenesis. The simple motif search (http://www.genome.jp/tools/motif/) with some databases available indicated the presence of interesting motifs such as Protein phosphatase inhibitor domain, and Type III secretion system inner membrane Q protein, and plexin repeat motif similarities as presented below. These putative functions can be tested.

A

Motif in your sequence

Blocks Name:

IPB011107C

Description:

Protein phosphatase inhibitor

Appearance (Score):

Position	Sequence	Score
47..56	GDDKSCAECH	1087

Sequence:

MKCFTFTAALLAAALIAGTSAGLVTEAESAAATSVKDVGEGAPEFGGDDKSCAECHRLGV QCMWCSR

B

Motif PSI in your sequence

Pfam ID:

PSI

Description:

Plexin repeat

Appearance (Independent E-value):

Position	Sequence (Upper = Query / Lower = Database)	i-Evalue
29..46	KSCAECHRLGV-QCMWCSR tsCssClaardpyCgWcsd	0.36

Sequence:

GLVTEAESAAATSVKDVGEGAPEFGGDDKSCAECHRLGVQCMWCSR

C

Motif in your sequence

Blocks Name:

IPB002191A

Description:

Type III secretion system inner membrane Q protein

Appearance (Score):

Position	Sequence	Score
4..25	FTFTAALLAAALIAGTSaGLVt	1062

Sequence:

MKCFTFTAALLAAALIAGTSAGLVTEAESAAATSVKDVGEGAPEFGGDDKSCAECHRLGV QCMWCSR

Figure 3.7 continued Motif search results of PstHa12h2 gene. Part A and C of the figure, the effector sequence was searched with its signal peptide, and in the B part, the sequence was searched without its signal peptide.

3.2 Cloning PstHa12h2 into pEDV6 vector

For delivering effector proteins directly into wheat, *Pseudomonas fluorescens* mediated gene transformation is used by pEDV6 vector. First, the gene of interest was amplified with PCR reaction for pENTR/D-TOPO cloning.

3.2.1 PCR amplification of PstHa12h2

The procedure and conditions for PCR reaction were described as in Table 2.2 and Table 2.3, in respective. Pst12h2-CACC-Fwd and Pst12h2-Stp-Rev primers were used listed in Table 2.1. This time, there was no need to eliminate the stop codon, because vector does not have an expression reporter. The delivery of the effectors can be detected by staining hydrogen peroxide accumulation instead of detection of a reporter gene expression like GFP. This is advantageous because this enables to detect hypersensitive response which is resulted from *Avr* gene interaction with corresponding *R* gene. After PCR reaction, the PCR products were analyzed on 1% agarose gel in 1X TAE buffer.

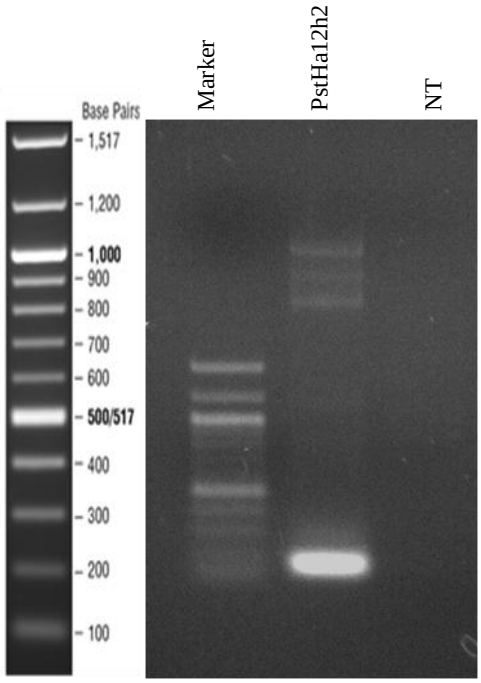

Figure 3.8 PCR amplification of PstHa12h2 with Pst12h2-CACC-Fwd and Pst12h2-Stp-Rev primers. 100 bp ladder (Marker) was loaded on the first well. In the second well, 4 µL of PCR product was loaded and no template PCR product was loaded on the last well. The band was observed on the expected size (148 bp).

3.2.2 pENTR/D-TOPO cloning

Fresh PCR product was used directly for cloning into entry vector. The same changes were applied in this cloning which was explained in Section 3.1.2 and efficient number of clones was observed on the plates. From the clones, colony PCR was performed for verification of positive clones. The PCR products were observed 1% agarose gel in 1X TAE buffer (Figure 3.8 A). The plasmid isolation was conducted which is mentioned in Section 2.4 from the first clone (Figure 3.8 B).

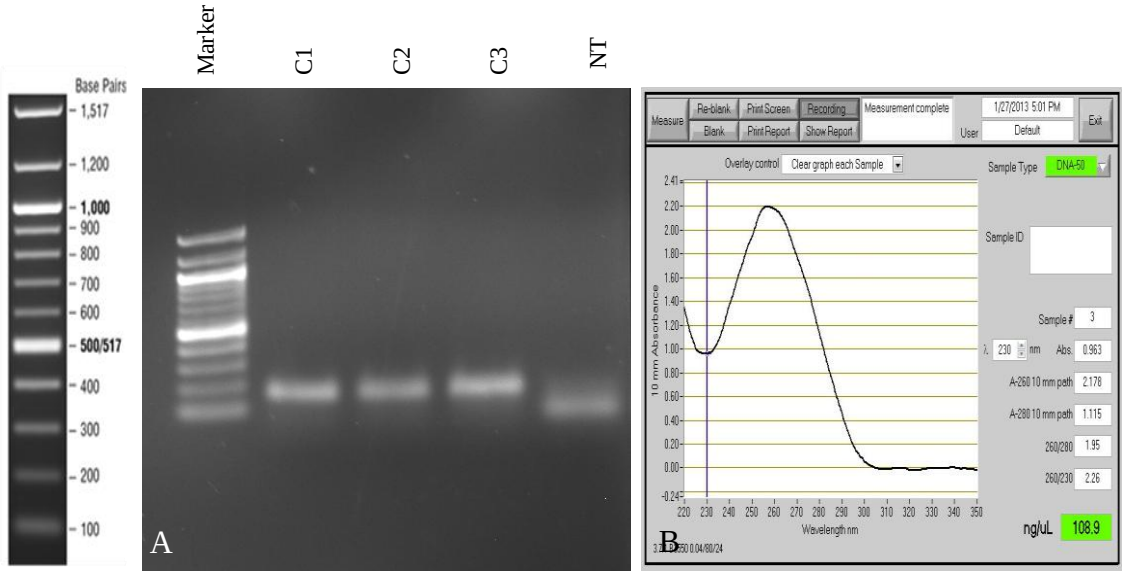

Figure 3.9 Verification of the positive clones after pENTR/D-TOPO cloning and concentration profile after plasmid isolation**. A)** In the first well, 100 bp ladder (Marker) was loaded. In the second, third and fourth lanes, 4 µL PCR products of clone 1, clone 2, and clone 3were loaded. No template PCR product was loaded on the last lane. The bands resulted from PCR amplification of the clones was in expected size (148 bp). **B)** Nanodrop result of the pENTR/D-TOPO clone was resulted in suitable concentration for LR reaction.

3.2.3 LR reaction for pEDV6 vector

Inserting PstHa12h2 to pEDV6 vector from pENTR/D-TOPO vector, LR reaction was performed as in Section 2.5.6. The same alterations were performed in the reaction. After selection and growth of the clones, colony PCR was conducted for confirmation of positive clones which is shown in Figure 3.9. After colony PCR, the plasmid isolation was conducted from clone 1 and 2, then, they were stocked at -80 ^{0}C for further use in *Pseudomonas fluorescens* mediated transformation and wheat infiltration assay.

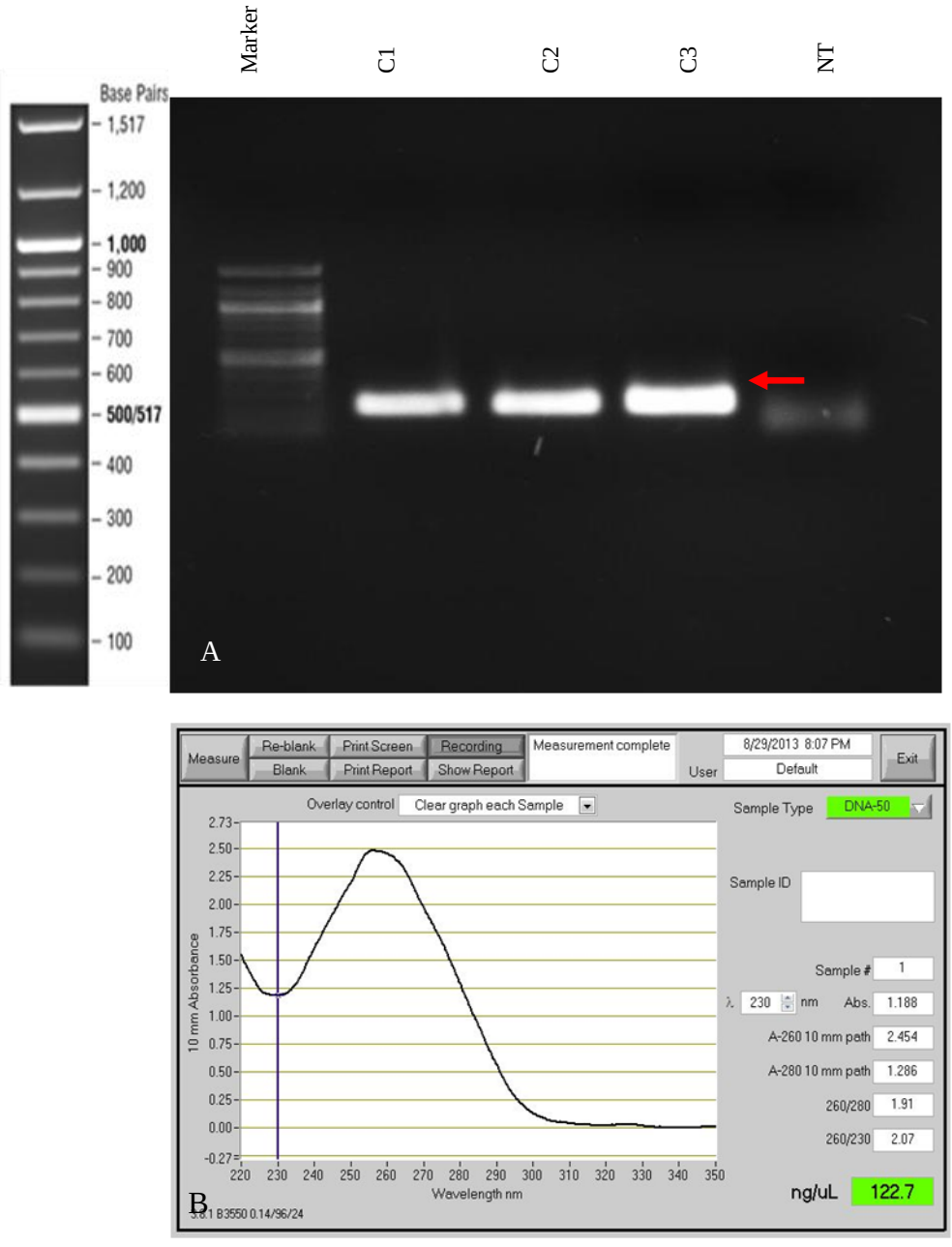

Figure 3.10 Positive clone verification after insertion of Pstha12h2 into pEDV6 and plasmid isolation result. **A)** 100 bp ladder (Marker) was shown in the first lane. PCR products of clones were loaded in the wells which were represented as C1,C2 and C3. NT was the negative control which has no template in the reaction. The bands were appeared as in the expected size (148 bp). **B)** The plasmid isolation was resulted succesfull enough according to its concentration (122.7 ng/µL).

3.3 Ligation of PstHa12h2 gene with pJL48-TRBO vector

Ligation of vector and insert with T4 DNA ligase (NEB) is a commonly used molecular technique. The critical step of ligation is optimization of the molar ratio of insert DNA and vector. Insert DNA molar ratio must be higher than the molar ratio of vector for obtaining efficient ligation. Commonly, vector: insert molar ratio should be 1:3 which is mostly indicated in ligation protocols. Also, there are other factors which influence the efficiency of ligation. One of them is in the gel extraction step. While cutting the bands of insert and vector from gel under UV light, it must be performed very quickly. DNA is damaged by high exposure of UV, so minimizing the UV exposure gives higher cloning results. Moreover, ligation can be effected by higher concentrations of salt. Thus, Buffer PE should be waited in spin column for extended time (3-5 min) in washing step of gel extraction to remove high concentration of salt. In the extended time, the salt dissolves in buffer and it can no longer reduce the ligation efficiency. The extended time for washing steps were conducted in gel extraction and in also plasmid isolation procedures. The other important factor is the double digestion of the vector and insert. When the vector does not digest completely, it results in false positive results. Despite low efficiency ligation, many colonies can be observed in the agar plates. Because of this reason, transformants must be verified for positive clones after ligation. Furthermore, when the insert is not fully digested, it cannot ligate with vector. It was observed that if PstHa12h2 gene was not fully digested, only false positive results were detected on the plates. Because PstHa12h2 gene was amplified with PCR reaction and it is a small sequence, fully digestion of the insert was a very hard step. For eliminating this problem, the insert was cloned into a different vector by an easy and efficient cloning system. GOI was cloned into pGEM-T-Easy vector by TA cloning. Then after verification of the cloning, the insert was double digested from this vector and after observing the digestion product in agarose gel, it was cloned into pJL48-TRBO vector. In the gel extraction after double digestion, the important steps were performed carefully which is mentioned above. Lastly, the transformation efficiency is also important for getting high number of positive clones. Too much ligation product decreases the transformation efficiency. So, appropriate amount of ligation product should be added into competent cells.

For the ligation procedure, first PCR reaction was performed by the procedure and conditions which are shown in Table 2.5 and Table 2.6, respectively (Figure 3.10). Then, TA cloning was performed (Section 2.8.1) and after transformation, the clones were verified by colony PCR. Then, the plasmid isolation of was conducted which is mentioned in Section 2.4 and it was double digested by *Pac*I and *Not*I restriction enzymes. After double digestion, the picture of the gel could not be taken because even taking image of the gel can give harm to DNA which leads to decrease efficiency after gel purification. After gel purification, the eluted DNAs were loaded on agarose gel again for controlling the concentration of them (Figure 3.11).

For preparation of the pJL48-TRBO vector, it was inoculated in four falcon tubes; because, this vector is a low copy number, high expression plasmid. To achieve desired concentration after

plasmid isolation, 4 falcon tubes containing 4 mL LB medium each were grown. They were pelleted at two separate 1.5 mL tubes and then they were transferred into one spin column in plasmid isolation. This increased the concentration of the plasmid. The vector was double digested by a plasmid which had another insert in it. Previously, pJL48-TRBO vector was ligated with another candidate effector gene (PstHa2a5). This made easy a little bit the double digestion. Normally, *Pac*I and *Not*I sites were very close each other in the vector and in double digestion, these restriction enzymes can encounter with each other which would result in less efficient digestion. The plasmid isolation result and gel extraction results after double digestion are shown at Figure 3.12.

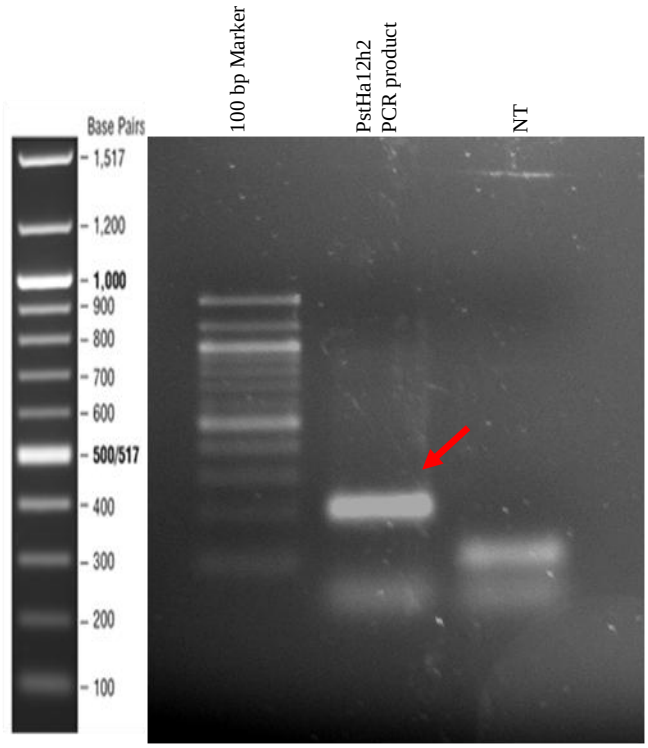

Figure 3.11 PCR amplification of PstHa12h2 gene with Pst12h2-Flag-Fwd and Pst12h2-Rev primers. 100 bp ladder was loaded in the first well. In the second well, 4µL of PCR product was loaded and it was observed in the expected size (208 bp). The size of the sequence was longer than its normal size because Flag-Tag and linker sequence (48 bp) was added to the GOI by Pst12h2-Flag-Fwd. In the last well, no template PCR product was loaded and Pst12h2-Flag-Fwd primer length was observed about 100 bp because of its long size (Electrophoresis was performed by 1% Agarose gel in 1X TAE buffer).

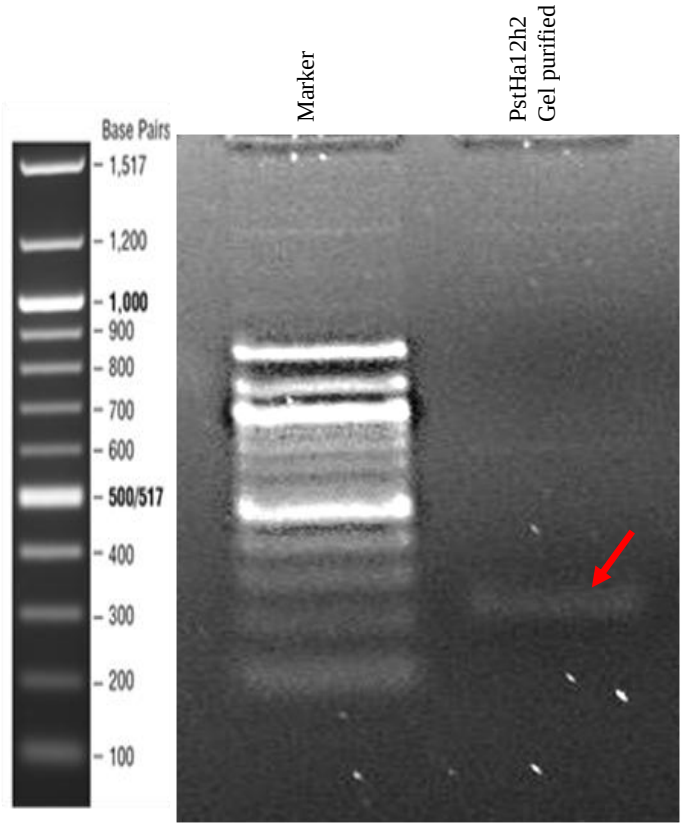

Figure 3.12 Visualization of PstHa12h2 double digested PCR product after gel purification (1% Agarose gel in 1X TAE buffer). In the first well, there was 100 bp Ladder (Marker). 1 µL of gel purified PstHa12h2 insert after double digestion was on the second lane. Despite its concentration was seen very low, it was sufficient to ligate with vector.

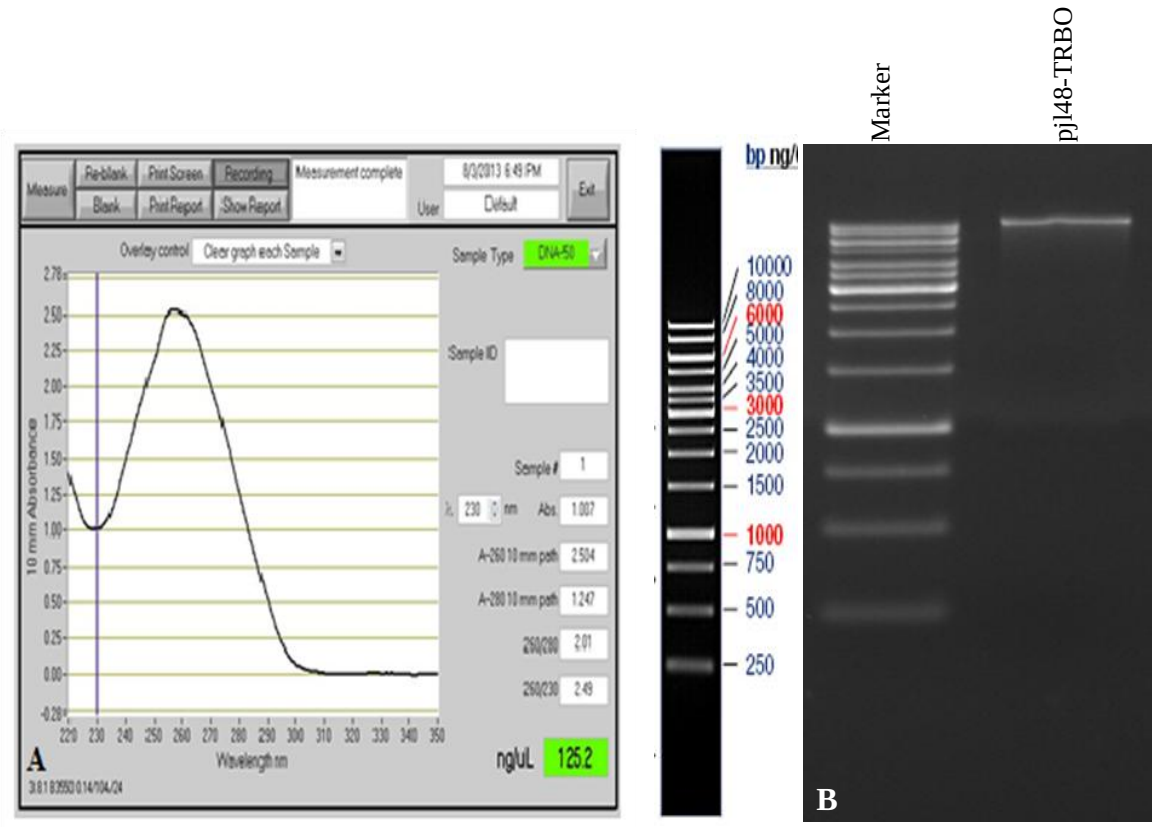

Figure 3.13 Preparation of pJL48-TRBO vector. **A)** The Nanodrop result after plasmid isolation (non-digested). **B)** Visualization of double digested product after gel purification. In the first lane, 1kb ladder (Marker) was loaded and in the second lane, eluted DNA was loaded. It was observed in the expected size (10,000 bp) which shows that cutting after digestion was performed successfully. (Electrophoresis was conducted by 1% Agarose gel in 1X TAE buffer.)

After ligation and transformation which is mentioned in Section 2.8.4, there were many colonies observed on the agar plates. Four or five colonies were verified by colony PCR reaction. After verification of positive clones, one of them was chosen and plasmid isolation was performed (Section 2.4) for double digestion of the clone as in Table 2.10 This was the second verification for the positive clone determination and the result was represented in Figure 3.13. From the grown cultures, the stock cells were prepared after second verification for further use in protein expression in *Nicotiana benthamiana*.

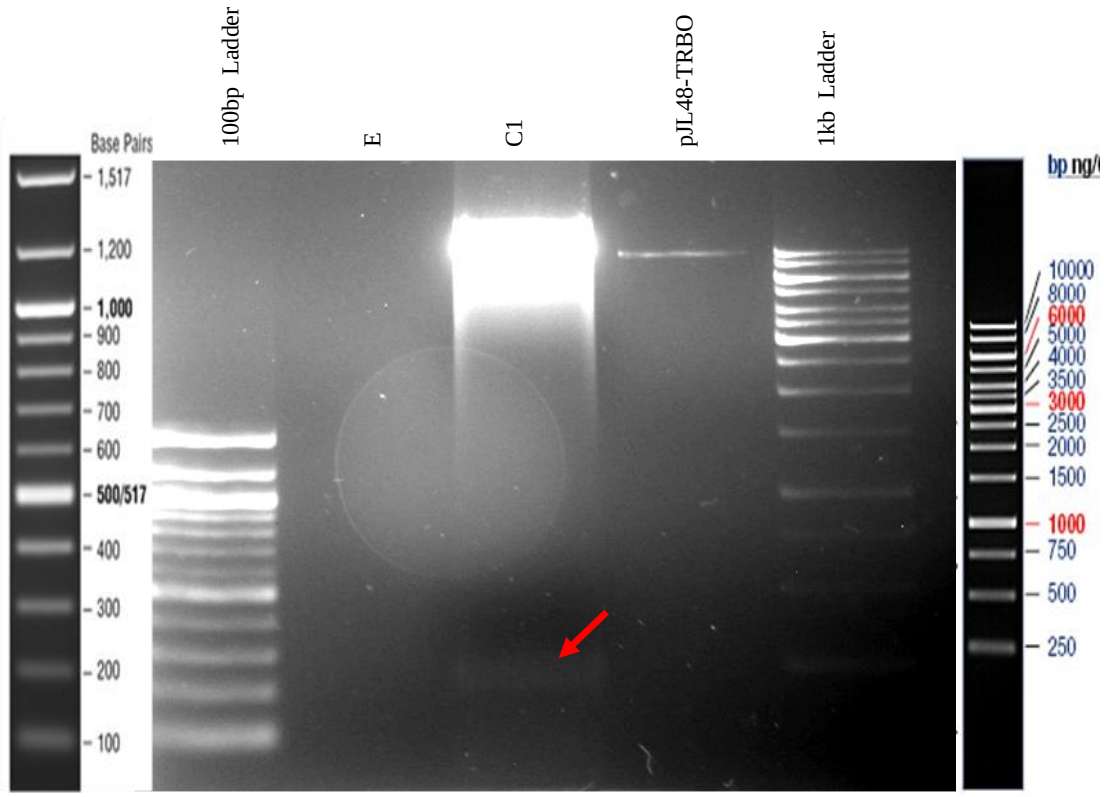

Figure 3.14 Verification of the ligation of pJL48-TRBO vector with PstHa12h2 gene. In the first lane, 100 bp ladder was loaded for easy detection of the insert size. Second lane was empty. In the third lane, all product of double digestion of the clone was loaded and the insert was observed as ~ 200 bp which was expected (indicated by an arrow). In the fourth lane, uncut vector (10,000 bp) was loaded for comparison and in the last lane 1 kb ladder was loaded for the identification of the vector size.

CHAPTER 4

CONCLUSION

In this research, a candidate effector gene of *Puccinia striiformis* f. sp. *tritici* was studied. The pathogen causes one of the main diseases of wheat called wheat yellow rust. In order to eliminate this disease effects, understanding the plant-pathogen interaction mechanism is a very crucial step. In the molecular perspective, there is very limited information about characterization of the effectors of this pathogen. The effectors are the major players of the pathogen, secreted into the host cell by the haustoria of the fungus to transform the host cellular transport mechanism for channeling nutrition for its development. Determination of the functions of each effector would be a main goal for sustaining resistance against them. An effector candidate gene, PstHa12h2, was chosen in this study, because it was shown that expression of was detected in all stages of fungus development with the highest level in infected leaves. Also, it is rich in cysteine residues (five cysteine residues) which are generally found in secreted pathogen proteins and no homology was found with other proteins with known functions in the databases, suggesting it is a novel pathogen protein.

We aimed to study its subcellular localization, determining its interacting proteins, and testing for avirulence activity. For all these goals, the effector gene was cloned into various vectors for differing purposes. It was cloned into three different vectors; one for detecting subcellular localization, by conducting bacterium mediated infiltration assay in *Nicotiana benthamiana*. The gene construct was cloned into pK7FWG2 destination vector to be expressed as GFP fusion protein. Based on the preliminary results presented in this thesis that PstHa12h2 is localized in nucleus and cytoplasm and but very clearly absent in nucleolus. Secondly, for avirulence testing, the effector was cloned into pEDV6 destination vector to deliver the gene directly into the wheat. The vector takes advantage of bacterial Type III secretion mediator gene promoter that if transformed into *P. fluorescens* which can be used to transiently transform the wheat by infiltration. The effector gene is fused with the *avr*RPS4 promoter in the pEDV6 vector. Although the construct made ready for avirulence tests on many yellow rust R-gene possessing differential wheat lines, the test itself was not conducted as part of this thesis study. The promise of the test is if the effector produces hypersensitive response by producing easily detectable hydrogen peroxide in any of the lines having a particular R-gene, this would be an indication of it being an avirulence gene against that particular R-gene.

Thirdly, the PstHa12h2 gene was cloned into pJL48-TRBO vector for protein expression in *N. benthamiana* plant. Apart from others, FLAG-Tag sequence was added to N-terminus of the sequence in the amplification step to later to conduct tagged-protein immunoprecipitation for identifying plant encoded interactors with the effector in study. Although, successful cloning was achieved, the tagged protein co-immunoprecipitation experiements were to be carried out later.

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APPENDIX A

PstHa12h2 SEQUENCE INFORMATION

(Retrieved from NCBI databases)

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DEFINITION  PSTha12h2 Puccinia striiformis f. sp. tritici haustoria cDNA
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            mRNA sequence.
ACCESSION   GH737173
VERSION     GH737173.1  GI:222429082
DBLINK      BioSample: LIBEST_024285
KEYWORDS    EST.
SOURCE      Puccinia striiformis f. sp. tritici
ORGANISM    Puccinia striiformis f. sp. tritici
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            Pucciniomycetes; Pucciniales; Pucciniaceae; Puccinia.
REFERENCE   1  (bases 1 to 493)
  AUTHORS   Yin,C., Chen,X., Wang,X., Han,Q., Kang,Z. and Hulbert,S.
  TITLE     Generation and analysis of expression sequence tags from haustoria
            of the wheat stripe rust fungus Puccinia striiformis f. sp. Tritici
  JOURNAL   BMC Genomics 10 (1), 626 (2009)
   PUBMED   20028560
COMMENT     Contact: Hulbert SH
            Department of Plant Pathology
            Washington State University
            345 Johnson Hall, Pullman, WA 99164, USA
            Tel: 509 335 3722
            Fax: 509 335 9581
            Email: scot_hulbert@wsu.edu
            Seq primer: M13 Forward.
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                     Site_2: Sfi I Forma specialis: tritici;"
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Amino Acid Sequence:

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APPENDIX B

pK7FWG2 VECTOR SEQUENCE

(Karimi et al.,2002)

>pK7FWG2 ;11880 bp

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//

APPENDIX C

pEDV6 VECTOR SEQUENCE

(sequence obtained from Peter Dodds)

>pEDV6;6985bp

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APPENDIX D

pJL48-TRBO VECTOR SEQUENCE

(Lindbo, 2007)

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